Nasal hyperreactivity and inflammation in perennial allergic rhinitis

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CATHARINA IN 'T VELD
geboren te Rotterdam

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geboren te Dordrecht

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Preface

Allergic rhinitis is a common disease resulting in a high morbidity. It is defined as an inflammation of the nasal mucosa and is characterised by symptoms such as itchy nose, sneezing, rhinorrhoea and nasal blockage. Repetitive allergen exposure gives rise to increased sensitivity to allergens (priming) and nonspecific stimuli. This nonspecific nasal hyperreactivity can be measured by history and by nasal challenge tests with methacholine or histamine. However, the pathogenesis of nasal hyperreactivity is still not clear.

In asthmatic patients bronchial hyperreactivity seems to be associated with the late phase response and allergic inflammation, suggesting that inflammation is involved in the pathogenesis of hyperreactivity. In analogy with the bronchial situation these associations were also studied in the nose. Until now, most studies investigating the relationship between nasal response to allergen and nasal hyperreactivity have been performed in patients with pollinosis. In these studies no relationship could be found between nasal hyperreactivity, nasal priming, the late phase response and nasal inflammation.

Only a few studies have been performed in patients allergic to house dust mites (HDM). In this subgroup it has been demonstrated that pre-existent nasal hyperreactivity can predict nasal response to allergen challenge, a finding more in agreement with the lower airways. In this thesis we further want to investigate the relationship between nasal hyperreactivity, nasal response to allergen and nasal inflammation in perennial rhinitis.

Hereto, we evaluated the effects of therapeutic intervention on nasal response to allergen and hyperreactivity and data obtained during the placebo treatment. Nasal response to HDM challenge was monitored by symptoms and mediators in nasal lavage fluid. Allergen-induced nasal hyperreactivity was determined 24 hours later by nasal challenge with histamine or methacholine.

It is expected that non-specific nasal hyperreactivity is particularly important in patients with perennial allergic rhinitis, since they are continuously exposed to allergen. However, the importance of routine histamine challenges in daily practice is not clear. Hereto, we studied nasal response to histamine and daily nasal symptoms. Inflammatory mediators in lavage fluid and eosinophils in cytospins were also determined in order to evaluate the involvement of inflammation in the ongoing allergic reaction.

In addition, we investigated the impact of rhinitis symptoms on day-to-day life. By means of a rhinitis quality of life (QOL) questionnaire, the relationship between nasal response to histamine, QOL and daily nasal symptoms was estimated in patients allergic to HDM.

Part one presents the definitions and clinical aspects of allergic rhinitis (chapter one), the cells and mediators involved in the allergic reaction (chapter two) and the aims of the study (chapter 3).

In **part two** the relationship between nasal hyperreactivity and inflammation is studied. Chapter 4 describes a nasal challenge study in patients with perennial rhinitis. Inflammatory mediators and nasal hyperreactivity were compared between patients with an early and late phase response and individuals expressing an isolated early response only. In chapter 5, inflammatory mediators in nasal lavage, eosinophils in nasal brushes and responsiveness to histamine were studied in non-challenged patients with perennial rhinitis

and healthy subjects.

Part three concerns the therapeutic intervention on nasal response to allergen challenge and hyperreactivity with a corticosteroid (fluticasone propionate) and an antihistamine (levocabastine). In chapter 7-10 the effect of two weeks topical fluticasone propionate on the immediate and late nasal symptoms, inflammatory mediators and nasal response to histamine is described. In chapter 11, the characteristics of the glucocorticoid receptor of the patients responsiveness or non-responsiveness to fluticasone propionate were compared. Chapter 13 describes the results of one week topical levocabastine on nasal response to allergen (symptoms and mediators), to histamine and to methacholine challenge. Chapter 14 discusses the results of an *in vitro* study. The effect of pre-incubation with levocabastine on mediator release of human leucocytes and on the contractile response of guinea-pig trachea and lungparenchyma is described.

Part four (chapter 15) discusses the relationship between nasal hyperreactivity, quality of life and daily nasal symptoms in 48 patients with perennial allergic rhinitis.

Part five is the general discussion and summary.

Nasal challenges were performed at the department of Allergology of the Academic Hospital Rotterdam and the *in vitro* studies and the assessment of mediators and cells at the department of Pharmacology of the Erasmus University Rotterdam.

PART ONE

General introduction

Chapter 1
Allergic rhinitis

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1.1 Historical review of allergy

The history of allergic diseases goes back to 1819, when Bostock described his own "periodical affection of the eyes and chest", which he called "summer catarrh". Since they thought it was produced by the effluvium of new hay, this condition was also called hay fever. Later, in 1873, Blackley[61] established that pollen played an important role in the causation of hay fever.

In 1902 Portier and Richet described the development of anaphylaxis in dogs a few minutes after reinjection with anemone toxine. By this experiment they demonstrated that, in this case, immunity was not protective but damaging to the individu. Arthus observed in 1903 that after repeated injections with substances which had not caused any reaction the first time, the injected tissues became inflamed. Von Pirquet[517] noted that under some conditions, patients, instead of developing immunity, had an increased reactivity to repeated exposure with foreign substances. By putting together the Greek words "allos" meaning different or changed, and "ergos" meaning work or action, he introduced in 1906 the term allergy. Both immunity and hypersensitivity were thought to have similar underlying immunologic mechanisms. Later, in 1923, Coca and Cooke[114] proposed the term atopy for the clinical forms of allergy, manifested by hay fever and asthma, in which "the individuals as a group possess a peculiar capacity to become sensitive to certain proteins to which their environment and habits of life frequently expose them". Thus, an inherited predisposition to become sensitized is a characteristic feature of atopy. Prausnitz and Küstner[518] demonstrated in 1921 that the serum of allergic individuals contained a humoral factor that caused specific allergen sensitiveness in a non-allergic individual. The factor responsible for the Prausnitz-Küstner reaction was named reagin by Coca and Cooke. These reagins were characterised by Ishizaka et al.[314] and independently at the same time also by Johansson and Bennich[322] as a new immunoglobulin class which they named immunoglobulin E (IgE). Gell and Coombs[222] subdivided allergy into 4 types: immediate IgE-dependent reaction (I), cytotoxic reaction (II), immune complex reaction (III), delayed cellular immune reaction (IV).

From a clinical view, Voorhorst[650] defined allergy in 1962 as an altered sensitivity, deviating from the norm (i.e. normergy) in a quantitative sense.

Nowadays, the definition of allergy as an "untoward physiologic events mediated by a variety of different immunologic reactions" described by Middleton, Reed and Ellis[441] is used. Accepting this definition, one should also keep in mind the following three criteria: (1) identification of the allergen, (2) establishment of a causal relationship between exposure to the antigen and occurrence of the lesion and (3) identification of the immunologic mechanism involved in the illness.

In this thesis, the term allergy will be restricted to the IgE-dependent reactions. The most important clinical manifestations of IgE-dependent reactions are allergic conjunctivitis,

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allergic rhinitis, allergic asthma and atopic dermatitis. However, we will restrict in this thesis to the allergic rhinitis.

1.2 Allergic rhinitis

1.2.1 Epidemiology

Allergic rhinitis is the most common manifestation of the IgE-mediated disorders, with a prevalence ranging from 2 to 20 percent.[167] The prevalence of allergic rhinitis seems to be increasing. In a study performed in Swedish army recruits, the prevalence of hay fever increased from 4.4% in 1971 to 8.4 % in 1981.[1] The prevalence of allergic skin test reactivity, i.e. atopy, increased from 39% to 50% in a community sample in the USA of individuals of all ages for a mean of 8 years.[34] Since skin reactivity and allergic disease are associated, this suggests that the prevalence of allergic rhinitis is also increasing.

1.2.2 Clinical aspects of allergic rhinitis

According to the international consensus rhinitis is defined as an inflammation of the nasal mucosa characterised by one of the following symptoms: nasal itchyness, sneezing, rhinorrhoea and nasal congestion.[311] Other symptoms, such as 'popping' of the ears, headache, throat clearing and coughing, are less common. In patients with allergic rhinitis these symptoms are due to the interaction between allergens and specific IgE antibodies. Allergic rhinitis can be subdivided in seasonal and perennial rhinitis. In seasonal rhinitis symptoms are triggered by exposure to tree, grass, and/or weed pollen. In non-tropical parts of the world, seasonal allergic rhinitis occurs during a defined period of the year, which implies that patients also have a symptom free period. In contrast, patients with perennial rhinitis suffer from almost continuous nasal symptoms throughout the year. The most common perennial allergens are indoor allergens like the house dust mite (Dermatophagoides pteronyssinus and D. farinae) and animal danders and in some areas also certain mould species and cockroaches. Although the house dust mite is regarded as a perennial allergen, an increased number of house dust mites are found in the autumn, which may result in an increase in symptoms in this period.[226,518,652]

1.2.3 Impact on daily activities and quality of life

However, patients with rhinitis are not only troubled by nasal symptoms. Nasal symptoms also interferes with their day-to-day lives and with their quality of life. Patients are limited in their daily activities, concentration and sleep are impaired. Associated symptoms as headache are troublesome, practical things such as the availability of a handkerchief and blowing the nose are a nuisance, social interaction is limited and there is an impact on emotional well-being.[335] In school aged children with pollinosis, learning abilities during the pollen season were reduced and this effect could only be partially counteracted by non-

sedating antihistamines.[654] This impact on day-to-day life and QOL is a major cause of morbidity. Due to impaired concentration and reduced productivity rhinitis patients may even lose earnings. So in addition to the costs of medication, health services and sick absence, this loss in personal income attributes to the economic impact of rhinitis. To measure the influence of nasal symptoms on day-to-day life, rhinitis quality of life (QOL) questionnaires have been developed.[71,335,336] These questionnaires are useful to study the effect of allergen exposure and therapeutic intervention in clinical trials. Juniper *et al.*[335,336] demonstrated that QOL deteriorated after allergen exposure (pollen season) and increased after symptomatic treatment.

1.3 Reactions of nasal mucosa on allergen exposure

Most studies concerning the pathophysiology of allergic rhinitis have been performed in patients with seasonal rhinitis. The effect of pollen exposure on the nasal mucosa can be determined during natural pollen season or by nasal challenge models performed outside the pollen season. In nasal allergen challenges studies, well-known amounts of standardised allergen are administered into the nose. In most studies, nasal challenges are used to investigate the pathophysiology of allergic rhinitis. However, one should keep in mind that the mode of exposure is not natural and that in a short time high concentrations of allergens are administered to elicit a clear nasal response instead of continuous exposure to lower and variable amounts of allergens. The problem of monitoring nasal response during natural exposure is the variable and unknown level and spectrum of allergen content.

Several methods have been used to perform nasal allergen challenges. Connell[119] developed a quantitated challenge with ragweed pollen. Later, standardised liquid allergen extracts were developed, which can be insufflated into the nose or can be administered by filter paper-discs or by special equipment like the 'nasal pool device.'[39,257,465,467]

Nasal response to allergen challenge can be determined by different methods. No standardised method is available. Usually, the symptomatic response is monitored by the number of sneezes, the amount of secretion, and nasal blockage. Sneezing and itchyness are the result of a central reflex elicited in the sensory nerve endings in the nasal mucosa. It is easy to grade the sneezing reflex by counting. Sneezing and itchyness can also be subjectively measured by symptom scoring. Nasal blockage is the result of pooling of blood in the capacitance vessels of the mucosa, and to some degree the result of tissue oedema. It can be assessed subjectively by means of symptom scoring. An objective estimation of nasal blockage can be made by methods such as rhinomanometry[112], nasal peak flow determination[233,685], acoustic rhinometry[181,287,381] and rhinostereometry[264]. Nasalsecretion can be assessed by weighing the blown secretion or by measuring the volume of secretion collected in a funnel equipped tube or syringe while the subject is bending her/his head forwards[68]. Several scoring methods have been developed: visual analogue scales[395], combined symptom scores taking nasal blockage, secretion and sneezes[70] and a combination of all signs and symptoms[373].

Nasal response can also be monitored by analysis of nasal biopsies[190,642], brushes[516], smears[432], or lavages[465]. These techniques can be used to make an estimation of nasal inflammation by determination of mediator levels and cell populations. The presence of inflammatory cells such as eosinophils, mast cells, T-cells and Langerhans cells has been

demonstrated in the nasal mucosa.[188,334,642] The inflammatory cells and mediators involved in allergic rhinitis are described in detail in chapter 2, allergic inflammation.

1.3.1 Immediate allergic reaction

When the nasal mucosa of patients with allergic rhinitis is exposed to allergen, allergen activates mast cells and basophils by bridging two or more IgE molecules on their surface. After being activated these cells produce and release biochemical mediators such as histamine, platelet activating factor (PAF), tryptase, leukotrienes, prostaglandin D_2 (PGD$_2$ only produced by mast cells), cytokines and chemotactic factors.[95,465] Gomez et al.[244] and Fokkens et al.[189] demonstrated in biopsy studies an increased percentage of degranulated mast cells at the surface of the nasal mucosa after nasal pollen challenge. The released substances act on the local cells, vessels and sensory nerve endings, leading to nasal itching, sneezing, rhinorrhoea and nasal blockage. In some patients after this immediate allergic reaction, a recurrence of symptoms can be demonstrated.

1.3.2 Late allergic reaction

Blackley[61] in 1873 was the first to describe the recurrence of symptoms several hours after the introduction of grass pollen in his nose. This recurrence of symptoms has been termed the late phase reaction.[176,505]
To define the late phase reaction in the nose is difficult. Mygind et al.[460] could not detect late phase reactions by means of symptom scores. Since it is hard for patients to make an estimation of their nasal patency and late phase responses are mainly characterised by nasal blockage and in a lesser extent by mild rhinorrhoea, this might declare the problems detecting clinical late phase responses. When estimating nasal obstruction by rhinomanometry, a recurrence of nasal blockage could be demonstrated.[176] In other studies late phase reactions were determined by measurement of nasal obstruction and analysis of nasal lavage fluid. Naclerio et al.[467] measured a second increase of histamine, but not of PGD$_2$. This suggests that basophils are, since they release histamine but no PGD$_2$, involved in the late phase. In nasal lavage fluid obtained during the late phase period an influx of eosinophils was demonstrated.[42] Also eosinophil derived mediators have been demonstrated during the late phase response, confirming the involvement of eosinophils in this period.[43,55] Immunohistology of the nasal mucosa demonstrated an increase of T-helper cells, eosinophils and neutrophils 24 hours after pollen challenge.[642]

1.3.3 Nasal priming

Connell[120,121] described in the sixties a phenomenon known as nasal priming: repetitive exposure to allergen causes an increased sensitivity to allergens. This has been demonstrated with repetitive exposures to pollenrich natural environment as well as by repetitive nasal provocation with allergen. This effect was confirmed by others by nasal challenge studies.[28,261] Wachs et al.[655] demonstrated that after repetitive pollen challenge,

increased levels of mast cell mediators could be detected in nasal lavage fluid, which may implicate an increase in the number and/or the releasability of mast cells. However, in a biopsy study by Fokkens et al.[189], no correlation was found between nasal priming by pollen challenge and the number of mast cells or amount of degranulation. Bentley et al.[49] demonstrated by immunohistology of the nasal mucosa an influx of inflammatory cells after allergen challenge. Since both systemic and topical corticosteroids abolish the primed response to allergen, this might suggest that priming is related to antigen-induced nasal inflammation.[515,516] However, the exact processes resulting in nasal priming remain unclear. In perennial rhinitis the priming phenomenon has only been examined in one study.[226] This Dutch study demonstrated an increased threshold sensitivity to house dust mite challenge in autumn, compared to spring months corresponding with the peak of house dust mite levels between August and October.

1.4 Allergen-induced nasal hyperreactivity

Hyperreactivity can be described as a clinical feature characterised by an exaggerated response of the nasal mucosa to everyday stimuli (perfume, tobacco smoke, change of temperature) as estimated by history (clinical hyperreactivity). In comparison to allergens, these stimuli are nonspecific, that is, they can affect the nasal mucosa of any individual, albeit to a different extent. In analogy with challenge studies in bronchial asthma, rhinitis patients were challenged with histamine[260,410] and methacholine[68,410] to measure nonspecific nasal hyperreactivity. It is well known that histamine and methacholine have different effects on the nose. Histamine, acting by H_1 and H_2 receptors, leads to vasodilatation and increased vasopermeability resulting in nasal congestion.[83,158] Nasal discharge is also increased and is believed to be caused by both transudation and glandular secretion.[532] The involvement of reflexes is demonstrated by the induction of sneezes[158,488] (see 2.2). Methacholine acts by the M_3 receptor and has a direct effect on glands only.[68,531]
Determination of nasal hyperreactivity by nasal provocation tests with pharmacological or physical agents assumes a relationship between this form of nasal hyperreactivity and hyperreactivity as estimated by history. Gerth van Wijk et al.[228] demonstrated that the amount of secretion and the number of sneezes in response to histamine challenge were associated with the clinical hyperreactivity assessed by a hyperreactivity score. It was also demonstrated that assessment of the number of sneezes and the amount of secretion is more appropriate in distinguishing patients from healthy subjects in terms of reproducibility and estimation of clinical hyperreactivity compared with assessment of nasal airway resistance after histamine challenge.[225]

In patients with allergic rhinitis, part of the symptoms is due to exposure to nonspecific stimuli. Repetitive exposure to allergen not only increases sensitivity allergens, but also to nonspecific stimuli. Borum and Mygind[69] demonstrated that in patients with seasonal allergic rhinitis during the pollen season nasal response to histamine and methacholine increased. Allergen challenge also increased nasal response to histamine[10,395] and methacholine[346]. In contrast, repeated challenges with histamine or methacholine did not increase nasal responsiveness to histamine.[261]

The observation that the increase of nasal sensitivity to histamine was very well correlated with the increase of sensitivity to allergen suggested a common pathogenesis.[10] However, the presence of increased numbers of mast cells might explain nasal priming[164], but not the closely related nasal hyperreactivity to histamine. The occurrence of certain cell populations together with the induction of nasal hyperreactivity after allergen challenge implies a time relationship. Inflammatory cells such as eosinophils could release mediators with toxic effects on the nasal mucosa, thereby causing nasal hyperreactivity. However, very few investigations have been designed to study the relationship between nasal hyperreactivity and the number of cells and or mediators. In one study only, a weak correlation between ECP and allergen-induced hyperreactivity to histamine could be demonstrated.[395] In another study no relation between eosinophil influx into lavage fluid and hyperreactivity could be demonstrated.[346] In a few studies evaluating the effect of topical corticosteroids, an effective anti-inflammatory drug, nasal hyperreactivity was reduced[348,356], which might indirectly give evidence of the involvement of inflammation in this process.

The importance of routine histamine challenges to assess nasal hyperreactivity in daily practice is not clear. Recently, a correlation between daily symptoms and response to histamine challenge was demonstrated in patients suffering from non-allergic nasal hyperreactivity.[265] It is expected that non-specific nasal hyperreactivity is particularly important in patients with perennial allergic rhinitis, since they are continuously exposed to allergen. Gerth van Wijk et al.[228] found in 12 selected patients with perennial allergic rhinitis, that nasal reactivity to histamine was associated with clinical symptoms and the sensitivity to everyday stimuli. However, such studies have not been performed routinely in daily practice.

Mechanisms of hyperreactivity
The exact mechanism of nasal hyperactivity is unknown. Although it is widely believed that allergen-induced mucosal inflammation provides the background for this condition, no evidence to support the role of a specific element or event of the allergic inflammatory process exists. Several hypotheses with respect to the mechanisms underlying hyperreactivity have been advanced. (1) Increased epithelial permeability, which would lead to an increased accessibility for stimuli to sensory nerve endings, vessels and nasal glands. An indirect support to this hypothesis has been delivered by Buckle and Cohen[78], who demonstrated that topically applicated ^{125}I-albumin penetrated better into the nasal mucosa in allergic rhinitis compared with healthy subjects. However, in more recent studies there is little evidence that the nasal epithelium suffers much damage in acute or chronic allergic rhinitis.[258,642] (2) Increased sensitivity of sensory nerve endings (irritant receptors) would induce an exaggerated response to normal stimuli. No firm data are available to confirm this theory. (3) Imbalance of the autonomic nerve regulation caused by changes of the neuroreceptors in the nasal mucosa. Megen et al.[430] demonstrated an increased sensitivity and a decreased number of muscarinic receptors in the nasal mucosa of allergic subjects. These findings may reflect the cholinergic-induced hypersecretion. In addition, a decreased number of β-adrenoreceptors was found which may be an indication of a β-adrenergic abnormality. Increased presence of the neuropeptide substance P or diminished levels of vasoactive intestinal peptide (VIP) might contribute to hyperreactivity.

Until now, evidence for this hypothesis has only been demonstrated in the lower airways.[35,490,491] The role of neuropeptides in allergic rhinitis is unclear.

1.5 Allergic rhinitis. A model to study airway inflammation?

Asthma and allergic rhinitis are common disorders, with a high socio-economic impact and causing a lot of morbidity. Many studies have been performed concerning the pathophysiological mechanisms. In the nose such studies are more easy to perform since the nose is readily accessible and biopsies and lavages are less accompanied by any risk and discomfort for the patient. It would therefore be easy if studies evaluating the pathophysiology and therapeutic intervention of asthma were to be replaced by studying the nasal mucosa. However, the upper and lower airways are not entirely similar since part of the symptoms in asthma are caused by smooth muscle tissue contraction, resulting in bronchoconstriction.

Repetitive allergen challenge causes an increased sensitivity to allergen and nonspecific stimuli. This phenomenon was first described for the lower airways[116] and could also be explored in the nasal situation[10,346,395]. In the lower airways, the late phase response to allergen challenge was found to be associated with inflammation and bronchial hyperreactivity[92,115,162,445] suggesting that inflammation is involved in the pathogenesis of hyperreactivity. Several studies have been performed to find out whether similar associations could also be shown in allergic rhinitis patients. However, no relation was found between nasal hyperreactivity and late nasal response[230], between nasal hyperreactivity and activation of eosinophils[346] or between nasal priming and late nasal response[310] in studies performed in pollinosis patients tested outside the pollen season.

In contrast, in a study with rhinitis patients allergic to house dust mite, an association between nasal responsiveness to allergen and pre-existent nasal hyperreactivity was found[229], a finding more in agreement with data from the lower airways. So this subpopulation might be more suitable to study the association between nasal hyperreactivity, nasal inflammation and the late phase response and might serve as a better model to study airway inflammation.

Chapter 2
Allergic inflammation

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2.1 Introduction

The histopathological features of allergic inflammation involve an increase in blood flow and in vascular permeability leading to plasma exudation and the formation of oedema. In addition, a cascade of events occur which involve a variety of inflammatory cells. These inflammatory cells migrate under the influence of chemotactic agents to the site of injury and induce the process of repair. Several types of inflammatory cells have been implicated in the pathogenesis of allergic rhinitis. What remains unclear is how the different cellular components interact with each other to induce the pathological symptoms of allergic rhinitis and the relationship between the inflammatory infiltration, cellular activation and hyperreactivity still need to be established. After specific or non-specific stimuli, inflammatory mediators are generated from cells normally found in the nose, such as mast cells, antigen-presenting cells and epithelial cells (primary effector cells) and from cells recruited into the nose, such as basophils, eosinophils, lymphocytes, platelets and neutrophils (secondary effector cells).

For inflammatory cells the primary function is phagocytosis and the secretion of inflammatory mediators. The phagocytic cells, by eliminating particles or organisms that have gained access to the host, act as a protective barrier between environment and the host. The secretory cells release inflammatory mediators, which increase vascular permeability and chemotactic factors, which results in recruitment of other inflammatory cells.

This chapter will describe the identification of each of the inflammatory cells and their mediators which play a role in the perennial allergic processes in the nose of rhinitis patients.

2.2 Cells in allergic rhinitis

2.2.1 Primary effector cells

Mast cells

Mast cells are polymorphonuclear leukocytes that differentiate in the bone marrow with a lifespan of approximately 10 days and are ordinarily distributed throughout normal body tissues. The maturation of human mast cells is known to be dependent on cytokines, such as Interleukin-3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF) and thus highly dependent on the local environment.[232,428,600]

Human mast cells can be characterised by the presence of tryptase on the one hand (MC_T) or tryptase and chymase (MC_{TC}) on the other. More than 95% of the epithelial mast cells

and 75% of the subepithelial mast cells in human airways are of the MC_T-subtype.[312,565] Binding of allergen to specific IgE-molecules on mast cells leads to a series of events finally resulting in allergic symptoms. IgE is able to bind to the high affinity IgE-receptors ($Fc_\varepsilon R_I$) on mast cells, which induces secretion of mediators.[122,623] These mast cell-derived mediators can be divided into two main categories: pre-formed or granule-associated mediators and the newly-formed or membrane-derived mediators. Pre-formed mediators include histamine, proteoglycans such as heparin and chondroitin sulphate, neutral proteases tryptase and chymase and acid hydrolases.[563] The membrane-derived mediators are mainly metabolites of arachidonic acid, such as prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4) and the cysteinyl leukotrienes C_4, D_4 and E_4, and platelet activating factor (PAF).[291,362,562] Mast cells additionally generated, adenosine[672], nitric oxide[24,57,422] and cytokines, such as IL-3, IL-4, IL-5, IL-6, GM-CSF and TNF-α[74,128,598,660,692], but not IL-2 and IFNγ.[107,519,689]

Rhinitis: Mast cells have been implicated in the pathogenesis of allergic diseases ever since histamine was localized to these cells.[539] The number of mast cells in the nasal mucosa is increased in allergic rhinitis.[49,189,276,302,486,487,495,648] Elevated levels of mast cell mediators are present in the nasal lavage fluid after experimental allergen challenge[95,465] and challenge with cold dry air[620], and experimental application of mast cell mediators to the nasal mucosa produces symptoms of rhinitis.[532,604] Several studies have demonstrated that the amount of mast cells in the epithelial layer is increased after allergen exposure, which can be interpreted as shift of cells from the lamina propria to the epithelium or proliferation of precursor cells in the epithelium.[164,401,486,487,496,647] Borres demonstrated that metachromatic cells can be found superficially in the nasal mucosa 5-24 hours after allergen challenge, with a correlation between the amount of cells and symptom score.[66] Mast cells are multifunctional cells which can play more than one role and can contribute to the chronic inflammation underlying allergic diseases by producing a number of immunomodulatory and proinflammatory cytokines and mediators.[108,215]

Antigen-presenting cells

Responses to most antigens require processing of the antigen by antigen-presenting cells (APC), because T-cells ordinarily recognize antigens only together with major histocompatibility complex (MHC; Human leukocyte antigen HLA-DR,-DQ,-DP) antigens on the surface of other cells. The first step in the immune response following entry of the antigen involves capture and processing of the antigen by APC and presentation of the processed form of the antigen to a subset of T-cells. These class II MHC proteins are expressed on the surface cell membrane of macrophages, dendritic cells in lymphoid tissue, Langerhans cells in the skin and the nose, Kupffer cells in the liver, microglial cells in the central nervous system tissue, epithelial cells and B-cells. These cells phagocytose the antigen from which fragments become noncovalently associated with class II molecules, whereafter the complex is transported to the cell surface, where it is then accessible to the T-cell. B-cells are relatively poor activators of T-cells when presenting antigens, possible because such T-cells require activating factors such as interleukins which B-cells fail to provide. Therefore, it is believed that macrophages or Langerhans cells probably play the dominant role as APCs in the initial or primary immune response whereas B-cells may dominate in

the memory or secondary response.[187,191,192,331]

Macrophages are differentiated from blood monocytes which are derived from bone marrow. Macrophages play a central role in host defense, which includes ingesting and killing invading organisms/antigens and releasing a number of factors involved in host defense and inflammation. They also function as antigen-presenting cells (APC) during the development of specific immunity.[288,423] Macrophages possess low affinity IgE-receptors and after binding of IgE they will release mediators.[330,411] They produce thromboxane A_2 (TxA$_2$), PGD$_2$ [407], PGF$_{2\alpha}$, LTB$_4$ [419,420,534], LTC$_4$ [178] and PAF.[15,580] They also release ß-glucuronidase, neutral proteases, lysosomal enzymes[212,624] and complement C3a.[30,471] Macrophages/monocytes produces cytokines, like TNF$_\alpha$, IL-1ß[65,166,585], IL-6[246], GM-CSF[79,86] and IL-10.[413] They release O_2^--radicals, which are inflammogen, bronchoconstrictor, and cause bronchial hyperresponsiveness.[340] Macrophages also are able to produce nitric oxide[182,527] and express iNOS in areas of acute and chronic inflammation.[353] However, other investigators showed that monocytes and macrophages failed to produce NO after stimulation with different cytokines.[325]

Rhinitis: Although macrophages are the most common cell type residing in the lumen of the lower airways, little is known about the presence and pathogenic implications of macrophages in the upper airways. Both local allergen challenge and natural exposure increase the number of macrophages on the mucosal surface during the immediate as well as late phase reactions, indicating that macrophages are involved in the inflammatory processes of allergic rhinitis.[26,331]

Langerhans cells are from dendritic morphology. The origin and relationship of dendritic cells to the monocytes/macrophages lineage are not established, it is likely that they originate from a common stem-cell precursor.[537] They contain IgE receptors (Fc$_\epsilon$R) on their surface.[192] It is important to note that the undoubtedly effective antigen-presenting ability of pulmonary interstitial dendritic cells may be limited to the interstitial lung-compartment[296,473]; these cells might, therefore, not be expected to come into direct contact with inhaled antigens. This is in contrast with other investigators, who found that Langerhans cells were found in the epithelium and lamina propria of the nasal mucosa.[192]

Rhinitis: Higher amounts of Langerhans cells were detected in nasal biopsies of allergic patients compared with controls.[192] During the grass-pollen season, the nasal epithelium of patients with an isolated grass-pollen allergy demonstrated more Langerhans cells than before and after the season.[191]

Epithelial cells

Epithelial cells play an important role in the defence of the airways and in inflammatory processes, it seems to be more than a protective barrier.

Immunohistochemical studies of human lung tissue have reported that epithelial cells have the ability to express the HLA-DR antigens, suggesting that these cells play an important role in the antigen presentation and immunoregulation.[355,544]

Epithelial cells produce mediators, like 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE; which is chemotactic[300,306,469,574,578,661]), 12-HETE, PGE$_2$ (which causes bronchodila-

tation and decreases mast cell mediator release[304,364]), small amounts of $PGF_{2\alpha}$ [109,683] and PAF.[409,549] Epithelial prostaglandin release can be stimulated by bradykinin[37,109,683], calcium ionophore A23187[109,683]; PAF[683], eosinophil major basic protein[320] and ozone[379,476]. Epithelial PGE_2 is also responsible for much of the transport of water and electrolytes by the airway epithelium.[6] Recent studies suggest that the release of 15-HETE is regulated by IL-4 (stimulation) and IFN-γ (inhibition) of the 15-lipoxygenase.[123] It is possible that IL-4 released by T_{H2} lymphocytes causes increased expression of 15-lipoxygenase.[543] The chemotactic substance LTB_4 is produced by dog epithelial cells, but not by human.[299] Airway epithelial cells are able to produce chemotactic cytokines, like TNF-α[357], IL-6[476], IL-8[470] and GM-CSF[110,485,497], which can be stimulated by TNF-α, IL-1 and histamine. The epithelial layer in the airways is enriched with nerve endings which contain tachykinins, such as substance P. This is chemotactic for neutrophils[416] and monocytes[548], and potentiates phagocytosis and lysosomal enzyme release by neutrophils and macrophages[33]. Substance P is mitogenic for T-lymphocytes[502] and stimulates histamine release by mast cells[197]. It also stimulates airway epithelial ion transport[5], causes airway smooth muscle contraction[609] and stimulates submucosal-gland secretion[67]. Epithelial cells are able to produce constitutive and inducible nitric oxide synthase.[19]
Several studies have demonstrated that exposure of the airways to irritant substances, such as allergens and ozone, leads to inflammation and damage of the airways in asthma.[366,687] Epithelial damage may contribute to bronchial hyperresponsiveness in asthma.[137,145,290,323]
Rhinitis: Although damage of the epithelial layer causes an increased permeability to antigens, exposure of sensory nerve fibres and actuation of local reflex mechanisms, changes in osmolarity of the bronchial surface lining fluid and a decreased production of epithelial relaxant factors[35,41,183], this has not been demonstrated in the nose. Epithelial cells may play an important role in the local recruitment, differentiation, and survival of inflammatory migrating cells[131], and contribute to the pathologic and clinical events which occur in allergic rhinitis.

2.2.2 Secondary effector cells

Basophils

Basophils are circulating leukocytes with many of the functional properties of tissue mast cells. They share with mast cells high affinity IgE-receptors (Fc_cRI) and cytoplasmic granules containing histamine, but differ from mast cells in that they differentiate and mature in the bone marrow, circulate in the blood, and are not normally found in connective tissue. IL-3 is a differentiation factor for human basophils.[636] Basophils express a low-affinity receptors for IgG ($Fc_\gamma R_{II}$).
Human basophils generate LTC_4 but no prostaglandins and LTB_4.[312,562,668] Despite previous controversy about the synthesis of PAF by basophils, recent studies have demonstrated that these cells are capable of producing PAF and its analog, 1-acyl-2-acetyl-sn-glycero-3-phosphocholine (1-acyl PAF).[629] It has been shown that basophils produce IL-4 and TNF_α[598], but failed to produce IL-5. Whether basophils secrete other cytokines is unknown.[3] Tryptase is present in negligable quantities in basophils and does not exceed 1% of the level seen in mast cell granules.[94]

IL-3, IL-5, IL-11, GM-CSF and IFN-γ can prime basophils for enhanced histamine release in response to anti-IgE or C5a.[53,426,449,557,669] In addition to histamine, LTC$_4$ is released in response to triggers like C5a. Some of these priming cytokines also upregulate adhesion molecules on basophils.[360]

Rhinitis: The blood basophil count increases during the pollen season, suggesting that basophilopoiesis may be influenced by environmental factors, such as allergens[100,489], but this has not been confirmed by others.[164,165] Several studies have demonstrated that the amount of basophils in the mucus and in the nasal lavage fluid is increased 4-11 hours after allergen exposure, which can be interpreted as shift of cells to the superficial layers of the mucosa.[44,487]

It has been suggested that basophils play an important role in the late phase of the allergic process, based on their release of lipid mediators.[3] However, whether basophils are associated with hyperresponsiveness is not known.

Eosinophils

Eosinophils are bone marrow derived granulocytes, with a short life span. They are present in large numbers in the circulation and tissues. Although a precise model of human eosinophil proliferation and differentiation *in vivo* is not known, *in vitro* studies suggest that GM-CSF, IL-3 and IL-5 are important in the regulation of eosinophilopoiesis.[210]

They have low-affinity receptors for IgE (Fc$_\varepsilon$R$_{II}$).[216]

The eosinophil possesses the capacity to produce and release several biological substances. *In vitro* experiments have shown that eosinophil-derived enzymes are capable of degrading mast cell products, such as histamine and leukotrienes.[673,693] Eosinophils have cytoplasmic granules which contain cytotoxic proteins, like eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN).[267,644] MBP and ECP can stimulate upregulation of intercellular adhesion molecule-1 (ICAM-1) on human nasal epithelial cells, which suggest a positive feedback mechanism, in which the products released from migrating eosinophils might promote additional human nasal epithelial cell-leukocyte adherence.[9] Eosinophils also produce the superoxide (.O$_2^-$), the hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (.OH), which contribute to eosinophil-mediated cytotoxicity.[406]

Although eosinophils are able to function as phagocytes, they are less efficient than neutrophils both in the rate of phagocytosis and in the quantity of material ingested. Eosinophils can be activated by factors derived from T-lymphocytes, mast cells, vascular endothelium, and monocytes, such as GM-CSF, IL-1, IL-3, IL-5 and TNF.

The major product of the eosinophil is the arachidonic acid metabolite LTC$_4$.[676,677] Eosinophils also produce other products of arachidonic acid, like PGE$_2$ [82,186,231], TxB$_2$ [186], 15-HETE[633], but no PGD$_2$ or PGI$_2$ [186]. However, these cells release quantitatively only a fraction of the cyclooxygenase products released by human macrophages [186]. PAF is another product of the eosinophil.[376] The eosinophil releases cytokines, like GM-CSF, IL-3[344], IL-5[152], IL-6[270], IL-8[76] and TNF$_\alpha$[129]. Priming of eosinophils via exposure to chemotactic stimuli, like LTB$_4$, PAF, IL-3, IL-5 or GM-CSF increases the formation of LTC$_4$.[676]

The role of eosinophilic inflammation in allergy has been studied most thoroughly in the pathogenesis of the airway inflammatory response in asthma. Cytotoxic proteins are

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elevated in sputum and bronchoalveolar lavage fluid of asthmatics, which are cytotoxic towards respiratory epithelium and causes histamine release.[204,205,235,366,481,665] Alveolar macrophages may be primed by EPO for more effective killing of micro-organisms.[533] There is evidence for eosinophil participation in the induction of airway hyperrreactivity in asthma.[268]

Rhinitis: A relationship between the influx of eosinophils into the nasal mucosa and allergic rhinitis was noted[169] and during asymptomatic periods, the eosinophils were absent from the nasal secretions.[396] The mechanisms underlying the accumulation of eosinophils in rhinitis are poorly understood. There are numerous factors, like GM-CSF, PAF and lymphocyte chemotactic factor (LCF), which have been shown to be chemotactic for eosinophils, to prolong eosinophil-progenitor multiplication, maturation and differentiation.[149,535] The eosinophils are probably in part derived from progenitors at the site of inflammation, which are in turn derived from the bone marrow via the circulation. The role of the eosinophil in perennial rhinitis has been rather less intensively studied than in seasonal rhinitis. It has been shown that the number of eosinophils is increased in the biopsies and secretions compared with controls.[49] An eosinophil infiltration has been identified in nasal secretions as early as 30 min. after nasal antigen-challenge and has been shown to persist as long as 48 hours.[42,506] The eosinophil influx of the nasal response to allergen has been shown to occur independently of the symptoms and hyperresponsiveness of the late reaction.[348]

Neutrophils

Polymorphonuclear neutrophils are formed in the bone marrow and the predominant leukocytes in the circulation. The neutrophil is not normally found in tissues unless recruited from the circulation to sites of tissue inflammation. They are primarily responsible for maintaining normal host defence and have phagocytic and microbicidal activity in the tissue sites of inflammation.

Their cytoplasmic granules contain a potent array of digestive enzymes, which may either remove tissue debris or kill and degrade micro-organisms, and myeloperoxidase, and O_2^--radicals and related products which may disrupt the airway epithelium.[31,570,667,675] Activated neutrophils have been shown to induce epithelial cell damage.[581]

Peripheral blood neutrophils have cell surface receptors for the Fc portion of IgG ($Fc_\gamma R$), but appear to lack Fc receptors for IgE, IgD and IgM. Neutrophils are able to produce metabolites of arachidonic acid, such as LTB_4 which is the major metabolite, LTC_4, 5-HETE, PGE_2 and TxA_2 [160,532,646] and also PAF[404]. Neutrophils produce cytokines, like IL-1, TNF-α, IL-8 and GM-CSF.[29,93] They are also capable of producing nitric oxide.[182,527]

One of the earliest events in acute inflammation is increased adherence of circulating neutrophils to vascular endothelium.[289] In response to bacterial lipopolysaccharides and cytokines, such as IL-1, TNF and IFN-γ, endothelial cells become adhesive for neutrophils.[274] There are a large number of chemotactic factors that can recruit neutrophils to sites of tissue inflammation. The best-characterized of these are f-Met-Leu-Phe (FMLP), LTB_4 and C5a. Cellular sources of factors chemotactic for neutrophils include bacteria, macrophages, lymphocytes, platelets and mast cells.

Rhinitis: Due to their ability to produce these inflammatory mediators, neutrophils could play an important role in allergic rhinitis, although the role of neutrophils is still unclear.[7] An increased influx of neutrophils is measured in nasal lavages of rhinitis patients after exposure to ozone.[255]

Monocytes/macrophages

In the bone marrow, over a period of approximately 6 days, a progenitor cell termed "colony forming unit granulocyte macrophage" (CFU-GM) differentiate under the influence of locally produced colony-stimulating factors (CSF) into a monoblast. The monoblast differentiates into a promonocyte and, subsequently, is released into the circulation as a monocyte, which are large cells with an oval, folded nucleus and containing fine azurophilic granules. Human monocytes, circulating in the bloodstream, have a half-life of about 1-3 days. The tissue macrophages arises either by immigration of monocytes from the blood (probably the predominant mechanism) or by proliferation of precursors in local sites. The monocyte cell surface membrane has less immunoglobulin and complement receptors than the macrophage. During differentiation of monocytes to macrophages, the azurophilic peroxidase-containing cytoplasmic granules are lost and lysosymes containing hydrolytic enzymes become prominent. Although monocytes produce myeloperoxidase, macrophages do not.[249,378] Other products released by monocytes and macrophages and their role in rhinitis are already presented in chapter 2, paragraph: antigen presenting cells.

Lymphocytes

On the basis of expression of cell surface markers called clusters of differentiation (CD) and by their antigen receptors, 3 distinct lineages of lymphocytes have been identified: Thymus-derived lymphocytes (T-cells), bone-marrow-derived lymphocytes (B-cells) and natural-killer (NK)-cells. Moreover the presence or absence of certain cell surface markers has been used to delineate stages of differentiation, states of cellular activation and functionally distinct subsets of lymphocytes. T-cells maturation involves T-cell progenitors which arise in the bone marrow, but further differentiate and proliferate in the thymus to give mature T-cells. B-cells complete their differentiation in the bone marrow, with final maturation in the peripheral lymphoid tissue, such as the spleen and lymph nodes. After direct interaction with antigen, B-cells can differentiate into plasma cells, which can secrete large amount of all immunoglobulin subclasses, including IgE. After the same exposure to antigen, some B-cells can differentiate to memory B-cells which are responsible for the rapid recall response observed after re-exposure to antigens previously recognized by the immune system. In addition to producing immunoglobulin, B-cells can secrete certain mediators, so called lymphokines, such as IL-6 that affect the growth and differentiation of B-cells and other lymphocytes.

Lymphocytes possess low-affinity IgE ($Fc_\varepsilon RII$) receptors.[89]

APC's present the processed antigen to the helper/inducer T-cells (T_H), expressing the surface protein CD4. The T-cell receptor complex on the cell surface of the T_H-cell binds

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to the peptide/MHC on the APC. This interaction generates an activation signal for the T-cells leading to differentiation and proliferation with the formation of T-lymphoblasts and the secretion of soluble mediators, such as IL-4 and IL-5 which augment to help B-cells to respond and regulate the IgE production.[606]
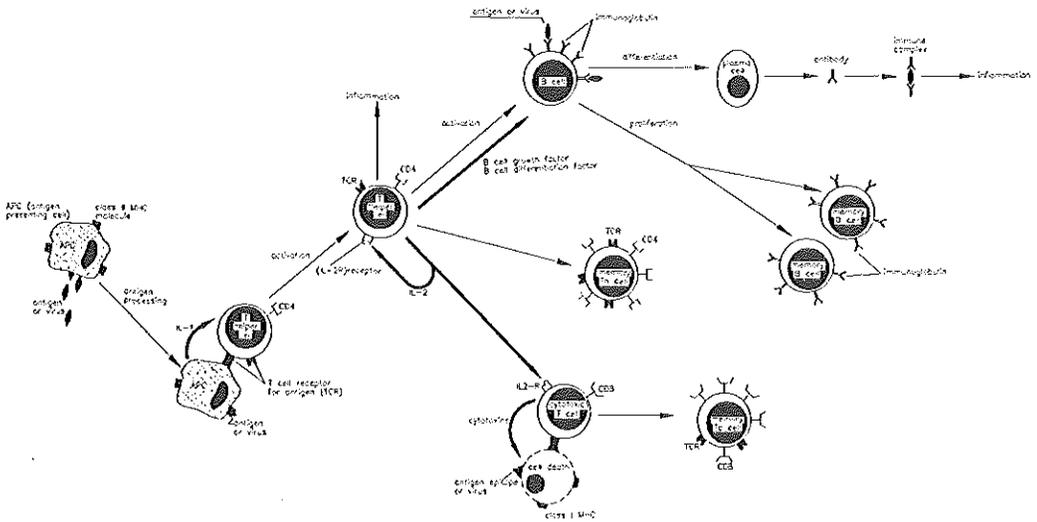
Figure 1: Response of the immune-system after antigen triggering

Two functional subclasses of murine T-helper clones have been described and are commonly designated T_{H1} and T_{H2}.[452] The murine T_{H1}-lymphocytes produce dominantly IL-2, IFN-γ and TNF-α, and they are thought to be involved in delayed-type hypersensitivity reactions and in the synthesis of IgM and some IgG subclasses. The murine T_{H2}-lymphocytes, on the other hand, have been shown to synthesize IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, and also TNF-α and are thought to be important in allergic-type inflammatory reactions and defense against parasites.[146,453] In humans, atopic allergic disorders seem to be related with the activation of T-helper lymphocytes with a type 2 cytokine secretion profile, including high levels of IL-4 ,IL-5, IL-6 , TNF-α and GM-CSF, minimal IFN-γ and little IL-2, although this profile is not as pronounced as in mice.[321,339,684] Non-atopic T lymphocytes resembled murine T_{H1}-cells by secreting IFN-γ, IL-2, IL-6, TNF-α and GM-CSF, little IL-5 and no IL-4. The atopics' T_{H2}-cells were excellent helper cells for IgE induction and the non-atopic T_{H1}-cells were cytolytic active towards autologous antigen presenting cells.[684]

Cytotoxic/suppressor T-cells ($T_{c/s}$), expressing the surface protein CD8, have the ability to kill other cells that are perceived as foreign, for example virus-infected cells. These $T_{c/s}$ cells recognize peptide antigens bound to class I MHC molecules on the cell surface of the target cell, whereafter the target cell is destroyed by the $T_{c/s}$-cell.

