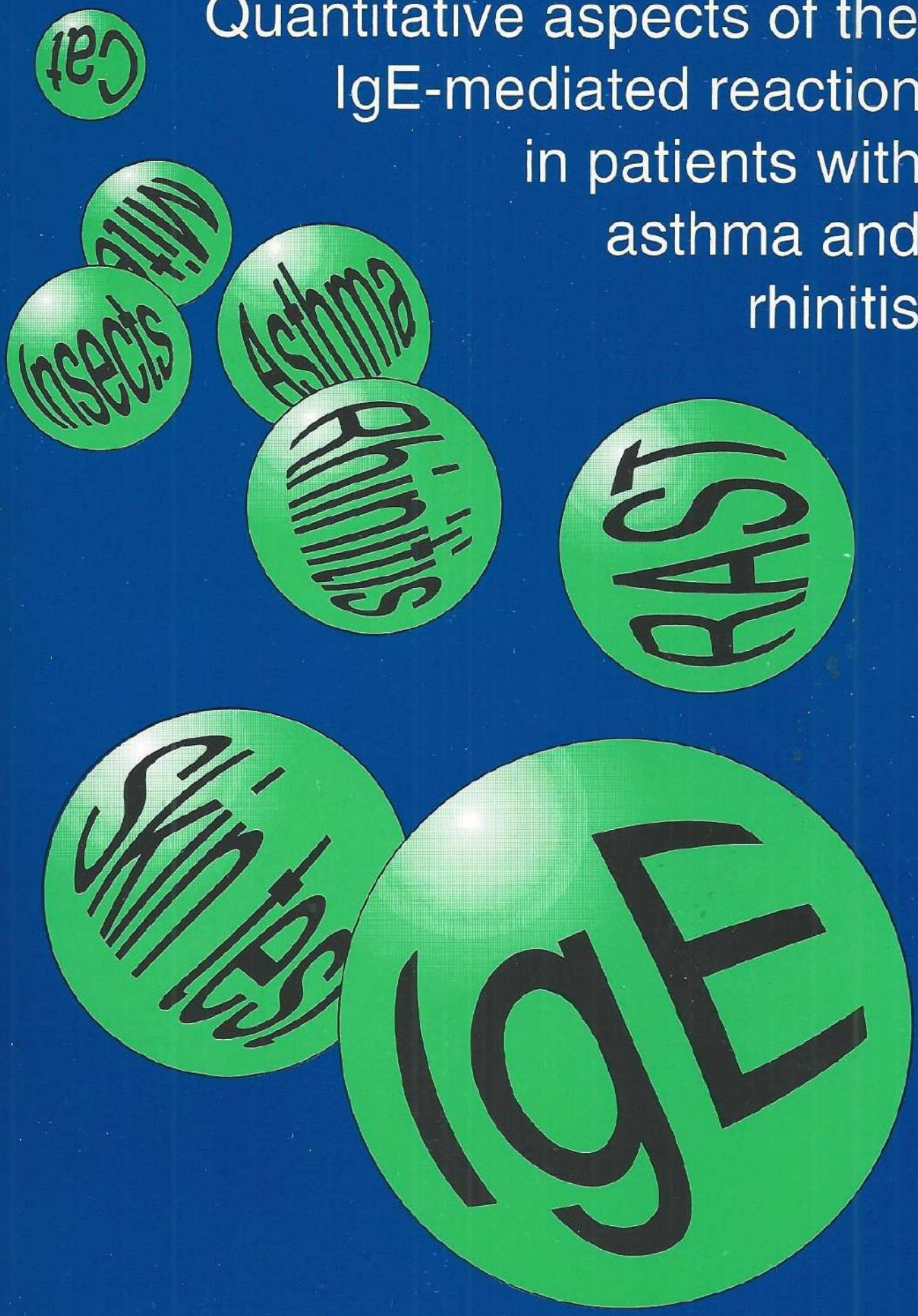
Quantitative aspects of the IgE-mediated reaction in patients with asthma and rhinitis



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Contents

Chapter 1

General Introduction

Allergic asthma and rhinitis, both inhalant allergies, are common diseases. Asthma is estimated to affect 2 to 5% of the population [1]. Estimates of the prevalence of allergic rhinitis vary from 2 to 15% [2]. There is evidence that the prevalence and severity of asthma and rhinitis are rising [3,4].