NK-cells are a subset of lymphocytes that arise from a precursor cell in the bone marrow. Mature NK-cells are present in blood, bone marrow and spleen, but are infrequent in the

lymph nodes or thymus. The exact development relationship of NK-cells to T- and B-lymphocytes is unknown. Most mature NK-cells are large granular lymphocytes. NK-cells were originally defined by their ability to kill certain tumour cells, but the most important role of NK-cells is probably in defense against viral infection, by killing virus-infected host cells. NK-cells mediate a variety of immune factors other than cytotoxity. After appropriate stimulation (e.g. interaction with certain tumors, lymphokines, immunoglobulin complexes) NK-cells secrete several cytokines, including interferon-gamma (IFN-γ), Tumour necrosis factor (TNF) and GM-CSF.

A few studies have shown that T-cell subsets changes in bronchoalveolar fluid and peripheral blood from asthmatic patients.[224,245,436] The production of IFN-γ, IL-4 and IL-5 is enhanced in asthmatics, showing an increased activity of T_H-cells.[201,399] IL-3, IL-5 and GM-CSF promote the production of eosinophils by the bone marrow and modulate mast cell differentiation.[285]

Rhinitis: It was recently demonstrated in biopsies from allergic patients and nonallergic controls that there were no differences between the number of T-helper cells and cytotoxic T-cells in the epithelium, but a higher number of activated T-cells expressing CD4 was found in the allergic group in the lamina propria.[292] Following a local allergen challenge of the nose, an increased number of CD4+ T-helper cells were found in the nasal submucosa.[319] Thus, it seems that allergen could promote T-cell activation and proliferation.

Platelets

Platelets arise in the bone marrow from megakaryocyte cytoplasm, which lack a nucleus, and circulate within the intravascular space during their 8-10-days life span. They have cytoplasmic granules which contain proteolytic enzymes and cationic substances.

Platelet activation is characterized by aggregation, by production of arachidonic acid metabolites, like TxA_2, PGD_2 and 12-HETE (a chemoattractant for neutrophils), and PAF, and by secretion of the contents from the granules, such as adenosine diphosphate (ADP), serotonin, platelet factor 4 (a chemoattractant for neutrophils, monocytes and eosinophils), ß-thromboglobulin and fibrinogen and lysosomal enzymes, such as ß-glucuronidase and acid phosphatase.[14,50,458,482,635] Platelets also have a membrane-bound enzymatic system able to generate and release hydrogen peroxide H_2O_2 after membrane stimulation.[179,456] Platelets produce Nitric Oxide (NO) by a calcium- and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent synthase.[182]

The role of platelets in inflammatory reactions is not as well defined as that of the neutrophil, eosinophil, macrophage or mast cell. However, activated platelets can release clotting and growth factors, amines and lipids with vasoactivity as well as neutral and acid hydrolases. Platelets are able to interact with the immune system through platelet cell surface $Fc_\gamma R$ and $Fc_\epsilon RII$.[284,329,450]

An increased number of platelets have been observed at the sites of the reaction in asthma after allergen-challenge.[46] Cooperation of platelets with basophils and/or mast cells was reported in the release of histamine during antigen challenge of asthmatics, which resulted in a potentiated sixfold increase of histamine release.[351]

Rhinitis: A significant increase in platelet volume and a shorter life-span (2-3 days) of

platelets was noticed in patients with allergic rhinitis compared with controls.[22] A potential role of platelet-release compounds in the development of delayed responses in allergic patients has been proposed.[147] These findings suggest that platelets participate in the pathogenesis in the allergic disease.

2.3 Products of allergic inflammation

The role of each product itself is complex and their interactions are even more complex. The most important features of the products relevant for rhinitis are reviewed in the following paragraphs.

Inflammatory products may have a large spectrum of effects on a variety of target cells in the airways, which are relevant in rhinitis. Some of them directly lead to contraction of smooth muscle or enhance muscle tone and indirectly, via secondary mediators or neuronal substances. They may also lead to oedema of the airways and exudation of plasma into the lumen. These inflammatory products can attract and activate inflammatory cells which thereafter can release mediators itself, consequently leading to ongoing inflammation.

Histamine

Histamine is formed by decarboxylation of the amino acid histidine by the pyridoxal-phosphate-dependent enzyme l-histidine decarboxylase. Human stores of histamine are predominantly contained within the cytoplasmic granules of basophils and mast cells.[539] Histamine may be released from mast cells[316] and basophils[134] by a number of immunologic, such as IgE, antigen and cytokines, and nonimmunologic, such as anaphylatoxins, peptides, e.g. substance P, drugs, e.g. opiates, and physical stimuli. After release from the storage granules, histamine rapidly diffuses into the surrounding tissues, and changes in blood concentration may be detected within minutes.[338]

Released histamine interacts with specific receptors on target cells. To date, three subtypes of histamine receptors have been characterized: H_1, H_2 and H_3 receptors. The first physiologic action of histamine to be described was smooth muscle contraction.[142] *In vitro* blockade of smooth muscle contraction by histamine H_1 receptor antagonists has clearly demonstrated that this effect is mediated predominantly via the H_1 receptor subtype.[20] In human airways smooth muscle contraction in response to histamine causes bronchoconstriction.[136,286] Histamine increases vascular permeability to macromolecules by the formation of intercellular gaps in the postcapillary venules.[47] In the nasal airways, the effect of histamine on vascular permeability is not clear. Changes are usually quantified by measurements of nasal airflow, which reflect mucosal oedema resulting from vascular leakage. Pretreatment with an oral or nasal H_1 receptor antagonist has been shown to attenuate the histamine-induced increase in nasal airway resistance (NAR), whereas H_2 receptor antagonists alone have no significant treatment effect.[277,568] Despite the ineffectiveness of pure H_2 receptor antagonism, when administered in combination with H_1 receptor antagonists a significant reduction in histamine-induced nasal airflow obstruction is seen. This supports a minor role for H_2 receptors, with most of the vascular permeability changes being mediated via the H_1 receptor. On human vessels histamine has a dual effect

with constriction mediated by H_1 receptors and vasodilatation via H_2 receptors.[63] Histamine effects both the quantity and viscosity of mucus secretion, mediated via H_2 [60,576] and H_1 [417] receptors, respectively. The H_1 receptor subtype is involved in the histamine-induced increase in action potentials in nerves.[550] The most important action of H_2 receptor antagonists is to reduce the secretion of acid in the stomach and relaxation of blood vessels. H_2-antihistamines enhance histamine release from human basophil leukocytes.[388,389,632] The H_2 receptors mediate the feed-back inhibition of histamine release from human basophils.[614] Histamine modulates the response of a number of subtypes of leukocytes. The chemotactic activity of eosinophils[111] and neutrophils[572] may be increased by histamine and the antigen-induced histamine release from basophils is controlled[632]. Histamine also modulates immunoglobulin synthesis, which includes interference with the maturation of antigen-stimulated B-cells[171], suppressing antibody secretion from plasma cells[431], and modulating immunoglobulin production of mature mononuclear cells[392]. The H_3 receptor has been identified using the H_3 receptor agonist R(-)-α-methylhistamine and the antagonist thioperamide, but the role in airways remains to be established.[18] H_3 receptors regulate the histamine synthesis and release from histaminergic nerve endings.[17,18,315]

Rhinitis: Nasal challenge of rhinitis patients with histamine induced nasal blockage, measured by NAR and is accompanied by dose-dependent sneezing and rhinorrhoea.[158,410] A greater change in NAR is found in rhinitis patients compared with controls, suggesting nonspecific hyperreactivity of the upper airways[125], which is in contrast with other investigations, in which an equal effect of histamine provocation on NAR in patients and controls was found.[225]

Thus, histamine derived from mast cells and acting via H_1 and H_2 receptors is responsible for much of the sneezing, nasal blockage and rhinorrhoea during the early response to nasal allergen challenge. Increased concentrations of histamine are found in nasal washings of rhinitis patients immediately after allergen-provocation.[230] Also during the late phase response histamine, released from basophils is found in increased concentrations in nasal washings.[44]

Tryptase

Proteases may be subdivided into those which cleave peptide bonds distant from the end of polypeptide chains (endopeptidases) and those which cleave only those bonds near to the end (exopeptidases).[40] Four groups of endopeptidases have been differentiated according to the nature of their catalytic site: serine, cysteine, aspartic and metalloproteases. The serine proteases are named after a serine residue in the catalytic site, and includes neutrophil elastase, mast cell tryptase and chymase. These enzymes are "neutral proteases", with pH optimal well suited to the neutral conditions of the extracellular environment of the respiratory tract.

Proteases are the major constituents of the mast cell secretory granule on a weight basis. To date three proteases have been isolated from human mast cells, tryptase[566,591], chymase[555,556], and carboxypeptidase[243]. Of these, tryptase is the most abundant with an estimated 10 pg per lung mast cell.[565] Tryptase appears to be present only in mast cells. Subsequent studies have demonstrated that there are major compositional differences

between mast cells from different species, and also between different tissues of the same species, such that proteases have become valuable markers of mast cell heterogeneity.

Tryptase has been found to cleave vasoactive intestinal peptide (VIP)[96,608], peptide histidine-methionine (PHM)[608], and calcitonin gene-related peptide (CGRP)[608,657], but does not hydrolyze substance P, neurokinin A or neurokinin B[96,608].

Dog mast cell tryptase has been reported to increase the contractile response of canine bronchial smooth muscle strips to histamine and other agonists. This effect appeared to be dependent on the enzymatic activity and was prevented by H_1 receptor antagonists and voltage dependent Ca^{2+} channel blockers. This observation has not been confirmed with human tissues, but raises the possibility that tryptase could contribute to bronchial hyperreactivity.[656] Tryptase has been found to increase vascular permeability, when injected into guinea-pig skin and stimulate neutrophil accumulation.[656,658]

Rhinitis: In rhinitis, comparatively little attention has been paid to the contribution of proteases in disease pathogenesis. However, the recent development of sensitive procedures for the detection of proteases from mast cells, and the discovery of their potent biologic effects has provoked interest in the potential of these enzymes to act as major mediators of allergic disease.[97,561] The mast cell is an important source of proteases in rhinitis. Increased levels of proteases have been detected in the nasal secretions of rhinitis patients. Provocation of acute rhinitis with allergen or cold dry air is associated with the rapid release of mast cell tryptase as well as histamine and other mast-cell-derived mediators into nasal fluid.[95,332,524]

Tryptase may thus participate in many of the processes of rhinitis and deserves attention beyond its role as a marker for mast cell activation in the airways.

Eicosanoids

Eicosanoids are biologically active lipids formed out of arachidonic acid. The principal sources of arachidonic acid are membrane bound phospholipids, primarily phosphatidylcholine, phophatidylethanolamine, and phosphatidylinositol. Arachidonic acid is released from the cell via hydrolysis of the phospholipids by phospholipase A_2, phospholipase C, and/or diglyceride lipase.[143,150,151,337] Activation of phospholipase A_2 may involve elevated concentrations of intracellular calcium, phospholipase C may involve generation of inositol phosphate and diacylglycerol.[177,313,579]

Free arachidonic acid may be enzymatically oxygenated by two major pathways: cyclooxygenase and lipoxygenase. The prostaglandins and thromboxane are generated through the cyclooxygenase pathway and the leukotrienes are derived via the lipoxygenase pathway.

Cyclooxygenase metabolites

Arachidonic acid is oxygenated by catalyzation via cyclooxygenase (prostaglandin endoperoxide synthetase) to the endoperoxide PGG_2, which is subsequently reduced to PGH_2. PGH_2 is transformed to PGD_2, PGE_2 by specific isomerases or non-enzymatic degradation, $PGF_{2\alpha}$ by reductase, PGI_2 by prostacyclin synthase and TxA_2 by thromboxane synthase. PGI_2 and TxA_2 are unstable biological active mediators which are rapidly

hydrolysed to 6-keto-$PGF_{1\alpha}$ and TxB_2 respectively.[359,384,592]

Cyclooxygenase (COX) has two isoforms: COX_1 and COX_2.[154] COX_1 is constitutively expressed in the stomach, intestine, kidney and platelets, while COX_2 expression is inducible in e.g. monocytes, macrophages, fibroblasts by stimuli, such as growth factors, cytokines and endotoxin.[153,638] COX_1 is involved in prostaglandin synthesis in cellular "housekeeping functions", while COX_2 is involved in inflammatory processes.[153]

Prostanoid receptors have been classified into five types, based on potencies of natural agonists and on identification of ligand-binding sites.[217,266] These receptor types are: TP, TxA_2/PGH_2; IP, PGI_2; EP_{1-3}, PGE_2, FP, $PGF_{2\alpha}$; and DP, PGD_2. These receptors differ greatly between species and within the same species, receptors may also vary between tissues.[266] All these receptors are G-protein coupled.

Almost all inflammatory cells are able to generate cyclooxygenase products. After stimulation with IgE, human mast cells produce PGD_2.[291,386,559] Human alveolar macrophages and airway epithelial cells generate PGE_2, $PGF_{2\alpha}$ and thromboxane.[242,472,677]

Cyclooxygenase products have effects on bronchial smooth muscle and vessels. $PGF_{2\alpha}$ and PGD_2 are potent bronchoconstrictors.[59,273,541,590] PGD_2 also has vasoactive properties, causing increase in pulmonary arterial pressure.[595] TxA_2 has bronchoconstrictor properties[538], stimulate airway smooth muscle cell proliferation[638], and causes vasoconstriction and platelet aggregation[553]. PGE_2 and PGI_2 are broncho and vasodilators.[307,359,595] However, inhaled PGI_2 may have bronchoconstrictor properties in some mild allergic asthmatics[272], this paradox has not been resolved. PGD_2, PGE_2 and PGI_2 inhibit platelet aggregation.[553] $PGF_{2\alpha}$, PGE_2 and PGI_2 are potent inducers of cough, perhaps through stimulation of irritant receptors and C-fibres.[117,130,540] PGE_2 inhibits phagocytosis, mediator function and cytotoxicity of macrophages, chemotaxis of macrophages and neutrophils and several lymphocyte functions.[610]

Increased concentrations of PGD_2 and TxA_2 were found in bronchoalveolar lavage fluid after antigen-induced bronchoconstriction in atopic asthmatics.[680] In addition to constrictor/dilator properties, prostanoids have also been demonstrated to induce airway hyperreactivity in asthma.[208,211,662]

Rhinitis: PGD_2 was released into nasal secretions during the immediate response to nasal challenge with pollen antigen, though not during the late phase response.[467,478] In another study with allergic rhinitis patients, increased concentrations of PGD_2 were reported to occur within minutes of and allergen-induced early nasal response.[223]

<u>Lipoxygenase metabolites</u>

In the presence of active 5-lipoxygenase unstable epoxide LTA_4 is formed via the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE).[551,552] LTA_4 is a intermediate in leukotriene synthesis for LTB_4 and the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4).[385,551] 12- or 15-lipoxygenase catalyzes the oxygenation of arachidonic acid to 12- or 15-HPETE, which is further converted into 12- and 15-HETE.[298]

In the guinea-pig lung distinct receptors mediating constriction for LTC_4 and LTD_4 were classified as LTR_c and LTR_d respectively.[318] In human bronchi such differentiation in LTC_4 and LTD_4 receptors appears to be absent.[84] LTD_4 receptors are found in human lung tissue, but the LTC_4 binding sites appear not to be receptors. LTC_4 binds to the ubiquitous human enzyme glutathione-S-transferase with characteristics that mimic receptor bindings.[159,494,602]

Thus, this suggest that there are specific receptors for LTD_4, but not for LTC_4 in human airways. LTE_4 appears to act as a partial agonist at the LTD_4 receptor, which can desensitize this receptor and displace radiolabeled LTD_4, but only can induce a fraction of the biological activity. The mechanism is unknown.[554] Specific binding sites for LTB_4 are identified on human neutrophils. Also these receptors are G-protein coupled.

Mainly, LTB_4 is produced by neutrophils, airway epithelial cells and alveolar macrophages, whereas the other leukotrienes and HETE's only are released in small quantities.[175,240,299,546] LTC_4, LTD_4 and LTE_4 are formed by eosinophils, basophils and mast cells.[499,559]

LTC_4, LTD_4 and LTE_4 have potent bronchoconstrictor properties, increase microvascular permeability in the airways and decrease blood pressure.[195,552] LTB_4 is a potent chemoattractant for neutrophils and monocytes, but is less effective for eosinophils.[193] LTB_4 also stimulates the release of lysosomal enzymes from macrophages and neutrophils[174], increases vascular permeability and releases oxygen radicals from neutrophils[573]. 5- and 15-HETE modestly contracted human bronchial muscle[124], and HETE's are chemotactic for neutrophils and eosinophils[239]. Neutrophils are degranulated by 5- and 12-HETE.[599]

Rhinitis: Increased concentrations of LTC_4, LTD_4 and LTE_4 were found in nasal lavages of rhinitis patients during allergen-induced early nasal response.[223]

As a consequence of their activity, the eicosanoids have been implicated as potential candidates in the pathogenesis of rhinitis.

Platelet activating factor

PAF was discovered during experiments designed to study cell-cell interactions following antigen challenge of rabbit leukocytes in the presence of IgE-sensitized basophils[51], capable of activating platelets. PAF is identified as a phospholipase-A_2-(PLA_2)-sensitive phospholipid and later as a 1-alkyl-2(R)-acetyl-sn-glycero-3-phosphorylchloride.[238] PAF can be synthesized by two distinct pathways. The first is a two-step pathway which involves the production of the biologically inactive intermediate lyso-PAF by phospholipase A_2. Lyso-PAF is converted into PAF by acetyl coenzyme A-dependent acetyltransferase enzyme. This pathway is only activated in response to inflammatory stimuli.[593] The second pathway involves cholinephosphotransferase which produces PAF indirectly from ether-linked phospholipides.[593] This pathway may be required to maintain physiological levels of PAF for normal cell function.[593]

There are specific G-protein linked PAF receptors on inflammatory cells, which activate PLA_2 and PLC.

Basophils, mast cells, neutrophils, eosinophils, monocytes and macrophages can synthesize PAF when stimulated with calcium ionophore or IgE.[75] PAF is released by macrophages from allergic patients stimulated by allergen.[16]

PAF is a potent *in vitro* activator of eosinophil, platelet, neutrophil, monocyte and macrophage chemotaxis and superoxide-anion production, and activator of the release of arachidonic acid metabolites, such as LTC_4, by neutrophils, eosinophils and macrophages.[51,75,81,194,275,283,380,483,639,666,691] PAF has been shown to be a potent mucus secretagogue for human airways *in vitro*[247] and stimulates the secretion of chloride ions and, thus, allows the movement of water toward the lumen.[622,678] Basophils are activated by

PAF and, thereafter, releases histamine and LTC_4 by a rise in calcium influx.[118] Intravenous injections of PAF to guinea-pigs leads to a bronchoconstriction and hypotension[640] as well as bronchial hyperreactivity to serotonin[641], histamine or acetylcholine[132,429]. In humans PAF induces bronchial hyperreactivity to methacholine in non-asthmatics.[104,105,138,547,664] This is in contrast with other investigators, who found that PAF failed to induce hyperreactivity to methacholine in normal subjects[103,363,596] and asthmatic patients[221,523].

Rhinitis: Lyso-PAF-acether, but almost no PAF was significantly increased in nasal secretions from allergic patients in the immediate reaction to antigen challenge.[438,626] Topical pretreatment with PAF of seasonal allergic rhinitis patients induced only minor changes in nasal respiratory peak flow rate and symptom score as compared with placebo. However, it induced an increase in responsiveness of the nasal vasculature to allergen challenge, measured as increased symptoms and nasal peak flow, but other parameters, such as sneezes and secretion remained identical.[13] Nasal challenge with PAF induced nasal obstruction, rhinorrhoea and itching in allergic rhinitis patients, but no increase in histamine levels were observed in nasal lavages. No changes were seen after challenge with lyso-PAF.[377] Topical nasal application of PAF induced an increase in eosinophils in the nasal lavage fluid and brushes of allergic rhinitis patients, but did not produce any changes in methacholine-induced secretory responsiveness.[345]

Thus, PAF may have pathogenetic and clinical relevance in allergic rhinitis.

Eosinophil-derived granule proteins

Activation of granulocytes, including eosinophils, can result in the release of granule contents, providing the cells with a very potent mechanism of inflammatory action. Specific eosinophil granule proteins include MBP, EPO, ECP and EDN. Degranulation of these cationic proteins has been correlated to several of the symptoms of asthma and rhinitis and hyperresponsiveness. MBP is toxic to many mammalian cells, such as human lung epithelium[203], and induces mast cell and basophil histamine release.[481] The EPO can stimulate mast cell secretion[281], inactivate mediators of immediate hypersensitivity[282], and is cytotoxic to various target cells[280]. ECP can inhibit T-lymphocyte proliferation in a non cytotoxic fashion, but the mechanisms involved are unclear.[508]

There appear to be two mechanisms of eosinophil granule release: 1) Selective release. Although residing in the same intracellular granule, eosinophils can selectively release specific proteins, while retaining others. After anti-IgE stimulation, eosinophils released EPO, but no ECP. IgG-dependent stimulation causes the opposite, while IgE-dependent stimulation releases both EPO and ECP.[90] Therefore, granular constituents can be solubilized within the eosinophil granule and then transported through the cell cytoplasm as molecules or, via the endoplasmic reticulum through the cell membrane.[597] 2) Exocytosis. In contrast, eosinophils stimulated with calcium ionophore result in the fusion of granules with each other and with the plasma membrane, suggesting the presence of a secondary mechanism of granule release, indicated as exocytosis.[279]

Eosinophil cationic protein

ECP is localized in the granules of eosinophils and has a molecular mass of 18-21 kDa.[236] Motojima *et al.* found that ECP caused dose-dependent damage to guinea-pig tracheal epithelium *in vitro*.[455] However, ECP had no effect on bronchoconstrictor or airway hyperresponsiveness of cynomolgus monkeys.[262]

Increased serum levels of ECP occur in allergen-provoked asthma.[140,643] Elevated levels of ECP have been found in bronchoalveolar lavage fluid of asthmatics obtained during the late phase reaction after allergen-inhalation challenge of asthmatics, as well as in unchallenged patients with chronic asthma.[445]

Rhinitis: In both allergic and nonallergic rhinitis increased serum levels of ECP are observed.[644] Lavage fluid from allergic rhinitis patients showed marked elevations of ECP after segmental bronchial antigen challenge.[569] An increased number of eosinophils and raised levels of ECP were found on the nasal mucosal surface during natural allergic rhinitis patients.[603] These changes were not accompanied by an increased secretory responsiveness of the nasal mucosa to methacholine.[348] In the lavage fluid of the patients with a late phase reaction, a significant eosinophilia was found, compared with controls and those patients who only demonstrated early responses. This suggested that eosinophils and their mediators might be involved in the development of the late phase reaction.

This suggests that the eosinophil influx and levels of ECP and the hyperresponsiveness following an allergen challenge are not necessarily integrally linked, but are possible simultaneous expressions of coincedental processes.

Cytokines

Cytokines function as up- and down-regulators of immunologic, inflammatory and reparative host responses to injury. Cytokines produced by lymphocytes are called lymphokines, whereas peptides produced by monocytes or macrophages are called monokines. Cytokines generally are synthesized and secreted peptides which are often glycolysed with molecular weights ranging from 6000 to 60000 Da. Cytokines are transmembrane glycoproteins with an extracellular amino-terminal ligand binding domain, a short hydrophobic transmembrane region and a carboxyl-terminal intracellular domain.[493]

Cytokines generally act in a paracrine (i.e. locally near the producing cell) or autocrine (i.e. directly on the producing cell) rather than in an endocrine manner on distant target cells. Cytokines modulate reactions of the host to foreign antigens or injurious agents by regulating the growth, mobility and differentiation of leukocytes and other cells. Normal resting cells must be stimulated to produce cytokines, and therefore usually no cytokines are normally present in serum. Many cytokines are simultaneously produced by activated cells. These cytokines interact with high affinity cell surface receptors specific for each cytokine. A single purified cytokine can have multiple effects on the growth and differentiation of many cell types. Consequently, cytokines may exhibit considerable overlap in their biologic effects on target cells. In addition, biologically distinct cytokines may have similar effects by initiating the production of a cascade of identical cytokines or of one another.

Some cytokines have direct histamine-releasing properties, such as IL-3, GM-CSF and IL-1 from basophils and mast cells.[263,408] Cytokines can prime basophils for enhanced histamine release in response to other secretagogues, such as anti-IgE and FMLP. This priming effect has been documented for IL-1-3[426,449,669], IL-5[53], IL-11, GM-CSF[54] and IFN-γ[557]. Some of these priming cytokines, such as IL-5, also upregulate adhesion molecules in nasal mucosa, including E selectin, P selectin, ICAM-1, ICAM-2 and VCAM-1.[360,520,616] The inducible expression of these molecules on endothelium directs the focal adherence of leukocytes to endothelium for extravasation at sites of inflammation.

Several investigators have suggested that the cytokines, IL-2, IL-3, IL-4 IL-5 and GM-CSF may be contributing to the occurrence of degranulation of cells in bronchial mucosa of asthmatics.[269,271]

Rhinitis: Durham *et al.*[163] showed with *in situ* hybridization messenger ribonuclear acid (mRNA) for IL-3, IL-4, IL-5 and GM-CSF in nasal biopsies 24 hours after allergen challenge, which is correlated with the number of activated T-cell and eosinophils. In addition to the work in nasal mucosal tissue, attempts have been made to quantitate cytokines in nasal secretions following antigen challenge.[4,27,202] In general, little success has been reported in nasal lavages, with some cytokines such as IL-1ß, IL-2 and IL-6 being detectable in higher levels than prechallenge fluids only in a subset of allergic subjects.[27] Increased levels of IL-1ß and GM-CSF have been detected by using strips of filter paper to collect secretions from the nose.[4]

Of these cytokines, IL-5 is highly important, because IL-5 alone is capable of inducing eosinophil degranulation in the absence of a ligand and greatly enhancing ligand-stimulated eosinophil degradation.[209]

Interleukin-5

IL-5 has a molecular weight of 18 kDa. It was initially detected and its gene was later cloned on the basis of its T-cell-replacing activity and B-cell-growth activity for the BCl-1 lymphoma line. Today, it is known that IL-5 is produced by mast cells[519], eosinophils[152] and mainly T_{H2}-cells[453].

IL-5 promotes the proliferation and differentiation of B-cells and promotes the antibody production by B-cells, particularly of the IgA isotype. IL-5 has modest mitogenic effects on T-cells. In addition, it induces the differentiation of bone marrow precursors into eosinophils and support the growth of eosinophilic cell lines and induction of cytotoxic T-cells. IL-5 enhances eosinophil development and differentiation[113,594] and prolong survival of eosinophils[545]. IL-5 can alter functional and immunologic properties of eosinophils. Data from patients with eosinophil-related disorders suggest that IL-5 produces "activated" eosinophils.[234] It has been observed that IL-5 increases eosinophil, but not neutrophil, adherence to vascular endothelium[659] and IL-5 is chemotactic for eosinophils[663]. Eosinophils can be primed by IL-5 for chemotaxis towards PAF.[670]

Thus, although the T-lymphocyte is considered to be a major source of IL-5, eosinophils contribute to the production of IL-5 in allergic airway inflammation.[58] This raises the possibility of an autocrine mechanism whereby stimulated eosinophils may both release and respond to cytokines, such as IL-5. Thus, there is the potential for a self-perpetuating cycle, with continuous eosinophil infiltration and activation and consequently chronic inflammation.

In humans, elevated serum IL-5 was noted in symptomatic asthmatics in association with activated T-lymphocytes and eosinophilia.[127]

Rhinitis: In allergic rhinitis patients, IL-5 levels were elevated 48 hours after antigen challenge and found to correlate strongly with eosinophil number, eosinophil granule proteins and LTC_4 levels.[569] Application of recombinant human IL-5 onto the nasal mucosa of patients allergic to pollen increased the numbers of eosinophils and epithelial cells and ECP and IgA in the nasal lavage fluid. Also the number of eosinophils in both the epithelium and lamina propria as well as in the lumens of the blood vessels in the nasal mucosa were increased. The responsiveness to histamine also enhanced after the application.[617,618]

Nitric oxide

Nitric oxide (NO) is generated from oxygen and L-arginine by nitric oxide synthase (NOS) in a complex nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction.[457,500] The NO is freely diffusible, highly reactive, and short-lived. In part of its reactivity, the actual molecules emanating from cells may include a complex mixture of nitrogen oxides, nitrite (NO_2^-), nitrate (NO_3^-), and the products of nitrosation of a variety of molecules. The interaction of NO with superoxide anion leads to the generation of hydroxyl radical, as well as peroxynitrite ($ONOO^-$).[526] In the presence of superoxide dismutase, the result of this interaction may be nitroxyl anion (NO^-).[457] Peroxynitrite and nitroxyl anion are very potently cytotoxic.

NOS present in various cells from different origin exists in two forms.[444] The first is a constitutive, cytosolic calcium dependent enzyme (cNOS), which releases nitric oxide after stimulation; this has been found in the vascular endothelium, platelets, neutrophils and neural tissue. The second subtype is an inducible, calcium independent enzyme (iNOS), which is found after induction with cytokines, in macrophages, vascular smooth muscle cells, cardiac myocytes, endothelial cells, fibroblasts, epithelial cells and adenocarcinoma cells.[326,328,443,444]

NO is produced by macrophages, neutrophils, mast cells and platelets.[57,527] NO has been identified as endothelium-derived relaxation factor (EDRF).[442] NO generated by intact endothelium not only induces smooth-muscle relaxation, but also appears to serve to inhibit further adhesion and aggregation of normal platelets, which suggest protective effects against inflammation.[8,427,645] NO has the ability to suppress leukocyte adherence and T-lymphocyte proliferation and to regulate the mitogen responses.[206] NO can modulate the release of histamine form mast cells.[421]

NO has been shown to be a potent bronchodilator in isolated guinea-pig trachea smooth muscle and in humans.[48,631] Probably, NO mediates airway smooth muscle relaxation by inhibiting the release of acetylcholine from the nerve terminal.[36] NO also leads to the production of cAMP.[259] The products of NO are extremely cytotoxic. Because epithelial damage is related to the development of bronchial hyperreactivity[365], NO may be greatly responsible for hyperresponsiveness in asthmatics. This is supported by Golden, who found that inhalation of nitrogen dioxide and ozone increases bronchial reactivity in healthy humans[241] and by Barnes, who suggested that free oxygen radicals from inflammatory cells increases the breakdown of NO, thus leading to exaggeration of the cholinergic reflex

bronchoconstriction.[36]

Rhinitis: Inhalation of ozone of allergic rhinitis patients caused an increase in symptoms after allergen challenge. Also an increase in nasal lavage neutrophils, eosinophils, mononuclear cells and epithelial cells was observed. The histamine and albumin concentrations in lavage fluid increased on the ozone exposure day.[41]

These findings suggests that NO could be associated with rhinitis and hyperreactivity.

2.4 Concluding remarks

Several inflammatory cells, such as mast cells, basophils, lymphocytes and eosinophils and their mediators released after specific or non-specific stimuli, have been demonstrated during the nasal allergic processes. Although some of these mediators, such as histamine, prostaglandins and leukotrienes may be biological active in allergic rhinitis, the role of others, such as PAF, IL-5 and nitric oxide still needs clarification. The interaction between these different cellular components to induce the clinical symptoms of allergic rhinitis remains unclear. Also the relationship between the inflammatory infiltration, cellular activation and hyperreactivity needs further establishment. We have investigated some of these cells and mediators in nasal lavages and brushes of perennial allergic rhinitis patients during allergen challenge or natural allergen-exposure. We have particularly investigated the role of tryptase, as a marker of activated mast cells, ECP, as a marker of activated eosinophils, and furthermore histamine, $LTC_4/D_4/E_4$, PGD_2, PAF, IL-5 and NO, which are involved in the immediate and late phase nasal reaction to allergen challenge, in hyperreactivity and in therapeutic intervention.

Chapter 3
Aim of the study

From chapter 1 and 2 it can be concluded that despite still poorly defined mechanisms, inflammation is clearly associated with the pathogenesis of allergic rhinitis. In order to investigate the association between nasal hyperreactivity, the late phase response and nasal inflammation in patients with perennial allergic rhinitis, several studies during allergen challenges or natural exposure, were performed.

Hereto, we tried to find an answer to the following questions:

1. Are nasal hyperreactivity and the immediate and late allergic reactions to allergen challenge associated?
2. Is nasal hyperreactivity related to the inflammatory response as measured by the release of inflammatory mediators in nasal lavage fluid after house dust mite challenge?
3. What are the effects of therapeutic interventions with a topical corticosteroid (fluticasone propionate) and a topical antihistamine (levocabastine) on nasal hyperreactivity, nasal inflammation, and the immediate and late response to nasal allergen challenge?
4. Is nasal hyperreactivity during natural allergen exposure associated with the ongoing allergic inflammation as determined by the amount of eosinophils (obtained by nasal brush) and the levels of inflammatory mediators in nasal lavage fluid?
5. Is nasal hyperreactivity associated with quality of life scores and with daily nasal symptoms?

PART TWO

Hyperreactivity and inflammation

Chapter 4
Nasal responsiveness to allergen and histamine are associated in patients with perennial allergic rhinitis

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4.1 Abstract

Background - In the lower airways an association has been found between early phase reaction (EAR), late-phase reaction (LAR) and bronchial hyperreactivity. However, this association could not be demonstrated for the upper airways, as shown in nasal pollen challenge studies. We have studied whether the EAR, LAR and nasal hyperreactivity are related in perennial allergic rhinitis.

Methods - Twenty-four rhinitis patients allergic to house dust mite (HDM) were challenged with HDM extract. Nasal response was monitored by symptom scores and nasal lavages for up to 9.5 hours after challenge. In lavage fluid albumin, tryptase and ECP were measured. Eleven patients (LAR+) demonstrated elevated symptom scores between 3.5 and 9.5 hours compared with the baseline score. The other 13 patients (LAR-) showed an isolated EAR only. Nasal hyperreactivity was determined by nasal histamine challenge 24 hours later.

Results - LAR+ responders showed a significantly higher symptom score, albumin influx and tryptase release during the EAR. During the late phase (3.5 -9.5 hrs) albumin influx was significantly increased at most time points and ECP release was significantly higher at 9.5 hours in the LAR+ group. LAR+ responders showed a significantly stronger response to all doses of histamine. The AUC of symptom scores during EAR, LAR and AUC of histamine dose-response were significantly correlated (EAR-LAR: r=0.49, p<0.01; EAR-histamine: r=0.75, p<0.001; LAR-histamine: r=0.66, p<0.001).

Conclusions - In patients with perennial allergic rhinitis the nasal responses to allergen and histamine are associated. LAR- and LAR+ responders show a different profile of mediator involvement.

Keywords: perennial allergic rhinitis; early allergic response; late allergic response; inflammation; nasal hyperreactivity

4.2 Introduction

In atopic patients, allergen challenge gives rise to increased responsiveness to allergen and non-specific stimuli. This phenomenon has been described for the upper as well as for the lower airways.[10,116,120,261]

In the lower airways, the immediate response to allergen challenge is often followed by a late-phase response (LAR). This bronchial late-phase reaction is associated with increased inflammation and bronchial hyperreactivity.[92,115,162,445] In the upper airways late-phase responses have also been described.[176,505] Several studies have been performed to find out whether similar associations could also be shown in allergic rhinitis patients. However, no relation was found between nasal hyperreactivity and late nasal response[230], between nasal hyperreactivity and activation of eosinophils[346] or between enhanced responsiveness to rechallenge with allergen and late nasal response[310]. These studies were performed in pollen sensitive patients tested outside the pollen season.

In contrast, in a study with rhinitis patients allergic to house dust mite (HDM), we have shown an association between nasal responsiveness to allergen and pre-existent nasal hyperreactivity[229], a finding more in agreement with data from the lower airways.

In the present study, patients with perennial rhinitis were challenged first with house dust mite and 24 hours later with histamine. Clinical response, mediators, and nasal hyperreactivity of patients with or without a LAR were compared in order to investigate if early (EAR), late-phase nasal reaction (LAR), inflammation and nasal hyperreactivity are associated in perennial allergic rhinitis.

4.3 Materials and methods

Subjects
Twenty-four patients participated in this study, 11 women and 13 men, mean age 34 yrs (range 21 to 50 yrs). All were characterised by a history of perennial rhinitis and a intradermal skin reaction of at least one plus-sign to 3 BU/ml HDM extract (ALK Benelux, Groningen, the Netherlands), according to the standardised plus-sign scoring system defined by Norman[477]. Patients with pollen allergy were tested outside the pollen season. Patients pet allergy were only included if they had no contact with pets.

Symptomatic medication for rhinitis were withdrawn: oral corticosteroids for two months, astemizole six weeks, nasal or inhaled corticosteroids, cromoglycate and nedocromil three weeks and antihistamines three days before the start of the study. Patients with nasal polyposis, nasal surgery less than three months before the study, nasal infection during the two weeks before the study or immunotherapy in the past were excluded.

Study design
Patients were challenged with three increasing doses HDM extract. Symptom scores were recorded and nasal lavage fluid was obtained for up to 9.5 hours after HDM challenge. Allergen-induced nasal hyperreactivity was determined by nasal histamine provocation 24 hours later.

During the study patients were not allowed any medication affecting nasal function. The study was performed during the period January-August. This period was chosen to

minimise natural exposure to HDM.

All patients gave their written informed consent and the study was approved by the Medical Ethics Committee of the University Hospital.

Nasal challenge with HDM and histamine

Challenges were performed in accordance with the methods described by Gerth van Wijk[229,230]. Before starting the nasal challenges, the patients waited for 30 minutes in order to give the nasal mucosa time to acclimatise.

Nasal challenges with HDM extract (100, 1000 and 10000 BU/ml, ALK Benelux, Groningen, the Netherlands) were performed at ten-minute intervals after challenge with phosphate-buffered saline (PBS), containing human serum albumin 0.03% and benzalkonium chloride 0.05% (ALK Benelux, Groningen, the Netherlands). The HDM extract was sprayed into each nostril by means of a nasal pump spray delivering a fixed dose of 0.125 ml solution. The nasal response was measured ten minutes after each challenge, 30 minutes after the last challenge (HDM 10000 BU/ml) and hourly for up to 9.5 hours after the last challenge. Nasal responsiveness was monitored by the number of sneezes, the amount of secretion collected according to Borum[68] and a symptom score according to Lebel[373].

Nasal challenge with histamine phosphate (0.25, 0.50, 1.0, 2.0 and 4 mg/ml) was performed at five-minute intervals after challenge with PBS. The amount of secretion, the number of sneezes and the symptom score according to Lebel[373] were used as nasal-response indicators.

Nasal lavage

Nasal lavages were performed as described by Naclerio[465]. This protocol comprises four pre-challenge lavages with isotonic saline solution to clear the nose from secretions and to obtain baseline levels of mediators. In order to prevent nasal congestion due to allergen challenge, oxymetazoline 0.1% (two 0.125 ml puffs) was applied in both nostrils. After five minutes the oxymetazoline was washed out just prior to the nasal challenge with PBS. A nasal lavage was performed ten minutes after PBS and after each allergen challenge, immediately before the subsequent challenge. Subsequently, lavage fluid was obtained hourly from 0.5 to 9.5 hours after the last allergen challenge. Nasal lavages were performed with 10 ml isotonic saline solution (0.9%) preheated to 37°C. In each nostril 5 ml saline was instilled with a pipette while the subject gently flexed his/her head backwards. After ten seconds, the lavage fluid was expelled and collected in tubes. This procedure has shown to produce a mean (SD) recovery of 7.7 (±1.2) ml.[229]

Symptom score

Symptoms were recorded using a scoring system according to Lebel *et al.*[373] at the time points of lavage. Symptom scores were graded in points (pts): 3-4 sneezes=1 pt, ≥5 sneezes=3 pts; anterior rhinorrhoea=1 pt; posterior rhinorrhoea=1 pt; difficult nasal breathing=1 pt, one nostril blocked=2 pts, both nostrils blocked=3 pts; pruritus of the nose=1 pt; pruritus of palate or ear=1 pt and conjunctivitis=1 pt (total score ranges from 0 till 11 pts). In addition, the number of sneezes and the amount of secretion were noted.

According to their symptom scores, patients were divided into two groups: those with an early response only (LAR-) and those with both early and late-phase symptoms (LAR+).[229]

Patients whose symptom scores were above baseline level at two consecutive time points (i.e. at least one hour) between 3.5 and 9.5 hr, were defined as LAR+ responders. The others with symptom scores equal or lower than the baseline score between 3.5 and 9.5 hours after challenge were assigned to the LAR- responder group. The LAR+ group included patients with either a dual response or prolonged/persistent response after challenge. The period from 3.5 to 9.5 hours post-challenge was chosen, since other investigators found a late-phase nasal response in the same period.

Mediator assays
Lavage fluid was stored on ice and centrifuged for ten minutes at 400 x g. The supernatant was stored at -20°C. Histamine was measured with an automated fluorometric assay[586]. Tryptase and ECP were determined by radioimmunoassay (RIA), according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden), albumin was determined by automatic kinetic nephelometry with use of the Array analyzer (Beckmann, Mijdrecht, the Netherlands) according to the manufacturer's instructions.

4.4 Statistical analysis

Patients were devided into the LAR- (n=13) or LAR+ group (n=11) according to their symptom scores. Between-group differences of clinical response, mediators in lavage fluid and histamine responsiveness were tested with the nonparametric Mann-Whitney U test. A two-tailed p-value < 0.05 was considered significant. The correlation of AUC of symptom scores during EAR (HDM 100, 1000, 10000 BU/ml), LAR (3.5 - 9.5 hrs) and AUC of histamine dose-response were tested with the Spearman correlation test.

4.5 Results

The clinical response to HDM challenge is shown in figure 1. LAR+ responders showed an immediate and late nasal response, while LAR- responders showed an immediate response only. The symptom score of the LAR+ group was significantly higher at most time points during both the immediate and late phases as compared with the LAR- group (fig. 1a). The number of sneezes was significantly higher in the LAR+ group at HDM 1000 BU/ml and at 3 time points during the late phase (fig 1b). LAR+ responders showed a significantly stronger secretory response at most time points during the late-phase response (fig. 1c).

Fig. 1a.
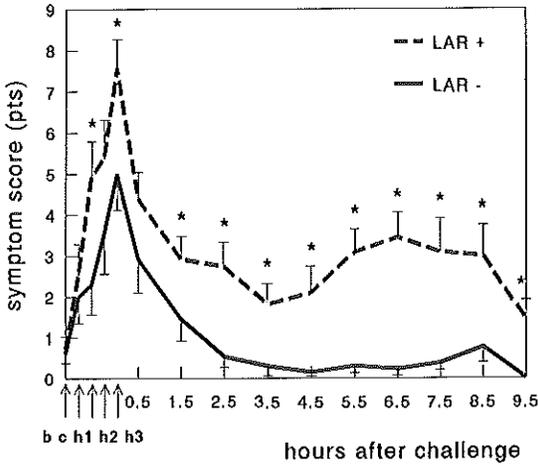
Fig. 1b.
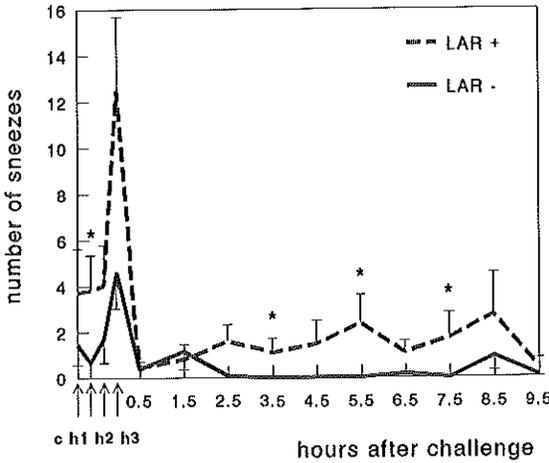
Fig. 1c.
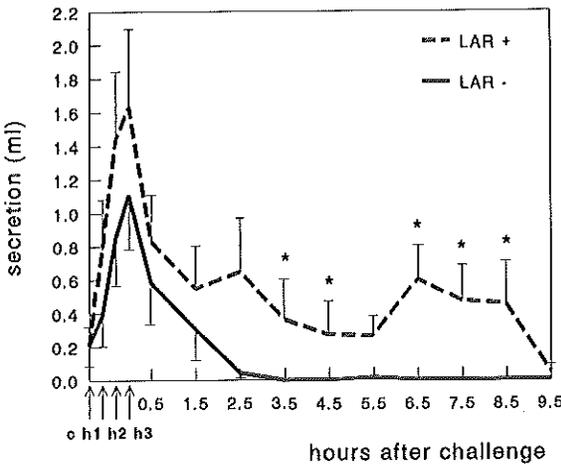
Figure 1.
Nasal clinical response of LAR+ and LAR- patients measured for up to 9.5 hours after HDM challenge. Abbreviations: b=before challenge, c=10 min after PBS, h1-h3=10 min after challenge with 100, 1000 and 10000 BU/ml HDM extract respectively. * = two-sided p-value≤0.05. Values are presented as mean ± standard error of the mean (SEM).

39

Fig. 2a.
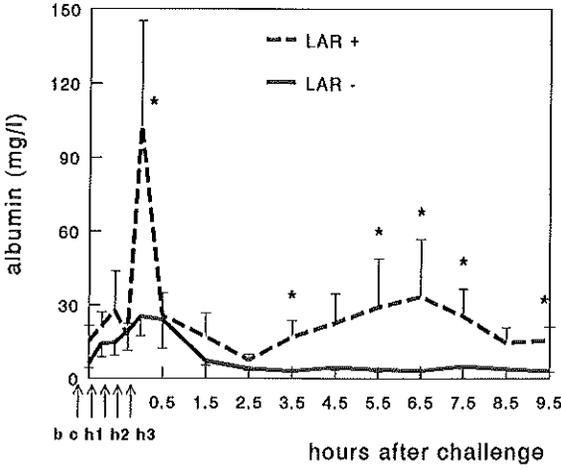
Fig. 2b.
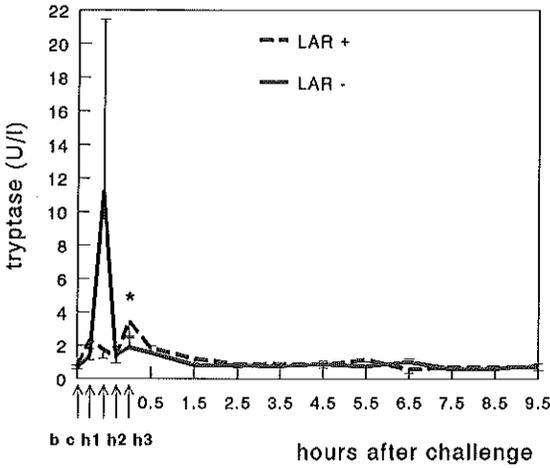
Fig. 2c.
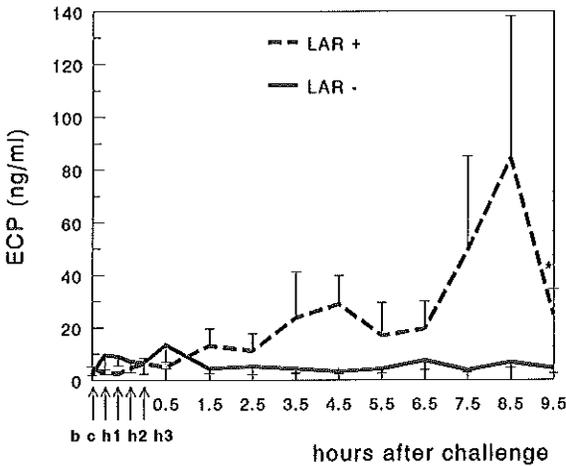
Figure 2.
Nasal response of LAR+ and LAR- patients measured for up to 9.5 hours after HDM challenge. Abbreviations: b=before challenge, c=10 min after PBS, h1-h3=10 min after challenge with 100, 1000 and 10000 BU/ml HDM extract respectively. * = two-sided p-value≤0.05. Values are presented as mean ± standard error of the mean (SEM).

In the analysis of nasal lavage fluid, albumin influx in the LAR+ group was significantly increased at HDM 10000 BU/ml and at most time points during the late phase as compared with the LAR- group (fig. 2a). ECP release during the late-phase response was significantly higher at 9.5 hour and tends to be higher at 4.5 (p=0.087) and 6.5 hour (p=0.099) in the LAR+ group (fig. 2b). Tryptase release was only observed immediately after HDM challenge. The high mean and standard error of the mean (SEM) at HDM 100 BU/ml are largely due to one patient. LAR+ responders showed a significantly higher release at HDM 10000 BU/ml (fig. 2c).

Allergen-induced nasal hyperreactivity was determined by nasal histamine challenge. Comparing the symptom scores, LAR+ responders showed an increased score at all histamine concentrations (fig. 3a). Although graphically the baseline symptom scores are different, statistically no significant difference was shown. When we expressed the response to histamine in percentage of the baseline, the LAR+ responders still did show significantly increased symptom scores to histamine at all doses. The numbers of sneezes of the LAR+ responders tended to be higher at 0.5 mg/ml (p=0.064) and 4 mg/ml histamine (p=0.09) (fig. 3b). The secretory response tended to be stronger at 0.25 mg/ml (p=0.079) and 1.0 mg/ml (p=0.079) and was significantly stronger at the two highest histamine concentrations (fig. 3c).

The symptom score and mediator release at HDM 10000 BU/ml, during 3.5-9.5 hours and nasal hyperreactivity (AUC of histamine dose response) are shown in table 1. LAR+ responders showed a significantly stronger response to HDM 10000 BU/ml (symptom score, tryptase release, albumin influx) and during the LAR (symptom score, albumin influx, ECP p=0.099). They also showed a significantly increased nasal hyperreactivity as compared with LAR- responders.

The AUC of symptom scores during EAR and LAR and the AUC of the histamine dose-response curves were significantly correlated (EAR-LAR: r=0.49, p<0.01; EAR-histamine: r=0.75, p<0.001; LAR-histamine: r=0.66, p<0.001).

Table 1. Profile of lar+ and lar- responders

	LAR+	LAR-	p-value
symptom score (10000 BU/mL)	7.50 ± 0.69	5.00 ± 0.026	0.026
albumin (10000 BU/mL)	102.72 ± 42.50	25.30 ± 7.89	0.022
tryptase (10000 BU/mL)	3.38 ± 0.59	1.90 ± 0.64	0.046
AUC symptom score (3.5-9.5 hr)	18.00 ± 2.50	2.20 ± 0.82	0.0001
AUC albumin (3.5-9.5 hr)	158.30 ± 68.80	27.67 ± 5.06	0.0056
AUC ECP (3.5-9.5 hr)	248.40 ± 135.90	35.03 ± 10.87	0.099
AUC histamine dose response	27.27 ± 2.49	13.31 ± 2.71	0.0006

Fig. 3a.
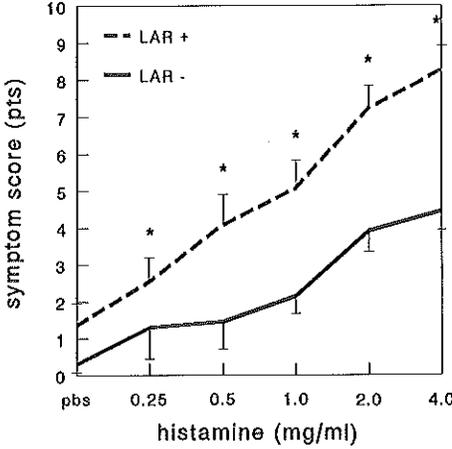
Figure 3. Histamine responsiveness
Nasal hyperreactivity in LAR+ and LAR-
patients. * = two-sided p-value≤0.05.
Values are presented as mean ± SEM.
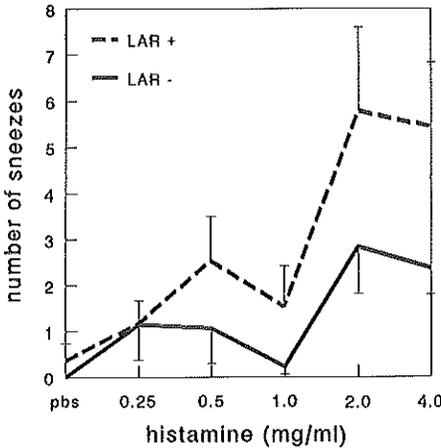
Fig. 3b.   Fig. 3c.
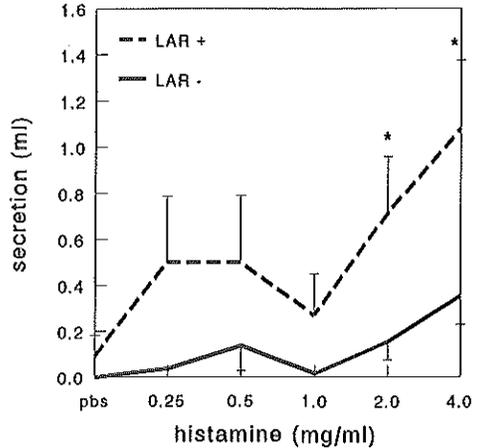
4.6 Discussion

Recent study of patients allergic to HDM revealed a relation between pre-existing hyperreactivity and LAR[229]. In view of this finding, we wanted to investigate the relation between EAR, LAR, inflammation and nasal hyperreactivity in patients allergic to HDM in more detail. Patients were challenged with high doses of HDM extract in order to obtain maximal nasal response.

To define the late-phase reaction in the nose is difficult. Mygind[461] could not detect late-phase reactions by means of symptom scores. In other studies late-phase reactions were determined by measurement of nasal obstruction and analysis of nasal lavage fluid.[176,467]

These studies concerned pollinosis patients. In the present study, patients were divided into LAR- or LAR+ responders according to their symptom scores during the late-phase period. The same approach has been used in other studies.[229,501]

When comparing the clinical response in both groups, apart from the difference in the late phase, the immediate response was significantly higher in the LAR+ group. The AUC of symptom scores during the EAR and LAR were significantly correlated, which implies that a strong immediate response is necessary to induce a late-phase response. Small[589] recently demonstrated in a pollen challenge study that the amounts of LTC_4 and PGD_2 during the EAR and LAR were correlated, also suggesting a relation between the two phases. However, nasal symptoms during the EAR and LAR were not correlated.

Apart from the clinical response, we also analyzed nasal lavage fluid for albumin, tryptase and ECP to monitor the nasal response. Albumin, being the major protein in plasma, can be used as a marker of increased vasopermeability.[45,604] Albumin influx showed a pattern comparable with the symptom score: a dual response in the LAR+ group and an immediate response only in the LAR- group.

In order to study the inflammatory response we measured tryptase and ECP in lavage fluid. Tryptase, a specific marker for mast cell activation[560], has proved to be a useful marker of the immediate nasal reaction[536]. The LAR+ responders showed an increased tryptase release as compared with the LAR- responders. The late-phase response in the upper and lower airways is associated with influx of eosinophils.[42,445] In the LAR+ group, ECP release, as a marker for activated eosinophils, could be detected during the late-phase. ECP release from 3.5-9.5 hours (AUC) tended to be higher in the LAR+ group than in the LAR- group.

Allergen-induced nasal hyperreactivity was determined 24 hours after HDM challenge. When comparing LAR+ and LAR- responders, the former showed a significantly higher clinical response at all histamine concentrations. The AUC of symptom scores during EAR, LAR and AUC of histamine dose-response were significantly correlated. This association between nasal hyperreactivity and EAR was not found in nasal pollen challenge studies[11,230,588] (tested outside the pollen season). The difference might be explained by patients allergic to HDM having more hyperreactive upper airways, due to the continuous natural allergen exposure.

The early and late phases might be influenced by the use of oxymetazoline. Svensson[605] showed that oxymetazoline did not influence histamine-induced plasma exudation, but an effect on nasal patency appears probable. However, in spite of this bias we found a clear-cut relationship between EAR, LAR and nasal hyperreactivity. This systemic error, made in all patients, probably did not influence this association.

In conclusion: In patients with perennial rhinitis due to HDM allergy, LAR+ and LAR-responders show an different profile of symptom score, mediator involvement and nasal hyperreactivity. Just as in the lower airways, the EAR, LAR and hyperreactivity are significantly correlated.

4.7 Acknowledgements

Chapter 5
Relationship between nasal hyperreactivity, mediators and eosinophils in patients with perennial allergic rhinitis and controls

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5.1 Abstract

Background: In perennial allergic rhinitis, patients are exposed almost daily to aeroallergens. This ongoing allergic reaction results in increased sensitivity to allergens and non-specific stimuli. It is generally known that inflammatory cells and mediators are involved in the pathogenesis of the allergic reaction.
Objective: To study the relationship between nasal hyperreactivity and nasal inflammation during natural allergen exposure.
Methods: In 48 patients with perennial allergic rhinitis and in 11 volunteers a nasal brush, a nasal lavage and a histamine challenge were performed. Nasal inflammation was estimated by the number of eosinophils, levels of albumin, tryptase, prostaglandin D_2 (PGD_2), eosinophil cationic protein (ECP) and leukotriene $C_4/D_4/E_4$ ($LTC_4/D_4/E_4$).
Results: In contrast to PGD_2 and tryptase, eosinophils (1.9 vs 0%, p=0.0023), $LTC_4/D_4/E_4$ (17.51 vs 1.43 pg/ml, p<0.0001) and albumin (8.61 vs 2.37 mg/ml, p=0.0008) were significantly increased in rhinitis patients as compared with controls. Patients also showed increased responses to nasal histamine challenge assessed using a composite symptom score (21.5 vs 4 pts, p<0.0001). The nasal response to histamine was weakly correlated with the number of eosinophils in the cytospin (correlation coefficient r=0.312, p=0.035).
Conclusion: Nasal hyperreactivity is correlated with the percentage of eosinophils in patients with perennial rhinitis. The patients' mediator profile suggest that eosinophils are more important than mast cells in the ongoing allergic reaction and nasal hyperreactivity.

Keywords: perennial allergic rhinitis; inflammation; nasal hyperreactivity

5.2 Introduction

Allergic rhinitis is a common problem with a prevalence ranging from 2 to 20 per cent[168]. The pathophysiology has mainly been studied in pollen allergy, and it has been demonstrated that inflammatory cells and their products are involved.[42,188,334,465,642] After exposure to the relevant allergen patients respond with sneezing, rhinorrhoea and nasal stuffiness. Nasal responsiveness to allergen increases after repetitive allergen exposure, described by Connell as nasal priming.[120,261] However, patients become more sensitive not only to allergens but also to non-specific irritants like perfumes and tobacco smoke.[10]

This non-specific nasal hyperreactivity is particularly important in patients with perennial allergic rhinitis, who are continuously exposed to allergens.

Both in the upper and in the lower airways, allergen challenge results in an immediate symptomatic response, often followed by a recurrence of symptoms 4-8 hours later.[115,176,505] In the lower airways, the late-phase response, inflammation and non-specific hyperreactivity are correlated[92,162,445], suggesting that inflammation is involved in the pathophysiology of non-specific hyperreactivity. These relationships could not be reproduced for the upper airways, as studied in seasonal rhinitis.[230,310,346] However, in recent nasal challenge studies concerning patients with perennial allergic rhinitis, we did find a relationship between nasal hyperreactivity, inflammation and the late-phase response.[229,253]

In the non-challenged nose Knani et al.[349] demonstrated the evidence of higher levels of inflammatory mediators and cells in perennial allergic rhinitis as compared with controls. The aim of our study was to investigate if in the daily situation (i.e. during natural allergen exposure) nasal hyperreactivity and inflammation are associated. To this purpose consecutive patients with perennial allergic rhinitis visiting our outpatient clinic routinely underwent a nasal lavage, a nasal brush and a nasal histamine challenge. Nasal inflammation was measured by nasal cytology and indirectly by mediators in nasal lavage fluid.

5.3 Material and Methods

Study design
Each adult patient with perennial allergic rhinitis visiting our outpatient clinic for the first time participated in this study. At the control visit (about 3 weeks later) they underwent a nasal lavage, a nasal brush, and half an hour later a nasal histamine challenge. Eleven non-atopic non-rhinitis volunteers underwent the same procedure. In the control group the procedure was performed twice with a one-week interval.

Subjects
Forty-eight patients (23 male, 25 female, aged 30 ± 10 years) with a history of perennial rhinitis participated in this study. They had to fulfil the following criteria: perennial rhinitis for at least one year; positive intradermal skin test for at least one perennial aeroallergen (house dust mites and/or moulds and/or domestic animal danders, ALK Benelux, Groningen, the Netherlands) and exposure to the allergen(s) involved. Of the included patients, 38 were allergic to house dust mites, 7 were allergic to house dust mites and pets (and had a pet at home), 2 were allergic to their pets only, 1 patient was allergic to moulds. Patients with a concomitant pollen allergy were included only outside the pollen

season. During the study they were not permitted to use any medication affecting nasal symptoms, with the exception of Acrivastine 8 mg (as escape medication). Acrivastine had to be withdrawn 3 days before the histamine challenge. Patients with a nasal infection were excluded. Eleven non-atopic, non-rhinitic volunteers (2 male, 9 female) participated in the control group.

Nasal lavage
Five ml isotonic saline solution (0.9%) preheated to 37°C was instilled into each nostril with a pipette while the subject gently flexed his/her head backwards. After 10 seconds, the lavage fluid was expelled and collected in plastic tubes.

Mediator assays
Lavage fluid was stored on ice and centrifuged for 10 minutes at 400 x g. The supernatant was stored at -20°C. Tryptase and eosinophil cationic protein were determined by radioimmunoassay (RIA), according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden) with sensitivities of 0.5 U/ml and 2 µg/l respectively. Albumin was determined by automatic kinetic nephelometry with use of the Array analyzer (Beckmann, Mijdrecht, the Netherlands) according to the manufacturer's instructions (sensitivity 2 mg/l). $LTC_4/D_4/E_4$ and PGD_2 levels were measured by Biotrak^R and radioimmunoassay (RIA) respectively (Amersham, UK). The limits of sensitivity of the assays were approximately 3.1 and 0.75 pg/100 µl respectively. Cross-reactivity of $LTC_4/D_4/E_4$ assay: LTC_4 (100%), LTD_4 (100%), LTE_4 (70%), LTB_4 (0.4%) and PGD_2 (<0.006%).

Nasal brush and staining procedure
In each patient a nasal brush was taken from the lower edge of the inferior turbinate, with a cytobrush (Cytobrush^R Plus, Medscand AB, Sweden); it was stored in phosphate buffered saline (PBS) at 7° C until cytospins (Hettich centrifuge) were made on microscope slides. Cytospins were dried, fixed in acetone for 10 min, and rinsed successively in PBS (pH 7.2) and in PBS-bovine serum albumin 1-2%. The cytospins were incubated for 10 min with normal rabbit serum (1:10) (CLB, Amsterdam, the Netherlands), subsequently incubated for 60 min with monoclonal antibody BMK13 (Sanbio, Uden, the Netherlands), rinsed twice in PBS, incubated for 30 min in Link biotinylated anti-mouse immunoglobulin (1:50) (StrAviGen Super Sensitive BioGenex Alkaline Phosphatase concentrated, catnr AZ000UM), rinsed twice with PBS again, incubated for 30 min in Label streptAP (1:50) (StrAviGen Super Sensitive BioGenex Alkaline Phosphatase concentrated, catnr AZ000UM), rinsed successively in PBS and Tris(hydroxymethyl)-aminomethane (TRIS) buffer (PH 8.0) respectively and incubated for 30 min with New Fuchsin solution (Chroma, Stuttgart, Germany). Finally the cytospins were rinsed with distilled water, counterstained with Mayer's haematoxylin, and mounted in glycerin gelatin.
The number of eosinophils was determined by counting the BMK13 positive cells, expressed as a percentage of the number of epithelial cells.

Nasal histamine challenge
Challenges were performed according to the methods described by Gerth van Wijk *et al.*[225,228] Patients waited for half an hour prior to the test in order to give the nasal mucosa time to acclimatise. Nasal challenge with histamine phosphate (0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) was performed at five-minute intervals after challenge with PBS. After each

challenge the symptom score[373] was recorded, sneezes were counted and nasal secretion was collected in a syringe-equipped funnel[68]. The areas under the curve ($AUC_{symptom\ score}$, $AUC_{sneezes}$ and $AUC_{secretion}$) of histamine dose response curves were used in the statistical analysis.

Symptom score
A composite symptom score according to Lebel *et al.*[373] was used: 3-4 sneezes=1 pt, ≥ 5 sneezes=3 pts; anterior rhinorrhoea=1 pt; posterior rhinorrhoea=1 pt; difficult nasal breathing=1 pt, one nostril blocked=2 pts, both nostrils blocked=3 pts; pruritus of the nose=1 pt; pruritus of the palate or ear=1 pt and conjunctivitis=1 pt. Total score ranges from 0 till 11 pts.

Statistics
For statistical analysis use was made of the SPSS/PC+ Statistical Package. Between-group differences were tested by the nonparametric Mann-Whitney U test. A two-tailed p value <0.05 was considered significant.
Correlations were tested with the Spearman correlation test.
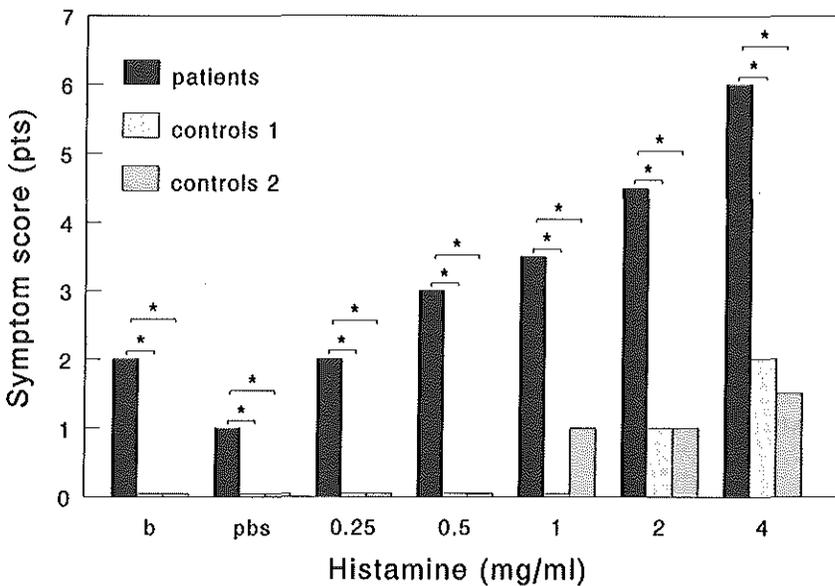
Figure 1. Nasal response to histamine
Nasal response to increasing doses of histamine phosphate in 48 patients and 11 healthy subjects. Nasal histamine challenge in the control subjects (control 1) was repeated one week later (control 2). For each histamine dose the data of the three groups were compared. Data are presented as medians. Abbreviations: b=baseline, pbs=phosphate buffered saline, * = p<0.001.

5.4 Results

Figure 1 shows the nasal response to histamine assessed with the symptom score. Rhinitis patients showed a significantly higher response to all histamine doses than the control group. An increased response was also found when nasal hyperreactivity was expressed as the $AUC_{symptom\ score}$ (21.5 vs 4 pts, p<0.0001). In the control group, nasal responses to the first and second histamine challenges were equal (4 and 3.5 pts respectively).

For the number of sneezes and the amount of secretion no significant differences were found apart from a higher secretory response after challenge with the highest histamine dose (data not shown). The $AUC_{secretion}$ was significantly increased in the patient group (0.1 vs 0 ml, p=0.0319), while the $AUC_{sneezes}$ was the same in both groups (both 6 sneezes).

In figure 2 median levels of mediators in nasal lavage fluid are demonstrated. Albumin, ECP, $LTC_4/D_4/E_4$ were significantly increased in the patient group (8.61 vs 2.37 mg/ml, p=0.0008; 1.34 vs 0.74 ng/ml, p=0.043; 17.51 vs 1.43 pg/ml, p<0.0001 respectively) when compared with the first control lavages. However, ECP levels in the second control lavages were significantly higher than in the first lavages (1.45 vs 0.66 ng/ml, p=0.0173) and equal to the patient levels. Tryptase levels did not significantly differ between the patient (2.11 U/l) and control group (2.17 U/l). PGD_2 levels were higher in the control group (27.77 vs 16.60 pg/ml, p=0.0084).
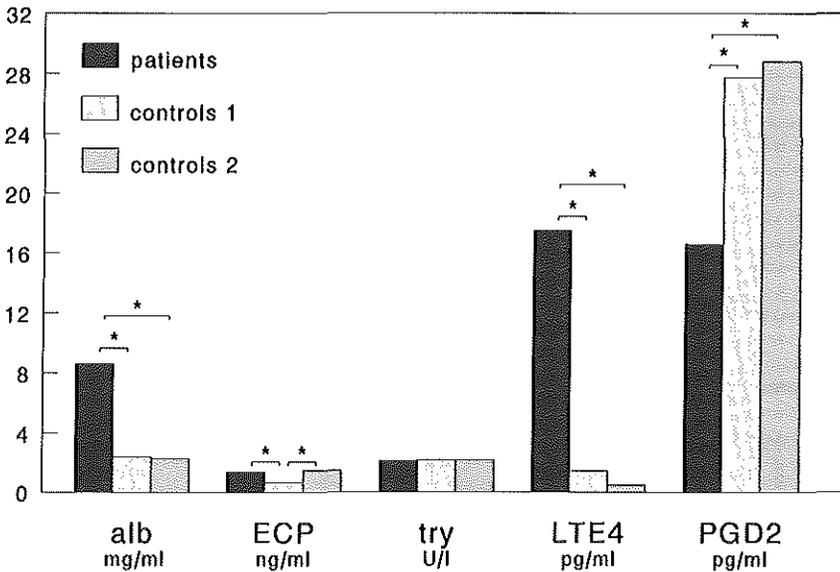
Figure 2. Mediators in lavage fluid
Levels of albumin, ECP, tryptase, $LTC_4/D_4/E_4$ and PGD_2 in nasal lavage fluid obtained from 48 patients and 11 healthy volunteers. Nasal lavages in the control group were repeated after one week (control 1 and control 2). The mediator levels of the three groups were compared. Data are presented as medians. * = p<0.05.

In the cytospins significantly (p=0.0023) more eosinophils could be detected in the patients than in the healthy volunteers (medians 1.9% and 0% respectively).

In the patient group, comparing nasal hyperreactivity with inflammatory mediators, no correlations were found. Nasal hyperreactivity ($AUC_{symptom\ score}$) correlated with the percentage of eosinophils in the cytospin (r=0.312, p=0.035) (fig. 3). When nasal hyperreactivity was expressed as $AUC_{sneezes}$ or $AUC_{secretion}$ no correlations were found. The percentage of eosinophils was not correlated with mediator levels.
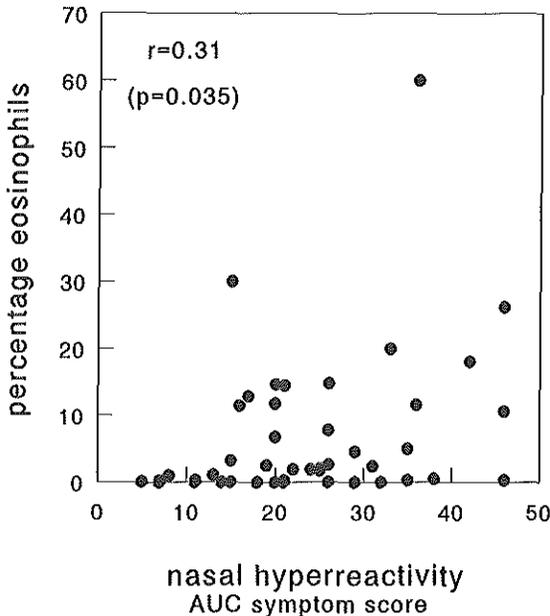
Figure 3. Nasal hyperreactivity versus nasal eosinophils

Nasal response to histamine ($AUC_{symptom\ score}$) is plotted against the percentage of eosinophils in patients with perennial allergic rhinitis.

5.5 Discussion

Nasal hyperreactivity, as determined by nasal histamine challenge, was significantly increased in the patient group. From nasal challenge studies it is known that repetitive allergen challenges result in increased sensitivity to non-specific stimuli[10]. Since patients with perennial allergic rhinitis are continuously exposed to perennial aeroallergens this increased hyperreactivity is in accordance with our expectations. In previous studies the amount of secretion and the number of sneezes proved to be useful tools to measure nasal response to histamine.[225,228] Recently, in some other studies, in addition to these response variables, nasal responsiveness to histamine was monitored by the symptom score of Lebel[373]. This symptom score turned out to be even more sensitive than the secretory and sneezing responses in detecting treatment effects and in discriminating patients with and without a late-phase response by their nasal hyperreactivity in nasal challenge

studies.[251,253,254] Comparing patients with a small control group the composite symptom score proved to be a more sensitive tool than the number of sneezes and the amount of secretion to measure differences in nasal response to histamine. This is probably due to the inclusion of a variety of symptoms such as itchiness and nasal blockage into the symptom score. To evaluate the reproducibility of this symptom score, 11 volunteers were challenged twice with histamine. The symptom scores of both histamine challenges were highly reproducible, even better than the reproducibility of the number of sneezes and the amount of secretion. Accordingly, the symptom score of Lebel[373] is a useful tool to discriminate patients and controls, treatment effects, and patient subgroups.

Nasal lavages and nasal brushes were performed to evaluate the presence of nasal inflammation. In nasal cytospins of patients with perennial allergic rhinitis significantly more eosinophils could be detected than in the control group. This might suggest that during natural perennial allergen exposure, the late-phase response plays an important role. In nasal HDM challenge studies, a relationship between nasal hyperreactivity, the late-phase response and inflammation has been found.[229,253] The present study was performed during natural allergen exposure, in non-challenged subjects. A moderate but significant correlation was found between nasal response to histamine, expressed as $AUC_{symptom\ score}$ and the percentage of eosinophils, suggesting that inflammation is involved in the pathogenesis of nasal hyperreactivity. The relatively low correlation and the absence of association between inflammatory mediators and nasal hyperreactivity are not surprising. Probably, non-specific hyperreactivity cannot be explained by activation of one cell or release of one specific mediator.

In lavage fluid several mediators were measured to gain an impression of the mediators involved in the ongoing allergic reaction. Furthermore, to study the reproducibility of the assays, nasal lavages in the control group were performed twice with a one-week interval. With the exception of ECP, levels of albumin, tryptase, $LTC_4/D_4/E_4$ and PGD_2 proved to be reproducible.

Albumin levels were higher in patients, demonstrating increased vasopermeability[45,604], confirming the presence of nasal inflammation. Tryptase, as a specific marker of activated mast cells[560], was the same in both groups. Since mast cells are involved in the immediate allergic response this might suggest that in the ongoing allergic reaction the late phase is more important than the immediate phase. PGD_2 is an inflammatory mediator released during the immediate allergic response by mast cells.[52,679] In the patient group we measured lower PGD_2 levels than in the control group. In the lungs and liver it has been demonstrated that PGD_2 is metabolized by a keto-reductase to the vasoactive metabolite $9\alpha,11\beta\text{-}PGF_2$.[571] Possibly, in the nasal mucosa of allergic rhinitis patients an increased number of leukocytes, containing keto-reductase, are present, resulting in an increased PGD_2 metabolism, which may explain the low PGD_2 levels in lavage fluid. However, we have no explanation for the differences with the results of Knani et al[349], who measured higher levels of PGD_2 in nasal lavage fluid of allergic patients than in controls. In lavage fluid we also measured $LTC_4/D_4/E_4$, arachidonate 5-lipoxygenase metabolites, predominantly produced by eosinophils, mast cells and basophils[385]. In the patients' lavages significantly higher levels of $LTC_4/D_4/E_4$ were demonstrated. This $LTC_4/D_4/E_4$ may be released by the increased number of eosinophils present in the nasal mucosa. Since in rhinitis patients the number of eosinophils was increased, ECP, derived from activated eosinophils[395], would be expected to be increased also. Initially, comparing patient lavages with the first control lavages, slightly but significant higher ECP levels were found for the

patient group. However, ECP levels of the second control lavages were as high as in the patient group. Measurement of ECP appears to be more variable than determination of the other mediators.

Conclusion: During natural perennial allergen exposure, eosinophils are involved in the ongoing allergic inflammation. The number of eosinophils correlated with the nasal response to histamine, suggesting that inflammation is involved in the pathogenesis of non-specific hyperreactivity. In addition, nasal responsiveness to histamine assessed with the symptom score of Lebel[373] proved to be a valid tool in discriminating rhinitic patients and healthy controls.

5.6 Acknowledgements

PART THREE

Therapeutic intervention

Chapter 6
Glucocorticosteroid intervention

Glucocorticosteroids, such as cortisone and hydrocortisone (cortisol) are hormones synthesized in the adrenal cortex. Synthetic compounds are also available, such as prednisolone, beclomethasone dipropionate and dexamethasone.

The receptor for steroids is an intracellular protein which exists as a soluble constituent of the cytosol or nucleus, and is also able to bind with high affinity to nuclear chromatin. The basic structure is large monomeric proteins of 400 - 1000 residues, containing a highly conserved region of about 60 residues in the middle of the molecule, which is believed to constitute the DNA-binding domain of the receptor. It contains two loops of about 15 residues each (zinc fingers), and the loops are believed to wrap around the DNA helix. The hormone-binding domain lies downstream of this central region. The steroid molecule crosses the cell membrane readily, being highly lipid soluble, and binds to the receptor which is believed to unfold, thus exposing the normally buried DNA binding domain. When this happens translocation of the receptor from the cytosol to the nucleus may occur. The receptor binds to certain well-defined regions of the nuclear DNA. An increase in RNA polymerase activity and the production of specific mRNA occurs within a few minutes of adding the steroid, though the physiological response may take hours to develop. The different steroid hormones are able to activate different genes, and thus initiate completely different patterns of protein synthesis, and produce different physiological effects.[167,447] Glucocorticoids enhance the production of lipocortin, which may account for their anti-inflammatory properties by inhibition of the activity and synthesis of PLA_2 [184] and by mechanisms independent of PLA_2 activity.[185,248] Whereas, mineralocorticoids stimulate the production of various transport proteins that are involved in renal tubular function.[167,447]

The anti-inflammatory effects are expressed by two mechanisms: decreased production of inflammatory mediators and cells and generation of anti-inflammatory mediators. The inflammatory mediators suppressed include histamine from basophils, complement components, and cytokines such as IL-1, IL-2, IL-4, IL-6, IL-8 and TNF-α by direct inhibition of transcription or interference with intracellular transduction events.[172,424] The inhibition of PLA_2 activity/synthesis by lipocortin causes a decrease in the production of PAF and mediators of arachidonic acid.[529] In addition, glucocorticoids have recently been shown to selectively inhibit the expression of COX_2.[358,688] It is known that glucocorticosteroids inhibit T-cell proliferation and cytokine production *in vitro*.[135] They decrease the number of Langerhans cells (in the skin).[21] Corticosteroids decrease the number of cells expressing IL-4 and IL-5 mRNA in asthma[542] and decrease the number and activation of mast cells and eosinophils in bronchial biopsies and lavages of asthmatics.[157] Corticosteroids relieve symptoms of asthmatics, however, some patients respond poorly to these drugs or need such high doses that unwanted side-effects become severe while symptoms do not further decrease, which is called corticosteroid-resistance.[690]

Rhinitis: Treatment with topical corticosteroids have been associated with a decrease in the number of nasal eosinophils, basophils, Langerhans cells and neutrophils of allergic rhinitis patients.[293,402,433] It has been suggested that corticosteroids may act by preventing activation of several cells and subsequent release of inflammatory mediators.[435] The effect of systemic steroids, such as prednisolone, reduce symptoms and mediator release in the late phase

reaction to allergen. They have little or no effect on the immediate reaction.[463,516] In contrast, anti-inflammatory therapy with topical corticosteroids have been shown to be effective on both the early and late phase reaction after allergen challenge, on nasal priming and on nasal hyperreactivity in rhinitis.[38,460,515] It is not known whether corticosteroid-resistance exists in rhinitis.

Chapter 7
Effect of intranasal fluticasone propionate on the immediate and late allergic nasal reaction and nasal hyperreactivity in patients with a house dust mite allergy

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7.1 Abstract

Background: Patients with perennial allergic rhinitis develop nasal symptoms not only after allergen exposure, but generally also after non-specific stimuli.
Objective: To evaluate the effect of two week's treatment with fluticasone propionate aqueous nasal spray (FPANS) on the nasal clinical response, inflammatory mediators and nasal hyperreactivity.
Methods: Twenty-four rhinitis patients allergic to house dust mite (HDM), participated in a double-blind, placebo-controlled crossover study. After a two week's treatment with placebo or 200 µg FPANS twice daily, patients were challenged with HDM extract. Symptoms were recorded and nasal lavages were collected for up to 9.5 hours after challenge. Nasal hyperreactivity was determined by histamine challenge 24 hours later.
Results: Because of a carry-over effect for the immediate symptom score, for this variable only the data from the first treatment period were used. FPANS treatment resulted in a significant decrease of nasal symptoms with 70%, 69% and 63% after 100, 1000 and 10000 Biological Units (BU)/ml of HDM extract respectively. Active treatment resulted in a 76% decrease of the late-phase symptoms. FPANS treatment significantly reduced albumin influx after HDM 1000 BU/ml with 62% and tended to reduce tryptase release after HDM 1000 BU/ml (p=0.0629). During the late phase FPANS treatment reduced albumin influx with 67% and eosinophil cationic protein (ECP) release with 83%. No effect of FPANS was seen on histamine levels. FPANS significantly decreased histamine-induced symptom score with 34%, secretion with 32% and sneezes with 41%.
Conclusion: FPANS significantly inhibits the immediate and late allergic response, and nasal hyperreactivity, probably by suppressing mast cells and eosinophils in the nasal mucosa.

Keywords: fluticasone propionate; perennial allergic rhinitis; house dust mite; nasal lavage; inflammatory mediators; early allergic response; late allergic response; nasal hyperreactivity

7.2 Introduction

Allergic rhinitis is a common problem (prevalence ranging from 2 to 20 percent[168]), characterized by symptoms as sneezing, itching, rhinorrhoea and nasal congestion. The pathophysiology is associated with the presence of inflammatory cells such as eosinophils, mast cells, T-cells and Langerhans cells[188,334,642] in the nasal mucosa. After re-exposure to allergen, mast cells are activated by crosslinking of allergen to mast cell-bound specific IgE. The activated mast cells release vasoactive mediators such as histamine and leukotrienes related to the early nasal symptoms, and chemotactic mediators that attract inflammatory cells such as eosinophils associated with the late-phase response.[42]
Connell[120] described in the sixties a phenomenon known as nasal priming: repetitive exposure to allergen causes an increased sensitivity to allergens. This effect was confirmed by others.[28,261] Exposure to allergen also increases nasal responsiveness to non-specific stimuli.[10] However, the pathophysiology of nasal hyperreactivity and nasal priming is still unclear.
In the lower airways, the late-phase reaction was found to be associated with inflammation and bronchial hyperreactivity[92,115,445], suggesting that inflammation is involved in the pathogenesis of hyperreactivity. These associations could not be confirmed in nasal challenge studies performed in patients with a pollen allergy[230,310,346], which makes the pathogenetic role of inflammation in nasal hyperreactivity less plausible. However, in a study with rhinitis patients allergic to HDM, Gerth van Wijk et al[229] has shown an association between nasal responsiveness to allergen and pre-existent nasal hyperreactivity, a finding more in agreement with data from the lower airways.
Anti-inflammatory therapy with intranasal corticosteroids have extensively been shown to be effective in the treatment of seasonal and perennial rhinitis.[460] It has been shown that topical corticosteroids have an effect on both the early and late-phase reaction after allergen challenge, on nasal priming and on nasal hyperreactivity in patients allergic to pollen.[38,516] In the present study we want to evaluate the anti-inflammatory effects of topical corticosteroid therapy on nasal response to HDM challenge, and on allergen-induced nasal hyperreactivity. In an attempt to provide further differentiation between the local anti-inflammatory effects and the unwanted systemic effects of corticosteroids, a new, topically active, fluorinated glucocorticosteroid: fluticasone propionate has been developed. It has shown to have twice the potency of beclomethasone dipropionate and to have minimal systemic effects[85,510], because of extensive first-pass metabolism by the liver.
In 24 rhinitis patients allergic to HDM the effect of two week's treatment with fluticasone propionate on nasal response to HDM challenge and nasal hyperreactivity was studied. Nasal response to allergen was monitored by symptoms and mediators in nasal lavage fluid (histamine, albumin as marker for vasopermeability, tryptase as mast cell marker and ECP as marker for activated eosinophils) for up to 9.5 hrs.

7.3 Materials and Methods

Subjects
Twenty-four rhinitis patients, 11 women and 13 men aged 21 to 50 years (mean 34 yrs) participated in this study. They had to fulfil the following criteria: a history of perennial

rhinitis; intradermal skin reaction of at least 1 plus-sign to 3 BU/ml HDM extract, (ALK Benelux, Groningen, the Netherlands), according to the standardised plus-sign scoring system defined by Norman[477]; age between 15 and 65 years. The only patient with a concomitant pollen allergy was tested outside the pollen season. Patients with concomitant allergy to pets were only included when they had no contact with pets.

To prevent interference with FPANS, symptomatic medication for rhinitis had to be withdrawn. Oral corticosteroids had to be withdrawn 2 months, astemizole 6 weeks, nasal or inhaled corticosteroids, cromoglycate and nedocromil 3 weeks and antihistamines 3 days before entering the study. Patients with nasal polyposis, nasal surgery less than 3 months before the study, nasal infection during 2 weeks before the study or immunotherapy in the past were excluded.

Study design

In 24 patients with perennial rhinitis a double-blind placebo-controlled crossover study was performed. Patients were treated with placebo or 200 µg FPANS twice daily (i.e. twice the recommended dosage) for two weeks. At visit 1 patients received a nasal spray containing 50 µg fluticasone propionate/actuation or placebo. After the first treatment period, followed by a wash-out period of 3 weeks, they received the complementary nasal spray (visit 4). After either treatment period patients were challenged with HDM extract. Symptom scores were recorded and nasal lavages were performed for up to 9.5 hours after HDM challenge (visit 2 and 5). Allergen-induced nasal hyperreactivity was determined by nasal histamine provocation 24 hours after HDM challenge (visit 3 and 6).

During the study patients were not allowed any medication affecting nasal function other than terfenadine 60 mg, which was provided as escape-medication. Terfenadine had to be withdrawn 3 days before the nasal challenges were performed.

The study was performed during the period January-August, to minimise natural exposure to HDM. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam-Dijkzigt and all patients gave written informed consent.

Nasal challenge with HDM and histamine

Challenges were performed in accordance with the methods described by Gerth van Wijk *et al.*[229,230] On each occasion the patients waited for half an hour before the test to give the nasal mucosa time to acclimatise.

Nasal challenges with HDM extract (100 BU/ml, 1000 BU/ml, 10000 BU/ml, ALK Benelux, Groningen, the Netherlands) were performed at ten-minute intervals after challenge with phosphate-buffered saline (PBS), containing human serum albumin 0.03% and benzalkonium chloride 0.05% (ALK Benelux, Groningen, the Netherlands). The HDM extract was sprayed into each nostril by means of a nasal pump spray delivering a fixed dose of 0.125 ml solution. Nasal response was monitored 10 minutes after each challenge, and hourly from 0.5 till 9.5 hours after the last challenge. Nasal responsiveness was monitored by the number of sneezes, the amount of secretion collected according to Borum[68] and a symptom score according to Lebel[373].

Nasal challenge with histamine phosphate (0.25, 0.50, 1.0, 2.0 and 4 mg/ml) was performed at five-minute intervals after challenge with PBS. The amount of secretion, the number of sneezes and the symptom score according to Lebel[373] were used as nasal-response indicators.

Nasal lavage
Nasal lavages were performed as described by Naclerio.[465] This protocol comprises 4 pre-challenge washings with isotonic saline solution to clear the nose from secretions and to obtain baseline levels of mediators. In order to prevent nasal congestion due to allergen challenge, oxymetazoline 0.1% (two 0.125 ml puffs) was applied in both nostrils. After 5 minutes the oxymetazoline was washed out just prior to the nasal challenge with PBS. A nasal lavage was performed 10 minutes after PBS and after each allergen challenge, immediately before the subsequent challenge. Subsequently, lavage fluid was obtained hourly from 0.5 to 9.5 hours after the last allergen challenge. Nasal lavages were performed with 10 ml isotonic saline solution (0.9%) preheated to 37°C. In each nostril 5 ml saline was instilled with a pipette while the subject gently flexed his/her head backwards. After 10 seconds, the lavage fluid was expelled and collected in plastic tubes. This procedure has shown to produce a recovery of 7.7 ± 1.2 ml.[229]

Symptom score
Symptoms were recorded by using a scoring system according to Lebel *et al.*[373] at the time points of lavage. Symptom scores were graded in points (pts): 3-4 sneezes=1 pt, ≥5 sneezes=3 pts, anterior rhinorrhoea=1 pt, posterior rhinorrhoea=1 pt, difficult nasal breathing=1 pt, one nostril blocked=2 pts, both nostrils blocked=3 pts, pruritus of the nose=1 pt, pruritus of palate or ear=1 pt and conjunctivitis=1 pt (total score ranges from 0 till 11 pts). In addition, the number of sneezes and the amount of secretion were noted.