1. The IgE-mediated reaction

The IgE-mediated allergic reaction can be distinguished in an early-phase reaction and a late phase reaction. Exposure to allergens in allergic patients results in cross-linking of receptor-bound specific IgE on mast cells and basophils, this will trigger the release of different preformed and newly synthesized mediators. Mediators with vaso-active and smooth muscle contracting properties (e.g. histamine, PGD_2, LTC_4) will induce immediate allergic responses in the target organ. In addition to various cytokines (e.g. IL-4, IL-5, TNF_α) produced during early reactions, some of the mast cell-derived mediators (e.g. LTC_4, PAF), have chemotactic and/or cell activating properties and will attract inflammatory cells like eosinophils, neutrophils, lymphocytes and basophils. Late-phase reactions start after a few hours and concur with the influx of inflammatory cells and subsequent release of mediators like eosinophil cationic protein, lysosomal enzymes, chemokines and histamine.

The early and late allergic reaction in the lower airways

Bronchial responses to allergen can be distinguished in early and late broncho-obstructive reactions. Early reactions generally occur within 15 minutes after allergen inhalation and may persist for as long as one hour [5]. Early reactions are mainly caused by IgE dependent mast cell degranulation [6]. A combination of mediators, including histamine and both lipoxygenase and cyclooxygenase products of the arachidonic acid metabolism, is likely to be responsible for what appears to be a predominantly bronchospastic response [7—9]. Early reactions can be prevented by administration of β-agonists before allergen exposure. Corticosteroids, however, do not have this effect. Moreover, the early asthmatic reaction reverses rapidly after administration of β-agonists. In many subjects, an early asthmatic reaction is followed by a late reaction; a recurrence of bronchial obstruction, which occurs between three and ten hours after allergen inhalation [10,11]. Occasionally, subjects will have a late reaction in absence of an early reaction. The late reaction is incompletely reversed with bronchodilators. This suggests that the airway obstruction may also be due to a non-bronchospastic mechanism, e.g., oedema or inflammation [12]. In contrast to the non-response in early reactions, glucocortico-steroids effectively prevent late reactions

[13]. Late allergic reactions are characterized by an influx of inflammatory cells into the airway wall. An increase of eosinophils, neutrophils, pulmonary macrophages and lymphocytes have been described in bronchial alveolar lavage fluid obtained 6—48 hours after allergen challenge [14—17]. Release of secondary mediators from these inflammatory cells, like LTC_4 and PAF, are considered to cause bronchial obstruction in patients with a late asthmatic response [18].

Cockcroft et al. [19] have stated that the allergen concentration causing a 20% fall in the first hour after allergen challenge can be predicted by knowing the amount of specific IgE antibodies, assessed by skin tests, and non-specific airway responsiveness as determined with histamine or metacholine. The factors which might predict the development of a late allergic reaction have been examined [20-23]. Important determinants appear to be high concentrations of specific IgE, the magnitude of the early allergic reaction and the initial level of bronchial responsiveness [20,21]. In this connection, the importance of the allergen dose is controversial. Some authors showed that inhalation of high doses of allergen results in an increased proportion of late reactions [22], whereas others indicated that isolated late allergic reactions may occur after repeated inhalation of low doses of allergen [23].

After allergen exposure there are also early and late responses in the nose. The main difference between reactions in the nose and in the bronchi can be explained by absence of smooth muscle in the nose. The nasal response after allergen challenge is characterized by induction of the sneeze reflex, stimulation of glands, dilatation of vessels and oedema. Research on nasal reactions after allergen provocations has, so far, been hampered by lack of well-defined and objective methods to determine early and late obstructive responses. Furthermore, small increases in nasal resistance during the late phase may be obscured by the nasal cycle. Methods to measure mediators in nasal lavage fluids as markers of an allergic response have facilitated the recognition of early and late responses in the nose [24].

Analogue to the lower airways, inflammatory cells such as mast cells, basophils, eosinophils and their mediators have been demonstrated in the nose after allergen exposure [24—27].

Allergen-induced non-specific bronchial hyperresponsiveness

Different studies point to a relation between allergy and non-specific bronchial hyperresponsiveness [28-31]. Non-specific bronchial hyperresponsiveness increases upon natural and experimental exposure to airborne allergens [28,29]. A decrease in

allergen exposure often results in a decrease in bronchial hyperresponsiveness [30,31].

In 1977, Cockcroft et al. suggested that bronchial hyperresponsiveness depends on airway inflammation and was exclusively associated with the late allergic reaction [28]. In 1982, Cartier and coworkers showed that the magnitude of the decrease in PC_{20} indeed correlated to that of the late allergic reaction [32]. Moreover, the duration of the decrease in PC_{20} also correlated to the magnitude of the late allergic reaction. More recently, detailed studies have demonstrated that induction of bronchial hyperresponsiveness actually precedes the late broncho-obstructive allergic reaction [33,34] and coincides with the inflammatory response [35,36]. There is a close relation between the degree of inflammation in the airway wall and the level of airway responsiveness [37]. Various cells may be involved in the inflammatory process, mast cells, macrophages, eosinophils, neutrophils, lymphocytes and platelets included [38]. The influx of these cells and the release of mediators damage the epithelium, stimulate smooth muscle and glands, and increase microvascular leakage and oedema of the airway wall [39].