Mediator assays
Lavage fluid was stored on ice and centrifuged for 10 minutes at 400 x g. The supernatant was stored at -20°C. Histamine was measured with an automated fluorometric assay[586] with a sensitivity of 0.1 ng/ml. Tryptase and ECP were determined by radioimmunoassay (RIA), according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden), with a sensitivity of 0.5 U/l and 2 µg/l respectively. Albumin was determined by automatic kinetic nephelometry with use of the Array analyzer (Beckmann, Mijdrecht, the Netherlands) according to the manufacturer's instructions (sensitivity 2 mg/l).

7.4 Statistical analysis

The effect of FPANS treatment on the immediate (HDM 100, HDM 1000 and HDM 10000 BU/ml) and late (area under the curve (AUC) between 3.5 and 9.5 hrs) nasal reaction and on nasal hyperreactivity (AUC of histamine dose response curve) was evaluated.
Thirteen patients received FPANS during the first treatment period and 11 during the second treatment period. For testing the carry-over effect, within patient totals over the two treatment periods were used. We considered there was no significant carry-over effect if the means of these within patients totals did not significantly differ between the two treatment-order groups. For this test a p-value < 0.1 was considered significant. In case of a carry-over effect, only the data of the first treatment period were used for statistical analysis.
For testing the effect of FPANS, within patient differences over the two treatment periods were used, provided that there was no carry-over effect. There was an effect of FPANS if
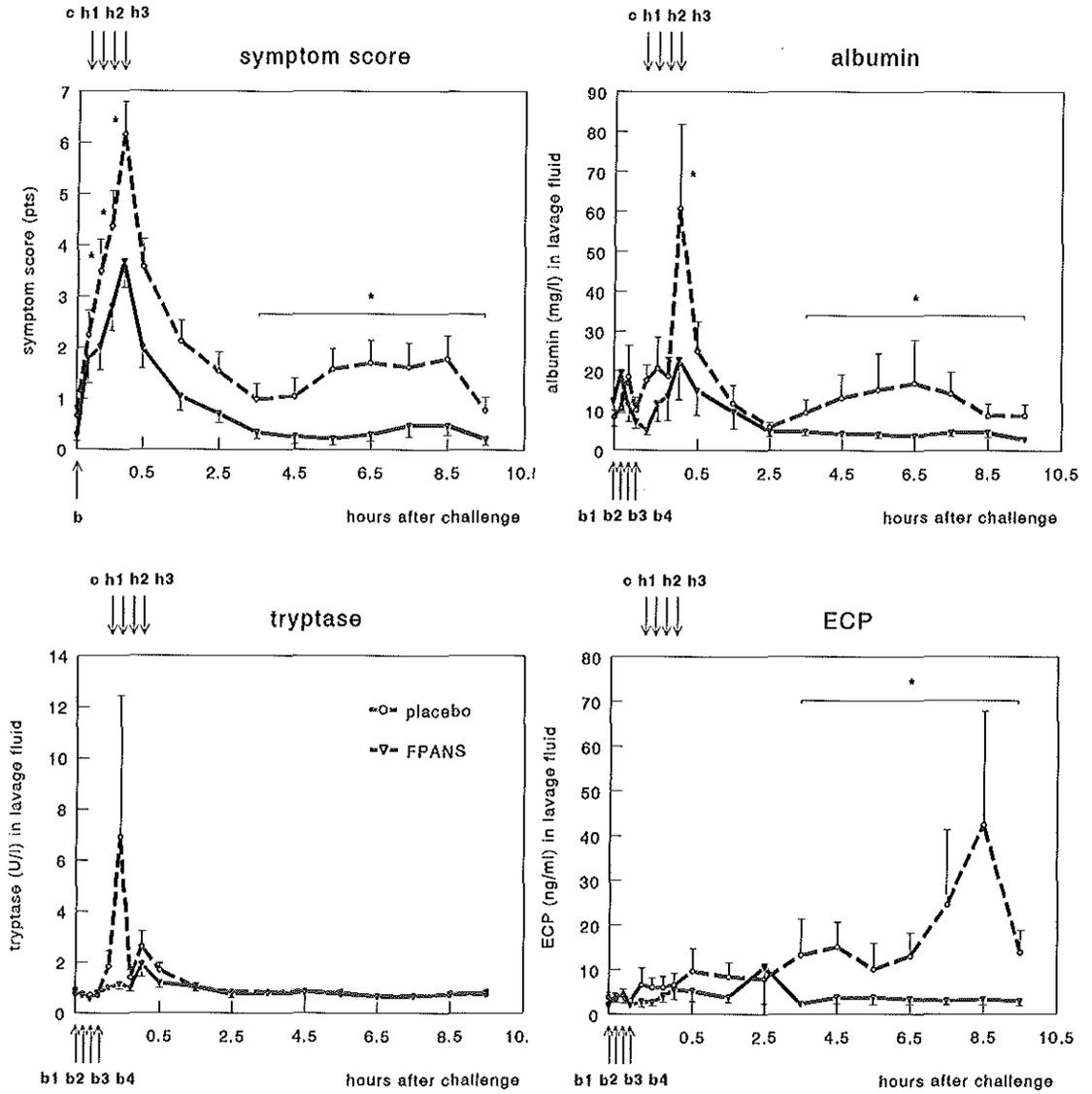
Figure 1. Nasal response of placebo and FPANS treated groups measured for up to 9.5 hours after HDM challenge. Abbreviations: b=baseline symptom score, b1-b4=pre-washings, c=10 min after challenge with PBS, h1-h3=10 min after challenge with 100, 1000 and 10000 BU/ml HDM extract respectively. * = two-sided p-value≤0.05. Values are presented as mean ± standard error of the mean (SEM).

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the means of these within patients differences did differ significantly between the two treatment-order groups. The nonparametric Mann-Whitney U test was used. A two-tailed p-value < 0.05 was considered significant.

7.5 Results

Figure 1 shows the results of FPANS treatment on nasal HDM challenge. Because a carry-over effect was found for the symptom score during the immediate reaction, for this variable only the data of the first treatment period were used for statistical analysis.
FPANS treatment significantly reduced the immediate symptom score with 70% (p=0.0104), 69% (p=0.0045) and 63% (p=0.0013) after 100, 1000 and 10000 BU/ml of HDM extract respectively and the late-phase symptom score with 76% (AUC from 3.5 till 9.5 hours, p=0.0003). The amount of secretion was significantly diminished during the immediate (HDM 100 BU/ml: 32% decrease, p=0.0105; HDM 1000 BU/ml: 49% decrease, p=0.0038; HDM 10000 BU/ml: 17% decrease, p=0.0297) and late response (AUC 3.5-9.5: 93% decrease, p=0.0464) (data not shown). For the number of sneezes only an effect was observed during the early response (HDM 100 BU/ml: 57% decrease, p=0.0477; HDM 1000 BU/ml: 77% decrease, p=0.0115; HDM 10000 BU/ml: 53% decrease, p=0.0019) (data not shown). Analysis of nasal lavage fluid showed a significant reduction of 62% in the amount of albumin during the early (HDM 10000 BU/ml, p=0.0011) and of 67% during the late phase (AUC 3.5-9.5 hrs, p=0.0019) after corticosteroid treatment. Tryptase release was only observed immediate after HDM challenge; treatment with FPANS tended to decrease this release (HDM 1000 BU/ml, p=0.0629). The high mean and standard error of the mean (SEM) at HDM 100 BU/ml are mainly caused by one patient. ECP release in the period 3.5-9.5 hours was almost completely inhibited (83% decrease, p=0.0024) by FPANS.
Nasal HDM challenge did not increase histamine levels in nasal lavage fluid. No effect of corticosteroid therapy was seen (fig. 2).
Allergen-induced nasal hyperreactivity was determined as AUC of histamine dose response curves. FPANS significantly decreased the symptom score with 34% (p=0.0162), the number of sneezes with 41% (p=0.0096) and the amount of secretion with 32% (p=0.0104) (fig. 3).

7.6 Discussion

Earlier studies mainly focused on seasonal allergic rhinitis. We studied the effect of a topical corticosteroid on the immediate and late sequelae after HDM challenge and on nasal hyperreactivity in 24 rhinitis patients allergic to HDM. Patients were challenged with high doses of HDM extract in order to obtain maximal nasal response. Since we wanted to evaluate the anti-inflammatory effects of FPANS, we were particularly interested in the effects on the late-phase response and inflammatory mediators.
Nasal symptom scores reached their maximum 10 minutes after challenge with HDM 10000 BU/ml and returned to baseline levels at 3.5 hours. Partial recurrence of symptoms was shown during 3.5 and 9.5 hours, the period of the late-phase response.[505]
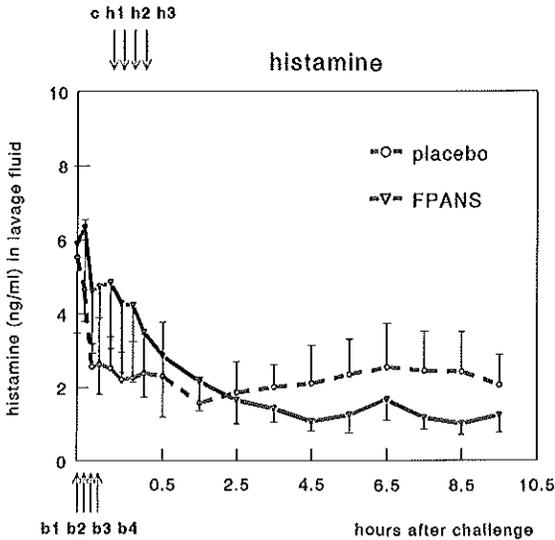
Figure 2.
Histamine in lavage fluid of placebo and FPANS treated groups in response to HDM challenge. Values are presented as mean ± SEM.

However, in the nose it is difficult to define the late-phase reaction. Mygind[461] could not detect a late-phase response, clearly separated from the early response, by means of symptom scores and measurement of nasal airway resistance. In other studies late-phase reactions were determined by measurement of nasal obstruction and analysis of nasal lavage fluid.[176,467] These studies concerned pollinosis patients.

Albumin, being the quantitative dominant protein in plasma, can be used as a marker of increased vasopermeability.[45,604] Assessment of albumin in lavage fluid demonstrated an increased influx during the immediate and the late-phase response.

To monitor the immediate response we assessed histamine and tryptase in lavage fluid. Although it is generally accepted that immediate reactions to allergen challenge are mast cell-dependent, histamine being the principal mediator, no increased release of histamine was found. This corresponds with a recent study by Gerth van Wijk et al.[229] also in patients allergic to HDM. Linder et al.[394] observed a decrease of histamine in nasal secretions during the pollen season and after pollen challenge. In contrast, Naclerio and co-workers[467] measured increased histamine release after nasal challenge with ragweed pollen. Davies[144] found an increase in histamine in response to nasal grass pollen challenge, however this release was not dose-dependent. Since the histamine levels we measured were above the detection limit of 0.1 ng/ml, our failure to detect an increased histamine release after allergen challenge is not due to insensitivity of our assay (automated fluorometric). Our failure to detect increased histamine levels may be attributed to our study design, because nasal secretion and nasal lavage fluid were collected separately, whereas others mixed secretion and lavage fluid. In addition, high levels of histamine have been detected in prechallenge lavages without association with the presence of symptoms.[56] Measurement of histamine in nasal lavage fluid as marker of the nasal allergic response seems, due to these inconsistent observations, not ideal. In contrast, tryptase, a specific marker for mast cell

activation[560], could be detected in measurable amounts during the immediate response. This finding is in agreement with the hypothesis that the immediate but not the late-phase response to allergen challenge is mast cell-dependent. A possible explanation for the discrepancy in tryptase and histamine release, might be that histamine is metabolized or removed more rapidly than tryptase, which has been demonstrated in the circulation[567]. In the present study as well as in a study performed by Rasp et al.[536] tryptase seems to be a useful marker of the immediate nasal reaction.

During the late-phase response activated eosinophils are involved[42]. As an indirect method to study the involvement of eosinophils, we measured ECP in lavage fluid. ECP, released from activated eosinophils was only detected during the late-phase response, suggesting that ECP can be used as a marker to detect the late-phase response.
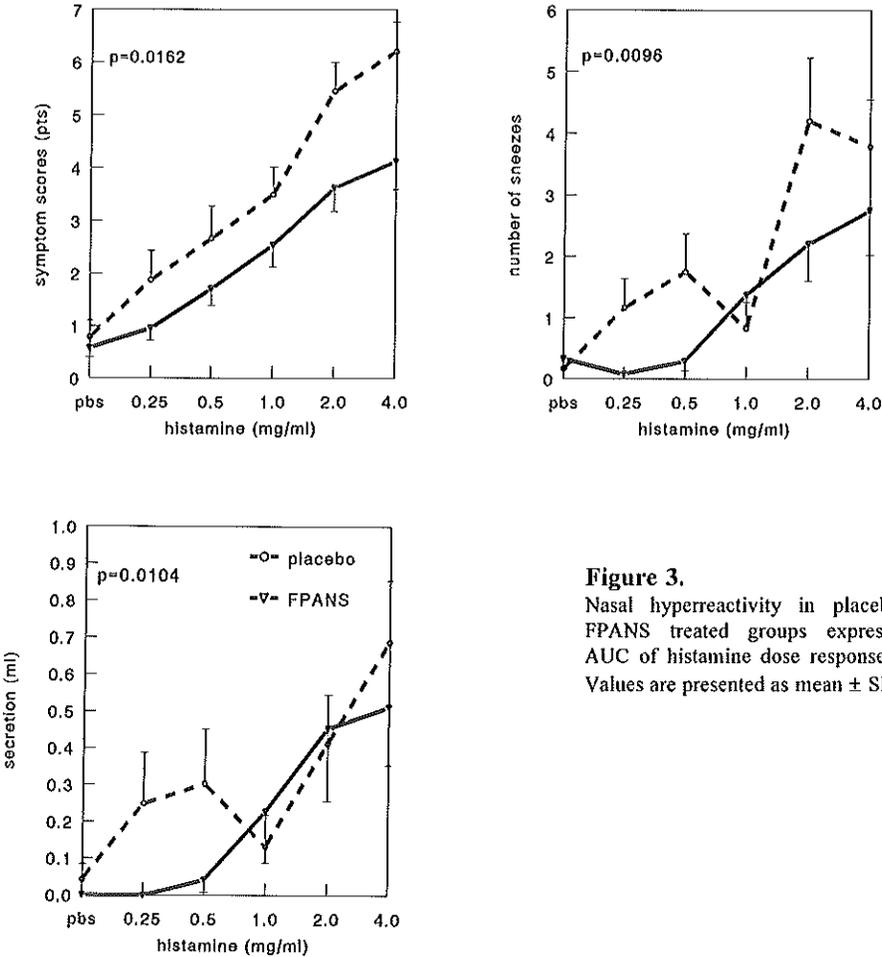
Figure 3.
Nasal hyperreactivity in placebo and FPANS treated groups expressed as AUC of histamine dose response curve. Values are presented as mean ± SEM.

Treatment with FPANS showed a partial but significant decrease of immediate symptoms and an almost complete inhibition of late symptoms after allergen challenge. The same was true for albumin influx in nasal lavage fluid. FPANS also reduced nasal inflammation as measured by ECP and tryptase. ECP release during the late phase was completely inhibited by topical fluticasone. This inhibition of ECP release was also shown by Lozewick[402] in 44 patients allergic to pollen. FPANS tended to decrease tryptase release. The tryptase values were widely dispersed. Probably with a larger number of patients the effect on tryptase would have reached significance. Juliusson[333] demonstrated a significant effect of FPANS on tryptase release after pollen challenge in 25 hay fever patients.

Inhibition of the symptoms during immediate and late-phase response by FPANS is assumed to be due to a reduction of the number of mast cells and eosinophils, with as consequence decreased levels of mediators.

In patients with perennial allergic rhinitis, it has been demonstrated that nasal hyperreactivity is associated with inflammation.[229] In this study two weeks' administration of FPANS significantly inhibited nasal response to histamine. This confirms the hypothesis that inflammation is involved in nonspecific nasal hyperreactivity.

Concluding: In patients with perennial allergic rhinitis, an immediate and a late allergic response was shown by nasal symptoms as well as by inflammatory mediators in lavage fluid. Two-week treatment with FPANS inhibits the immediate and in particular the late allergic response as well as allergen-induced nasal hyperreactivity. This may occur as a consequence of a reduction of mast cells and eosinophils in the nasal mucosa as indirectly measured by tryptase and ECP in lavage fluid.

7.7 Acknowledgements

Chapter 8

Effect of fluticasone propionate aqueous nasal spray treatment on platelet activating factor and eicosanoid production by nasal mucosa in patients with a house dust mite allergy

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8.1 Abstract

Objective: The relationship between the release of platelet activating factor [PAF], leukotriene $C_4/D_4/E_4$ [$LTC_4/D_4/E_4$] and prostaglandin D_2 [PGD_2] from nasal mucosa *in vivo* was examined in 24 rhinitis patients allergic to the house dust mite (HDM).

Methods: During a double blind placebo controlled cross-over study 200 µg fluticasone propionate aqueous nasal spray (FPANS) was administered twice daily for two weeks.

In response to allergen provocation (100, 1000, 10000 Bu/ml) and during the 9.5 hours after this challenge the nasal fluid was obtained by washing the nose with saline and the levels of PAF, $LTC_4/D_4/E_4$ and PGD_2, as indicators of mediator release, were measured at the following time-points: baseline ($t=-\frac{1}{2}$), allergen provocation with 10000 Bu/ml ($t=0$), 3.5 and 7.5 hours (late phase).

Results: After allergen provocation the levels of the mediators increased in the nasal fluids of placebo treated patients (increase from baseline: PAF: 15-fold; $LTC_4/D_4/E_4$: 12-fold; PGD_2: 1.5-fold). In fluids of patients treated with FPANS these levels tended to decrease. At the time of provocation the levels of PAF, $LTC_4/D_4/E_4$ and PGD_2 showed a significant correlation.

Conclusion: The results indicate that these mediators can be used as markers of allergic reactions against house dust mites and that fluticasone propionate aqueous nasal spray tended to reduce the release of mediators of inflammation correlated with beneficial effects on clinical symptoms in this type of allergic reactions.

Keywords: fluticasone propionate aqueous nasal spray; house dust mite; platelet activating factor; eicosanoids

8.2 Introduction

In the 1920s house dust allergy was recognized when dust extracts from mattresses and vacuum cleaners were found to give relevant positive reactions in skin test on asthmatics.[343,451] Since 1964 it has been known that the majority of house dust sensitive patients show positive skin reactions to the mites of the genus Dermatophagoides farinae (Df) and D. pteronyssinus (Dp) as a major source in house dust.[651] The faeces particles, in particular, contain allergenic material in a concentrated form. Practicable control measures, such as chemicals, cleaning, ventilation and temperature regulation have only been able to reduce the number of mites in houses to some extent but the clinical effect has been disappointing.[278,370,451,651,653] As long as these methods are insufficient other forms of therapy are needed, such as immuno-therapy and symptomatic medication.[77,198,213,214]

House dust mites are the major cause of perennial rhinitis. The pathophysiology of allergic rhinitis, however, has been mainly studied in pollen allergy. Naclerio et al.[465] developed a model to explore the role of inflammatory mediators in ragweed pollinosis. As a consequence of cross-linking of IgE on mast cells and basophils by antigen mediators, such as prostaglandin D_2 [PGD_2], tryptase and histamine are released in the so-called early phase of the allergic process. These mediators cause sneezing, rhinorrhoea and nasal congestion, which are the main symptoms of allergic rhinitis when they interact with neural elements, mucosal gland and blood vessels. After a quiescent period a second phase of the allergic process occurs. In this so-called late phase mediators are released again and symptoms recur.[465,466,463,625] The effect of systemic steroids, such as prednisone, reduce symptoms and mediator release in the late phase of the process. They have little or no effect on the early phase.[463,515] In contrast, topical steroids, such as flunisolide, used in the nose reduce symptoms and mediator release in the early phase as well in the late phase of the allergic process in a study with patients challenged with pollen antigens.[463,516] The corticosteroid, fluticasone propionate [FP] has potent topical anti-inflammatory activity coupled with the low systemic activity. It has more than nine times the anti-inflammatory activity of fluocinolone acetonide and twice the activity of beclomethasone dipropionate.[85,510]

The present study uses a nasal challenge model developed by Naclerio et al.[465] to explore the role of PAF and eicosanoids in the early and late phase of the allergic process in patients with allergic rhinitis against house dust mites. PAF could be involved in respiratory allergies because PAF is a potent eosinophil chemotactic factor.[666] However, to the authers'knowledge, there are so far no available data regarding in vivo PAF generation by human nasal mucosa of patients allergic to house dust mites. In this report, the effect of fluticasone propionate aqueous nasal spray, a new and potent corticosteroid, on the levels of platelet activating factor (PAF), leukotriene $C_4/D_4/E_4$ ($LTC_4/D_4/E_4$) en prostaglandin D_2 (PGD_2) after nasal challenge with house dust mite extract, is also described.

8.3 Materials and methods

Patients

This study was performed in 24 patients. There were 11 women and 13 men aged 21 to 50 years (mean, 34 years). All were characterized by a history of perennial rhinitis, and by a positive skin test to house dust mite extract. All patients showed a skin reaction rated as at

least one "+"-sign to 0.3 to 3 BU/ml extract, according to the standardized plus-sign scoring system defined by Norman.[477] Six of the 24 patients were allergic to grass pollen or animal dander as well. The nasal lavage experiments were performed between January and August to minimize exposure to house dust mites. The only patient with a concomitant pollen allergy was tested outside the pollen season. None of the patients allergic to animals had pets in their home. Antihistamines were withdrawn 72 hours before testing. The antihistamine astemizole, topical corticosteroids, cromoglycate or nedocromil were not used for 3 weeks before the tests were performed. Oral corticosteroids had to be withdrawn 2 months before the study. Patients who developed a nasal infection during the 2-weeks period before entering the study were excluded. None had undergone immunotherapy previously.

The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam-Dijkzigt and all patients gave written informed consent.

Nasal challenge and lavage

After the positive skin test the subjects entered the double blind placebo-controlled crossover phase of the study. Each underwent two allergen challenges, performed after 2 weeks pretreatment with 200 µg fluticasone propionate aqueous nasal spray (FPANS) (Glaxo, GRD) or placebo spray twice daily. A 3-weeks washout period separated the two challenges.

Before nasal challenge with house dust mite extract a nasal lavage was performed four times to obtain baseline mediator levels and to clear the nose from secretions. To prevent nasal congestion caused by the allergen challenges 0.250 ml oxymetazoline (0.1%) was sprayed into each nostril 5 min before the first challenge. Nasal lavage was performed as described by Naclerio *et al.* and Gerth van Wijk *et al.*[229,230,465] Both nostrils were washed with 5 ml saline, prewarmed to 37°C. Lavage fluid was collected in plastic tubes that were kept on ice. These lavage fluids were centrifuged for 10 min at 400 x g and the supernatants were stored at -70°C until detection of PAF or eicosanoids. To obtain a control challenge, 0.125 ml phosphate buffered saline [PBS] was sprayed in each nostril and a nasal lavage was performed. For allergen challenge 0.125 ml allergen extract was sprayed in each nostril and 10 min thereafter a nasal lavage was performed. Allergen doses of 100, 1000, 10 000 Biological Units [BU]/ml (extract of Dermatophagoides pteronyssinus; ALK, Groningen, The Netherlands) were administered. From 30 min up to 9.5 hours after this challenge the nasal fluid was obtained every hour by washing the nose with saline. Allergen-induced secretion collected before nasal lavage was not used for analysis. From the series of lavages the levels of PAF, $LTC_4/D_4/E_4$ and PGD_2, as indicators of mediator release, were measured at the following time-points: baseline (t=-½), allergen provocation with 10 000 BU/ml (t=0), 3.5 and 7.5 h. These time-points were chosen based on recently described studies[229,230], in which it was shown that between 3 and 10 hours after antigen challenge the late phase reaction occurred.

Symptom Score

Symptoms were scored according to a scoring system described by Lebel *et al.*[373] These symptoms were observed in order to study the correlation between these clinical symptoms and the inflammatory mediators. The score was compiled before each lavage and after PBS and each allergen insufflation.

Mediator assays

The levels of PAF, $LTC_4/D_4/E_4$ and PGD_2 were measured by Scintillation Proximity Assay (SPA), Biotrak[R] and RadioImmunoAssay (RIA) respectively (Amersham, U.K.) The limits of sensitivity of the assays were approximately 20, 3.1 and 0.75 pg/100 µl respectively.
Cross-reactivity (50% B/B_0 displacement) of: PAF-assay: 1-Hexadecyl-2-acetyl GPC-PAF(C16:0) (100%), 1-Octadecyl-2-acetyl GPC-PAF(C18:0) (40%), rac PAF (29%), 1-Hexadecyl-2-lyso GPC-Lyso-PAF(C16:0) (<0.01%); $LTC_4/D_4/E_4$-assay: LTC_4 (100%), LTD_4 (100%), LTE_4 (70%), LTB_4 (0.4%) and prostaglandins (<0.006%); PGD_2-assay: PGD_2 (100%), PGJ_2 (7%), TxB_2 (0.3%), $PGF_{2\alpha}$ (0.04%) and other prostaglandins (<0.02%).

8.4 Statistical analysis

Statistical analysis was performed with the Friedman Two-way ANOVA followed by the Wilcoxon Matched-pairs Signed-ranks Test. The Kruskal-Wallis rank test was used for correlations. For testing equality of the carry-over effect, within-patient totals over the two treatment periods are used. There is said to be no significant carry-over effect if the means of these within-patient totals do not significantly differ between the two treatment-order groups. For this test a p-value < 10% is considered significant.

8.5 Results

Nasal mediator release
The levels of the inflammatory mediators, PAF, $LTC_4/D_4/E_4$, and PGD_2, in nasal washings from allergic patients to house dust mites with and without fluticasone propionate aqueous nasal spray [FPANS] are presented in Table 1. No significant carry-over effect was observed. The baseline-levels of the placebo-group and FPANS-group respectively are: PAF: 907 ± 177 [range 147-3172] and 780 ± 316 [range 95-7272] pg/ml; $LTC_4/D_4/E_4$: 112 ± 10 [range 37-233] and 106 ± 9 [range 10-209] pg/ml and PGD_2: 94 ± 26 [range 21-592] and 92 ± 30 [range 3-734] pg/ml. Because these baseline-levels are in a large range, the levels are recalculated in percent change to baseline.
Nasal challenge with house dust mite extract caused an immediate influx of these inflammatory mediators. After allergen provocation the levels of the mediators <u>increased</u> in the nasal fluids of placebo-treated patients (x-fold increase to baseline: PAF: 15; $LTC_4/D_4/E_4$: 12 and PGD_2: 1.5). In fluids of patients treated with FPANS these levels tended to decrease (x-fold increase to baseline: PAF: 6; $LTC_4/D_4/E_4$: 4 and PGD_2: 1.1). P-value between the placebo and FPANS-group after the primary trigger initiated after challenge with 10 000 BU/ml house dust mite extract of PAF: 0.2124, $LTC_4/D_4/E_4$: 0.1618 and PGD_2: 0.2227.

Table 1: Platelet activating factor (PAF), Leukotriene $C_4/D_4/E_4$ (LTC$_4$/D$_4$/E$_4$) and Prostaglandin D_2 (PGD$_2$) production in nasal lavages at the following time-points: allergen provocation with 10 000 BU/ml house dust mite (HDM) extract (t=0), 3.5 and 7.5 hours (late phase) of allergic patients treated with Placebo or with fluticasone propionate aqueous spray (FPANS). Because of scattered individual data values are expressed as % change of baseline ± S.E.M. Statistical significant decrease to the HDM 10 000 is shown as * according to the Wilcoxon Matched-pairs Signed-ranks Test (p≤0,05).

	HDM 10 000 BU/ml		3.5 hours		7.5 hours	
	Placebo	FPANS	Placebo	FPANS	Placebo	FPANS
PAF	1490±479	554±283	5± 19*	29± 37*	19±20*	1±28*
LTC$_4$/D$_4$/E4	1115±518	355±190	-4± 9*	-21± 7*	-6± 7*	-9± 9*
PGD$_2$	50± 26	8± 16	-42±11*	-47± 10*	-39± 8*	-29±12

At 3.5 and 7.5 hour after this challenge a significant <u>decrease</u> of PAF, LTC$_4$/D$_4$/E$_4$ and PGD$_2$ was observed in both groups compared with the value at the time-point of allergen provocation with 10 000 BU/ml house dust mite extract. The p-values of the placebo-group and the FPANS-group at 3.5 hour are respectively: PAF: 0.0004 and 0.0010, LTC$_4$/LTD$_4$/LTE$_4$: 0.0003 and 0.0003; PGD$_2$: 0.0126 and 0.0116. At 7.5 hour the p-values of the placebo-group and the FPANS-group are respectively: PAF: 0.0116 and 0.0184, LTC$_4$/LTD$_4$/LTE$_4$: 0.0300 and 0.0025; PGD$_2$: 0.0023 and 0.0936.
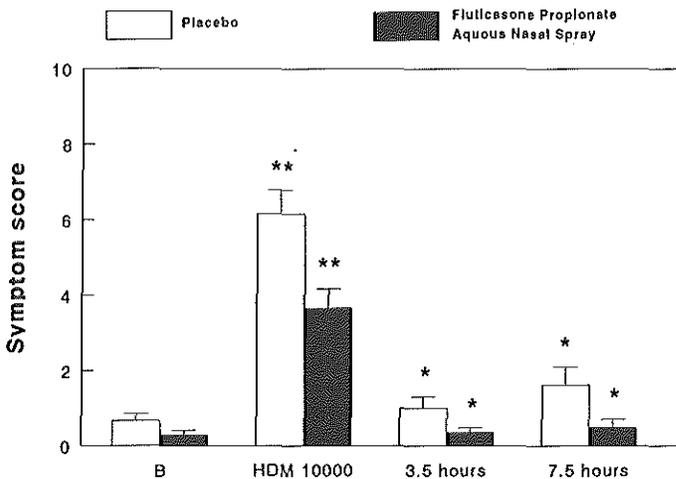
Figure 1.
Symptom score at the following time-points: baseline (B), allergen provocation with 10 000 BU/ml house dust mite extract (t=0), 3.5 and 7.5 hours (late phase) of allergic patients treated with or without FPANS. Values are expressed mean ± S.E.M. Statistical significant difference to the HDM 10000 is shown as * (p≤0.05). Statistical significant difference to the baseline is shown as ** (p≤0.05).

Because a significant carry-over effect was observed, only the results of the first treatment period was used. A significant _increase_ is observed immediately after the challenge with house dust mite extract in the placebo and FPANS-group. At 3.5 and 7.5 hours after this challenge a significant _decrease_ of the symptom score is observed as compared to the level at the time-point of the challenge in both groups (p=0.001). The symptom score of patients treated with FPANS is decreased in comparison to the placebo-group.

Correlation between inflammatory mediators and symptom score
A significant correlation (P≤0.05) is found immediately after the challenge with 10000 BU/ml house dust mite extract between: (1). the release of $LTC_4/D_4/E_4$ and PGD_2 in the placebo-group (correlation coefficient (c) = 0.656) and in the FPANS-group (c=0.776), (2). the release of PAF and $LTC_4/D_4/E_4$ in the placebo-group (c=0.719) and in the FPANS-group (c=0.990), (3). the release of PAF and PGD_2 after the administration of placebo (c=0.466) or FPANS (c=0.740), (4). the release of $LTC_4/D_4/E_4$ and symptom score in the placebo-group (c=0.547) and in the FPANS-group (c=0.545) and (5). the release of PAF and symptom score of the FPANS-group (c=0.598).

8.6 Discussion

Lavage of the nasal mucosa appears to be a convenient model for measuring inflammatory mediator release during an allergic reaction to the house dust mite.
In agreement with other investigators it was found that within a few minutes of exposure to an allergen leukotrienes and prostaglandins can be measured in nasal washings.[56,80,465,478,492,682]
This is the first study in which PAF could be measured in nasal lavages in detectable amounts seen within a few minutes after nasal provocation with _house dust mite_ extract. Other investigators found lyso-PAF but almost no PAF by bioassay in nasal washings after nasal challenge of patients with a _pollen_ allergy.[435,438] _In vitro_ studies have demonstrated that PAF is released by alveolar macrophages[16], eosinophils[375], monocytes and endothelial cells[87,88] and platelets[101]. It has now been demonstrated that PAF is present in nasal lavages of patients with house dust mite allergy; however, the origin of PAF is uncertain. In the early phase of the allergic process IgE crosslinks by antigen challenge on mast cells and basophils, which release primary mediators. After a quiescent period a late phase allergic reaction occurs, in which eosinophils and macrophages are involved, releasing secondary mediators.[463,465,466,625] It has been shown that PAF is released during the early phase reaction as primary mediator and not as a secondary mediator. The present study also shows that PGD_2 is released only during the early phase of the allergic process as a primary mediator. This is in agreement with other investigators, who found that PGD_2 is produced by mast cells.[52,679] Sulphidopeptide-leukotrienes are known to be released by eosinophils[448,575,677] and macrophages[32,64,498], which indicated that $LTC_4/LTD_4/LTE_4$ are secondary mediators. However, these sulphidopeptide-leukotrienes were released during the early phase reaction and not during the late phase reaction, which indicated that these sulphidopeptide-leukotrienes are also primary mediators. The generation of the mediators PAF, $LTC_4/D_4/E_4$ and PGD_2, reached baseline levels after 3.5 hrs. During the late phase reaction symptoms partially recurred, but surprisingly PAF, $LTC_4/D_4/E_4$ and PGD_2 were not

released again. This symptoms should be due to other mediators. Furthermore it was clearly demonstrated that the release of PAF, $LTC_4/D_4/E_4$ and PGD_2 correlated with each other immediately at the time-point of the nasal challenge with house dust mite extract. Previous reports of studies with patients allergic to grass-pollen and ragweed described a correlation between $LTC_4/D_4/E_4$ and PGD_2.[223,373]

We also found a significant correlation between the release of $LTC_4/D_4/E_4$ and PAF with the symptom score at the time-point of challenge, but not between the release of PGD_2 and the symptom score. However, Lebel et al.[373] who studied patients with a grass-pollen allergy observed that the release of PGD_2 was well correlated with the symptom score.

In this study pretreatment of the patients with FPANS for two weeks twice daily greatly reduced the development of symptoms. In a study performed with 17 atopic patients, during a 2 weeks pretreatment with FPANS 200 µg/day the immediate increase in nasal airway resistance was not inhibited.[619] In another study the dose of ragweed pollen required to produce a standardised response was unchanged after 4 weeks of treatment with FPANS 200 µg/day in 49 patients during the ragweed season.[587] However, FPANS improved the symptom score after 2 and 4 weeks of treatment in 24 patients with seasonal allergic rhinitis after nasal challenge with allergen.[295]

It has been suggested that the number of eosinophils and basophilic cells (basophils and mast cells) increase following allergen challenge and that this factor is responsible for the initiation of the allergic vascular response.[301,514] Treatment with FPANS 50 to 800 µg/day administered for 2 weeks to 6 months was associated with a significant decrease in the number of nasal eosinophils, basophils and neutrophils compared with placebo patients with seasonal allergic rhinitis.[433] It has been proposed that FPANS may act by preventing activation of several cells and subsequent release of inflammatory mediators.[435]

The present findings indicate that FPANS reduces not only the allergen-induced symptoms (ratio placebo/FPANS: 1.68) but also tended to reduce the release of PAF (ratio placebo/FPANS: 2.69), $LTC_4/D_4/E_4$ (ratio placebo/FPANS: 3.14) and PGD_2 (ratio placebo/FPANS: 6.25) after the primary trigger initiated after challenge with 10000 BU/ml house dust mite extract at the time-point of challenge.

In conclusion, the results indicate that the inflammatory mediators platelet activating factor, leukotriene $C_4/D_4/E_4$ and prostaglandin D_2, can be used as markers of allergic reactions to house dust mites and that fluticasone propionate aqueous nasal spray counteracts the release of mediators of inflammation, correlated with beneficial effects on clinical symptoms in this type of allergic reaction.

8.7 Acknowledgements

Chapter 9
Interleukin-5 and eosinophil cationic protein in nasal lavages of rhinitis patients

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9.1 Abstract

Objective: The production of interleukin-5 and eosinophil cationic protein (ECP) in the nasal cavity was examined in 24 patients with rhinitis who were allergic to the house dust mite.
Methods: During a double-blind placebo-controlled cross-over study, fluticasone propionate aqueous nasal spray (200 µg) was administered twice daily for two weeks. After four basal nasal lavages provocation with house dust mite extract was performed and nasal lavages were collected every hour for 9.5 h.
Results: Interleukin-5 was present in detectable amounts in nasal lavages from patients allergic to house dust mite. Nasal challenge with house dust mite extract caused immediate nasal symptoms and increased levels of interleukin-5. Between 3.5 and 8.5 hours after the challenge symptoms recurred and interleukin-5 levels increased, reflecting a late phase reaction. Eosinophil cationic protein, a marker of activated eosinophils, was released between 6.5 and 9.5 hours after challenge.
Conclusion: Treatment with fluticasone propionate (as an aqueous nasal spray) significantly decreased the evoked interleukin-5 and ECP levels in the late phase reaction. This response was correlated with an improved symptom score. This could indicate that the number and activity of eosinophils are increased during the late phase allergic reaction, a response that is inhibited by corticosteroids.

Keywords: interleukin-5; eosinophil cationic protein (ECP); house dust mite; fluticasone propionate aqueous nasal spray; rhinitis

9.2 Introduction

Human allergen-induced responses in the nose are used as a model for allergic inflammation.[230,465] After provocation with antigen, mediators, such as prostaglandin D_2, leukotriene E_4, tryptase and histamine, are released during the so-called early allergic reaction, which causes sneezing, nasal blockage and rhinorrhoea. After a quiescent period, a late allergic reaction occurs and symptoms recur.[309,465,505] Studies on human nasal late responses have shown an influx of eosinophils and an increase in the content of eosinophil cationic protein (ECP) in nasal fluids 3-11 hours after challenge. The presence of mononuclear cells in lavage fluid during the late responses has also been recognized.[309] Several studies have suggested a role for cytokines, particularly granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 and interleukin-5, in the initiation of allergic inflammation. These cytokines promote the survival and activation of eosinophils *in vitro*, enhance adhesion to microvascular endothelial cells *in vitro* and induce eosinophilia *in vivo*. Mosmann and colleagues described two classes of T helper lymphocytes on the basis of their profile of cytokine release. T_{H1}-cells produce interleukin-2, interleukin-3, interferon-γ and GM-CSF but no interleukin-4 or interleukin-5, whereas T_{H2} clones produce interleukin-3, interleukin-4, interleukin-5, interleukin-10 and GM-CSF but no interleukin-2 or interferon-γ.[452] However, the exact cells from which GM-CSF, interleukin-4 and interleukin-5 are released remain to be determined, because other studies have shown that mast cells also produce interleukin-4 and interleukin-5 whereas eosinophils may produce GM-CSF and interleukin-5.[689]

In our study, the nasal challenge model developed by Naclerio *et al*.[465] was used to correlate clinical symptoms and the release of biochemical markers such as interleukin-5 and ECP and to establish whether these markers are formed predominantly in the early or late phase of the allergic process in patients with allergic rhinitis. Furthermore, the effect of fluticasone propionate, a new and potent corticosteroid, on the levels of interleukin-5, ECP and on symptom scores after nasal challenge with allergen was investigated.

9.3 Materials and methods

Patients
This study was performed with 24 patients. (11 women and 13 men, aged 21 to 50 years, mean: 34 years). All patients had a history of perennial rhinitis and a skin reaction to extract of house dust mite as an inclusion criterion. The skin reaction was rated as at least one "+"-sign in response to 0.3 or 3 biological units (BU)/ml extract, according to the standardized plus-sign scoring system. Six of the 24 patients were also allergic to grass pollen or animal dander. The nasal lavage experiments were performed in January-August to minimize exposure to house dust mite. The patients with a concomitant pollen allergy were tested outside the pollen season. None of the patients allergic to animals had pets in their home. Anti-histaminic drugs were withdrawn 72 hours before testing. The antihistamine astemizole, topical corticosteroids, cromoglycate or nedocromil were not used for 3 weeks before the start of the study and oral corticosteroids were withdrawn at least 2 months before the study. Patients who developed a nasal infection during the 2-week period prior to the study were excluded. None of the patients had immunotherapy previously.

The study was approved by the Medical Ethics Committee of the University Hospital Rotterdam-Dijkzigt and all patients gave written informed consent.

Nasal challenge and lavage
After the positive skin test the subjects entered the double blind placebo-controlled cross-over phase of the study. Each underwent two allergen challenges, performed after a twice daily pretreatment with 200 µg fluticasone propionate (aqueous nasal spray (Glaxo, U.K.)) or placebo spray for 2 weeks. A three-weeks washout period separated the two treatment periods.
Before nasal challenge with house dust mite extract, a nasal lavage was performed 4 times to obtain baseline levels and to clear the nose of secretions (b). To prevent nasal congestion, 0.25 ml oxymetazoline (0.1%) was sprayed into each nostril 5 min before the first challenge (prescribed by the Hospital Ethical Committee). Nasal lavage was performed as described before.[219,230,465] Both nostrils were washed with 5 ml saline, prewarmed to 37°C. Lavage fluid was collected in plastic tubes and kept on ice. After centrifugation (10 min, 400 x g) the supernatants of lavage fluids were stored at -20°C until determinations. Due to the low number of cells in the lavages we were not able to count the number of eosinophils and perform statistical analysis. To obtain a control challenge (c), 0.125 ml phosphate-buffered saline (containing 0.03% human serum albumin and 0.05% benzalkonium chloride) was sprayed in each nostril and a nasal lavage was performed. For allergen challenge 0.125 ml allergen extract was sprayed in each nostril and 10 min thereafter a nasal lavage was performed. Allergen doses of 100, 1000, 10 000 (h1-h3) BU/ml (extract of D. Pteronyssinus; ALK, Groningen, The Netherlands) were administered. From 30 min up to 9.5 hours after the last challenge (given at h3) the nasal washings were collected every hour by washing both nostrils with saline (s1-s10).

Symptom Score
Symptoms were scored to assess the correlation between clinical symptoms and interleukin-5 production. The score was compiled before each lavage and after phosphate-buffered saline challenge and after each allergen spray. Symptom scores were graded in points: 3-4 sneezes = 1 point; ≥5 sneezes = 3 points; rhinorrhoea anterior = 1 point; rhinorrhoea posterior = 1 point; difficult nasal breathing = 1 point; one nostril blocked = 2 points; both nostrils blocked = 3 points; pruritus of the nose = 1 point; pruritus of palate or ear = 1 point and conjunctivitis = 1 point (total score ranged from 0 to 11 points).

Interleukin-5 assay

Material
Tween 20 (Merck, Darmstadt, Germany); bovine serum albumin fraction V (Euromex, Schiltigheim, France); horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) F(ab')₂ (Biosys, Compiègne, France); TMB microwell peroxidase substrate system (Kirkegaard & Perry Lab., Gaithersburg MD, USA); polyclonal rabbit anti-human-interleukin-5 (Genzyme, Cambridge, MA, USA); anti-human-interleukin-5 monoclonal antibody and recombinant human-interleukin-5 were kindly provided by Drs R. Devos and J. Tavernier (Roche, Gent, Belgium).

Method
ELISA plates (96-well Immuno Plate MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100 μl of the anti-human-interleukin-5 monoclonal antibody (1μg/ml in phosphate-buffered saline, pH7.4) at 37°C for 2 h. The wells were washed 5 times with phosphate-buffered saline containing 0.1 % Tween 20. Then, 100 μl of the sample or recombinant-human-interleukin-5 standard (0.2-200 ng/ml) in phosphate buffered saline-Tween and 3 % bovine serum albumin was added to each well and incubated at 37°C for 1 h. The wells were washed 3 times with phosphate-buffered saline-Tween and incubated with polyclonal rabbit anti-human-interleukin-5 (10 μg/ml in phosphate-buffered saline-Tween-bovine serum albumin) at 37°C for 1 h. After the wells were washed 3 times with phosphate-buffered saline-Tween, horseradish peroxidase-conjugated goat anti-rabbit IgG F(ab')$_2$, at a dilution of 1/4000 in phosphate-buffered saline-Tween-bovine serum albumin, was added for 1 h. Then, 100 μl of freshly prepared substrate solution (TMB microwell peroxidase substrate) was added to each well. After a 10-min incubation, the reaction was stopped by the addition to each well of 100 μl of a 2 M solution of HCl. Absorbance was read at 450 nm. The lower limit of sensitivity of the assay was 0.18 ng/ml.

Eosinophil Cationic Protein assay
ECP was determined in the lavages by radioimmunoassay (Kabi Pharmacia Diagnostics, Uppsala, Sweden).

9.4 Statistical analysis

Statistical analysis was performed with the Friedman two-way analysis of variance (ANOVA) followed by the Wilcoxon matched-pairs signed-ranks test. The effect of fluticasone propionate nasal spray on the early phase reaction (area under the curve (AUC) between h1 and s1) and the late phase reaction (AUC: between s4 and s10) was assessed. These areas under the curve are based on the outcome of the symptom score. A two-tailed P value ≤ 0.05 was considered significant.

9.5 Results

Eleven patients (out of 24) showed *both an early and a late phase* reaction to the house dust mite extract, as assessed by their symptom scores (Fig. 1a: Symptom score, Fig. 1b: interleukin-5 and Fig. 1c: ECP), whereas the other 13 patients showed *only an early phase* reaction (Fig. 2).

Symptom score
The effects of placebo and fluticasone propionate nasal spray on the symptom scores of the 11 patients who showed *both an early- and a late phase* reaction to the house dust mite with treatment are shown in Fig. 1a. A significant increase was observed immediately after the challenge with house dust mite extract in comparison with baseline levels in the placebo group (h1-s3; P≤0.05) and in the fluticasone group (h2 and h3; P≤0.05). A late phase reaction occurred only in the placebo group, when symptoms recurred (s6-s9;

P≤0.05). The symptom score of patients treated with fluticasone propionate nasal spray was significantly lower than that of the placebo group in the early phase reaction (AUC h1-s1; P≤0.05) as well as in the late phase reaction (AUC s4-s10; P≤0.05).
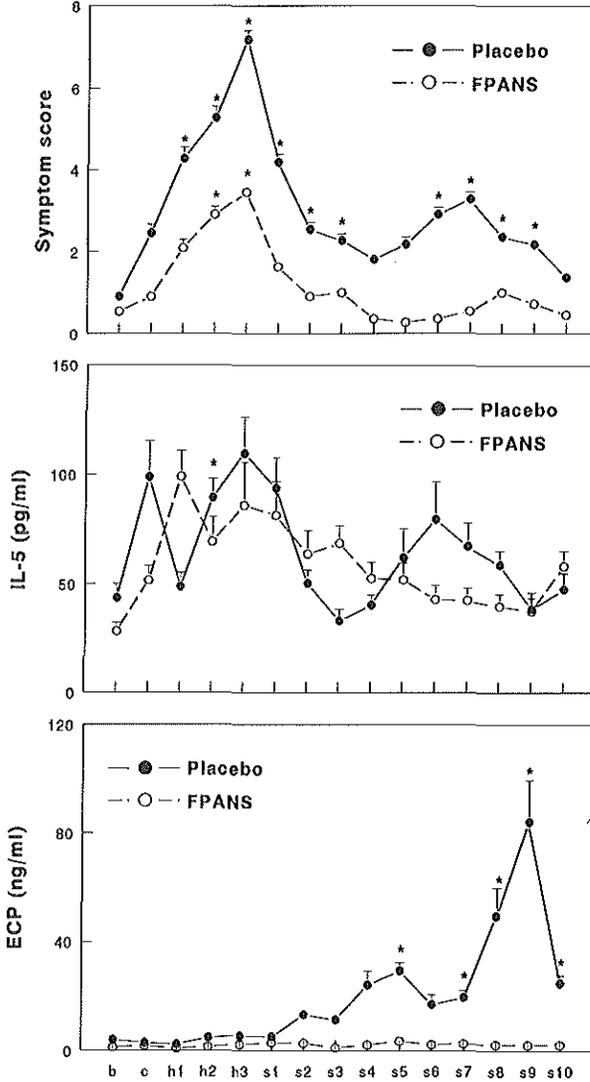
Figure 1. Effect of placebo and fluticasone propionate aqueous nasal spray on the symptom scores, interleukin-5 levels in nasal lavages and eosinophil cationic protein levels in nasal lavages of the 11 patients allergic to house dust mite who showed *both an early and a late phase reaction.* Values are expressed as means ± S.E.M. Significant differences in comparison to baseline: *, P≤0.05. b: baseline, c: challenge with phosphate-buffered saline; h1,h2 and h3, challenge with 100, 1000 and 10000 BU/ml house dust mite extract respectively; s1-s10, lavage performed every hour after the challenge with 10000 BU/ml house dust mite extract at h3.

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The results of the 13 patients who showed *only an early phase* reaction are shown in Fig. 2. A significant increase was observed immediately after the challenge with house dust mite extract in comparison with the baseline levels in the placebo group (h1-s1; $P \leq 0.05$) and in the fluticasone group (pbs-s3; $P \leq 0.05$). No late phase reaction occurred. The symptom score of patients treated with fluticasone propionate nasal spray was significantly lower than that of the placebo group in the early phase reaction (AUC h1-s1; $P \leq 0.05$).

Nasal interleukin-5 release
The effects of placebo and fluticasone propionate nasal spray on the levels of interleukin-5 in nasal washings from the 11 patients with *both an early and a late phase* allergic reaction to house dust mite are presented in Fig. 1b. Interleukin-5 levels varied between 50 and 100 pg/ml before provocation, with a tendency towards an increase in comparison with the baseline level in the placebo group (h2; $P = 0.0702$), a tendency which was not seen in the fluticasone propionate group. During the late phase reaction interleukin-5 was released again in the placebo group. Fluticasone propionate nasal spray significantly inhibited the release of interleukin-5 in the late phase reaction in comparison with placebo (AUC s4-s10; $P \leq 0.05$).
The 13 patients with *only an early phase* allergic reaction to house dust mite who were treated with placebo or fluticasone propionate nasal spray showed neither an immediate nor a late phase release of interleukin-5 (Fig. 2).

Eosinophil Cationic Protein
The effects of placebo and fluticasone propionate nasal spray on the ECP levels of the 11 patients who showed *both an early and a late phase* reaction to house dust mite are shown in Fig. 1c. A significant increase in ECP was observed in comparison with the baseline levels during the late phase reaction (s7-s10; $P \leq 0.05$). The ECP level in nasal lavages was significantly decreased by treatment with fluticasone propionate nasal spray in comparison with those of the placebo during the late phase (AUC s4-s10; $P \leq 0.05$).[251]
The 13 patients with *only an early phase* allergic reaction to house dust mite did not show a late phase release of ECP (all levels were below 10 ng/ml; Fig. 2) after placebo or fluticasone propionate.

9.6 Discussion

This is the first study demonstrating that interleukin-5 is present in detectable amounts in nasal lavage obtained from patients allergic to house dust mites who show both an early and late phase reaction. Nasal challenge with house dust mite extract immediately caused nasal symptoms and tended to increase levels of interleukin-5 in the placebo group in the early phase reaction. Nasal challenge with phosphate-buffered saline also caused an immediate increase in the level of interleukin-5 in the placebo group, which may indicate a nonspecific reaction due to the human serum albumin in the phosphate-buffered saline. Between 3.5 and 8.5 hours after the challenge with house dust mite extract symptoms recurred and interleukin-5 levels increased, reflecting a late phase reaction.
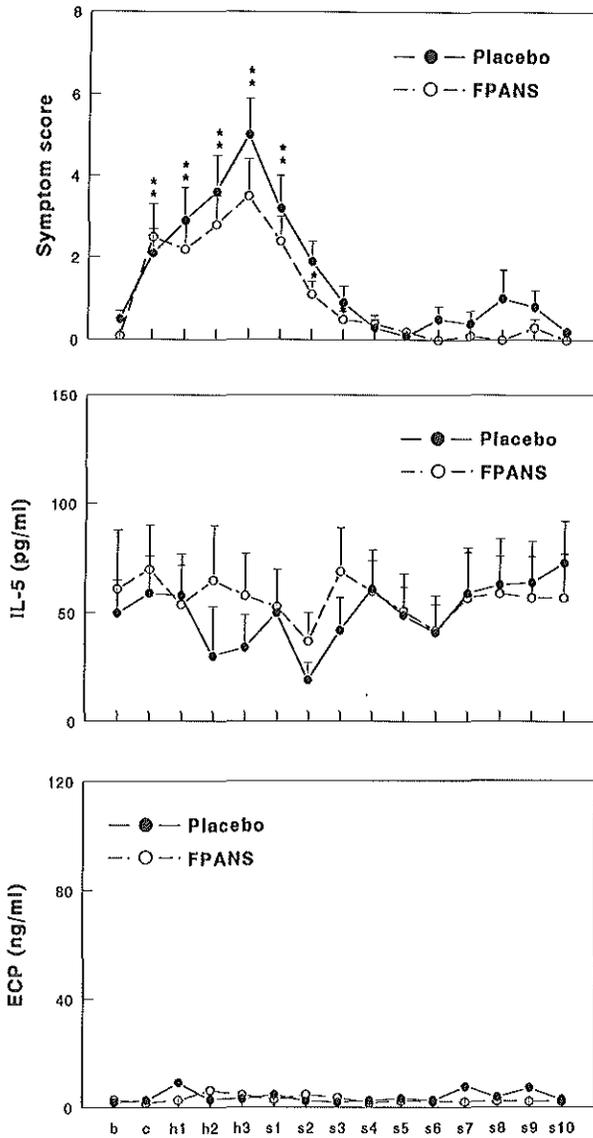
Figure 2. Effect of placebo and fluticasone propionate aqueous nasal spray on the symptom scores, and interleukin-5 and eosinophil cationic protein levels in nasal lavages of the 13 allergic patients to house dust mite who showed *only an early phase reaction*. Values are expressed as means ± S.E.M. Significant differences in comparison to baseline: *, P≤0.05. b: baseline, c: challenge with phosphate-buffered saline; h1,h2 and h3, challenge with 100, 1000 and 10000 BU/ml house dust mite extract respectively; s1-s10, lavages performed every hour after the challenge with 10000 BU/ml house dust mite extract at h3.

Interleukin-5 was released in the lungs of rhinitis patients allergic to ragweed 24 hours after segmental antigen challenge, and this release correlated with eosinophil recruitment, degranulation, and lung injury.[484] In inferior turbinate biopsies from patients with perennial allergic rhinitis interleukin-4 and interleukin-5 were localized by immunohistochemical staining of mast cells and interleukin-5 immunoreactivity was also localized in eosinophils. No immunoreactivity for cytokines was found in T-cells.[73] In studies with grass pollen-sensitive patients 24 hours after local provocation with antigen, nasal biopsies showed an increase in cells with positive mRNA hybridization signals for interleukin-3, interleukin-4, GM-CSF and interleukin-5[163] when allergen-challenged sites were compared with control sites. It has been recently demonstrated by Terada *et al.* that interleukin-5 is released in nasal fluid in the late phase reaction after challenge with Japanese cedar.[615]

In this study we demonstrated that ECP was released a few hours later (between 6.5 and 9.5 h) than interleukin-5 (between 3.5 and 8.5 h), which may indicate that the number of eosinophils was increased and/or that the eosinophils present were more activated during the late allergic reaction. Due to the low numbers of cells in the lavages we were not able to perform statistical analysis of eosinophil numbers.

Our study showed a significant decrease in interleukin-5 and ECP levels during the late phase reaction after treatment with fluticasone propionate nasal spray. This decrease correlated with an improved symptom score in the 11 patients with an early and late phase reaction. A decreased release of ECP may indicate that the number of eosinophils was decreased and/or that the eosinophils were less active during the late allergic reaction after treatment with fluticasone propionate nasal spray. The protective effects of fluticasone propionate nasal spray on interleukin-5 were detected in the late phase reaction and failed to affect the initial (baseline) release of interleukin-5 as well as the early phase response to house dust mite provocation. Other factors involved in allergic rhinitis, such as albumin, tryptase, leukotriene E_4, prostaglandin D_2 and platelet activating factor, are also increased after house dust mite provocation and are inhibited by fluticasone propionate nasal spray.[219,251] Pretreatment with fluticasone propionate nasal spray improved the symptom score of patients with seasonal allergic rhinitis after nasal challenge with allergen and decreases the number of nasal eosinophils, basophils and neutrophils.[333] It has been proposed that fluticasone propionate nasal spray may act by preventing the activation of these cells and the subsequent release of (chemotactic) inflammatory mediators and further influx of cells. Several *in vitro* studies have shown that corticosteroids inhibit T-cell proliferation and cytokine production[135], decrease the number of cells expressing interleukin-4 and interleukin-5 mRNA *in vivo* [542] and decrease the number and activation of mast cells and eosinophils in bronchial biopsies and lavages.[157]

In summary, the results of this study show that interleukin-5 was found in detectable amounts in nasal lavages of patients with a house dust mite allergy before and after challenge with house dust mite extract, which caused immediately nasal symptoms. Between 3.5 and 8.5 hours after the last challenge symptoms recurred and interleukin-5 levels increased, reflecting a late phase reaction. ECP was released between 6.5 and 9.5 hours after challenge. A two-week pretreatment with 200 µg fluticasone propionate aqueous nasal spray resulted in a significant decrease in interleukin-5 and ECP levels in the late phase reaction. This decrease was correlated with an improved symptom score.

Although it is not fully understood how the mechanism of interleukin-5 release is modulated, the programmed release of this cytokine would be of interest in other allergic

reactions.

9.7 Acknowledgements

Chapter 10
Nitric oxide metabolites in nasal lavage fluid of patients with house dust mite allergy

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10.1 Abstract

Objective: The role of nitric oxide in the early and late phase of the allergic process was investigated in patients with allergic rhinitis against house dust mite and the effect of fluticasone propionate aqueous nasal spray was determined.

Methods: Production of nitric oxide (measured as nitrite+nitrate) *in vivo* in nasal mucosa was examined in 24 patients with rhinitis allergic to the house dust mite. In a double blind placebo controlled crossover study fluticasone propionate 200 μg aqueous nasal spray was administered twice daily for two weeks. In response to provocation with house dust mite extract (after four basal nasal lavages) nasal lavages were performed every hour for 9.5 hours by washing the nose with saline. In addition, a similar lavage protocol was performed in healthy volunteers with or without challenge with phosphate buffered saline.

Results: Nitric oxide is present in nasal lavage fluid in detectable amounts (range 10-50 μM), the level gradually increasing with time in both patients and controls after a decrease during the four basal lavages. Treatment with fluticasone propionate aqueous nasal spray did not affect initial basal production of nitric oxide nor production following provocation with house dust mite extract.

Conclusion: Production of nitric oxide in nasal mucosa determined in sequential nasal washings is not affected by therapeutic doses of intranasal steroids.

Keywords: nitric oxide; house dust mite; fluticasone propionate aqueous nasal spray

10.2 Introduction

House dust mites are the major cause of perennial rhinitis. The pathophysiology of allergic rhinitis, however, has been studied mainly in pollen allergy.[343,451,651] To study the role of inflammatory mediators in ragweed pollinosis Naclerio et al developed a control model.[465] After provocation with antigen mediators such as prostaglandin D_2 (PGD_2), leukotriene E_4 (LTE_4), tryptase, and histamine are released in the early phase of the allergic process, causing sneezing and rhinorrhoea. After a quiescent period a late phase occurs and symptoms recur when mediators are again released.[465,466,463,625]

It has recently been claimed that nitric oxide (NO) is an important mediator in bronchial inflammation.[36,145,161,328,387,631] NO synthase (NOS) is the enzyme responsible for the generation of nitric oxide from L-arginine. The highly reactive and unstable nitric oxide rapidly decomposes to nitrogen oxides such as nitrite and nitrate.[443] Nitric oxide synthase present in various cells from different (embryological) origin exists in two different forms[444] -the constitutive, calcium dependent enzyme (cNOS) which releases nitric oxide after stimulation and the inducible, calcium independent enzyme (iNOS) which is found after induction with cytokines.[326,443,444]

The function of nitric oxide in the bronchoalveolar compartment may be either brochodilatory (nitric oxide gas retains bronchodilating proporties) or regulatory. Little is known of the regulatory function of nitric oxide.

In patients challenged with pollen antigens topical nasal steroids such as flunisolide reduce symptoms and mediator release in both the early and late phases of the allergic process.[463,516] The corticosteroid fluticasone propionate has potent topical anti-inflammatory activity coupled with low systemic activity.[85,510]

Our study design is based on the nasal challenge model developed by Naclerio et al[465] which enables us to explore the role of nitric oxide in the early and late phase of the allergic process in patients with allergic rhinitis against house dust mites. The effect of fluticasone propionate aqueous nasal spray on the levels of nitrite + nitrate after nasal challenge with house dust mite extract was investigated and the effect of challenge with phosphate buffered saline on normal levels of nitrite + nitrate in healthy volunteers was determined.

10.3 Materials and Methods

House dust mite experiment

Patients
The study was performed in 24 patients (13 men) of mean age 34 (range21-50) years with a history of perennial rhinitis and skin reaction to house dust mite extract. All patients showed a skin reaction rated as at least one plus-sign to 0.3 or 3 biological units (BU)/ml extract according to the standardised plus-sign scoring system defined by Norman.[477] Six of the 24 patients were also allergic to grass pollen or animal epithelia. The nasal lavage experiments were performed between January and August to minimise exposure to house dust mites. The patients with a concomitant pollen allergy were tested outside the pollen season. None of the patients allergic to animals had pets in their home. Anti-histamine

drugs were withdrawn 72 hours before testing. The antihistamine astemizole, systemic corticosteroids, and topical corticosteroids were not used later than three weeks, two months, and three weeks respectively before the tests were performed. Patients who developed a nasal infection during the two weeks period before the study were excluded. None had received immunotherapy.

The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam-Dijkzigt and all patients gave written informed consent.

Nasal challenge and lavage

After the positive skin test the subjects entered the double blind, placebo controlled, crossover phase of the study. Each underwent two allergen challenges performed after pretreatment with fluticasone propionate aqueous nasal spray (Glaxo,UK), 200 µg twice daily for two weeks, or a placebo spray. A three weeks washout period separated the two challenges.

Before nasal challenge with house dust mite extract a nasal lavage was performed four times to obtain baseline mediator levels (b1-b4) by the method described by Naclerio *et al.*[465] Both nostrils were washed with 5 ml saline prewarmed to 37°C. Lavage fluid was collected in plastic tubes and kept on ice. After centrifugation (10 min, 400 g) supernatants of lavage fluids were stored at -20°C until assay of NOx. To prevent nasal congestion 0.25 ml oxymetazoline (0.1%) was sprayed into each nostril five minutes before the first challenge. To obtain a control challenge 0.125 ml phosphate buffered saline (PBS) was sprayed into each nostril and a nasal lavage was performed. For allergen challenge 0.125 ml allergen extract was sprayed into each nostril and a nasal lavage was performed after 10 minutes. Allergen doses of 100, 1000, 10 000 (h1-h3) Biological Units (BU)/ml (ALK, Groningen, The Netherlands) were administered. From 30 minutes up to 9.5 hours after the last challenge (time point h3) the nasal washings were performed every hour by washing both nostrils with saline (s1-s10).

Symptom Score

Symptoms were scored to study the correlation between clinical symptoms and production of nitric oxide as described by Lebel *et al.*[373] The score was compiled before each lavage and after PBS and each allergen insufflation.

Control experiments with healthy volunteers

Two experiments were performed

Experiment 1: Nasal challenge with PBS and nasal lavage

This experiment was performed with 11 healthy volunteers (six men) of mean age 31 (range 25-40) years. The same protocol as described above for patients was used, but instead of nasal challenges with house dust mites extract, challenges with PBS were performed. From 30 minutes up to 7.5 hours after the last challenge (time-point h3) the nasal fluid was obtained by washing the nose with saline.

Experiment 2: Nasal lavage without challenge
This experiment was performed with six healthy volunteers (three men) mean age 29 (range 25 - 41) years. The same protocol as with healthy volunteers as described in experiment 1 was followed but without PBS challenge.

Nitric oxide assay
The nitric oxide metabolites nitrate (after reduction to nitrite) + nitrite were assayed colorimetrically after the Griess reaction as described by Phizackerly and Al-Dabbagh[512] with the following modifications:
Collection and deproteinization of samples:
200 µl of nasal lavage fluid or standard was deproteinated by adding 20 µl NaOH (1.0 M, 4°C; Merck, Germany) and 30 µl $ZnSO_4$ (1.3 M, 4°C; Merck, Germany). Samples were mixed and allowed to stand on ice for 15 minutes.
Conversion of nitrate to nitrite:
After centrifugation (5 min, 4°C, 2600 g) 100 µl of the supernatant was mixed with 10 µl Klebsiella-pneumoniae-suspension (7.5 mg/ml protein), 20 µl 0.2 M N-tris (hydroxy-methyl)-methyl-2-amino-ethane sulfonic acid (TES, pH7.0; Sigma, U.S.A.) and 20 µl 0.5 M sodium formate (Merck, Germany). After 20 minutes anaerobic incubation at room temperature for 20 minutes 1.0 ml water was added to the samples and nitrite was assayed in supernatants obtained by centrifugation (5 min, 2600 g).
Estimation of nitrite:
Deproteinised samples or standard (200 µl) were mixed with 20 µl 1% sulphanilamide (Sigma, U.S.A.) in 15% phosphoric acid (Merck, Germany). After 10 minutes 20 µl 0.1 % N-(1-naphtyl)ethylenediamine (Sigma, U.S.A.) was added and the absorption at 540 nm was determined (595 nm was used as a reference wavelength).

10.4 Statistical analysis

The within patient totals over the two treatment periods were used to test equality of the carry-over effect. Thirteen of the 24 patients received fluticasone propionate aqueous nasal spray during the first treatment period and 11 patients during the second treatment period. It was assumed that no significant carry-over effect occurred if the means of these within patient totals were not significantly different in the two treatment groups. For this test a p-value < 10% was considered significant. In case of a carry-over effect, only the data of the first treatment period were used for statistical analysis. Values are expressed as means (SE).
Statistical analysis was performed with the non-parametric Mann-Witney U test (for comparison of placebo and fluticasone treatment) and the Wilcoxon unpaired Test (for comparison between patients and controls).

10.5 Results

House dust mite experiment

Symptom score
Symptom scores are shown in the Table 1. Because a significant carry-over effect was observed, only the results of the first treatment period were used. A significant increase was observed immediately after the challenge with house dust mites extract in both groups. A late phase reaction occurred in both groups, after which symptoms recurred. The symptom score of patients treated with fluticasone propionate aqueous nasal spray was significantly decreased in comparison with the placebo group at all time points examined.

Table 1: Mean (SE) symptom scores of patients allergic to house dust mites treated with placebo or with fluticasone propionate aqueous nasal spray. b=baseline before challenge; PBS=lavage after challenge with phosphate buffered saline; h1,h2 and h3=lavages after challenge with resp. 100, 1000 and, 10 000 biological units (BU)/ml house dust mite extract; s1-s10=lavages obtained every hour after the challenge with 10 000 BU/ml house dust mite extract.

	Placebo	Fluticasone propionate
Baseline	0.67 (0.19)	0.29 (0.13)
PBS	2.25 (0.48)	1.79 (0.49)
h1	3.50 (0.61)	2.00 (0.46)
h2	4.38 (0.69)	2.79 (0.47)
h3	6.17 (0.63)	3.67 (0.51)
s1	3.58 (0.55)	2.00 (0.41)
s2	2.13 (0.41)	1.04 (0.28)
s3	1.54 (0.37)	0.71 (0.19)
s4	1.00 (0.30)	0.35 (0.14)
s5	1.04 (0.36)	0.26 (0.14)
s6	1.58 (0.39)	0.22 (0.13)
s7	1.71 (0.44)	0.30 (0.13)
s8	1.63 (0.47)	0.48 (0.23)
s9	1.79 (0.45)	0.48 (0.19)
s10	0.71 (0.25)	0.22 (0.13)

Nasal nitric oxide levels

The levels of nitric oxide in nasal washings from patients allergic to house dust mite treated with fluticasone propionate aqueous nasal spray and with placebo are presented in the Figure 1 (part A). No significant carry-over effect was observed, but because a significant carry-over effect was observed for the symptom score only the results of the first treatment period were used. The levels of nitric oxide decreased significantly after the four basal washings. Nasal provocation with house dust mite extract caused no immediate release of nitric oxide but levels increased during the hours thereafter. No effect of fluticasone propionate on production of nitric oxide was observed in comparison with the placebo-group, nor was there any effect on the nitric oxide content in the first lavage sample (b1).

Control experiments

The levels of nitric oxide in nasal washings from healthy volunteers, with and without nasal challenge with PBS, (Figure 1, part B) were significantly decreased after the four basal washings. Nasal challenge with PBS caused no immediate release of nitrix oxide but levels increased during the subsequent hours in controls both with and without PBS challenge.

10.6 Discussion

Lavage of the nasal mucosa after nasal challenge appears to be a convenient model for the determination of inflammatory mediator release during an allergic reaction to house dust mite. Exposure to the antigen in the nose triggers the formation of IgE antibodies which bind to receptors on several cells in the respiratory epithelium in the nasal cavities. These antibodies crosslink to the antigen, and inflammatory mediators such as histamine, tryptase, leukotriene E_4, and eosinophil cationic protein are released. It has been suggested that the number of eosinophils and basophilic cells (basophils and mast cells) increase following allergen challenge and that this factor is responsible for the initiation of the allergic vascular response.[301,514]

Our results show that nitric oxide (nitrite + nitrate) is detectable in nasal lavage fluid and shows little individual variation. Levels of nitric oxide decreased significantly during the first four basal lavages, indicating that nitric oxide present in the nostrils is washed out. Nasal challenge with house dust mite extract in patients with a history of perennial rhinitis caused no immediate synthesis of nitric oxide but the levels gradually increased during the hours after the challenge. A similar increase was also observed in the lavage fluid of the healthy volunteers, with or without challenge or challenge with PBS. Sequential nasal washings probably initiate migration of monocytes from the circulation to the nasal compartment or (re)activation of the monocytes present in the nasal mucosa. These monocytes may well be responsible for the gradual increase in endogenous nasal production of nitrix oxide observed. Another possible explanation is plasma exudation of nitric oxide.
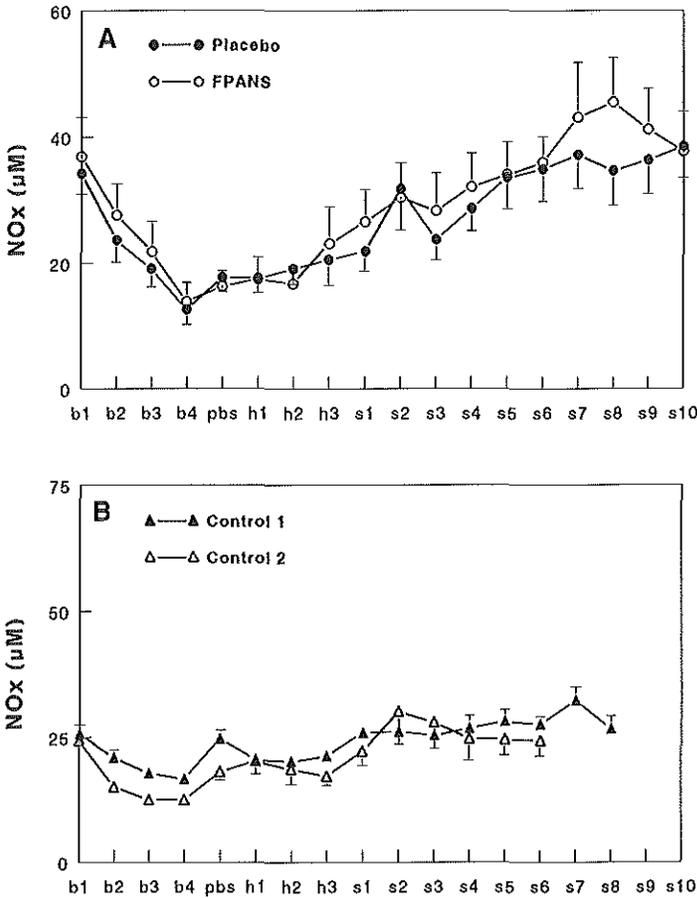
Figure 1. (A) Mean (SE) levels of nitric oxide metabolites in nasal lavage fluid of patients allergic to house dust mite treated with placebo or with fluticasone propionate aqueous nasal spray (FPANS).
b1-b4=four basal lavages; PBS=lavage after challenge with phosphate buffered saline; h1, h2 and h3=lavages after challenge with 100, 1000, and 10 000 BU/ml house dust mite extract respectively; s1-s10=lavages obtained every hour after the challenge with 10 000 BU/ml house dust mite extract (time-point: h3).
(B) Mean (SE) levels of nitric oxide metabolites in nasal lavage fluid of controls. b1-b4=four basal lavages, PBS, h1, h2 and h3=lavage after challenge with (control 1) or without (control 2) phosphate buffered saline; s1-s10=lavages obtained every hour after time point h3.

In another study we have measured plasma levels of nitric oxide in healthy volunteers by the same method and have found levels of 24.5 (3.4) μM.
In contrast, in the same 24 patients levels of albumin, tryptase, leukotriene E_4, prostaglandin D_2, and platelet activating factor increased immediately after provocation with house dust mite allergen (early phase reaction) and during the late phase reaction

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eosinophilic cationic protein is released. These mediators were inhibited by treatment with fluticasone propionate aqueous nasal spray.[219,251]

Pretreatment of the patients with fluticasone propionate aqueous nasal spray twice daily for two weeks greatly reduces or prevents the major events in the allergic process - the development of symptoms and release of inflammatory mediators. In a study performed in 17 atopic patients the immediate increase in nasal airway resistance was not inhibited by two weeks pretreatment with intranasal fluticasone propionate 200 µg/day.[619] In another study the dose of ragweed required to produce a standardised response was unchanged after four weeks of treatment with intranasal fluticasone propionate 200 µg/day in 49 patients during the ragweed season.[587] However, in 24 patients with seasonal allergic rhinitis the symptom score was improved after nasal challenge with allergen after two and four weeks of pretreatment with fluticasone propionate.[333] Longterm treatment with intranasal fluticasone propionate was associated with a significant decrease in the number of nasal eosinophils, basophils, and neutrophils compared with placebo patients with seasonal allergic rhinitis. It has been proposed that fluticasone propionate may act by preventing activation of these cells and the subsequent release of (chemotactic) inflammatory mediators and further influx of cells.[619]

Glucocorticoids are able to inhibit the induction of nitric oxide synthase in the lung *in vivo* after lipopolysaccharide treatment[352] and they block the synthesis of nitric oxide in alveolar macrophages *in vitro*.[327] Glucocorticoids inhibit induction of nitric oxide synthase in murine macrophages[156] and the anti-inflammatory effects of glucocorticoids could partly result from this inhibition.

In contrast to the above findings, we did not observe any effect of topically applied fluticasone propionate on production of nitric oxide in a cross-over study in patients allergic to house dust mites. Both in patients and in controls nasal levels of nitric oxide gradually increased with time after a decrease during the four basal lavages.

Systemically applied glucocorticoids may act by preventing activation of monocytes and the subsequent production of nitric oxide.

In conclusion, synthesis of nitric oxide can be monitored in nasal lavage fluid. Although intranasally applied fluticasone propionate aqueous nasal spray successfully reduced the allergen-induced symptoms, the production of nitric oxide after challenge with house dust mite extract was not affected.

10.7 Acknowledgements

Chapter 11

The reduction of symptoms to the corticoid fluticasone propionate of patients with perennial allergic rhinitis is not associated with glucocorticoid receptor binding characteristics of peripheral blood mononuclear cells

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11.1 Abstract

Background - The reduction of symptoms due to treatment with corticosteroids varies among patients with perennial rhinitis. Most of the patients will respond, however, a few patients respond less to these drugs.

Objective - To investigate the association in reduction of symptoms due to glucocorticoids and glucocorticoid receptor characteristics in patients with perennial allergic rhinitis, *in vitro* glucocorticoid receptor binding studies were performed with peripheral blood mononuclear cells using dexamethasone and *in vitro* production of mediators were measured.

Methods - During a double blind placebo-controlled cross-over study 200 µg of the glucocorticosteroid fluticasone propionate aqueous nasal spray (in the active treatment period) and a placebo (in the placebo treatment period) were administered twice daily for two weeks to 22 patients allergic to house dust mite. At the end of either treatment period, symptoms were scored after an allergen provocation (100, 1000, 10000 BU/ml) and during the 9.5 hours after this challenge. In patients the symptom scores will differ between the active and placebo treatment period. Interest thus is in the partial correlation of the receptor characteristics with the symptom score after allergen provocation at the end of the active treatment period and adjusted for the symptom score after allergen challenge at the end of the placebo treatment period. Binding studies with dexamethasone were performed with peripheral blood mononuclear cells. Leukotriene B_4 produced by monocytes *in vitro* and soluble interleukin-2 receptor released by lymphocytes *in vitro* and cortisol levels in plasma were determined.

Results - No significant partial correlations of the number of the peripheral blood mononuclear cell glucocorticoid receptors and the affinity for the glucocorticoid receptors with the symptom score after active treatment were found. Also no significant partial correlations of the levels of leukotriene B_4 produced by monocytes *in vitro*, soluble interleukin-2 receptor released by lymphocytes *in vitro* and cortisol levels in plasma with the symptom score after active treatment were found.

Conclusions - The reduction of symptom due to the topical corticosteroid fluticasone propionate of rhinitis patients allergic to house dust mite is not correlated with the characteristics of the glucocorticoid receptor.

11.2 Introduction

Perennial rhinitis, which is characterized by chronic nasal symptoms of obstruction, rhinorrhoea, sneezing and itching is caused by house dust mite. Treatment with intranasal corticosteroids relieves symptoms of rhinitis in the majority of the patients, however, some patients respond less to these drugs. In other diseases, such as asthma and eosinophilic gastritis symptoms sometimes do not reduce, which is called corticosteroid-resistance.[341,.525,686,690] In allergic rhinitis this phenomenon is not known. A group of chronic asthmatic patients who were clinically resistant to treatment with prednisolone (40 mg daily for two weeks) were described by Carmichael in 1981 and corticosteroid resistance was defined as an improvement of less than 15% in the forced expiratory volume in one second (FEV$_1$).[91]

In addition to our earlier study to explore the effect of fluticasone propionate in the early and late phase of the allergic process[218,251], we have investigated whether a relationship exists between the reduction in symptoms due to corticosteroid treatment and the glucocorticoid receptor characteristics in these same rhinitis patients. During this double blind placebo-controlled cross-over study the glucocorticosteroid fluticasone propionate aqueous nasal spray was administered to patients allergic to house dust mite outside the season. Symptom scores were measured in response to allergen provocation and during the 9.5 hours following this challenge.[218,251] In addition, we have performed binding studies with dexamethasone to determine peripheral blood mononuclear cell glucocorticoid receptor number and binding affinity for dexamethasone to investigate if there is a partial correlation between the symptom score concluding the active treatment period and the receptor characteristics. Mediator release by peripheral blood monocytes and lymphocytes were also investigated *in vitro* to measure the activity of these cells.

11.3 Materials and methods

Patients[218,251]

This study was performed in 22 patients (10 women and 12 men, aged 21 to 50 years (mean: 34 years), with a history of perennial allergic rhinitis, and a positive skin test to house dust mite extract. All patients showed a skin reaction rated as at least one "+"-sign to 0.3 or 3 BU/ml extract, according to the standardized plus-sign scoring system defined by Norman.[477] Six of the 22 patients were allergic to grass pollen or animal dander as well. The experiments were performed in January-August to minimize exposure to house dust mite. The only patient with a concomitant pollen allergy was tested outside the pollen season. None of the patients allergic to animals had pets in their home. Antihistamines were withdrawn 72 hours before testing. The antihistamine astemizole, topical corticosteroids, cromoglycate or nedocromil were not used for 3 weeks before the tests were performed. Oral corticosteroids had to be withdrawn 2 months before the study. Patients who developed a nasal infection during the 2-weeks period before entering the study were excluded. None had undergone immunotherapy previously.

The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam-Dijkzigt and all patients gave written informed consent.

Nasal challenge and symptom score

After the positive skin test the subjects entered the double blind placebo-controlled crossover phase of the study. Each underwent two allergen challenge-periods, performed after 2 weeks pretreatment with 200 µg fluticasone propionate aqueous nasal spray (Glaxo, U.K.) or placebo spray twice daily. A three weeks washout period separated the two challenges. Nasal challenges were performed as described before.[219,230,465] The baseline symptoms (b) were scored before nasal challenges and were performed as described by Lebel *et al.*[373] Symptom scores were graded in points: 3-4 sneezes = 1 point; ≥5 sneezes = 3 points; rhinorrhoea anterior = 1 point; rhinorrhoea posterior = 1 point; difficult nasal breathing = 1 point; one nostril blocked = 2 points; both nostrils blocked = 3 points; pruritus of the nose = 1 point; pruritus of palate or ear = 1 point and conjunctivitis = 1 point (total score ranges from 0 till 11 points). To prevent the most severe nasal congestion caused by the allergen challenges 0.250 ml oxymetazoline (0.1%) was sprayed into each nostril 5 min before the first challenge. To obtain a control challenge, 0.125 ml phosphate buffered saline (PBS, containing 0.03% human bovine serum and 0.05% bezalkoniumchloride; ALK, Groningen, The Netherlands) was sprayed in each nostril and the symptoms were scored. For allergen challenge, 0.125 ml allergen extract was sprayed in each nostril and 10 min thereafter the symptoms were scored. Allergen doses of 100, 1000, 10000 Biological Units (BU)/ml (extract of Dermatophagoides pteronyssinus; ALK, Groningen, The Netherlands) were administered (h1-h3). From 30 min up to 9.5 hours after this challenge the symptoms were scored every hour (s1-s10).

Blood donors

At the time point of blood-collecting none of the patients had used pharmaceutical compounds as described before, which could have interfered with the herewith described *in vitro* studies.

In total 64 ml venous blood was collected into lithium-heparin-coated tubes (5 x 10ml, heparinized-blood; Vacutainer[R], Becton-Dickinson, France) and Ethylenediamine tetra-acetic acid (EDTA K$_3$)-coated tubes (2 x 7ml, EDTA-blood; Vacutainer[R], Becton-Dickinson, France) from each patient. An aliquot (1 ml) of the heparinized-blood was centrifugated at 4000 rpm for 10 min at 20 °C and the plasma was stored at -80 °C until measurement of cortisol levels.

Peripheral blood mononuclear cell (PBMC) isolation and glucocorticoid receptor characteristics

The method used was described before.[126,367,368] Heparinized-blood (50 ml) was mixed with an equal volume of PBS. Aliquots (20 ml) were layered onto 10 ml Ficoll-Paque (Pharmacia Biotech., Uppsala, Sweden) in 50 ml sterile polypropylene tubes. After centrifugation at 1000 x g for 15 min at 20 °C peripheral blood mononuclear cells (PBMC) were removed from the plasma/Ficoll interphase using gentle suction, transferred to sterile 50 ml polypropylene tubes, and washed twice with 15 ml DMEM (Dulbecco's modified Eagles medium containing 25 mM HEPES (GIBCO, Life Technologies, The Netherlands), supplemented with 2% Penicillin-Streptomycin (ICN Biochemicals, Inc) and 10% Foetal Calf Serum (GIBCO, Life technologies, The Netherlands). Total PBMC isolated (approx. 5-10 x 10^7 cells) were resuspended in 15 ml DMEM and incubated for 30 min in a shaking waterbath at 37 °C to remove endogenous bound glucocorticoid.[133] After centrifugation (5

min at 400 x g) the cells were resuspended in 15 ml DMEM and incubated again for 30 min under the same conditions in a waterbath. This was repeated once. After centrifugation PBMC were resuspended in DMEM at $1x10^7$cells/ml.

Stock solution of ^{3}H-dexamethasone (specific activity 89 Ci/mmol; total concentration 1.0 mCi in 1 ml ethanol; Amersham, The Netherlands) was further diluted in DMEM to final concentrations of 64, 32, 16, 8, 4, 2, 1 and 0.5 nM. Non-labelled, "cold" dexamethasone (Sigma, U.S.A.) was diluted in DMEM to final concentrations of 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 µM. For measurements of non-saturable binding (in duplicate), aliquots (100 µl) of PBMC suspensions were mixed with 50 µl aliquots of the ^{3}H-dexamethasone dilutions and also 50 µl aliquots of the "cold" dexamethasone. For measurements of the total binding (in threefold), aliquots (100 µl) of PBMC suspensions were mixed with 50 µl aliquots of the ^{3}H-dexamethasone dilutions and also 50 µl aliquots of DMEM. After incubation for 1 hour at 30 °C in a shaking waterbath the reaction was stopped by adding 2 ml ice-cold PBS. The cells were harvested onto glass microfibre filters using a cell harvester apparatus (Millipore Corporation, U.S.A.) and washed twice with 4 ml ice-cold PBS. These glass fibre filters were added to 7 ml Emulsifier-scintillator-plus^R liquid (Packard, U.S.A.), incubated over night and the incorporated radiolabel counted using a betacounter (Packard, U.S.A.). To measure the amount of added tritiated dexamethasone, aliquots of 50 µl were added to 7 ml scintillation liquid and counted.

Data analysis was performed by construction of a Scatchard plot after subtraction of nonspecific dexamethasone binding calculated from the tubes containing the cold dexamethasone, assuming that nonspecific binding was nonsaturable and linearly related to the concentration of free glucocorticoid in the supernatants.

PBMC isolation and culture for leukotriene B$_4$ (LTB$_4$) and soluble interleukin-2 receptor (IL-2 SR) measurements

EDTA-blood (14 ml) was mixed with an equal volume of PBS. Aliquots (14 ml) were layered onto 7 ml Ficoll-Paque (Pharmacia Biotech., Uppsala, Sweden) in 50 ml sterile polypropylene tubes. After centrifugation at 1000 x g for 15 min at 20 °C, PBMC were removed from the plasma/Ficoll interphase using gentle suction, transferred to sterile 15 ml polypropylene tubes, and washed twice with 14 ml DMEM, (containing 25 mM HEPES, 2% Penicillin-Streptomycin and 10% Foetal Calf Serum). PBMC isolated were resuspended in DMEM at $1x10^7$cells/ml. Aliquots (2ml) of this cell suspension were added together with 8 ml DMEM in sterile flatbottom culture flasks (75 cm^2; Costar) and were incubated for 1 hour at 37 °C, 7.5% CO$_2$. After this hour a) the supernatant (containing primarily the lymphocytes) was collected and b) to the adherent mononuclear cells consisted primarily of monocytes, 5 ml DMEM with or without 1 µM Calcium-ionophore (A23187, Sigma U.S.A.) was added. After 15 min incubation at 37 °C, 5% CO$_2$ the supernatant of the monocytes-suspension was collected and stored at -80°C until measurements of LTB$_4$, as a marker of the activity of monocytes. The lymphocytes-suspension was centrifugated at 400 x g for 5 min at 20 °C and aliquots (1ml) of this supernatant were stored at -80°C (t=0) until measurements of IL-2 SR. The cells were resuspended in DMEM at a concentration of $2x10^6$ cells/ml and aliquots with and without Phytohemagglutinin (PHA, 20 µg/ml; Difco Laboratories, U.S.A.) were transferred to flatbottom 24 wells cell-dishes (Costar, U.S.A.). Culture plates were incubated for 48 hours at 37 °C, 7.5% CO$_2$, whereafter the supernatants were collected and stored at -80°C until determination of IL-2 SR, as a

marker of the activity of lymphocytes.

Assays of LTB$_4$, IL-2 SR and cortisol
The levels of LTB$_4$, IL-2 SR and cortisol were measured by respectively RadioImmunoAssay (RIA; ^{3}H-LTB$_4$: Amersham, U.K.; antibody: Advanced Magnetics Inc., U.S.A.), Enzyme-Linked ImmunoSorbent Assay (ELISA; soluble IL-2 receptor; Eurogenetics, The Netherlands) and RIA (Coat-a-count cortisol; Diagnostic Products Corporation (DPC), The Netherlands). The limits of sensitivity of the assays were respectively 7.5 pg/ml, 20 U/ml and 5.5 pmol/ml. Cross-reactivity (50% B/B$_0$ displacement) of the LTB$_4$ assay: Leukotrienes (<1.00%) and Prostaglandins (<1.00%) and of the cortisol assay: Prednisolon (76%), 11-Deoxycortisol (11.4%) and Prednisone (2.3%).