In summary, there appears to be a positive feed-back loop between the IgE-mediated reaction and the non-specific bronchial responsiveness. The IgE-mediated reaction leads to an influx of inflammatory cells, associated with an increase in non-specific bronchial hyperresponsiveness. As a consequence, allergens will probably more easily penetrate the damaged epithelial layer and meet more IgE-bearing inflammatory cells in the bronchial wall.

Allergens

Common inhalant allergens in The Netherlands are house dust mite (*Dermatophagoides pteronyssinus*), animal dander, pollen of grass and trees, and fungal spores. In particular, exposure to indoor allergens appears to be associated with bronchial asthma [40].

The immediate allergic reaction depends on the amount of allergen inhaled and the physical properties of the allergen. Important factors are environmental presence of allergen, particle size, solubility in mucosal lining fluid, permeation through the mucosal barrier and rate of degradation of the allergen. Possibly, enzyme activity of allergen is important for permeation through the mucosal barrier. Recently, indications were found that Der p 1 (a major allergen from house dust mite) increases the permeability of the bronchial epithelium [41]. More than half of the major allergens from house dust mites appears to have an enzymatic activity [42].

Large allergens probably penetrate the epithelial layer with more difficulty than

smaller allergens. However, small molecules (with few epitopes) are in general less immunogenic and/or less able to induce cross-linking of receptor bound IgE. Probably, the location of the IgE-binding epitopes is relevant as well.

IgE

Vital to the allergic reaction are specific IgE antibodies bound to mast cells and basophils. Probably, also the ratio specific IgE/total IgE is relevant. The mechanism of inhibition via saturation of mast cell Fcε receptors by irrelevant IgE was established *in vitro*. Lung fragments first exposed to IgE-rich serum became resistant to further passive sensitization with serum which contained grass-pollen specific IgE [43]. In another study, the Prausnitz-Kustner titre of a donor serum in different recipients was inversely related to the total serum IgE level of the recipients [44]. There are several studies about a lower allergic reactivity of patients in a tropical environment where helminthic infections induce high total serum IgE levels [45—47].

The biologic activity of IgE antibodies requires binding to the high affinity IgE-receptor on mast cells and basophils as well as crosslinking by allergen. The latter will only occur if IgE antibodies are directed against allergen molecules with multiple epitopes. Repetitive epitopes are required for a monospecific IgE antibody response. Generally, IgE antibody responses in allergic patients are directed against different epitopes on major allergens doing away with the need of repetitive epitopes.

Diversity in the biologic activity of IgE-allergen interactions is probably relevant. For instance, IgE directed against cross-reacting carbohydrate determinants from different vegetable sources was not able to induce the release of histamine from basophils [48].

Other factors may interfere with the interaction of allergen and cell-bound IgE. The IgG antibodies directed against the same molecules as IgE antibodies, may have an inhibitory effect on the IgE-mediated reaction. The inhibitory effect of IgG antibodies could be demonstrated clinically and experimentally [49—53].

Mediator release from mast cells and basophils

The term 'releasability' refers to the intrinsic capability of mast cells and basophils to release mediators after a specific stimulus [54]. Releasability of mast cells and basophils is assumed to be related to allergic disease. Several authors demonstrated an enhanced releasability of basophils from patients with allergic asthma [55—57]. Casolaro et al. [58] studied the releasability of human basophils and mast cells and found that anti-IgE induced histamine release in mast cells in the bronchial alveolar lavage

fluid of patients with asthma was significantly higher compared to that in cells of controls. There are clear differences between releasability of mast cells and basophils. In the study of Casolaro et al. [58] releasability of lung mast cells and basophils was compared in 52 normal and asthmatic donors. No correlation could be found in release of anti-IgE or IgE-independent secretogogues between mast cells from the lung and basophils.

2. Tests for studying the IgE-mediated reaction to inhalant allergens

Most tests in which the allergic reaction is studied, measure an overall effect of several factors. In table I it is schematically shown for different tests which factors they include.

Radio allergosorbent test (RAST)

The concentration of allergen specific IgE in serum is measured by the RAST. Only IgE bound to the mast cell is essential for the immediate allergic reaction. Van Toorenenbergen et al. [59] showed an equilibrium between circulating IgE and cell-bound IgE. Only in case of rapid changes in the concentration of circulating IgE antibodies, the concentration of cell-bound IgE lags behind.