11.4 Statistical analysis

Statistical analysis was performed with the Friedman two-way analysis of variance (ANOVA) followed by the Wilcoxon matched-pairs signed-ranks test for the symptom score and the Students T-test for the mediators and other parameters. Values are expressed as mean (standard deviation: SD).
The mean of the symptom score during the early phase reaction (EAR: h1 to s1) is calculated for each patient in the placebo and fluticasone treatment period as a marker of responsiveness. Interest thus is in the partial correlation of the receptor characteristics with the symptom score after allergen provocation at the end of the active treatment period and adjusted for the symptom score after allergen challenge at the end of the placebo treatment period. The partial correlation coefficient (R) is estimated and tested, after logarithmic transformation of the K$_d$, number of receptor sites, cortisol levels and LTB$_4$ levels, because of their positive skewness; the levels of Il-2 is not transformed.
A p-value of ≤0.05 was considered significant.

11.5 Results

Symptom score
The means (SD) of the symptom score of the patients are shown in Figure 1. A significant increase of the symptom score was observed immediately after the challenge with house dust mite extract in the placebo and fluticasone propionate-group (EAR) and also a late phase reaction was seen (LAR). The means (SD) of the mean-symptom scores during the EAR of the placebo and fluticasone treatment period were 4.3 (2.45) pts [range 0.75 - 8.75; median: 4.4] and 2.4 (1.55) [range 0 - 5.8; median: 2.3] respectively.

Glucocorticoid receptor characteristics
The Scatchard plot was linear and the mean (SD) of K$_d$ for dexamethasone was calculated 16.5 (13.51) nmol/l [range 2.28 - 51.4; median: 11.3]. Furthermore, the mean (SD) of receptor numbers were 6821 (5669) binding sites per cell [range 397 - 21468; median: 6010].
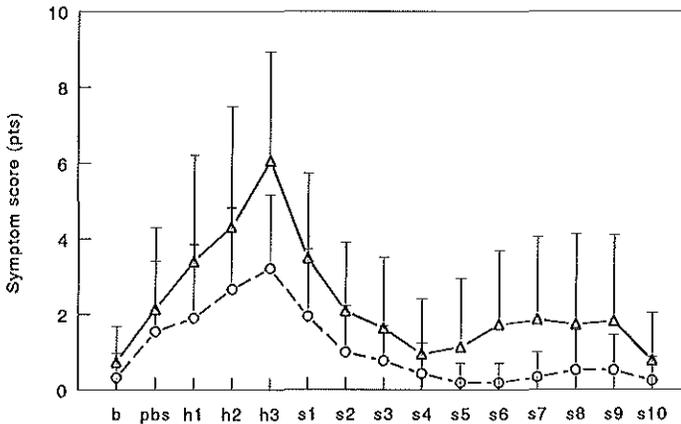
Figure 1. Means (SD) of symptom scores of the patients allergic to house dust mite at the end of the placebo treatment period and the fluticasone propionate treatment period. b: baseline score, pbs: score after challenge with phosphate buffered saline, h1, h2, and h3: score after allergen provocation with 100, 1000 and 10000 BU/ml house dust mite extract respectively, s1-s10: scores obtained every hour after time point h3.

Measurements of LTB₄, IL-2 SR and cortisol

Mean (SD) LTB_4 production as a marker of the activity of monocytes *in vitro* after addition of 1.0 μM A23187 for 15 min was 45.6 (105.3) [range 2.49 - 457.4; median: 13.1] ng/10^6 monocytes. LTB_4 levels without stimulation with A23187 for 15 min were not detectable. Mean (SD) IL-2 SR levels as a marker of the activity of lymphocytes *in vitro* after stimulation with 20 μg/ml PHA for 48 hours were 734 (237) [range 342 - 1245; median: 689] ng/10^6 lymphocytes. IL-2 SR levels at t=0 and without stimulation with PHA for 48 hours were not detectable. Mean (SD) cortisol levels in plasma were 571 (236) [range 248 - 1186; median: 541] ng/ml.

Associations

No significant partial correlations of the number of receptor sites (R=-0.2518; p=0.2984) and K_d (R=-0.3412; p=0.1528) with the symptom score concluding the active treatment period were found. Also no significant partial correlations of LTB_4 (R=-0.0812; p=0.7412), IL-2 SR (R=-0.0892; p=0.7165) and cortisol levels (R=0.0047; p=0.9849) with the symptom score after active treatment were found.

Only a significant correlation was found between the means of the symptom score during EAR of the placebo treatment period and the means of the symptom score during EAR of the fluticasone treatment period (R=0.5590; p=0.007).

11.6 Discussion

In this study treatment of rhinitis patients allergic to house dust mite with fluticasone propionate for two weeks twice daily in general reduces the development of symptoms due to a provocation test. Response to treatment with the corticosteroid varies among these patients. Most patients responded, however, a few patients responded less to this drug. Thomas and coworkers performed a study with 17 atopic patients in which the immediate increase in nasal airway resistance tended to decrease after a 2 weeks pretreatment with fluticasone propionate 200 µg/day (p=0.089).[619] However, the symptom score was decreased in 24 patients with seasonal allergic rhinitis after nasal challenge with allergen and 2-4 wks pretreatment with fluticasone propionate.[294]

One possible mechanism for the failure of cells not to respond to glucocorticoids could be an abnormality in the affinity ($1/K_d$) or a lower number of glucocorticoid receptors. In order to assess whether an association exists between glucocorticoid receptor affinity and number and the reduction of symptoms due to glucocorticoid in rhinitis patients, we have determined the K_d and number of receptor sites per cell of the glucocorticoid receptor of PBMC in perennial rhinitis patients. There was no significant partial correlation of the K_d or number of receptor sites with the symptom score concluding the active treatment period with fluticasone propionate, while adjustment had been made for the symptom score concluding the placebo treatment period. The K_d in PBMC from rhinitis patients was higher than those reported previously for monocytes and total mononuclear cells from normal individuals (2-8 nmol/l), but the number of receptor sites per cell (1-9 x 10^3) was similar [17-19].[102,397,681] Previous studies in our laboratory showed that normal individuals (n=9) have a K_d for dexamethasone of 11.0 ± 1.30 nmol/l and 4430 ± 340 receptor sites per cell of PBMC[412], from which it was concluded that no significant difference exists between rhinitis patients and normal individuals. These results suggest that the glucocorticoid receptors are not associated with responsiveness of rhinitis patients to the corticosteroid, which is supported by the finding that there is no significant partial correlation found of plasma levels of cortisol and responsiveness to fluticasone propionate.

In asthmatic patients other investigators were unable to demonstrate any significant difference in either glucocorticoid receptor density or binding affinity to dexamethasone in PBMC between corticosteroid-sensitive and -resistant patients, suggesting that the molecular defect was not due to abnormal receptor number or function.[368] This is in contrast with Corrigan et al., who did not observe a difference in dexamethasone receptor density, but found that glucocorticoid receptors from PBMC in corticosteroid-resistant asthmatics had a significantly lower binding affinity for dexamethasone than that in corticosteroid-sensitive patients.[126] Leung et al. performed a study with asthmatics and found prior to prednisone therapy a significant greater number of bronchoalveolar cells expressing IL-2 mRNA and IL-4 mRNA in resistant asthmatics compared with sensitive patients. After one week treatment with prednisone the sensitive patients had a significant decrease in the number of cells expressing mRNA for IL-4 and IL-5 and a rise in the numbers of Interferon-gamma mRNA expressing cells. The resistant asthmatics had no significant change. This indicated that steroid-resistant asthma is associated with a dysregulation of the expression of the genes encoding for cytokines.[382] This is supported by the findings of Sher et al, who found that 15 of the 17 resistant asthmatics demonstrated a significant reduced glucocorticoid receptors binding affinity as compared with sensitive

patients. This defect was localized to T-cells, which reverted to normal in culture media. However, incubation with a combination of IL-2 and IL-4 sustained this abnormality. The other two resistant patients had an abnormally low glucocorticoid receptor number with normal binding affinity that was not limited to T-cells, which failed to normalize after incubation in media alone or IL-2 and IL-4. Sher suggested that corticosteroid resistant-asthma may be due to more than one abnormality, the majority related to a reversible cytokine-induced reduction in glucocorticoid receptor binding affinity and the second related to an irreversible reduction in glucocorticoid receptor number.[577]

Recent studies in our laboratory showed that corticosteroid-resistant ulcerative colitis patients had a significant lower number of glucocorticoid receptors from PBMC.[412]

We also performed an *in vitro* study with PBMC of the rhinitis patients to investigate if there is an association between the activity of monocytes and lymphocytes and responsiveness to the corticoid. LTB_4 production in monocytes after stimulation with A23187 and IL-2 soluble receptor release in the lymphocytes after stimulation with PHA were not significantly partial correlated with the reduction of symptoms after allergen challenge. Lane *et al.*, who found that the generation of cytokines, such as TNF-α, IL-1ß and GM-CSF, from monocytes was not affected by treatment with hydrocortisone in either corticosteroid-sensitive or -resistant asthmatic patients.[369] It is known that corticosteroids decrease T-lymphocyte proliferation and activation, which is mediated by the inhibition of IL-2 production. Induction of lipocortin synthesis, an immunomodulatory protein which is capable of suppressing phospholipase A_2, by corticosteroids inhibits the formation of arachidonic acid-derived metabolites.[139,219]

In conclusion, The reduction of symptoms due to treatment with fluticasone propionate of rhinitis patients allergic to house dust mite is not correlated with the characteristics of the glucocorticoid receptors of their PBMC.

11.7 Acknowledgements

Chapter 12
Antihistaminic intervention

Histamine produces its action by an effect on specific histamine receptors, which are of three main types, H_1, H_2 and H_3, distinguished by means of selective antagonists. H_1 receptors are found in human and guinea-pig bronchial muscle, and in guinea-pig ileum; stimulation causes contraction of the muscle. H_1 receptors are linked to transduction systems which increase intracellular Ca^{2+}. Occupancy of H_1 receptors results in the stimulation of phosphatidyl inositol turnover and the subsequent production of the second messengers inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG), which results in an increase in intracellular calcium.[256,440]

In asthma patients they have a mild, dose-related bronchodilator effect, and they protect against bronchoconstriction by stimuli such as exercise, hyperventilation, inhalation of cold air, histamine or antigen.[155,528,628]

Rhinitis: The second-generation histamine H_1 receptor antagonists are now widely used for the prevention and relief of sneezing, itching and rhinorrhoea in patients with allergic rhinoconjunctivitis and rhinitis[199,342,434], but they did not improve nasal obstruction.[354] In addition to their H_1-blocking activities, some of the second-generation compounds have antiallergic properties as shown by a reduction in the release of mediators of inflammation, such as histamine, PGD_2 and LTC_4 from mast cells and basophils.[99,398,462,464,621] They also inhibit recruitment of inflammatory cells such as eosinophils, neutrophils and basophils to the site of the allergic reaction, suppressing both the early and late phase response to allergen.[99,170,439] Levocabastine inhibited the allergen-induced nasal reactivity to histamine and the cellular influx in patients with perennial rhinitis[503], which suggest that levocabastine could also have anti-inflammatory properties.

Chapter 13
Effect of topical levocabastine on nasal response to allergen challenge and nasal hyperreactivity in perennial rhinitis

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13.1 Abstract

Background: It has been demonstrated that some oral antihistamines reduce non-specific nasal reactivity and that topical levocabastine reduces cellular influx after nasal allergen - challenge. This suggests that antihistamines possess other properties besides the classical histamine H_1 receptor antagonism.

Objective: To evaluate the effect of one week's treatment with topical levocabastine on the nasal clinical response, inflammatory mediators and nasal hyperreactivity.

Methods: In a double-blind, placebo-controlled, two-period, two-treatment, crossover study, 21 rhinitis patients allergic to house dust mite participated. After each treatment period patients were challenged with house dust mite extract. Symptom scores and nasal lavages were collected for 9.5 hours after challenge.

Allergen-induced nasal hyperreactivity was determined by nasal methacholine challenge 24 hours after alllergen challenge. A nasal histamine challenge was performed as well.

Results: Patients showed only an immediate nasal response. Levocabastine significantly reduced the symptom score after 100 (p=0.0063), 1000 (p=0.0035) and 10,000 biological units (BU)/ml (p=0.0013) of house dust mite extract. Albumin influx and tryptase release were not significantly reduced by levocabastine. No release of histamine and eosinophil cationic protein was seen. Levocabastine did not reduce nasal response to methacholine. Active treatment significantly reduced histamine-induced nasal secretion (p=0.0009) and the number of sneezes (p=0.0001).

Conclusion: A significant effect of levocabastine was shown on the immediate clinical response to house dust mite and to histamine challenge only. Our findings suggest that levocabastine is an effective H_1 receptor antagonist without anti-inflammatory properties.

Keywords: levocabastine; perennial allergic rhinitis; nasal hyperreactivity; inflammation

13.2 Introduction

It is generally accepted that histamine, released locally from mast cells, is one of the major mediators[459] responsible for most symptoms in allergic rhinitis. In the symptomatic treatment of allergic rhinitis, oral H_1 receptor antagonists are widely used.[582] Topical treatment is preferred however, to prevent systemic side-effects and to obtain locally therapeutic drug concentrations. Oral antihistamines administered locally are not very effective.[507,511] In contrast, topical levocabastine, a new selective H_1 receptor antagonist[23], has proved to be very effective in suppressing sneezing and rhinorrhoea in pollinosis patients[354]. In a recent study, Pazdrak[503] demonstrated an inhibitory effect of levocabastine on allergen-induced nasal reactivity to histamine and cell influx in patients with perennial rhinitis. This suggests that levocabastine possesses other properties apart from being a H_1 receptor antagonist. Klementsson[347] showed that some oral antihistamines inhibit the increased response to a non-specific stimulus (methacholine).

To determine if levocabastine has other properties besides the H_1 receptor antagonism, we studied its effect on the release of inflammatory mediators in lavage fluid in response to nasal house dust mite challenge. Allergen-induced nasal hyperreactivity was determined by nasal methacholine challenge.

Nasal histamine challenge was also performed. However, since levocabastine is a H_1 receptor antagonist, nasal histamine challenge cannot be used to study the effect of levocabastine on nasal hyperreactivity.

13.3 Methods

Subjects
Twenty-one patients, 13 women and 8 men aged 19 to 54 years (mean 33 yrs), with a history of perennial rhinitis participated in this study. They all had a positive intradermal skin test at 3 BU/ml of house dust mite extract (Dermatophagoides pteronyssinus extract, ALK Benelux, Groningen, the Netherlands), according to the standardised plus-sign scoring system defined by Norman[477]. If patients had a concomitant allergy, they were only included if exposure to the allergen could be avoided.

Symptomatic medications for rhinitis had to be discontinued: oral corticosteroids 2 months, astemizol 6 weeks, nasal or inhaled corticosteroids, cromoglycate and nedocromil 3 weeks and antihistamines 3 days before the start of the study. Patients with nasal polyposis, nasal surgery in the 3 months preceding the study, nasal infection during the 2 weeks preceding the study or immunotherapy in the previous year were excluded.

Study design
A double-blind, placebo-controlled, two-period, two-treatment, crossover study comparing levocabastine with placebo was performed. Patients used a nasal spray containing either levocabastine (0.5 mg/ml solution) or placebo, twice daily for one week. The treatment periods were separated by 2 weeks. After either treatment period patients were challenged with house dust mite extract. Symptom scores were recorded and nasal lavages were performed up to 9.5 hours after allergen challenge. Nasal methacholine challenge was performed 24 hours after allergen challenge, followed one hour later by nasal histamine

challenge. During the provocation days, patients continued their study medication.

The study was performed during the period January-August, to minimise natural exposure to house dust mites. All patients gave written informed consent. The study protocol was approved by the Medical Ethics Committee of the University Hospital.

Nasal challenge tests

Challenges were performed in accordance with the methods described by Gerth van Wijk *et al*[230]. On each occasion the patients used their nasal spray and subsequently waited for half an hour before the test in order to acclimatise the nasal mucosa.

Challenge with house dust mite extract

Challenges with house dust mite extract (100, 1000, and 10,000 BU/ml, ALK Benelux, Groningen, the Netherlands) were performed at ten-minute intervals after challenge with phosphate-buffered saline, containing human serum albumin 0.03% and benzalkonium chloride 0.05% (ALK Benelux, Groningen, the Netherlands).

House dust mite extract was sprayed into each nostril with a nasal pump spray delivering a fixed dose of 0.125 ml solution. Nasal response was monitored 10 minutes after each challenge, and hourly from 0.5 till 9.5 hours after the last challenge. Nasal response was monitored by the number of sneezes, the volume of secretion collected according to Borum[68], a symptom score according to Lebel[373], and mediators in nasal lavage fluid.

Methacholine challenge

Methacholine bromide (32 mg/ml) was sprayed in each nostril, 15 minutes after nasal challenge with phosphate-buffered saline. Nasal response was measured by the volume of secretion[68] and albumin influx in nasal lavage fluid.

Histamine challenge

Nasal challenge with histamine phosphate (0.25, 0.50, 1.0, 2.0 and 4 mg/ml) was performed at five-minute intervals after challenge with phosphate-buffered saline. The volume of secretion and the number of sneezes were used as indicators of nasal response. We assume the histamine challenge is not influenced by the preceding methacholine challenge, since Grønborg[261] demonstrated that repeated nasal challenges with histamine and methacholine do not increase nasal reactivity.

Nasal lavage

Nasal lavages were performed as described by Naclerio.[465] This protocol comprises 4 pre-challenge lavages to clear the nose of secretions and to obtain baseline levels of mediators. To maintain nasal patency after allergen challenge, oxymetazoline 0.1% (two 0.125 ml puffs) was sprayed into both nostrils.

In each nostril 5 ml isotonic saline solution (0.9%) preheated to 37°C was instilled with a pipette while the subject gently flexed his/her head backwards. After 10 seconds, the lavage fluid was expelled and collected in tubes. The recovery was 7.8 ± 0.9 ml.

Symptom score

Symptoms were recorded using a scoring system according to Lebel *et al.*[373], immediately before the corresponding lavage. Symptom scores were graded in points (pts): 3-4

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sneezes=1 pt, ≥5 sneezes=3 pts; anterior rhinorrhoea=1 pt; posterior rhinorrhoea=1 pt; difficult nasal breathing=1 pt, one nostril blocked=2 pts, both nostrils blocked=3 pts; pruritus of the nose=1 pt; pruritus of palate or ear=1 pt and conjunctivitis=1 pt (total score ranges from 0 till 11 pts).

Mediator assays
Lavage fluid was stored on ice and centrifuged for 10 minutes at 400 x g. The supernatant was stored at -20°C. Histamine was measured with an automated fluorometric assay.[586] Tryptase and eosinophil cationic protein were determined by radioimmunoassay (RIA), according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden), albumin was determined by automatic kinetic nephelometry with use of the Array analyzer (Beckmann, Mijdrecht, the Netherlands) according to the manufacturer's instructions.

13.4 Statistical analysis

Ten patients received the levocabastine nasal spray during the first treatment period and 11 during the second treatment period. There was no carry-over effect between the two groups. The effect of levocabastine on the immediate and late (area under the curve (AUC) between 3.5 and 9.5 hrs) nasal reaction, on nasal hyperreactivity (response to methacholine) and response to histamine (AUC of histamine dose response curve) was tested by the nonparametric Mann-Whitney U test. A two-tailed p-value < 0.05 was considered significant.

13.5 Results

Nasal allergen challenge resulted in an immediate clinical response only. Levocabastine significantly reduced the symptom score after 100 (p=0.0063), 1000 (p=0.0035) and 10,000 BU/ml (p=0.0013) of house dust mite extract (fig. 1a). The secretory response was significantly reduced after 1000 (1.0±0.28 vs 0.83±0.24, p=0.0206) and 10,000 BU/ml (1.91±0.33 vs 1.45±0.38, p=0.0376), the same was true of the number of sneezes (2.52±0.83 vs 1.57±0.58, p=0.0185; 8.14±1.17 vs 3.1±0.9, p=0.0007). Albumin influx was seen after nasal challenge with the highest allergen dose; Levacobastine did not reduce this influx (fig. 1b). Patients showed increased histamine levels after phosphate-buffered saline, but not after allergen challenge. There was no significant treatment effect (fig. 1c). No significant release of eosinophil cationic protein in response to allergen challenge was detected; no effect of levocabastine on it was seen (fig. 1d).
Since in a previous study, tryptase release could not be detected during the late-phase response[250], we measured tryptase during the immediate response only. Tryptase release was observed after the highest allergen dose; treatment with levocabastine did not decrease this release (fig. 2).
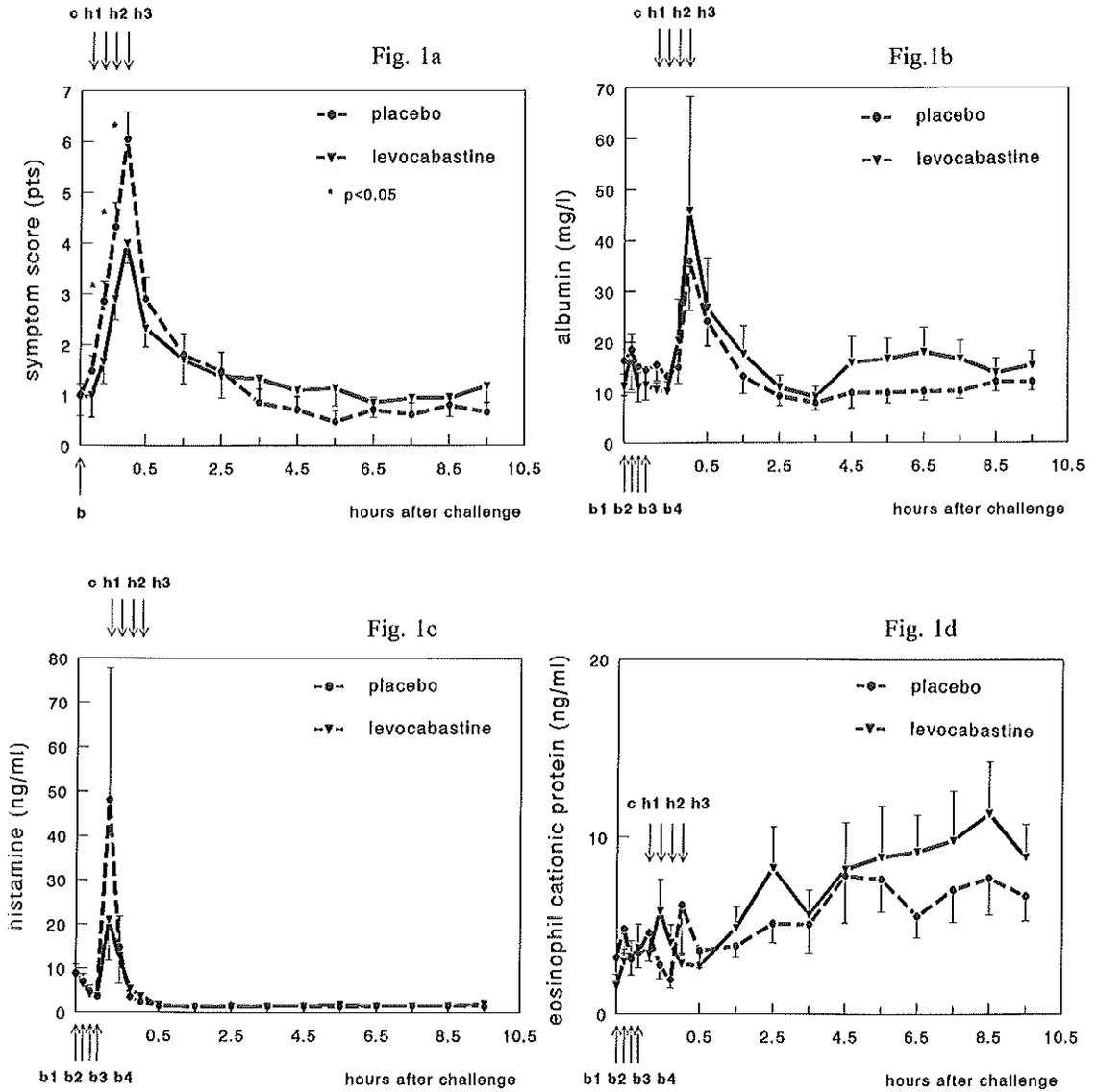
Figure 1. Nasal response to HDM challenge

Figure 1a: symptom scores; b: albumin; c: histamine; d: eosinophil cationic protein. Nasal response to allergen challenge in placebo and levocabastine treated groups measured for 9.5 hours after allergen challenge. Values are presented as mean ± standard error of the mean (SEM). Abbreviations: b: baseline symptom score, b1-b4: four pre-lavages, c: 10 min after challenge with phosphate-buffered saline, h1-h3: 10 min after challenge with 100, 1000 and 10,000 BU/ml house dust mite extract respectively. * = two-sided p-value≤0.05.
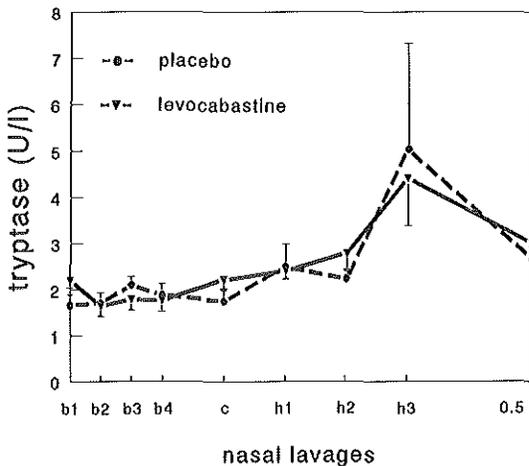
Figure 2.
Nasal tryptase release of placebo and levocabastine treated groups measured for 0.5 hour after allergen challenge. Abbreviations: b1-b4: four pre-lavages, c: 10 min after challenge with phosphate-buffered saline, h1-h3: 10 min after challenge with 100, 1000 and 10,000 BU/ml house dust mite extract respectively. Values are presented as mean ± SEM.

In figure 3 the results of methacholine provocation are demonstrated. Patients showed a secretory response (fig. 3a) and a slight albumin influx (fig. 3b) after phosphate-buffered saline; methacholine did not further increase this response. Levocabastine did not reduce the amount of secretion or the albumin influx.

In figure 4 histamine dose response curves are shown. One week on levocabastine significantly decreased the AUC of the number of sneezes (p=0.0001) (fig. 4a) and the amount of secretion (p=0.0009) (fig. 4b).

13.6 Discussion

Nasal response to allergen challenge was monitored by a nasal symptom score up to 9.5 hrs. Sypmtoms reached a maximum 10 minutes after the highest allergen dose and returned to baseline levels at 3.5 hours. No recurrence of symptoms was observed during 3.5 and 9.5 hours, the period of the late-phase response.[505] The allergic response was also monitored by assessment of mediators in nasal lavage fluid. Albumin influx, as a marker of increased vasopermeability[45,604], could be detected only during the immediate response.

To study if levocabastine has any anti-inflammatory effects, we paid particular attention to the mediators histamine, tryptase and eosinophil cationic protein. It is generally accepted that mast cell derived histamine is the principal mediator causing most of the symptoms during the immediate allergic reaction. However, we observed an increase of histamine in response to phosphate-buffered saline only; no increase of histamine was seen after allergen challenge. Results concerning histamine release in lavage fluid are not consistent: some studies did not show histamine release either[229,394], while others showed an significant histamine release[144,467] after allergen challenge. Assessment of histamine in nasal lavage fluid therefor appears unsuitable for monitoring the allergic response. Tryptase, as a specific marker for mast cell activation[560], was only measured during the immediate response. In other studies[536,625], tryptase was also found to be a useful marker of the immediate nasal reaction. Eosinophil cationic protein, released from activated eosinophils, can be used to measure the late-phase response.[42] However, in this study no significant

Fig. 3a
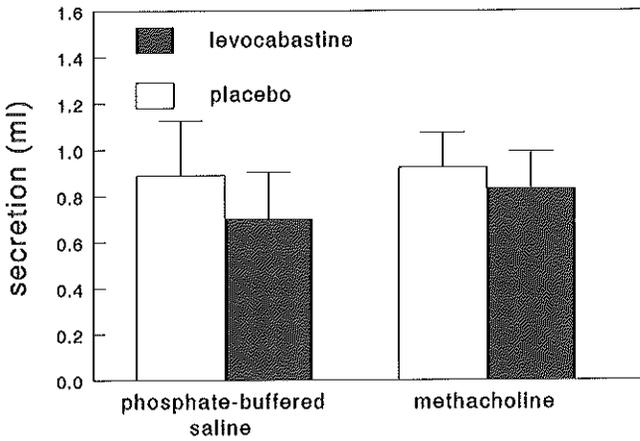
Fig. 3b
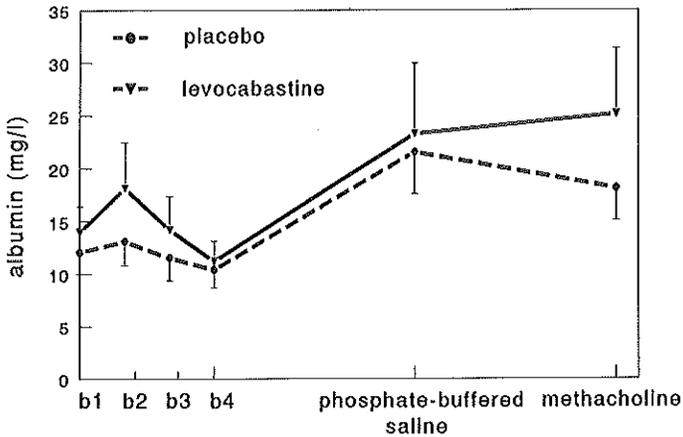
Figure 3. Secretory response (Figure 3a) and albumin influx (Figure 3b) after nasal challenge with methacholine. Response to methacholine was not significantly increased as compared with response to phosphate-buffered saline. Levocabastine treatment did not significantly reduce the secretory response or the albumin influx. Abbreviations: b1-b4: four pre-lavages. Values are presented as mean ± SEM.

Fig. 4a

Fig. 4b
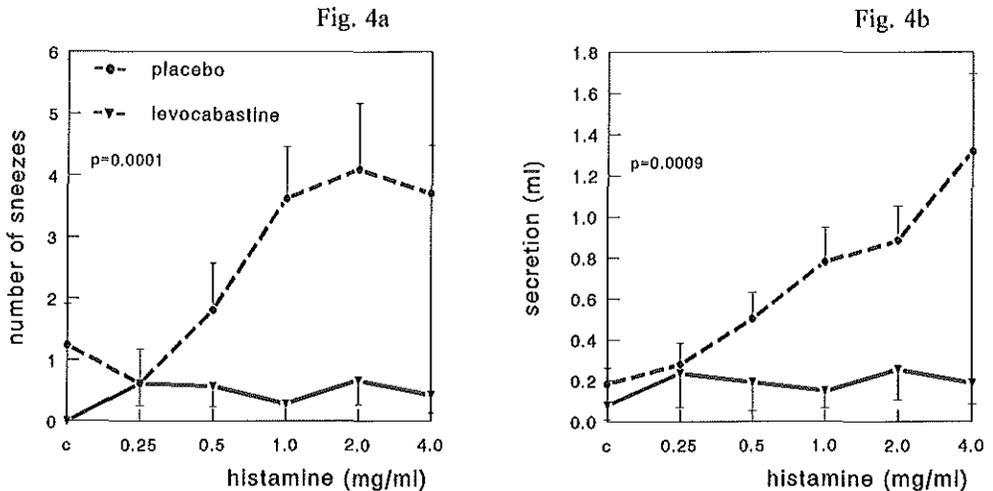
Figure 4. Nasal reactivity (Figure 4a: sneezes; Figure 4b: secretion) to histamine in placebo and levocabastine treated groups. Values are presented as mean ± SEM. The AUCs of the two treatments groups are compared.

release was measured during 3.5 and 9.5 hrs, corresponding to the absence of albumin influx and recurrence of symptoms during the late-phase period.

We monitored the allergic response for 9.5 hrs, because in case of a late-phase response an increase of inflammatory cells and mediators is expected. Therefore, this period is suitable to study anti-inflammatory effects of drugs. In contrast to previous studies[250,465], this study revealed no increased nasal symptoms, eosinophil cationic protein release or albumin influx during this period. Since the patients also showed a milder immediate response than in previous studies[229,465], the threshold for developing a late-phase response may not have been reached.

During the immediate response the influx of albumin and the release of tryptase after the highest allergen dose were not inhibited by levocabastine. Schwartz *et al.*[564] recently developed a new, more sensitive immunoassay for tryptase. Using more sensitive assays, a treatment effect might be more easily detectable. Levocabastine significantly inhibited the clinical response after allergen challenge. Since no release of histamine and eosinophil cationic protein was measured in lavage fluid, an effect of levocabastine could not be evaluated. Accordingly, in this study levocabastine appeared to have no effect on nasal inflammation (i.e. on tryptase during the immediate response). This in contradiction to Pazdrak[503], who described an inhibitory effect of levocabastine on influx of inflammatory cells 2 hours after house dust mite challenge. However, since Pazdrak did not study inflammatory mediators or activation of cells the results of our studies are difficult to compare. Furthermore, since we used half the dose Pazdrak used, our dose of levocabastine

may not have been sufficient to affect the inflammatory markers.

Allergen-induced nasal hyperreactivity was determined by nasal challenge with methacholine. Patients already showed a clear secretory response to the preceding phosphate-buffered saline (0.89 ± 0.24 ml) without additional increase after the methacholine challenge (0.92 ± 0.15 ml). The same was true of albumin influx in lavage fluid. This increased response to phosphate-buffered saline suggests that the nasal mucosa is reactive to the challenge (by nasal pump spray) itself. This may reflect a form of nasal hyperreactivity, perhaps an increased responsiveness to mechanical stimulation. When we compare this response with the other phosphate-buffered saline challenges in this study, preceding the house dust mite and the histamine challenge, nasal secretory responses were only 0.32 ± 0.16 ml and 0.17 ± 0.08 ml, respectively. Probably the nasal mucosa had become so reactive due to the combination of the preceding allergen challenge and the 4 prewashings immediate prior to the phosphate-buffered saline challenge. Levocabastine did not prevent this nasal reactivity.

Provocation with histamine was performed 1 hour after the methacholine challenge. Secretion and number of sneezes were both significantly decreased by levocabastine treatment, which clearly demonstrates the H_1 receptor antagonist effect.

In conclusion: Levocabastine significantly decreased the clinical nasal response to house dust mite and histamine challenge, as expected by histamine inhibition, but it did not decrease albumin, tryptase and nasal hyperreactivity as measured by methacholine challenge.

13.7 Acknowledgements

Chapter 14
Selective effect of levocabastine on histamine receptor and histamine release from human leukocytes and guinea-pig airways

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14.1 Abstract

Background: Levocabastine is a potent histamine H_1 receptor antagonist which is used topically in the treatment of patients with allergic rhinitis. It has been suggested that antihistamines also have anti-inflammatory properties.
Objective: The present study was performed to investigate whether levocabastine, in addition to the anti-H_1 receptor activity, has anti-inflammatory properties and thus is able to modulate the release of histamine and cytokines, such as interleukin-5 from human leukocytes and isolated tissues.
Methods: Leukocyte suspensions were prepared by dextran sedimentation of peripheral venous blood drawn from allergic and healthy volunteers. Leukocytes obtained from allergic volunteers were preincubated for 30 min with levocabastine (doses 10^{-8} M to 10^{-6} M) and thereafter incubated with allergen. Leukocytes obtained from healthy volunteers were incubated for 0 to 3 hours with levocabastine (doses 10^{-14} M to 10^{-3} M). Histamine release was measured by an automated fluorometric method. Interleukin-5 release was measured by Enzyme Linked Immuno Assay. Contractile responses to histamine on guinea-pig trachea and lung parenchyma as well as the release of histamine and interleukin-5 by the tissues were investigated in the absence or presence of levocabastine and/or the histamine H_2 receptor antagonist, cimetidine.
Results: Levocabastine did not influence the allergen-induced histamine release from leukocytes obtained from allergic volunteers. However, high concentrations (10^{-4} and 10^{-3} M) of levocabastine caused release of histamine from leukocytes obtained from healthy volunteers as well as guinea-pig airways. Pretreatment with levocabastine dose-dependently decreased the contractile response to histamine, showing an irreversible competitive mechanism. Interleukin-5 release from human leukocytes and guinea-pig airways was not detectable.
Conclusions: These findings indicate that the H_1 receptor blocker, levocabastine, has probably no anti-inflammatory properties, as indicated by its inability to inhibit the allergen-induced histamine release, and that the histamine release from both human leukocytes and guinea-pig trachea and lung parenchyma only is significantly increased by the drug at high concentrations.

Keywords: levocabastine; histamine receptor; human leukocytes; guinea-pig tissue; histamine release

14.2 Introduction

Levocabastine is a selective histamine H_1 receptor antagonist.[601] There are several reports in literature showing the effectiveness of levocabastine in allergic processes.[148,583] Allergic rhinitis is characterized by symptoms such as sneezing, itching, rhinorrhoea and congestion. It is well accepted that histamine released locally in the early phase of the allergic reaction from mast cells and in the late phase from basophils is one of the major mediators responsible for most of these symptoms.[459,468,637] Topically applied levocabastine has been demonstrated to protect pollinosis patients against allergen-induced rhinorrhoea and sneezing; however, levocabastine failed to influence nasal obstruction.[354] The inhibitory effect of levocabastine on allergen-induced nasal reactivity to histamine and cell influx in patients with perennial rhinitis was investigated by Pazdrak et al., who suggested that levocabastine possesses properties other than H_1 receptor blockade.[503] In an earlier study we demonstrated that while treatment with levocabastine significantly decreased the symptoms after nasal challenge with house dust mite extract in allergic rhinitis patients, the release of inflammatory mediators in nasal lavage fluid was not affected.[254]
Basophils and mast cells are important cells which are involved in the allergic reactions, since they express high affinity IgE receptors on their membranes and release potent inflammatory mediators, such as histamine, interleukin-5 and leukotrienes, following membrane IgE cross-linking by antigen.[317,519,558] Mast cells seem to be important in the early antigen-induced reaction[244] and basophils are believed to act in the late reaction[308]. Although a number of publications have appeared concerning the clinical efficacy of levocabastine, little is known about the mechanism of anti-allergic activity. In order to examine whether levocabastine has anti-inflammatory properties besides the classical H_1 receptor antagonism, we studied the effect of levocabastine on the release of histamine and interleukin-5 from human blood leukocytes as well as from guinea-pig trachea and lung parenchyma during registration of the contractile function.

14.3 Materials and methods

Materials
Levocabastine, methacholine hydrobromide and histamine hydrochloride were provided by Janssen Pharmaceutica B.V. (Tilburg, The Netherlands) and dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis USA). DMSO was used as vehicle control. The final concentration of DMSO was <10% in all experiments and did not affect histamine release or cell viability. Cimetidine was from BUFA B.V. (Uitgeest, The Netherlands) and dissolved in Krebs-Henseleit buffer. The EDTA-vacutainer tubes for collecting blood were from Becton Dickinson (Meylan Cedex, France). Dextran was purchased from Pharmacia (Upssala, Sweden). Glucose, NaCl, KCl, NaOH, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $MgSO_4$, KH_2PO_4, $NaHCO_3$ and Hemacolor Färbetest were from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), EDTA and Trypan Blue solution were from Sigma (St Louis, USA). Piperazine-1,4-bis-2-ethanesulfonicacid (PIPES) was from Janssen Chimica (Geel, Belgium). Human-interleukin-5 Enzyme-linked Immuno-assay was from Pharmingen (San Diego, U.S.A.). This ELISA assay also was used in the organ bath experiments, because no guinea-pig-IL-5 assay is available and there could be a cross-reactivity with the

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human-IL-5.

Blood sample collection and preparation of leukocyte suspension
Peripheral venous blood was obtained from four allergic volunteers (two were allergic against house dust mite, one against grass pollen and one against dog-dander; mean age 45 years) and from six healthy volunteers (mean age 31 years) in EDTA-vacutainer tubes (7 ml) with approbation of the local Medical Ethics Committee. Leukocyte suspensions were prepared by dextran sedimentation of peripheral blood as described by Lichtenstein and Osler[390] and by Siraganian and Hook[586]. Leukocytes were finally resuspended at a concentration of 2×10^6 cells/ml. The viability of all leukocyte suspensions, assessed by trypan blue exclusion was greater than 95%. Cells were stained by Hemacolor to examine viability of basophils, which was greater than 95%.

Effect of levocabastine on the release of histamine and interleukin-5 by leukocytes obtained from allergic volunteers
In the experiments the histamine release was determined after incubation of the leukocyte suspension with vehicle control (DMSO) or with three concentrations of levocabastine (10^{-8} to 10^{-6} M) for 30 min and thereafter incubation with allergen (10^{-7}, 10^{-5}, 10^{-3} and 10^{-1} mg/ml) at 37°C as described by Siraganian and Hook.[586] Histamine concentrations in the supernatants were measured by an automated fluorometric method.[586] Interleukin-5 concentrations in the supernatants were measured by ELISA.

Effect of levocabastine on the release of histamine and interleukin-5 by leukocytes obtained from healthy volunteers
Two series of experiments were performed. In the first series of experiments the histamine release was determined after incubation of the leukocyte suspension with DMSO or with different concentrations of levocabastine (10^{-14} to 10^{-3} M) for 1 min or 1 hour at 37°C. In the second series of experiments the histamine release was determined after incubation of the leukocyte suspension with DMSO or with three concentrations of levocabastine (10^{-5} to 10^{-3} M) for different time-intervals (0 to 3 hours) at 37°C. These relatively high concentrations of levocabastine were used because a) we found in the first series of experiments a release of histamine after incubation with high concentrations of levocabastine and b) it has also been reported that antihistamines have a dual action on cells: at high concentrations they enhance histamine release from mast cells while at low concentrations they inhibit the secretion of histamine.[674,371,372,454] Histamine concentrations in the supernatants were measured by an automated fluorometric method.[586] Interleukin-5 concentrations in the supernatants were measured by ELISA. Levocabastine or DMSO, at the concentrations used, neither interfered with the fluorometric determination of histamine nor interleukin-5 ELISA nor modified cell viability, as assessed by trypan blue exclusion.

Effect of levocabastine on the contractile response to histamine on guinea-pig trachea and lung parenchyma
After cervical dislocation (with approbation of the local Animal Ethics Committee) of the guinea-pigs (male and female, 400-600 g; CPB-TNO, Zeist, The Netherlands), the trachea and lungs were taken out and washed in Krebs-Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.5) which was

aerated with 95% O_2, 5% CO_2. Two series of experiments were performed using four guinea-pigs each. In the first series of experiments, three trachea strips (four cartilage-rings for each strip) and three lung parenchyma strips of approximately 20 mm length were prepared. In the second series of experiments, four lung parenchyma strips of approximately 20 mm length from each guinea-pig were prepared. The tissue preparations were mounted in 10 ml double-jacketed organ baths containing aerated buffer at 37°C. They were allowed to equilibrate under an isotonic tension of 500 mg which resulted in optimal contractility, using Harvard model 386 heart-smooth muscle transducers.[324] All strips were primed by adding methacholine (10^{-6} to 10^{-4} M) in 10-fold concentration steps followed by washout, because this ensured a stable function for the rest of the day. The response to methacholine (10^{-5} M) was used as a reference for the histamine curve. After return to baseline, a cumulative concentration response curve (CCRC) was made using histamine (10^{-8} to 10^{-3} M) on each strip. From the histamine-CCRCs the -log EC_{50} (concentration producing 50% of the maximal effect) was derived. After washout, in the first series of experiments levocabastine (10^{-6}, 10^{-7} or 10^{-8} M) was added and preincubated for 15 min, whereafter a second histamine-CCRC was generated. In the second series of experiments, after washout, cimetidine (H_2 receptor antagonist; 10^{-6} M) was added and preincubated for 15 min. Thereafter levocabastine (10^{-6}, 10^{-7} or 10^{-8} M) was added and preincubated for 15 min, whereafter a second histamine-CCRC was generated.

Effect of levocabastine on the release of histamine and interleukin-5 by guinea-pig trachea and lung parenchyma

After priming by adding methacholine to the organs and returning to baseline, the organs were allowed to equilibrate for 15 min, whereafter samples of 800 µl were taken from the bath solution for measurements of histamine and interleukin-5 concentrations. In the series of experiments in which cimetidine and/or levocabastine and/or histamine were added and preincubated for 15 min, samples were taken for histamine and interleukin-5 measurements. To examine whether the endogenous histamine release after incubation with exogenous histamine for 15 min was influenced, samples were taken for histamine measurements. Histamine concentration in the bath solutions was measured by an automated fluorometric method. Interleukin-5 concentration were measured by ELISA. Cimetidine, levocabastine, DMSO or Krebs-Henseleit buffer, at the concentrations used, did neither interfere with the fluorometric determination of histamine nor with the interleukin-5 ELISA.

14.4 Statistical analysis

The results were expressed as means ± S.E.M. ANOVA for multiple comparisons followed by a Students t-test for paired data was used to analyze the effect of levocabastine on histamine release, on interleukin-5 release, on -log EC_{50}, and on the CCRC's. P-values < 0.05 were considered significant.

14.5 Results

Effect of levocabastine on the release of histamine and interleukin-5 by leukocytes obtained from allergic volunteers

Incubation with 10^{-8} to 10^{-6} M levocabastine did not influence the release of histamine by leukocytes after stimulation with allergen (10^{-7}, 10^{-5}, 10^{-3} and 10^{-1} mg/ml) compared with the incubation with DMSO. The allergen-induced histamine release from allergic patients are shown in Figure 1.

Interleukin-5 release from the leukocytes were not detectable.
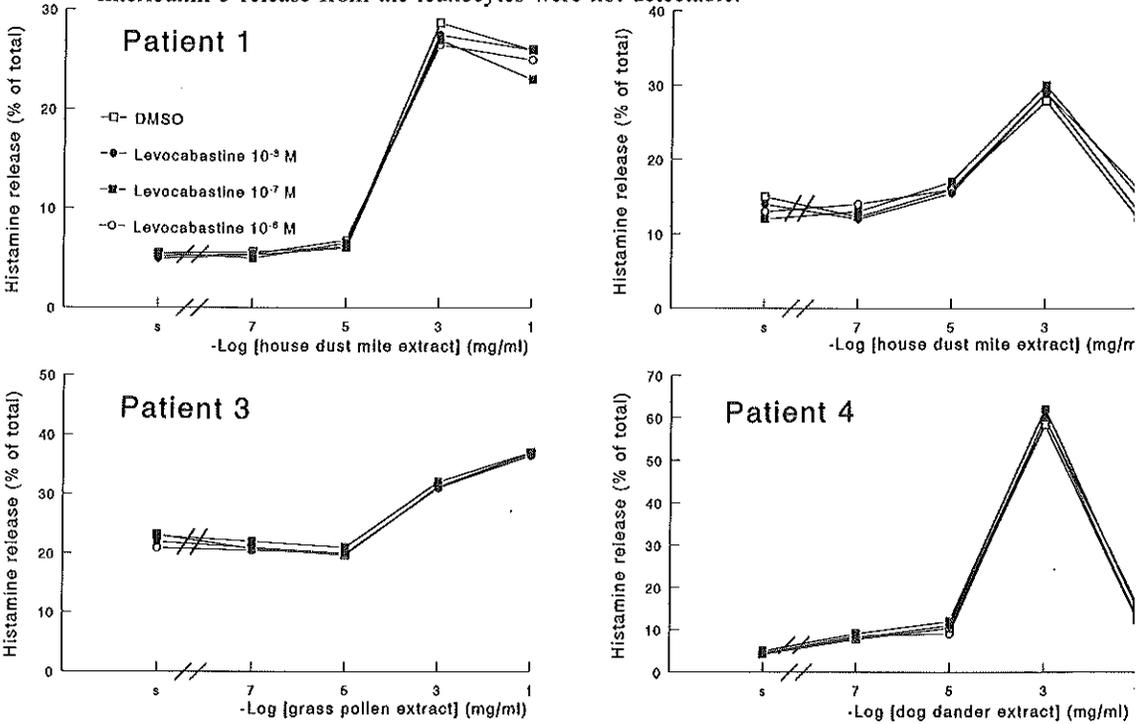
Figure 1. Effect of preincubation with levocabastine or vehicle control (DMSO) on spontaneous (s) or allergen-induced histamine release from human leukocytes obtained from allergic patients for 30 min. Values are expressed as % histamine release of the total amount of histamine present in the leukocytes.

Effect of levocabastine on the release of histamine and interleukin-5 by leukocytes obtained from healthy volunteers

The results of the histamine release in the first series of experiments (n=6) after incubation of the leukocyte suspension with DMSO or with different concentrations of levocabastine (10^{-14} to 10^{-3} M) for 1 min or 1 hour at 37°C are shown in Figure 2. Incubation for 1 hour significantly increased the histamine release in comparison with 1 min. Incubation with 10^{-4} and 10^{-3} M levocabastine significantly increased the release of histamine by leukocytes compared with the incubation with DMSO.

The results of the histamine release in the second series of experiments (n=6) after incubation of the leukocyte suspension with DMSO or with three concentrations of levocabastine (10^{-5}, 10^{-4} and 10^{-3} M) for different time intervals (0, 1, 2 and 3 hours) at 37°C are presented in Figure 3. The release of histamine by leukocytes was significantly increased by incubation with 10^{-3} M levocabastine for 1 to 3 hours.

Interleukin-5 release from the leukocytes was not detectable in either the first or the second series of experiments.
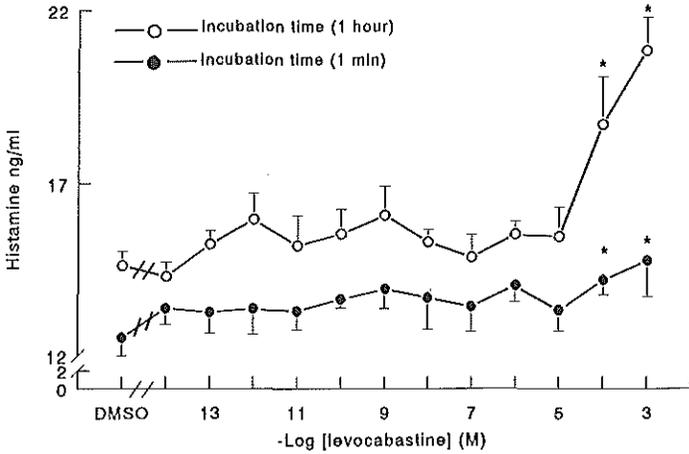
Figure 2. Histamine release by human leukocytes obtained from healthy volunteers after incubation for 1 min or 1 hour with vehicle control (DMSO) or with levocabastine (10^{-14} to 10^{-3} M). Values are expressed as means ± S.E.M. * P<0.05 vs histamine release after incubation with vehicle control.
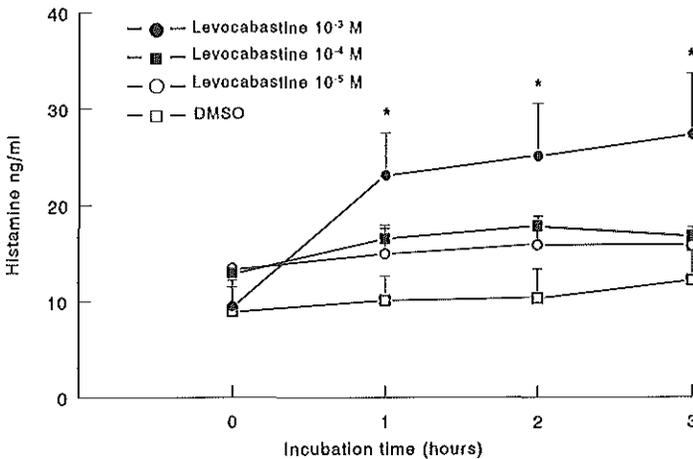
Figure 3. Histamine release by human leukocytes obtained from healthy volunteers before (0 hour) or after incubation for 1, 2 or 3 hours with vehicle control (DMSO) or levocabastine. Values are expressed as means ± S.E.M. * P<0.05 vs histamine release at 0 hour. When no error bar is visible, error falls within the limits of the symbol.

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Effect of levocabastine on the contractile response to histamine on guinea-pig trachea and lung parenchyma

The mean CCRCs for histamine with DMSO or with levocabastine for the guinea pig trachea and lung parenchyma in the first series of experiments are shown in Figure 4. The mean -log EC_{50} ($\pm$ S.E.M.) of the CCRC of histamine in the trachea and lung parenchyma were 5.62 $\pm$ 0.25 and 5.75 $\pm$ 0.06 (n=4) respectively, which were not significantly different. In both preparations, levocabastine decreased the E_{max} of histamine dose-dependently.
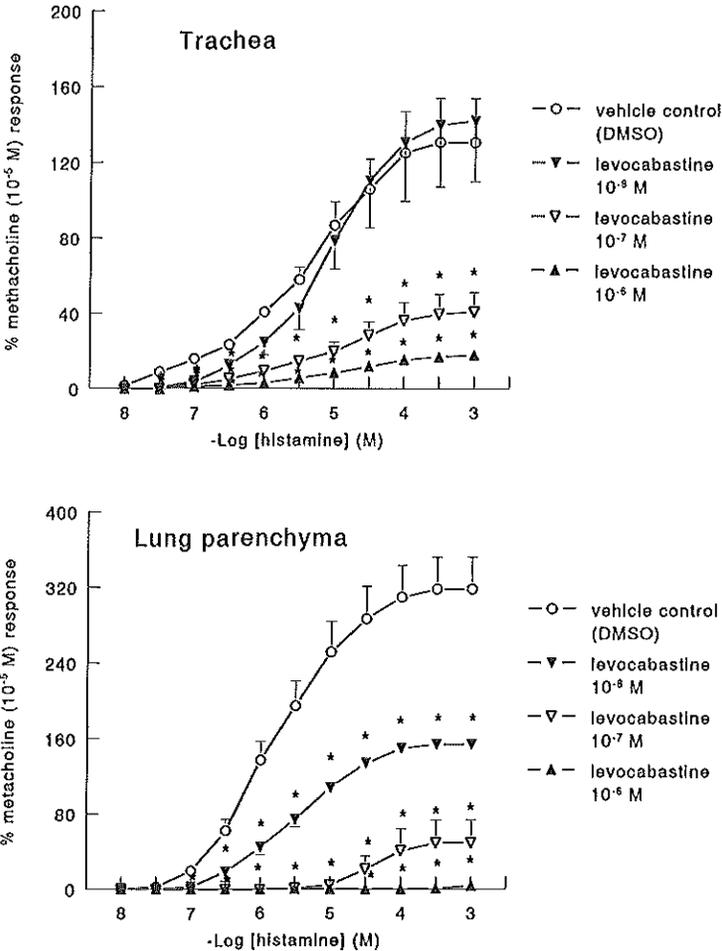
Figure 4. Effect of levocabastine on the histamine-induced contractions of the guinea-pig isolated trachea (upper curve) and lung parenchyma (lower curve). Values are expressed as means $\pm$ S.E.M. * P<0.05 vs control response. When no error bar is visible, error falls within the limits of the symbol.

The mean CCRCs for histamine with DMSO or with preincubation with the H_2 antagonist cimetidine and with DMSO or with levocabastine for the guinea-pig lung parenchyma in the second series of experiments are shown in Figure 5. The mean -log EC_{50} (± S.E.M.) of the CCRC with histamine in the presence of cimetidine on lung parenchyma was 5.53 ± 0.04 (n=4), which was not significantly different from the mean -log EC_{50} of the histamine-CCRC with DMSO. Cimetidine decreased the E_{max} of histamine, which was further decreased dose-dependently in the presence of levocabastine.
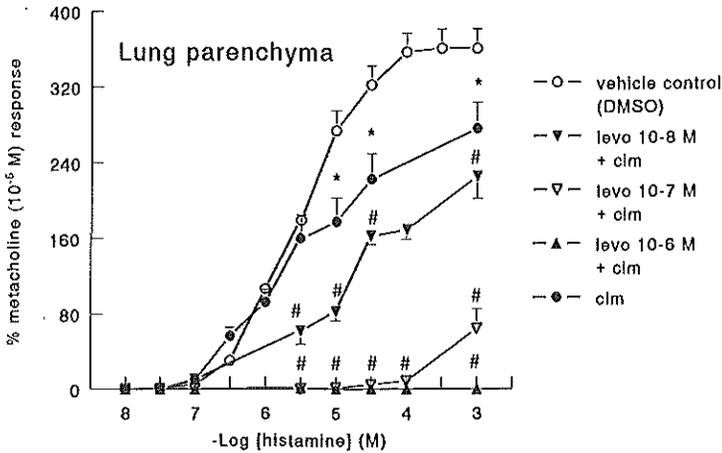
Figure 5. Effect of cimetidine (cim; 10^{-6} M) alone and levocabastine (levo) in combination with cimetidine (cim; 10^{-6} M) on histamine-induced contraction of the guinea-pig isolated lung parenchyma. Values are expressed as means ± S.E.M. * P<0.05 vs control response and # P<0.05 vs the response in presence of cimetidine. When no error bar is visible, error falls within the limits of the symbol.

Effect of levocabastine on the release of histamine and interleukin-5 by guinea-pig trachea and lung parenchyma
The results of the measurements of histamine concentrations in the organ baths of the first series of experiments are shown in Table 1.

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Table 1: Histamine concentration in guinea-pig organ bath solutions with DMSO and with incubation of levocabastine (10^{-8} to 10^{-6} M) for 15 min. Values are expressed as means ± S.E.M.

Levocabastine (M)		Histamine (ng/ml)
Trachea	vehicle control	0.36 ±0.06
	10^{-8}	548 ± 359
	10^{-7}	607 ± 367
	10^{-6}	55.1 ±26.6
Lung parenchyma	vehicle control	0.33 ±0.15
	10^{-8}	38.4 ±17.2
	10^{-7}	217 ± 79
	10^{-6}	139 ± 48

Table 2: Histamine concentration in guinea-pig lung parenchyma bath solutions with vehicle control and with incubation of cimetidine (10^{-6} M) for 15 min and with vehicle control and with levocabastine (10^{-8} to 10^{-6} M) for 15 min. Values are expressed as means ± S.E.M.

Levocabastine (M)	Cimetidine (M)	Histamine (ng/ml)
vehicle control	vehicle control	< 0.2
vehicle control	10^{-6}	10.0 ± 2.5
10^{-8}	10^{-6}	21.5 ± 5.1
10^{-7}	10^{-6}	23.8 ± 8.7
10^{-6}	10^{-6}	103 ± 58

Histamine concentrations in the bath solutions of the second series of experiments are shown in Table 2.

Krebs-Henseleit buffer, DMSO, levocabastine (10^{-8}, 10^{-7}, 10^{-6} M) and cimetidine (10^{-6} M) did almost not interfere with the fluorometric determination of histamine; measured concentrations were all < 0.2 ng/ml.

Incubation with levocabastine (10^{-8} to 10^{-6} M) increased the histamine release by guinea-pig trachea and lung parenchyma. After preincubation with 10^{-6} M cimetidine and thereafter incubation with levocabastine (10^{-8} to 10^{-6} M) the histamine release was increased in a dose-dependent manner. Incubation with exogenous histamine (10^{-8} to 10^{-3} M) did not

influence the endogenous histamine release by guinea-pig organs (data not shown).

Interleukin-5 concentrations in the bath solutions of both the first and the second series of experiments were not detectable by the human-interleukin-5 ELISA. This assay was used because no guinea-pig-interleukin-5 assay is available, but probably no cross-reactivity with human-interleukin-5 occurs.

14.6 Discussion

It is well accepted that histamine released locally from mast cells and basophils is one of the major mediators responsible for most of the symptoms, such as sneezing, itching, rhinorrhoea and congestion in allergic rhinitis. Mast cells seem to be important in the early antigen-induced reaction[244] and basophils seem to be involved mainly in the late reaction[308,317,558].

Topical application of levocabastine has been demonstrated to protect pollinosis patients[354] and patients with house dust mite allergy[254] against allergen-induced symptoms. The inhibitory effect of levocabastine on allergen-induced nasal reactivity to histamine and cell influx in patients with perennial rhinitis was investigated by Pazdrak *et al.*, which suggested that levocabastine possesses other properties than being only a selective H_1 receptor antagonist.[503] However, in our earlier studies the release of inflammatory mediators, such as histamine and tryptase, in nasal lavage fluid of patients allergic to house dust mite was not affected by treatment with levocabastine.[254] Several investigators have found that the most recent generation of antihistamines, such as terfenadine, cetirizine, astemizole and loratadine, possesses anti-inflammatory activities *in vivo* and *in vitro*, such as inhibitory effects on histamine release from mast cells and/or basophils.[12,72,106,173,425,437,462,464,504] Tasaka *et al* found that levocabastine did not inhibit histamine release from rat peritoneal mast cells induced by compound 48/80, A23187 or concanavalin A. In sensitized rat peritoneal mast cells, however, the drug caused a significant inhibition of histamine release, though relatively high concentrations were necessary.[613]

In the present study, we examined whether levocabastine has anti-inflammatory properties besides the classical H_1 receptor antagonism. We studied the effect of levocabastine on the release of histamine from human blood leukocytes and from guinea-pig isolated trachea and lung parenchyma.

Leukocytes from allergic volunteers: Our results showed that the allergen-induced release of histamine from leukocytes obtained from allergic volunteers was not influenced by levocabastine (10^{-8} to 10^{-6} M).

Leukocytes from healthy volunteers: Though lower concentrations (10^{-14} to 10^{-5} M) of levocabastine did not affect spontaneous release of histamine from leukocytes obtained from healthy volunteers, high concentrations (10^{-4} and 10^{-3} M) of the drug, not likely to be encountered in clinical situations, increased histamine release from these leukocytes. It is possible that incubation of leukocytes, obtained from allergic patients, with levocabastine (10^{-4} and 10^{-3} M) increase the release of histamine, but such high concentrations were not used in these experiments.

Guinea-pig isolated airways: Incubation with levocabastine (10^{-8} to 10^{-6} M) induced histamine release from guinea-pig isolated trachea and lung parenchyma in the organ bath

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solution with DMSO and with preincubation with the H_2 receptor antagonist cimetidine.

Thus levocabastine stimulated both the histamine release by human peripheral leukocytes (basophils) and of mast cells in the guinea-pig organs. It has been suggested that the histamine releasing properties of antihistamines are due to direct cytotoxic action and consequently cell lysis[371,372,454], but in our experiments levocabastine at the concentrations used did not modify cell viability, as assessed by trypan blue exclusion and Hemacolor (see Material and Method section), which suggests that the intracellular granules of the mast cells or basophils did not release histamine caused by mortality of the cells. Furthermore, it has also been reported that antihistamines have a dual action on cells: at high concentrations (>10mM) they enhance while at low concentrations they inhibit the release of histamine from mast cells.[674,371,372,454]

We did not study the intracellular mechanism of action of histamine release by levocabastine in these experiments. However, inhibitory effects of anti-histamines on intracellular Ca^{2+} release from the sequestered Ca^{2+} store and Ca^{2+} influx into the cell have been reported.[437,462,612] Other investigators have shown that a relationship exists between Ca^{2+} influx into the cell and histamine release.[196,405] Some antihistamines can inhibit histamine release by inducing membrane stabilization.[2] The dual action on cells by antihistamines could be caused by a biphasic action on Ca^{2+}: low concentrations of antihistamines inhibit intracellular Ca^{2+} release and Ca^{2+} influx into the cell, while high concentrations probably induce intracellular Ca^{2+} release and Ca^{2+} influx. Another hypothesis is that the release of histamine by the H_1 antagonist levocabastine could be due to a feedback mechanism regulated by the H_2 receptor[614]. Therefore, we performed the experiments with the H_2 antagonist cimetidine, but no effect was seen on the histamine release from the guinea-pig airways after preincubation with cimetidine.

Although levocabastine caused a dose-dependent release of histamine of human leukocytes and guinea-pig smooth muscle tissues, the clinical relevance is negligible. In a recent study we have demonstrated that nasal provocations with high doses of histamine of rhinitis patients allergic to the house dust mite have no systemic effects [De Graaf-in't Veld, personal communication].

In this study, we could not show that levocabastine, modulated the release of the cytokine interleukin-5, because interleukin-5 was not detectabled in the supernatant of the leukocyte suspensions. In an earlier study, we demonstrated that interleukin-5 plays an important role in allergic rhinitis. We found that interleukin-5 was present in detectable amounts in nasal lavages of patients with a house dust mite allergy before and after challenge with house dust mite extract, which immediately caused nasal symptoms, and in the late phase reaction.[220] It is known that T_{H2}-cells[452], mast cells and eosinophils[519] can produce interleukin-5. However, Alam and Grant demonstrated that basophils failed to produce interleukin-5.[3] The interleukin-5 concentrations in the organ baths were not detectable, because the organs were from guinea-pigs and the ELISA-kit was based on human-interleukin-5; there was no cross-reactivity between the two species.

In this study we showed that the selective H_1 receptor antagonist levocabastine significantly reduced the contractile response of guinea-pig isolated trachea and lung parenchyma caused by histamine in an irreversible competitive manner. After preincubation with levocabastine higher doses of histamine were necessary to obtain the same contractile responses, and the histamine-CCRC with levocabastine did not reach the same E_{max} in comparison to the histamine-CCRC with DMSO. After washing with Krebs-buffer for 2 hours levocabastine

was not diffused from the histamine receptors; furthermore new incubations with histamine resulted in the same histamine-CCRC in comparison to preincubation with levocabastine for 15 min. Thus, levocabastine reduced the contractile response of the guinea-pig tissues in an irreversible manner. Before and at the end of each experiment with levocabastine we performed CCRCs with methacholine. The same contractile responses were observed with methacholine before and after the experiments with levocabastine, which suggests that levocabastine is selective for histamine receptors. High concentrations of histamine caused a relaxation of the guinea-pig organs, which is not diminished by preincubation with the H_2 receptor antagonist cimetidine. The E_{max} of the histamine-CCRC with cimetidine did not reach the same contractile-response as the histamine-CCRC with DMSO, which is probably caused by the small H_1 receptor activity of cimetidine; K_B: H_1: 4.5×10^{-4} M, H_2: 0.8×10^{-6} M and H_3: 3.3×10^{-5} M.[530]

In conclusion, in addition to H_1 receptor blocking activity levocabastine has probably no anti-inflammatory properties. Levocabastine significantly reduced the contractile response of guinea-pig isolated trachea and lung parenchyma caused by histamine in a irreversible competitive manner and the histamine release from both human leukocytes and guinea-pig trachea and lung parenchyma only was significantly increased after incubation with levocabastine at high concentrations.

14.7 Acknowledgments

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PART FOUR

Hyperreactivity and quality of life

Chapter 15
The relationships between nasal hyperreactivity, quality of life and nasal symptoms in perennial allergic rhinitis

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15.1 Abstract

Background: It would be useful to have a clinical test that would be able to inform the clinician about the severity of the patients nasal symptoms and health-related quality of life (QOL).

Objective: We investigated if in patients with perennial allergic rhinitis nasal challenge with histamine may be used to estimate daily symptoms and QOL.

Methods: Forty-eight patients with perennial allergic rhinitis were challenged with histamine to determine nasal hyperreactivity. Nasal response was monitored by the number of sneezes, the amount of secretion and a symptom score. Daily nasal symptoms were recorded during the two preceding weeks. Patients also completed a rhinitis QOL questionnaire (according to Juniper).

Results: Responsiveness to histamine and total daily nasal symptoms were moderately correlated ($r=0.51$, $p=0.001$). Comparing total daily nasal symptoms with the overall quality of life score showed a moderate correlation ($r=0.59$, $p<0.001$). Nasal response to histamine and overall QOL score were also correlated ($r=0.43$, $p=0.002$). However, overall QOL and daily nasal symptoms could be predicted by wide 95% prediction intervals only for each decade of nasal responsiveness to histamine (expressed by a composite symptom score).

Conclusion: In patients with perennial allergic rhinitis nasal hyperreactivity as determined by histamine challenge, quality of life and daily nasal symptoms are moderately correlated. Therefore, nasal histamine challenge can be used as a tool to estimate the severity of daily nasal symptoms and QOL, although it can not predict nasal symptoms and QOL very accurately.

Keywords: perennial allergic rhinitis; daily symptoms; quality of life; nasal hyperreactivity; histamine challenge

15.2 Introduction

Patients with allergic rhinitis suffer from sneezing, nasal itchyness, rhinorrhoea and nasal blockage after exposure to the relevant allergen. After repetitive exposure to allergen, nasal responsiveness to allergens increases. This phenomenom, known as nasal priming, was first described by Connell[120] and was confirmed by others[28,261]. Exposure to allergen also increases nasal responsiveness to non-specific stimuli.[10] This non-specific nasal hyperreactivity is particularly important in patients with perennial allergic rhinitis, since they are continously exposed to allergen.

Hyperreactivity can be described as a clinical feature characterized by occurence of symptoms on exposure to daily-life stimuli as estimated by history (clinical hyperreactivity). Determination of nasal hyperreactivity by nasal provocation tests with pharmacological or physical agents assumes a relationship between this form of nasal hyperreactivity and hyperreactivity as estimated by history. Gerth van Wijk et al.[228] demonstrated that the amount of secretion and the number of sneezes in response to histamine challenge were associated with the clinical hyperreactivity assessed by a hyperreactivity score.

The importance of routine histamine challenges in daily practice is not clear. In a previous study, we showed that assessment of nasal airway resistance after histamine challenge is less appropriate in distinguishing patients from healthy subjects in terms of reproducibility and estimation of clinical hyperreactivity compared with assessment of number of sneezes and amount of secretion.[225] Recently, we demonstrated that reactivity to histamine expressed as area under the curve with a composite symptom score (according to Lebel[373]) as response variable is a valid method to detect treatment effect.[251] In addition the method is valid to distinguish between patients with and without a late-phase allergic reaction.[227] Moreover, this method appears to be reproducible.[252]

Recently, a correlation between daily symptoms and response to histamine challenge was demonstrated in patients suffering from non-allergic nasal hyperreactivity.[265] We investigated if in patients with perennial allergic rhinitis nasal hyperreactivity (responsiveness to histamine) corresponds with daily nasal symptoms. Besides the amount of secretion and the number of sneezes, the symptom score of Lebel[373] was used to monitor the nasal responsiveness to histamine.

Patients' suffering from nasal symptoms, also interferes with their day-to-day lives. To measure the influence of nasal symptoms on day-to-day life, rhinitis quality of life (QOL) questionnaires have been developed.[71,335,336] Juniper et al. demonstrated that QOL deteriorated after allergen exposure (pollen season) and increased after symptomatic treatment.[335,336]

We studied the questions if in patients with perennial allergic rhinitis the daily nasal symptoms are correlated with QOL, and also if nasal hyperreactivity, as measured by nasal histamine challenge, is correlated with QOL scores and nasal symptoms. Furthermore, we studied whether nasal histamine challenges can be used as a tool to estimate the severity of patients daily nasal symptoms and health-related QOL.

15.3 Materials and Methods

Study design
Each adult patient with perennial allergic rhinitis visiting our outpatient clinic for the first time completed a nasal symptom diary during the two weeks preceding the follow-up visit. At this second visit, patients completed a rhinitis QOL questionnaire and underwent a nasal histamine challenge.

Subjects
Forty-eight patients (23 males, 25 females, aged 30 ± 10 years) with a history of perennial rhinitis participated in this study. They had to meet the following criteria: perennial rhinitis for at least one year; positive intradermal skin test with at least one perennial allergen (house dust mites and/or moulds and/or animal dander, ALK Benelux, Groningen, the Netherlands) and exposure to the allergen(s) involved. Of the patients included, 38 were allergic to house dust mites, 7 were allergic to house dust mites and pets (and had a pet at home), 2 were allergic to their pets only, 1 patient was allergic to moulds. Patients with a concomitant allergy to pollen were only included outside the pollen season. During the study they were not permitted to use any medication affecting nasal symptoms, apart from Acrivastine 8 mg (as escape medication). Acrivastine had to be withdrawn 3 days before the histamine challenge. Patients with a nasal infection were excluded.

Nasal symptom diary
Patients recorded their nasal symptoms during two weeks on a modified diary card according to the symptom score system of Norman[479]. Sneezes/itching nose, nasal blockage, and rhinorrhoea were scored according to their daily duration (0 = no symptoms, 1 = symptoms<1 hour, 2 = symptoms between 1 and 4 hours, 3 = symptoms>4 hours). The sum of the individual symptoms during the 2 weeks and the total nasal symptom score (sneezing + rhinorrhoea + nasal blockage) during these 2 weeks were used for statistical analysis.

Rhinitis quality of life questionnaire
We translated the rhinitis QOL questionnaire developed by Juniper *et al.*[336] Patients had to score 25 items subdivided into the following domains: sleep (n=3), nasal symptoms (n=5), practical problems (n=3), emotions (n=4), activities (n=3), and nonrhinitis symptoms (n=7). They were asked how much they were troubled in each item as a result of their nasal symptoms during the previous week. The score of these items ranged from 0 (not troubled) to 6 pts (extremely troubled). In case of the items concerning emotions, patients were asked how often they were troubled in each of the items as a result of their nasal symptoms. The score also ranged from 0 pts (never troubled) to 6 pts (constantly troubled). In the analysis the mean score of the items in each domain was used. In addition, the total score of the means of the six domains was used to calculate the overall QOL score.

Nasal histamine challenge
Challenges were performed according to the methods described by Gerth van Wijk *et al.*[225,228] Patients waited for half an hour prior to the test in order to give the nasal mucosa time to acclimatise. Nasal challenge with histamine phosphate (0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) was performed at five-minute intervals after challenge with phosphate-buffered saline (PBS). The PBS and the increasing doses of histamine were sprayed into the nostrils with a nasal pump spray delivering a fixed dose of 0.125 ml solution. After each challenge the symptom score[373] was recorded, sneezes were counted and anterior rhinorrhea was measured by collecting nasal secretion in a syringe-equipped funnel[68]. The areas under the curve of histamine dose response were used in the statistic analysis.

Symptom score
The symptom score according to Lebel *et al.*[373] was used: 3-4 sneezes=1 pt, ≥5 sneezes=3 pts; anterior rhinorrhoea=1 pt; posterior rhinorrhoea=1 pt; difficult nasal breathing=1 pt, one nostril blocked=2 pts, both nostrils blocked=3 pts; pruritus of the nose=1 pt; pruritus of the palate or ear=1 pt and conjunctivitis=1 pt. Total score ranges from 0 till 11 pts.

Statistics
40 patients completed their nasal symptom diary correctly; in comparing nasal symptoms with QOL scores and nasal hyperreactivity we used data of these patients only (n=40). When testing the correlation between QOL scores and nasal hyperreactivity all data could be used (n=48).
Because not all variables were normal distributed, relationships between the variables were tested by Spearman rank correlations. A two-tailed p-value <0.05 was considered significant. Furthermore, the mean predicted values and 95% prediction intervals of overall QOL and total nasal symptoms for each decade of nasal response to histamine challenge were determined by multiple regression analysis (this method could be used since these three variables were normal distributed).

15.4 Results

Table I shows correlation coefficients of daily nasal symptoms compared with nasal response to histamine. Histamine responsiveness is expressed as the AUC of the amount of secretion, the number of sneezes and the symptom score of Lebel after nasal histamine challenge. Moderate, but statistical significant correlations were found between histamine responsiveness, expressed as symptom score of Lebel[373] and daily nasal symptoms. The secretory response to histamine is moderately correlated with daily secretion only. Expressing histamine responsiveness as the number of sneezes no correlations were found. Table II shows the correlation coefficients of nasal response to histamine and QOL domains. The symptom score of Lebel[373] correlates moderately with most QOL domains with exception of sleep and emotions. However, the correlations with sleep and emotions tended to be significant ($r=0.27$, $p=0.067$ and $r=0.24$, $p=0.098$ respectively). The number of sneezes is correlated with the activities and with overall QOL score only. Secretory response to histamine is correlated with sleep, nonrhinitis symptoms and the overall QOL score.

Comparing daily nasal symptoms with the QOL domains, moderate, but statistical significant correlations are shown for all domains and the overall QOL score (table III). Of the individual symptoms, itchyness/sneezing is correlated with five of the six QOL domains and the overall QOL, congestion with three domains and the overall QOL, and secretion with two domains and the overall QOL only.

When daily Acrivastine intake was added to the total score of the diary, as Norman did[479], the correlations of total daily symptoms with hyperreactivity were 0.05 (sneezes), 0.26 (secretion) and 0.51 (symptom score) compared to 0.10, 0.24 and 0.51 without Acrivastine.

Table I. Nasal hyperreactivity versus daily nasal symptoms (n=40). Associations are presented by Spearman rank correlation coefficients (r). *= p-value< 0.05.

Daily nasal symptoms	Nasal hyperreactivity		
	Sneezes	Secretion	Symptom score
Itchyness/Sneezing	r=0.00	r=0.22	r=0.49*
Secretion	r=0.04	r=0.41*	r=0.39*
Congestion	r=0.14	r=0.05	r=0.31*
Total symptoms	r=0.10	r=0.24	r=0.51*

Table II. Quality of life versus nasal hyperreactivity (n=48). Associations are presented by Spearman rank correlation coefficients (r). *= p-value< 0.05.

Quality of life	Nasal hyperreactivity		
	Sneezes	Secretion	Symptom score
Sleep	r=0.15	r=0.36*	r=0.27
Nonrhinitis symptoms	r=0.28	r=0.34*	r=0.29*
Practical problems	r=0.10	r=0.25	r=0.34*
Nasal symptoms	r=0.26	r=0.18	r=0.47*
Activities	r=0.37*	r=0.21	r=0.32*
Emotions	r=0.07	r=0.16	r=0.24
Overall QOL	r=0.31*	r=0.32*	r=0.43*

Correlations of total daily symptoms with QOL domains ranged from 0.37 to 0.60 compared to 0.35 to 0.59 with or without Acrivastine respectively. So Acrivastine intake did not change the correlation coefficients

In figure 1 the association between overall QOL and total daily symptoms is shown (r=0.59, p<0.001).

In table IV predicted values and 95% prediction intervals of overall QOL and total nasal symptoms for each decade of nasal response to histamine are demonstrated. Nasal response to histamine explained 21% of the variance of nasal symptoms and 21% of the variance of QOL.

Table III. Quality of life versus daily nasal symptoms (n=40). Associations are presented by Spearman rank correlation coefficients (r). *= p-value< 0.05.

Quality of life	Daily nasal symptoms			
	Itchyness/ Sneezing	Secretion	Congestion	Total
Sleep	r=0.33*	r=0.25	r=0.29	r=0.35*
Nonrhinitis symptoms	r=0.37*	r=0.19	r=0.29	r=0.36*
Practical problems	r=0.57*	r=0.37*	r=0.39*	r=0.56*
Nasal symptoms	r=0.61*	r=0.43*	r=0.23	r=0.54*
Activities	r=0.46*	r=0.14	r=0.52*	r=0.45*
Emotions	r=0.29	r=0.19	r=0.36*	r=0.36*
Overall QOL	r=0.59*	r=0.36*	r=0.47*	r=0.59*
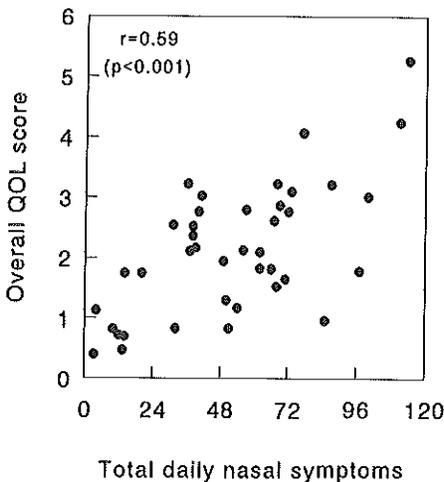
Figure 1. Overall quality of life score plotted against total daily nasal symptoms recorded during two weeks (n=40).

Table IV. Predicted nasal symptoms (n=40) and QOL (n=48) for each decade of the hyperreactivity range

Hyperreactivity symptom score	Predicted nasal symptom score		Predicted QOL score	
	mean	95% prediction interval	mean	95% prediction interval
10	34.98	0.00 - 88.34	1.43	0.00 - 3.44
20	48.46	0.00 - 100.79	1.89	0.00 - 3.88
30	61.94	0.31 - 114.57	2.36	0.37 - 4.35
40	75.42	21.19 - 129.65	2.81	0.79 - 4.85
50	88.90	31.88 - 145.93	3.29	1.18 - 5.39

15.5 Discussion

We investigated if routine nasal histamine challenges in patients with perennial allergic rhinitis can be used to give an impression of the severity of patients' daily nasal symptoms. Nasal response to histamine, if expressed as the symptom score of Lebel et al.[373], correlated moderately with sneezes/itchyness, rhinorrhoea, congestion, and total nasal symptoms. Secretory response to histamine correlated only weakly with nasal symptoms, wheras the number of sneezes to histamine challenge was not correlated with daily nasal symptoms at all. This is in contrast with a previous study of Gerth van Wijk et al., who demonstrated that histamine-induced sneezes and secretion correlated well with daily nasal symtoms.[228] However, the diary he used contained, besides the symptom score system of Norman[479], also specific questions about nasal hyperreactivity, so the two diaries are not completely comparable. This might suggest that routine histamine challenges in daily practice, if monitored by the symptom score of Lebel et al.[373], give a good impression of the severity of patients' daily symptoms. The correlations, however, were only moderate and daily nasal symptoms could be predicted by wide 95% prediction intervals only. It can not be expected from one clinical test to be able to predict exactly daily nasal symptoms. It might be used however, in addition to a carefully taken history, to make a better estimation of the severity of patients' symptoms.

Rhinitis patients not only suffer from the nasal symptoms themselves, their day-to-day lives can also be impaired by their nasal symptoms. We used the rhinitis QOL questionnaire developed by Juniper et al.[336] to measure the effect of rhinitis on day-to-day life. Juniper has validated this questionnaire and has shown that it is a useful instrument in clinical trials to detect within-subject changes over time (treatment effect, allergen exposure). We did not study changes in QOL but we investigated if QOL scores are correlated with the daily nasal symptoms and nasal response to histamine.

Malo et al. demonstrated that in patients with asthma, QOL and bronchial hyperreactivity were correlated.[414] Since the relationship between nasal hyperreactivity and QOL has not been studied yet, we investigated if in patients with perennial allergic rhinitis, such a

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relationship could be demonstrated also. Nasal hyperreactivity was determined by nasal response to histamine. Expressing histamine responsiveness by the symptom score a significant correlations were found with all QOL domains with exception of emotions and sleep. The correlation coefficients found in our rhinitis study were moderately ($0.24 < r < 0.47$), however they were equal or higher than in asthma studies. Correlation coefficients between bronchial responsiveness to methacholine found by Malo et al.[414] ranged from -0.1 to -0.24. Marks et al.[418] studied the relationship between changes in bronchial hyperreactivity and changes in QOL; correlation coefficients ranged from 0.14 to 0.47.

The number of sneezes and the amount of secretion in response to histamine challenge correlated only slightly with some of the QOL domains. So when comparing nasal hyperreactivity with QOL, nasal hyperreactivity expressed as the symptom score of Lebel[373] is a better variable than the number of sneezes and/or the amount of secretion.

Comparing QOL with nasal symptom diary, QOL scores (all six domains and the overall score) correlated with total nasal symptoms, indicating that in patients with more severe rhinitis symptoms, QOL is more impaired. The moderate correlations between nasal symptoms and the nasal symptom domain of QOL might be due to the indivudual perception of the degree of impairment caused by the nasal symptoms. Some patients might be impaired a lot by only a few nasal symptoms, while other might be less impaired in spite of more severe nasal symptoms. The correlation coefficients we found (0.35 to 0.59) are about in the same range as those found by Juniper et al.[335] (0.31 to 0.59), however the results are not totally comparable, since Juniper compared changes in nasal symptoms with changes in QOL.

Comparing QOL scores with the individual nasal symptoms, a better correlation was seen with sneezes/itchyness than with rhinorrhoea and congestion, so that it seems that sneezing/itchyness causes the most impairment of day-to-day life. Perhaps patients become used to a stuffy nose, and blowing their nose, but sneezing and nasal itchyness are hard to ignore and more difficult to accept. Since the Dutch version of the by Juniper developed and validated rhinitis QOL questionnaire correlates well with daily nasal symptoms, it can also in the Netherlands be used as a valid tool to measure rhinitis QOL.

When Acrivastine intake was added to the total nasal symptoms, as Norman did[479], the correlations between total nasal symptoms and QOL or responsiveness to histamine challenge did not change. So it seems to make no difference whether Acravistine intake is included or not. Norman also found that by using the symptom diary to interpret the clinical effectiveness of immunotherapy it made no difference when the use of antihistamines was included or not.

To conclude: In patients with perennial rhinitis nasal hyperreactivity, QOL and daily nasal symptoms are moderately correlated. Therefore, routine histamine challenge can be used as tool to obtain an impression of daily nasal symptoms and rhinitis QOL, although it can not predict nasal symptoms and QOL very accurately.

15.6 Acknowledgements

PART FIVE

General discussion and summary

Chapter 16
General discussion

The investigations described in this thesis were initiated to study the involvement of inflammation in nasal hyperreactivity in allergic rhinitis. Several methods can be used to investigate the presence of inflammation in the nasal mucosa. Since the study-design was to investigate (the dynamics of) nasal inflammation at different time points over a period of 9.5 hours after allergen challenge, a non-invasive method was needed, causing as little as possible damage to the nasal mucosa and with only little inconvenience for the patients. Therefore, the nasal lavage method according to Naclerio et al.[465] was used instead of methods like nasal biopsies or brushes. However, this method has also some disadvantages; for example, the levels of inflammatory mediators are diluted by the large lavage volume and only small amounts of inflammatory cells can be detected in the nasal lavage fluid. Since the mucous secretion could not be dissolved with the lavage fluid to a homogenous manner to detect mediators and cells, nasal lavage fluid and nasal secretion were separately collected. Because only a few inflammatory cells could be detected in lavage fluid, nasal inflammation was estimated by the levels of inflammatory mediators only. Nasal symptoms to HDM challenge were recorded according to a scoring system described by Lebel et al.[373], which consists of both objective and subjective symptoms. Earlier studies have shown that this scoring system is suitable to measure both the early and late phase response to allergen challenge.[229] This symptom score was used in order to study the correlation between the clinical response and the inflammatory mediators and nasal hyperreactivity.

Since it is generally known that during the late phase response inflammatory cells [42,642] and mediators[55] are involved, the first goal was to investigate if nasal hyperreactivity was correlated with the immediate and the late allergic response after allergen challenge, which might indirectly give evidence for the involvement of inflammation. The second goal was to investigate the relationship between nasal hyperreactivity and inflammatory response, as measured by inflammatory mediators in nasal lavage fluid. According to the symptom scores patients were divided in two groups. The first group consisted of patients with only an early clinical response, the second consisted of patients with both an early and late phase clinical response to allergen provocation. The early and late allergic reaction were correlated with each other and also with nasal hyperreactivity. Patients with a late phase response demonstrated increased levels of tryptase and albumin during the early response and higher levels of albumin and ECP during the late phase response in comparison with patients with an early response only. Also nasal response to histamine challenge was more severe in the group with both an early and late phase response. Our findings suggest that a strong early response gives rise to increased amounts of chemotactic mediators with as a consequence an increased amount of inflammatory cells a few hours later, during the late phase response, and an increased hyperreactivity 24 hours later. The results suggested that nasal hyperreactivity could be influenced by the degree of nasal inflammation.

Mechanisms of allergic rhinitis and nasal hyperreactivity can also be studied by therapeutic intervention. The third goal was to study the effect of two different drugs on the clinical immediate and late phase response to HDM challenge, on the nasal inflammation and on the allergen-induced nasal hyperreactivity in perennial allergic rhinitis patients.

First, the effects of intervention with the topical glucocorticoid fluticasone propionate were investigated. Both the clinical immediate and late phase response to nasal provocation with HDM extract were inhibited by fluticasone propionate. Assessment of albumin in nasal lavage fluid demonstrated an increased influx during both the immediate and late phase reaction, which indicated an increased vasopermeability.[604] Tryptase, a specific marker of mast cells[560], was only detected during the immediate nasal response, whether ECP as a marker of activated eosinophils[267] only could be measured during the late nasal response. PAF, PGD_2 and $LTC_4/D_4/E_4$ only were released immediately after allergen challenge. As described in chapter 2, mast cells and macrophages are able to release these mediators. Basophils, eosinophils and neutrophils are known to produce LTC_4 and PAF, whereas platelets can release PGD_2 and PAF. Epithelial cells are also able to produce PAF. The levels of IL-5 in the nasal lavage fluids was also measured, which was increased during the immediate and late phase reaction. It is known that IL-5 is produced by mast cells, eosinophils and T_{H2}-cells. Nasal challenge with allergen did neither cause a release of NO during the immediate nor in the late phase reaction, which suggests that NO plays no important role in hyperreactivity in the nose. The release of inflammatory products in the nasal lavage fluid during the immediate and late phase reaction to HDM challenge indicates that inflammation is involved in this allergic process. Fluticasone propionate decreased the release of these inflammatory products, which suggested that the nasal inflammation was inhibited. Allergen-induced nasal hyperreactivity was investigated by the clinical response described by Lebel et al.[373] to histamine provocation, which was decreased by fluticasone propionate. These findings suggest that fluticasone propionate may act by preventing activation of several cells during the early response and subsequent release of inflammatory mediators, which could be chemotactic to other cells and could cause the late phase response. The results showed that the late allergic reaction and hyperreactivity are associated and prevention of the late reaction resulted in a decreased nasal hyperreactivity. So, indirectly, it is demonstrated that inflammation and nasal hyperreactivity are associated.

In addition, the relationship between the reduction of symptoms due to treatment with the glucocorticosteroid and the glucocorticosteroid receptor characteristics was investigated, because a few patients respond less to these drugs. No correlation between the reduction of symptoms due to fluticasone propionate and the glucocorticoid receptor characteristics of the perennial allergic rhinitis patients was found. This suggests that the extent of reduction of symptoms due to corticoids is neither dependent on the number nor on the affinity of the corticoid receptors, but probably on other (intracellular) pathways.

Secondly, the effects of intervention with the topical antihistamine levocabastine were investigated. Another study with perennial rhinitis patients was performed to investigate 1) the association between inflammation and nasal hyperreactivity and 2) whether levocabastine has anti-inflammatory properties. Treatment with the histamine receptor antagonist levocabastine decreased the immediate clinical response to allergen challenge and histamine provocation, but did not decrease the nasal hyperreactivity (assessed by nasal response to methacholine). Because in this study no late nasal response was measured (symptom score and release of ECP), no effect of levocabastine on the late allergic response was detected. Levocabastine did not decrease the levels of albumin and tryptase in the nasal lavage fluids. This study suggested that levocabastine has no anti-inflammatory properties, besides its selective histamine H_1 receptor antagonism. To investigate further

whether levocabastine, also has anti-inflammatory properties an *in vitro* study was performed. The results showed that levocabastine, in contrast to other antihistamines[12,173,464], did not seem to possess anti-inflammatory properties. The histamine release from human leukocytes and guinea-pig trachea and lung-parenchyma was increased by the drug in relatively high doses, which are very unlikely to be encountered in clinical situation, particularly after topical application. These studies also showed that levocabastine has no anti-inflammatory properties and has no effect on nasal hyperreactivity, which also indirectly demonstrates an association between inflammation and nasal hyperreactivity.

The fourth goal was to study the relation between hyperreactivity and inflammation in the non-challenged situation, i.e. during natural HDM exposure. In this study only one lavage and one brush to measure inflammation was performed. Albumin, ECP, tryptase, PGD_2 and $LTC_4/D_4/E_4$ in lavage fluid did not correlate with nasal hyperreactivity. In contrast to nasal lavage fluid, sufficient cells in the brushes were detected. Eosinophils were present in increased amounts in comparison to healthy volunteers. The percentage of eosinophils showed a mild correlation with nasal responsiveness to histamine, indicating that eosinophils are involved in hyperreactivity.

As a final goal, the relationship between nasal hyperreactivity in perennial allergic rhinitis patients and their quality of life was studied. Mild correlations were found between quality of life scores and nasal hyperreactivity, assessed by nasal response to histamine according to the symptom score of Lebel *et al.*[373] Therefore routine nasal histamine challenges can probably be used to estimate health related quality of life and daily nasal symptoms.

The investigations described in this thesis demonstrated an association between nasal hyperreactivity and inflammation in perennial allergic rhinitis patients. In the lower airways the late phase response to allergen challenge was found to be associated with inflammation and bronchial hyperreactivity[96,115,162,445], suggesting that inflammation is involved in the pathogenesis of hyperreactivity. Patients with seasonal allergic rhinitis showed an increased response to histamine and methacholine during the pollen season[119], as well as after nasal allergen challenge[10,346,395]. However, no relation was found between nasal hyperreactivity and late phase response[230], and activation of eosinophils[346] respectively and between nasal priming and late phase reaction in patients allergic to pollen outside the pollen season. Patients with perennial allergic rhinitis suffer from almost continuous nasal symptoms throughout the year, this situation is in agreement with asthmatics. The nasal lavage model with perennial allergic rhinitis patients is more suitable to study the association between nasal hyperreactivity and inflammation than with pollinosis patients.

Our studies deal with several inflammatory cells and their products which play a predominant role in the modulation of perennial allergic rhinitis. Although some of these mediators, such as histamine, prostaglandins and leukotrienes may be biologically active in rhinitis, the role of others needed clarification.
The studies presented in this thesis showed that tryptase, PAF, PGD_2, $LTC_4/D_4/E_4$ and IL-5 are released in the nasal lavage fluid immediately after allergen challenge of patients allergic to HDM. This indicates that primary cells such as mast cells, antigen-presenting cells and epithelial cells and secondary cells such as basophils, eosinophils, neutrophils, T_H-

cells and platelets are important in the early clinical response. The results of this study demonstrated that ECP and IL-5 are released several hours after allergen challenge, which suggests that eosinophils and T_{H2}-cells play an important role during the late phase response. However, in the nasal lavage method only low amounts of inflammatory cells could be detected in the nasal lavage fluid, therefore, it was not possible to confirm that these cells were responsible for the production of the inflammatory mediators. This could be due to the fact that the mucous secretions were not dissolved in the lavage fluid, whereas others made a miscellaneous mixture of the secretion and lavage fluid.

In a biopsy study an increased percentage of mast cells was found at the surface of the nasal mucosa after allergen challenge[189] during the immediate allergic reaction. During the late phase response, an influx of eosinophils[42] and basophils[44] was demonstrated in the nasal lavage fluid and also an increase of T_{H}-cells, eosinophils and neutrophils was found in the nasal mucosa[642]. During the pollen season, more Langerhans cells were demonstrated in the nasal mucosa[191] and an increased level of blood basophils[100] was found compared to outside the season. Our results demonstrated that only the amount of eosinophils was increased in the nasal brushes of patients during natural HDM exposure compared with healthy volunteers, which showed a correlation with nasal responsiveness to histamine. This indicated that eosinophils play an important role in perennial rhinitis and hyperreactivity. This is in contrast with other investigators, who found no relation between eosinophils and hyperreactivity.[346] Others demonstrated an increased influx of neutrophils in nasal lavages of rhinitis patients during hyperreactivity.[255] Also the involvement of mast cells in hyperreactivity could be of importance.[620] Damage to the epithelial layer contributes to an increased bronchial hyperreactivity[145], because neuroendopeptidase is decreased, which resulted in an increased level of substance P (SP), but this is not demonstrated in the nose.[391,642]

Increased concentrations of histamine have been found in nasal lavages of rhinitis patients during the immediate[230] as well as the late phase[44,467] responses to allergen challenge. In our studies no release of histamine was detected in the nasal lavage fluid during either the early or late phase reaction. This difference is probably due to the fact that the mucous secretions were not dissolved in the lavage fluid, whereas others made a miscellaneous mixture of the secretion and lavage fluid. However, another mast cell product, tryptase, was released during the immediate response. A possible explanation might be that histamine is metabolized or removed more rapidly than tryptase, which has been demonstrated in the circulation.[567] Histamine increases the chemotactic response of eosinophils[111] and neutrophils[572], controls the histamine release from basophils[632], modulates the immunoglobulin production[171,392] and suppresses the antibody secretion from plasma cells[431]. Tryptase is released immediately in nasal lavage fluid after allergen or cold dry air challenge of rhinitis patients[95,524], who indicates an important role of mast cells in the early clinical response and in hyperreactivity. Tryptase is not released during the late allergic response, but still histamine is found by Naclerio et al.[467], which indicated that basophils play an important role in the late allergic response. It is known that tryptase could cleave VIP and CGRP, but not SP, NK_A and NK_B.[96,608] Our results showed only an increase in PGD_2 release during the immediate response to allergen challenge, which is also found by other investigators.[478] Only mast cells are known to produce PGD_2 in the nasal mucosa[559], which suggests that mast cells are only of importance during the early phase response. The cysteinyl leukotrienes are only found in the nasal lavage fluids during the immediate

response to provocation with HDM, which is also found by Georgitis et al.[223] In contrast to other investigators[438], our results demonstrated increased levels of PAF during the immediate allergic reaction to allergen challenge. Application of PAF in the nose caused an increase in eosinophils in the nasal lavage fluid of rhinitis patients.[345] PAF is an activator of eosinophil, platelet, neutrophil, monocyte and macrophage chemotaxis[283,483,639,666,691] in vitro, superoxide-anion production[275] and activator of the release of eicosanoids[81] and histamine[118] from several cells. It is not known whether PAF is correlated with hyperreactivity, because in humans PAF induced bronchial hyperresponsiveness to methacholine[104], but other investigators found that PAF failed to induce hyperreactivity[363,523]. However, prior inhalation of the PAF antagonist BN52021 inhibited the bronchoconstriction induced by PAF and allergen in asthmatics.[303] During natural allergen exposure increased levels of ECP were found in the nasal mucosa.[603] After nasal allergen challenge of rhinitis patients increased levels of eosinophil-derived mediators, such as ECP, were found in the nasal lavage fluid during the late phase response[43,55], which was confirmed by our results. These eosinophil-derived granule proteins can stimulate mast cell secretion[281], are cytotoxic to several cells[280] and can inhibit T-lymphocyte proliferation[508]. Increased levels of IL-5 were demonstrated in the nasal lavage fluids during both the immediate and late phase response. After allergen challenge IL-3, IL-4 and IL-5 were found in nasal biopsies, which was correlated with the number of activated T-cells and eosinophils[163] and eosinophil granule proteins and LTC_4 levels[569]. Application of IL-5 in the nose of allergic rhinitis patients increased the numbers of eosinophils and epithelial cells, ECP and IgA in nasal lavage fluid as well as the hyperreactivity.[591,617]

Our study was confined to inflammatory cells and their products which certainly play a predominant role in the modulation of perennial allergic rhinitis, but it should be kept in mind that allergic inflammation and hyperreactivity as mentioned before are not induced by the action of one single cell type or one mediator, but by a variety of cells and mediators.

Concluding remarks

In answer to the aims of the study it is shown that in nasal hyperreactivity the clinical immediate and late allergic response and the inflammatory reaction to allergen challenge were associated in patients with perennial allergic rhinitis. Anti-inflammatory therapy with the glucocorticosteroid fluticasone propionate reduced the nasal hyperreactivity, nasal inflammation, and the immediate and late clinical response to allergen provocation. Intervention with the antihistamine levocabastine, which has no anti-inflammatory properties, inhibited the immediate nasal response, but had no effect on nasal hyperreactivity and nasal inflammation after allergen challenge. During natural allergen exposure, nasal hyperreactivity was associated with nasal inflammation. Nasal hyperreactivity was associated with the quality of life and daily nasal symptoms.
In addition, the symptoms score according to Lebel et al.[373] is suitable to monitor the clinical immediate and late phase response to allergen challenge and responsiveness to histamine provocation; furthermore, it is sensitive to detect treatment effects.

For treatment of perennial allergic rhinitis several unresolved mechanisms, which also could play a role, should be investigated, such as the effect of other inflammatory products,

i.e. neuropeptides, adhesion molecules and second messengers.

In recent years several studies have examined the role of neural mechanisms in asthma and, in a lesser extent, also in rhinitis. The sensory nerves in the airways may be stimulated by inhalation of non-specific irritants.[403] The neural control is complex, comprising cholinergic, adrenergic and non-adrenergic non-cholinergic (NANC) mechanisms. Many neuropeptides, such as VIP, SP and CGRP, have been identified in the human airways.[521] It has been suggested that they are involved in controlling airway tone and secretion. Since the NANC system has longer-acting effects on blood vessels and submucosal glands (causing vasodilatation, transudation and secretion) than the cholinergic and adrenergic system, the neuropeptides of this system seems to be more important in rhinitis. Neural and neuropeptide mechanisms might be affected by inflammation.[35] Of the NANC system, the neuropeptide SP, a mediator of C fibres, has been examined extensively. After nasal allergen challenge in patients allergic to pollen, an immediate release of SP was found in lavage fluid.[474] In allergic patients increased levels of SP were present in lavage fluid, which might indicate an increased activation of the NANC system.[475] Nasal challenge with SP resulted in an increased nasal airway resistance, an increased influx of albumin (i.e. increased vasopermeability), and an increased influx of neutrophils in nasal lavage fluid.[475]

In several studies the effect of capsaicin, a neurotoxin on sensory nerves has been investigated. Capsaicin's site of action is mainly the C fibres.[297] Nasal challenge with capsaicin leads to airway sensory nerve activation and induces burning, rhinorrhoea and lacrimation.[509] Capsaicin initially stimulates the C fibres, however, after several stimulations, this is followed by depletion of these nerve fibres, resulting in desensitisation to capsaicin and other sensory stimuli.[297] These desensitising properties have been demonstrated to reduce nasal symptoms in patients with nonallergic rhinitis.[62,361,415] It would be interesting to study if capsaicin has also therapeutic effects in allergic rhinitis.

Nasal epithelial cells represent the first barrier against noxious agents and allergens. In allergic rhinitis, in contrast to asthma, epithelial cells are not shed, but are activated. The epithelial cells in allergic patients can be activated by histamine, cytokines and ECP, and leads to increased expression of surface markers like HLA-DR and ICAM-1.[9,616,649] ICAM-1 binds with leukocytes, and might, as a consequence, induce migration of inflammatory cells to the epithelial layer in allergic rhinitis.[9] Endothelial cells also play an important role in the migration of inflammatory cells from the intravascular to the extravascular sites. In allergic rhinitis, increased expression of adhesion molecules on endothelial cells has been found, which are able to bind leukocytes.[446] VCAM-1 seems to be an important adhesion molecule which predominantly binds eosinophils. Its expression can be induced by allergen challenge and is accompanied by increased nasal submucosa eosinophilia.[374] Allergic rhinitis might be treated by preventing migration of inflammatory cells to the site of action (i.e. the nasal mucosa) by reducing the expression of adhesion molecules on endothelial and epithelial cells.

The cyclic nucleotides, cAMP and cyclic guanosine monophosphate (cGMP) are important second messengers and play a key role in the regulation of cell activity. The intracellular concentration of these nucleotides is regulated by stimulation of surface receptors and intracellular breakdown by phosphodiesterases (PDEs), such as theophylline. In the airways these nucleotides regulate the smooth muscle tone, mediator secretion and activation of inflammatory cells. There is evidence that atopic disease may be associated with abnormal expression of PDEs in inflammatory cells resulting in increased breakdown of cyclic

nucleotides.[25,98] *In vitro* and animal studies with selective PDE inhibitors have shown anti-inflammatory effects by inhibition of mast cells, macrophages, eosinophils and CD4+ lymphocytes.[627] A study in monkeys has demonstrated that PDE inhibitors inhibited allergen-induced eosinophilic inflammation and airway hyperresponsiveness.[634] Whether selective PDE inhibitors will prove to be useful new anti-asthma and may be also an anti-rhinitis compound is not yet certain. Clinical studies with PDE inhibitors have to be performed to study both the therapeutic and side effects.

Furthermore, the treatment of allergic rhinitis with new 5-lipoxygenase inhibitors should be investigated. In antigen sensitized guinea-pigs the 5-lipoxygenase inhibitor E6080 inhibited bronchospasm, decreased bronchoepithelial damage and inhibited infiltration of leukocytes.[630] The leukotriene production and nasal congestion and airway responses after allergen challenge in asthmatics were decreased after treatment with the 5-lipoxygenase inhibitor Zileuton (A 64077).[305,350] Also the treatment with specific receptor antagonists could be usefull to modify the condition of rhinitis patients, because several studies with asthmatics showed an improvement of their symptoms. The novel leukotriene antagonist ONO 1078 reduced the severity of bronchial hyperresponsiveness of asthmatics after challenge with allergen, histamine or methacholine.[207,607,611] The early airway reaction to cumulative bronchial allergen challenge of asthma patients was inhibited by the leukotriene-antagonist ICI-204,219.[141,180] In asthmatics prior inhalation of the PAF antagonist BN52021 inhibited the bronchoconstriction induced by PAF and allergen, but not by methacholine.[303] This was in contrast with other investigators who found that treatment with the PAF-antagonist WEB 2086 did not attenuate allergen induced early or late responses or airway hyperresponsiveness in asthmatics.[200] Treatment with a combination of therapeutic drugs, such as receptor antagonists, should be investigated, because this may have a synergistic beneficial effect on the inflammatory process. The antigen-induced airway microvascular leakage in guinea-pigs was decreased by a combination of LTD_4 and PAF antagonists, Ro 24-5913 and Ro 24-4736.[671] Simpson described a study with hay fever patients treated with budesonide and terfenadine, separately, and in combination.[584] Budesonide was more effective than terfenadine in reduction of the symptoms, such as nasal blockage. Although the symptom scores tended to be lower with combined budesonide and terfenadine treatment than with either drug alone, the global assessments of combination therapy and budesonide alone were very similar.

Finally, not only treatment of allergic rhinitis should be investigated, also the analysis of treatment failure should have our attention.

Chapter 17
Summary

In patients with allergic rhinitis nasal symptoms are elicited by exposure to specific antigens and often also by non-specific stimuli. The pathophysiology of this non-specific nasal hyperreactivity is still not clear. In the lower airways it has been demonstrated that inflammation is involved in bronchial hyperreactivity. Most rhinitis studies have been performed in patients allergic to pollen. In these studies no clear relationship was found between nasal hyperreactivity, inflammation and the late phase response. Since in a study performed in patients allergic to house dust mite nasal hyperreactivity was associated with nasal response to allergen, a finding more in agreement with the bronchial situation, we further investigated the relationship between nasal hyperreactivity, inflammation and the late phase response in this patient group.

Hereto, patients were challenged with house dust mite and 24 hour later with histamine and/or methacholine. We investigated (1) if nasal hyperreactivity and the clinical immediate and late allergic response are associated, (2) if nasal hyperreactivity is associated with the inflammatory mediators in lavage fluid, (3) if these associations are influenced by treatment with a topical corticosteroid and a topical antihistamine. Further, we investigated in perennial allergic rhinitis during natural exposure (4) if nasal hyperreactivity is associated with inflammatory mediators and eosinophils and (5) if nasal hyperreactivity is associated with daily nasal symptoms and quality of life.

Part one consists of a general introduction which contains an overview of allergic nasal reactions in rhinitis, the role of inflammatory cells within this process and their inflammatory products.

In **chapter 1** a short historical review is given about allergy. Also the prevalence and clinical and socio-economic aspects of allergic rhinitis are described. Further, nasal response to allergen challenge and the present ideas about the pathophysiology, in particular the involvement of inflammation, of allergic rhinitis and allergen-induced non-specific hyperreactivitity are discussed. Most of these studies have been performed in patients with seasonal rhinitis.

Chapter 2 focuses on the role and interactions of inflammatory cells, their typical functions and their release of mediators within the process of allergic rhinitis. To understand the mechanism of allergic rhinitis, information on the reactivity and the ability to respond to stimuli of the involved cells, and the source, secretion, breakdown and site of activity of their mediators is essential. Allergic rhinitis is not induced by the action of one single cell type or one mediator, but by a variety of cells and mediators.

Chapter 3 summarizes the aims of the study.

In **part two** the relationship between the allergic response and hyperreactivity is studied.

In **chapter 4** twenty-four patients with perennial allergic rhinitis are challenged with house dust mite and 24 hours later with histamine. Thirteen patients showed an early response only (LAR-), 11 patients an early as well as a late allergic response (LAR+). LAR- and LAR+ responders showed a different profile of symptom score, mediator involvement (albumin, ECP, tryptase) and nasal hyperreactivity. In analogy with the lower airways, the EAR, LAR, and hyperreactivity are significantly correlated.

In **chapter 5** nasal hyperreactivity and the involvement of inflammation during the ongoing allergic reaction is investigated. Hereto, a nasal brush, a nasal lavage and a nasal histamine challenge was performed in 48 patients with perennial allergic rhinitis and in 11 healthy controls. Nasal hyperreactivity, assessed by a composite symptom score was significantly higher in patients. In contrast to PGD_2 and tryptase, eosinophils, $LTC_4/D_4/E_4$ and albumin were significantly increased, which suggest that eosinophils are important in the ongoing allergic reaction. Nasal hyperreactivity was weakly correlated with the percentage of eosinophils.

Part three describes the effects of therapeutic intervention on the immediate and late phase nasal response, nasal inflammation and nasal hyperreactivity to nasal allergen challenge.
Chapter 6 describes the mechanisms of action of glucocorticosteroids in allergic rhinitis.
In **chapter 7** the effects of the topical glucocorticoid fluticasone propionate aqueous nasal spray on the immediate and late phase allergic reaction and nasal hyperreactivity was investigated. Twenty-four rhinitis patients allergic to HDM were treated with fluticasone propionate or with placebo for two weeks twice daily. Thereafter, the patients were challenged with HDM extract. Symptoms were recorded and nasal lavages were collected every hour up to 9.5 hours after challenge. Nasal hyperreactivity was determined by histamine challenge 24 hours later. The results of this study showed that fluticasone propionate significantly inhibits the immediate and late allergic response and nasal hyperreactivity, probably by suppressing mast cells and eosinophils in the nasal mucosa.
In **chapter 8** the relationship between the release of PAF, $LTC_4/D_4/E_4$ and PGD_2 from nasal mucosa in rhinitis patients allergic to HDM was investigated in the same experiment as described in chapter 7. After allergen provocation the levels of PAF, $LTC_4/D_4/E_4$ and PGD_2 increased in the nasal lavage fluid, which were significantly correlated. This indicates that these mediators can be used as markers of allergic reactions against HDM. Furthermore, fluticasone propionate tended to reduce the release of these mediators, which was correlated with beneficial effects on clinical symptoms.
Chapter 9 deals with the effect of fluticasone propionate on IL-5 production in the nasal cavity of the rhinitis patients allergic to HDM. Nasal challenge with HDM caused immediate nasal symptoms and increased levels of IL-5 in the nasal lavages. IL-5 and ECP are released during the late phase reaction. Treatment with fluticasone propionate significantly decreased the evoked IL-5 and ECP levels in the late phase reaction. This could indicate that the number and activity of eosinophils are increased during the late allergic reaction, a response that is inhibited by corticosteroids.
In **chapter 10** the role of nitric oxide in the early and late phase of the allergic process was investigated and the effect of fluticasone propionate was determined. The production of nitric oxide metabolites were measured in the nasal lavages of the rhinitis patients described in chapter 7 and of healthy volunteers with or without challenge with PBS. This study showed that nitric oxide metabolites are present in nasal lavage fluid in detectable amounts, the level gradually increasing with time in both patients and controls. Treatment with fluticasone propionate did not affect initial basal production of nitric oxide metabolites nor production following provocation with HDM extract.
In **Chapter 11** the association in reduction of symptoms due to treatment with a glucocorticoid and the glucocorticoid receptor characteristics in the rhinitis patients allergic to HDM was investigated. *In vitro* glucocorticoid receptor binding studies with

dexamethasone were performed with peripheral blood mononuclear cells of these patients. LTB$_4$ produced by monocytes *in vitro* and IL-2 SR released by lymphocytes *in vitro* were determined. The number of peripheral blood mononuclear cell glucocorticoid receptors and the affinity of dexamethasone for the receptors were not significantly partially correlated with the reduction of the symptoms after the active treatment period. Also no partial correlation was observed of the levels of LTB$_4$ or IL-2 SR release by monocytes and lymphocytes, respectively, with the reduction of the symptoms. These results suggest that the extent of reduction of symptoms due to the topical glucocorticosteroid fluticasone propionate of the rhinitis patients is not associated with the characteristics of the glucocorticoid receptor.

Chapter 12 describes the mechanisms of action of antihistaminics in allergic rhinitis.

Chapter 13 reports the results of a study with the topical antihistamine levocabastine (one week twice daily) in 21 patients with perennial allergic rhinitis. Patients responded to HDM challenge with an early response only. Levocabastine significantly reduced the clinical response to HDM and histamine challenge. Since it has been suggested that antihistamines possess other properties besides the classical H$_1$ receptor antagonism, the effect of levocabastine on inflammatory mediators and on nasal hyperreactivity (nasal response to methacholine) was investigated. Levocabastine pretreatment did not reduce inflammatory mediators or nasal hyperreactivity, suggesting that it is an effective H$_1$ receptor antagonist, without anti-inflammatory properties.

In **chapter 14** an *in vitro* study with levocabastine is described to investigate further, whether levocabastine, in addition to the anti-H$_1$ receptor activity, has anti-inflammatory properties. Peripheral leukocytes of allergic volunteers were preincubated with levocabastine and thereafter incubated with specific allergens. Leukocytes of healthy volunteers were incubated for different time-intervals with different concentrations of levocabastine. Histamine release was measured. The effect of levocabastine on the contractile responses to histamine on guinea-pig trachea and lung-parenchyma as well as the histamine release were investigated. The results indicate that the H$_1$ receptor blocker levocabastine has probably no anti-inflammatory properties, measured as histamine release, and that the histamine release from both human leukocytes and guinea-pig isolated tissue is significantly increased by the drug in relatively high doses.

In **part four** a study to investigate the association between hyperreactivity and the quality of life is described.

In **chapter 15** the importance of routine histamine challenges in daily practice was investigated. Hereto, 48 patients with perennial allergic rhinitis were challenged with histamine and completed a nasal symptom diary during the 2 preceding weeks. Since it has been demonstrated that rhinitis also has an impact on quality of life, the patients were asked to complete a rhinitis QOL questionnaire also. Nasal hyperreactivity, quality of life and daily nasal symptoms were significantly correlated. Therefore, routine histamine challenge is a useful tool to obtain an impression of daily nasal symptoms and rhinitis quality of life.

Samenvatting

De neusklachten van patiënten met allergische rhinitis worden veroorzaakt door blootstelling aan specifieke antigenen en vaak ook door blootstelling aan aspecifieke prikkels. De pathofysiologie van deze aspecifieke hyperreactiviteit is nog steeds niet duidelijk. In de lagere luchtwegen is het aangetoond dat ontsteking betrokken is bij bronchiale hyperreactiviteit. De meeste onderzoeken betreffende rhinitis zijn uitgevoerd bij patiënten met een allergie voor pollen. In deze studies werd geen duidelijke relatie gevonden tussen nasale hyperreactiviteit, ontsteking en de late fase reactie. In een eerder onderzoek uitgevoerd bij patiënten met een huisstofmijt allergie bleek dat nasale hyperreactiviteit geassocieerd was met de nasale reactie op allergeen. Deze bevinding is in overeenstemming met de bronchiale situatie. We hebben de relatie tussen nasale hyperreactiviteit, ontsteking en de late fase reactie in deze patiënten groep nader onderzocht.

Hiervoor werden patiënten geprovoceerd met huisstofmijt en 24 uur later met histamine en/of methacholine. We hebben onderzocht (1) of nasale hyperreactiviteit en de directe klinische en late allergische reactie geassocieerd zijn, (2) of nasale hyperreactiviteit geassocieerd is met ontstekingsmediatoren in neusspoelvloeistof en (3) of deze relaties beïnvloedbaar zijn door behandeling met een lokaal toegediend corticosteroïd en een antihistaminicum. Verder hebben we bij patiënten met chronische allergische rhinitis tijdens natuurlijke blootstelling onderzocht (4) of nasale hyperreactiviteit gerelateerd is met ontstekingsmediatoren en eosinofielen en (5) of nasale hyperreactiviteit geassocieerd is met de dagelijkse neusklachten en de kwaliteit van leven.

Deel een bestaat uit een algemene inleiding, welke een overzicht geeft van de nasale reacties in allergische rhinitis, de rol van ontstekingscellen in dit proces en hun ontstekingsprodukten.

In **hoofdstuk 1** wordt een kort historisch overzicht gegeven over allergie. Ook worden het voorkomen van en de klinische en sociaal-economische aspekten van allergische rhinitis beschreven. De nasale reactie op allergeen provokatie en de huidige ideeën over de pathofysiologie, in het bijzonder de betrokkenheid van ontsteking, van allergische rhinitis en allergeen-geïnduceerde aspecifieke hyperreactiviteit worden besproken. De meeste studies zijn echter uitgevoerd in patiënten met hooikoorts.

Hoofdstuk 2 richt zich op de rol en interakties van ontstekingscellen, hun typische funkties en het vrijkomen van mediatoren in het proces van allergische rhinitis. Om het mechanisme van allergische rhinitis te begrijpen, is er informatie nodig over de mate van reaktiviteit en de gevoeligheid voor bepaalde stimuli van de betrokken cellen. Ook is de herkomst, uitscheiding, afbraak en plaats van werking van hun mediatoren van essentieel belang. Allergische rhinitis wordt niet geïnduceerd door de werking van een enkele cel of mediator, maar door een samenspel van meerdere cellen en mediatoren.

Hoofdstuk 3 vat de doelstellingen van de studie samen.

In **deel twee** is de relatie tussen de allergische reaktie en hyperreactiviteit bestudeerd.

In **hoofdstuk 4** zijn 24 patiënten met chronische allergische rhinitis geprovoceerd met huisstofmijt en 24 uur later met histamine. Dertien patiënten vertoonden alleen een vroege reaktie (LAR-), 11 patiënten vertoonden zowel een vroege als een late allergische reaktie

(LAR+). LAR+ en LAR- responders lieten een verschillend profiel van symptoom score, betrokken mediatoren (albumine, ECP, tryptase) en nasale hyperreactiviteit zien. In overeenstemming met de lagere luchtwegen, waren de vroege allergische reaktie (EAR), de late allergische reactie (LAR) en nasale hyperreactiviteit gecorreleerd.

In **hoofdstuk 5** is de nasale hyperreactiviteit en de rol van ontsteking gedurende de chronische allergische reaktie onderzocht. Hiervoor werd een uitstrijkje van de neus, een neusspoeling en een nasale histamine provokatie uitgevoerd bij 48 patiënten met chronische allergische rhinitis en 11 gezonde vrijwilligers. Nasale hyperreactiviteit, gemeten door middel van een samengestelde symptoom score, was hoger bij de patiënten. In tegenstelling tot PGD_2 en tryptase waren de eosinofielen, $LTC_4/D_4/E_4$ en albumine verhoogd, wat suggereert dat eosinofielen belangrijk zijn in de chronische allergische reaktie. De nasale hyperreactiviteit was matig gecorreleerd met het percentage eosinofielen.

Deel drie beschrijft het effekt van geneesmiddelen op de onmiddellijke en late nasale reaktie, ontsteking en nasale hyperreactiviteit ten gevolge van nasale allergeen provokatie.

In **Hoofdstuk 6** wordt het werkingsmechanisme van glucocorticosteroïden in allergische rhinitis weergegeven.

In **hoofdstuk 7** werd het effekt van het lokaal toegediende glucocorticosteroïd fluticasone propionaat op de onmiddellijke en late fase allergische reaktie en nasale hyperreactiviteit onderzocht. Vierentwintig rhinitis patiënten met een allergie voor huisstofmijt werden gedurende 2 weken, 2 maal daags, behandeld met fluticasone propionaat of placebo. Hierna werden deze patiënten geprovoceerd met huisstofmijt extrakt. De symptomen werden geregistreerd en de neusspoelingen werden elk uur tot 9,5 uur na provokatie uitgevoerd. De nasale hyperreactiviteit werd 24 uur later gemeten door middel van provokatie met histamine. De resultaten lieten zien dat fluticasone propionaat de onmiddellijke en late allergische reaktie en nasale hyperreactiviteit verminderde, hetgeen waarschijnlijk veroorzaakt wordt door het verminderen van het aantal en de activiteit van mest cellen en eosinofielen in het neusslijmvlies.

In **hoofdstuk 8** wordt de relatie beschreven tussen het vrijmaken van PAF, $LTC_4/D_4/E_4$ en PGD_2 uit het neusslijmvlies van rhinitis patiënten met een allergie voor huisstofmijt (zie ook hoofdstuk 7). Na provokatie met allergeen namen de concentraties van PAF, $LTC_4/D_4/E_4$ en PGD_2 in de neusspoelvloeistof toe en waren gecorreleerd. De resultaten tonen aan dat deze mediatoren gebruikt kunnen worden als markers voor de allergische reaktie op huisstofmijt. Fluticasone propionaat verlaagde de vrijmaking van deze mediatoren, welke gecorreleerd waren met verbeteringen van de klinische symptomen.

In **hoofdstuk 9** wordt het effekt van fluticasone propionaat op de IL-5 produktie in de neusholte van chronische allergische rhinitis patiënten onderzocht. Neusprovokatie met huisstofmijt veroorzaakte onmiddellijke neusklachten en een verhoogde concentratie van IL-5 in de neusspoelvloeistof. IL-5 en ECP werden vrijgemaakt gedurende de late fase reaktie. Behandeling met fluticasone propionaat verminderde de verhoogde IL-5 en ECP concentraties in de late fase reaktie. Deze resultaten zouden aan kunnen tonen dat, gedurende de late fase reaktie het aantal én de aktiviteit van eosinofielen zijn verhoogd, welke afnemen door behandeling met een corticosteroïd.

In **hoofdstuk 10** werd de rol van stikstofmonoxide (NO) tijdens de vroege en late fase reaktie in het allergische proces bestudeerd. Ook het effekt van fluticasone propionaat is beschreven. De produktie van de afbraakprodukten van NO is gemeten in de

neusspoelingen van rhinitis patiënten (zie hoofdstuk 7) en in neusspoelingen van gezonde vrijwilligers, zowel met als zonder provokatie met PBS. De resultaten tonen aan dat er afbraakprodukten van NO aanwezig zijn in de spoelvloeistof en dat de concentratie toeneemt in de tijd, zowel bij de patiënten als bij de controles. Behandeling met fluticasone propionaat had geen enkel effekt op de basale produktie en op de produktie na allergeenprovokatie.

In **hoofdstuk 11** wordt de relatie tussen de verlaging van klachten na behandeling met fluticasone propionaat met de glucocorticoïd receptor karakteristieken van de rhinitis patiënten met een allergie voor huisstofmijt onderzocht. Op de enkelvoudige-kern cellen uit het bloed van deze patiënten zijn glucocorticoid receptor binding-studies gedaan met dexamethason. De LTB_4 produktie door monocyten en het vrijmaken van de oplosbare IL-2 receptor (IL-2 SR) door lymfocyten is onderzocht. Het aantal glucocorticoïd receptoren op de enkelvoudige-kern cellen en de affiniteit voor dexamethason van deze receptoren was niet gecorreleerd met de verlaging in symptomen na behandeling met fluticasone propionaat. Ook de produktie van LTB_4 en IL-2 SR door monocyten en lymfocyten was niet gecorreleerd met de verlaging in symptomen na behandeling. Deze resultaten suggereren dat de mate van verlaging van de symptomen na het lokaal toegediende glucocorticoïd fluticasone propionaat niet geassocieerd kan worden met karakteristieken van de glucocorticoïd receptor.

Hoofdstuk 12 beschrijft in algemene zin de werkingsmechanismen van antihistaminica in allergische rhinitis.

In **hoofdstuk 13** worden de resultaten besproken van een studie met het lokaal toegediende antihistaminicum levocabastine (gedurende 1 week, 1 maal daags) bij 21 rhinitis patiënten die allergisch waren voor huisstofmijt. De patiënten vertoonden na provokatie met allergeen alleen een vroege fase reaktie. Levocabastine verlaagde significant de klinische reaktie op huisstofmijt- en histamine provokatie. Het effect van levocabastine is ook onderzocht op de ontstekingsmediatoren en op nasale hyperreactiviteit, omdat antihistaminica behalve het klassieke H_1 receptor antagonisme ook andere effekten kunnen hebben. Echter, door behandeling met levocabastine werden noch de ontstekingsmediatoren noch de nasale hyperreactiviteit verlaagd. Dit suggereert dat levocabastine een effektieve H_1 receptor antagonist is, zonder ontstekings-remmende eigenschappen.

In **hoofdstuk 14** wordt een *in vitro* studie met levocabastine beschreven om nader te onderzoeken of levocabastine ontstekings-remmende eigenschappen bezit, aansluitend op de H_1 receptor blokkende werking. Leukocyten uit het bloed van allergische vrijwilligers werden voorgeïncubeerd met levocabastine en daarna geïncubeerd met een allergeen. Leukocyten van gezonde vrijwilligers werden voor verschillende tijdsintervallen met oplopende concentraties levocabastine geïncubeerd. De histamine produktie werd gemeten. Het effect van levocabastine op de contractie van gladde spieren van de cavia luchtpijp en longparenchym op histamine werd onderzocht, alsmede de produktie van histamine. De resultaten suggereren dat de H_1 receptor blokker levocabastine waarschijnlijk geen ontstekings-remmende eigenschappen heeft, gemeten in histamine produktie. De histamine produktie door zowel humane leukocyten alsmede door geïsoleerd weefsel van cavia's werd in relatief hoge doseringen aanmerkelijk verhoogd door het geneesmiddel.

In **deel vier** wordt een studie beschreven waarin de associatie tussen nasale hyperreactiviteit en de kwaliteit van leven werd onderzocht.

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In **hoofdstuk 15** is het belang van routinematige histamine provokatie in de dagelijkse praktijk onderzocht. Hiervoor werden 48 patiënten met een chronische allergische rhinitis geprovoceerd met histamine. Er is aangetoond dat rhinitis een grote invloed op de kwaliteit van leven heeft. Daarom is de patiënten gevraagd om een vragenlijst over de kwaliteit van leven in te vullen. Tevens hebben ze gedurende de 2 voorafgaande weken, hun neusklachten in een klachtendagboek genoteerd. Nasale hyperreactiviteit, kwaliteit van leven en de dagelijkse neusklachten waren significant gecorreleerd. Daarom is de routinematige histamine provokatie een doeltreffend middel om een impressie te krijgen van de dagelijkse neusklachten en kwaliteit van leven.

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Abbreviations

ANOVA	analysis of variance
APC	antigen presenting cell
AUC	area under the curve
B cell	bone marrow derived lymphocyte
BSA	bovine serum albumine
BU	biological units
cAMP	cyclic 3',5' adenosine monophosphate
cGMP	cyclic 3',5' guanosine monophosphate
CCRC	cumulative concentration-response curve
CFU-GM	colony forming unit granulocyte macrophage
CGRP	calcitonin gene-related peptide
cNOS	constitutive nitric oxide synthase
COX	cyclooxygenase
COX_1	constitutive cyclooxygenase
COX_2	inducible cyclooxygenase
CSF	colony stimulating factor
DAG	1,2 diacylglycerol
Df	dermatophagoides farinae
DMEM	dulbecco's modified eagles medium
DMSO	dimethylsulfoxide
Dp	dermatophagoides pteronyssinus
EAR	early allergic reaction
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDRF	endothelium-derived relaxation factor
EDTA	ethylenediamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
$Fc_\varepsilon R_I$	high affinity IgE receptor
$Fc_\varepsilon R_{II}$	low affinity IgE receptor
$Fc_\gamma R_I$	high affinity IgG receptor
$Fc_\gamma R_{II}$	low affinity IgG receptor
FEV_1	forced expiratory volume in one second
FPANS	fluticasone propionate aqueous nasal spray
GM-CSF	granulocyte macrophage-stimulating factor
HDM	house dust mite
HETE	hydroxy-5,8,11,13 eicosatetraenoic acid
HLA	human leukocyte antigen
H_2O_2	hydrogen peroxide
HPETE	hydroperoxyeicosatetraenoic acid
H receptor	histamine receptor
ICAM	intercellular adhesion molecule
IgA	immunoglobulin A
IgD	immunoglobulin D

IgE	immunoglobulin E
IgM	immunoglobulin M
IFN-γ	interferon-γ
IL	interleukin
IL-2 SR	soluble interleukin-2 receptor
iNOS	inducible nitric oxide synthase
IP$_3$	inositol 1,4,5-triphosphate
LAR	late allergic reaction
LAR+	patients with both EAR and LAR
LAR-	patients with only EAR
LCF	lymphocyte chemotactic factor
LT	leukotriene
LTC$_4$/D$_4$/E$_4$	cysteinyl leukotrienes
LTR	receptor for leukotriene
MBP	major basic protein
MC$_T$	mast cell containing only tryptase
MC$_{TC}$	mast cell containing tryptase and chymase
MHC	major histocompatibility complex
mRNA	messenger ribonuclear acid
NANC	non-adrenergic non-cholinergic
NADPH	nicotinamide adenine dinucleotide phosphate
NAR	nasal airway resistance
NK cell	natural killer cell
NK$_A$	neurokinin A
NK$_B$	neurokinin B
NO	nitric oxide
NO.	nitroxyl anion
NO$_2^-$	nitrite
NO$_3^-$	nitrate
NOS	nitric oxide synthase
.O$_2^-$	superoxide
.OH	hydroxyl peroxide
ONOO$^-$	peroxynitrite
PAF	platelet activating factor: 1-alkyl-2(R)-acetyl-sn-glycero-3-phosphorylchloride
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDE	phosphodiesterase
PG	prostaglandin
PGI$_2$	prostacyclin
PHA	phytohemagglutinin
PHM	peptide histidine-methionine
PIPES	piperazine-1,4-bis-2-ethane sulfonic acid
PLA$_2$	phospholipase A$_2$
PLC	phospholipase C
QOL	quality of life

RIA	radio immuno assay
SD	standard deviation
SEM	standard error of mean
SP	substance P
T cell	thymus derived lymphocyte
$T_{c/s}$	cytotoxic/suppressor T cell
T_H	T helper cell
TES	N-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid
TNF-α	tumor necrosis factor-α
TRIS	tris(hydroxymethyl)aminomethane
Tx	thromboxane
VCAM	vascular cell adhesion molecule
VIP	vasoactive intestinal peptide

Dankwoord

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Curriculum vitae

Catharina (Tineke) in 't Veld was born on April 27th, 1967, in Rotterdam, the Netherlands. She attended primary school in Alblasserdam, and finished her secondary school (VWO) in Papendrecht in 1985. The same year medical studies were started at the Medical Faculty of the Erasmus University Rotterdam, the Netherlands. In 1989 she obtained her doctoral degree. From November 1991 to August 1992 she worked on a research project (supported by the Dutch Asthma Foundation) at the department of Allergology of the University Hospital Rotterdam, the Netherlands (supervised by Dr. R. Gerth van Wijk). After having obtained her medical degree in July 1992, she worked from September 1992 to October 1993 at the same department of Allergology (headed by Dr. P.H. Dieges) as a clinical fellow. She also continued working on the research project together with Ingrid Garrelds (Department of Pharmacology. Erasmus University Rotterdam), which resulted in this thesis. Since September 1995 she is in training for specialist in Allergology at the department of Allergology of the University Hospital Rotterdam-Dijkzigt, the Netherlands. She is married to Erik Wilfred de Graaf.

Ingrid Martine Garrelds was born on October 12th, 1968, in Dordrecht, the Netherlands. She attended primary school in Rijsoord and secondary school in Ridderkerk (VWO), the Netherlands and passed her exams in 1987. In the same year she started at the Higher Laboratory School (Van 't Hoff Institute) in Rotterdam and obtained her BSc degree in Delft in June 1991 (Hogeschool Rotterdam e.o., Polytechnical Faculty). During this study she was involved in a research project at the Erasmus University of Rotterdam in the Department of Pharmacology (headed by Prof. Dr. I.L. Bonta) under supervision of Dr. F.J. Zijlstra. The pharmacological intervention on the production of arachidonic acid metabolites during inflammation in the mouse-colon was investigated. At april 1991 she started as a research analist at the same Department (headed by Prof. Dr. P.R. Saxena). The role of eicosanoids, TNF-α and PAF in lipopolysaccharide-induced shock in pigs was investigated together with T. Mózes MD and Dr. F.J. Zijlstra. Also the effect of orally given carnitines on the production of eicosanoids and TNF-α by peritoneal rat macrophages was studied under supervision of Dr. F.J. Zijlstra. In April 1992 she started together with Tineke de Graaf-in't Veld (Department of Allergology, Academic Hospital Rotterdam) a PhD project, which resulted in this thesis.
She lives with Jeroen Ernst Alexander van den Berg.

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3.   Annual meeting of European Academy of Allergology and Clinical Immunology, September 12-15 1993, Rotterdam, the Netherlands.
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-   De Graaf-in 't Veld C, Garrelds IM, Gerth van Wijk R, van Toorenenbergen AW, Jansen APH, Meeuwis J. The effect of intranasal fluticasone propionate (FP) on the immediate and late allergic nasal reaction and nasal hyperreactivity in patients with a house dust mite (HDM) allergy.
4.   Scientific Meeting of the Dutch Association of Lung Diseases and Tuberculosis, the Netherlands Association of Allergology, and the Pulmonology Department of the Dutch Association of Pediatrics, October 9 1993, Utrecht, the Netherlands.
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7.   International Conference on Prostaglandins and Related Compounds, June 6-10 1994, Florence, Italy.
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-   De Graaf-in 't Veld C, Koenders S, Gerth van Wijk R. Nasal hyperreactivity, quality of life and nasal symptoms are correlated in perrennial rhinitis.

Co-author at congresses

1. Dutch Association of Gastro-enterology, March 21-22 1991, Veldhoven, the Netherlands.
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2. Third Meeting on Side effects of Anti-Inflammatory Analgesic Drugs and 13th European Workshop on Inflammation, May 8-11 1991, Verona, Italy.
   - Zijlstra FJ, Garrelds IM, van Dijk APM, Wilson JHP. Experimental colitis in mice, effects of olsalazine on eicosanoid production in colonic tissue.
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3. Dutch Foundation for Pharmacological Sciences - Pharmacological days, December 13-14 1993, Noordwijkerhout, the Netherlands.
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7. ATS International Conference, March 21-24 1995, Seattle, U.S.A..
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