The RAST gives limited information about the biologic activity of the IgE. Because of an excess of allergen in the RAST, the outcome is less affected by the affinity of the IgE antibodies.

The level of IgG antibodies to inhalant allergens without prior immunotherapy does not influence the RAST outcome. Generally, the level of total IgE does not influence the RAST results either [60,61].

Table I. Schematic representation of the factors that directly contribute to the results of tests used in allergy

	RAST	histamine release test	skin test	bronchial allergen challenge	bronchial histamine challenge
allergen					
concentration	—	+	+	+	—
MW[1]	—	—	—	+	—
valency	—	+	+	+	—
specific IgE					
concentration	+	+	+	+	—
biologic activity	—	+	+	+	—
total IgE	—	+	+	+	—
specific IgG	—	—	+	+	—
releasability					
basophil	—	+	—	—	—
TC[2] mast cell	—	—	+	—	—
T mast cell	—	—	—	+	—
mediator sensitivity (histamine)					
vasopermeability/ vasodilatation	—	—	+	+	+
airway smooth muscle	—	—	—	+	+

1. MW = molecular weight; 2. TC = tryptase chymase

Basophil histamine release test

For this *in vitro* test, different concentrations of allergen are added to washed leukocytes from peripheral blood. Histamine is released from the basophils as a consequence of the interaction of allergen with cell-bound IgE antibodies. Histamine in the supernatant fluid can be measured, e.g. spectrofluorometrically. Advantages of the test are that besides the concentration of specific IgE, information about the biological activity of the IgE antibodies, is obtained. The test is generally performed under conditions of a limited amount of allergen in a fluid phase similar to the allergic reaction *in vivo*. Disadvantages are that in a significant percentage of patients the release of histamine from basophils cannot be interpreted. In about 20% of patients, basophils do not release histamine if challenged *in vitro* [62,63]; this in spite of membrane-bound IgE antibodies, a positive skin test and allergic symptoms. The mechanism of this phenomenon is, as yet, unclear [64]. Probably, releasability is changed by an unknown mechanism outside the body.

Total IgE can probably influence the histamine release test by competition with specific IgE for the IgE receptor.

Furthermore, the concentration and the valency of the allergen are important; histamine release can be initiated with a minimum of 100 to 200 allergen molecules per basophil, this suggests a lower limit for IgE-crosslinking to induce mediator release [65,66].

Skin test

A small volume of allergen extract is intracutaneously injected in the skin. Wheal and flare reaction are recorded after 15 minutes. The skin reaction to full strength extract is mostly used for routine allergy testing. The concentration of allergen extract is crucial for the results. In diagnostic tests the concentration of the extract is chosen to reach a balance between false-negative and false-positive results in individuals who are considered allergic or non-allergic based on the clinical history or results of provocation tests. However, there is no consensus as to which test represents the gold standard for the diagnosis of allergy.

The outcome of the skin test depends on the sensitivity of the skin for mast cell mediators and the amount of mast cells at the test site. Skin reactions to full strength extract in highly allergic patients, are partly determined by the plateau of histamine sensitivity of the skin. Measuring of threshold (endpoint) dilutions with the results expressed as the lowest concentration of allergen inducing a positive skin test is less influenced by the skin sensitivity for histamine. Another way to circumvent this

problem is correction for skin reactivity to histamine. Theoretically, however, the system only gives optimal results with a dilution curve of histamine.

In contrast to the histamine release test from washed leucocytes, plasma factors which include IgG antibodies may influence the skin test results [67].

Several studies on immunotherapy failed to show a close correlation between the decrease in skin reactivity and the concentration of IgG antibodies induced by therapy [68–72]. This possibly because the authors measured IgG antibodies against total allergen extracts. The results will then be confounded by the inclusion of IgG antibodies with irrelevant specificities. IgG antibodies to irrelevant, that is non-allergenic material in allergen extracts, or to allergens against which IgE antibodies are not directed are not expected to contribute to the blocking of the binding of the IgE antibodies.

The skin test depends on activation of skin mast cells while the histamine release test depends on activation of basophils. As mentioned earlier, there is no correlation between mast cell and basophil releasability.

With high concentrations of allergen extract, the skin test can be positive by non-IgE mediated mechanisms. Toxic effects, complement activation or kallikrein activation possibly play a role in the mechanisms of these reactions. This is less likely to occur with the concentration of allergen extract usually applied in diagnostic tests. Van der Zee et al. [63] have studied patients with a negative RAST result and a positive skin test. In 82% of the patients (23/28) they found induction of histamine release from washed leucocytes with the relevant allergen. This suggests an IgE mediated reaction in the majority of discrepant results with allergen extract of usual strength.

Bronchial challenge test

In bronchial challenge tests, allergen is in general directly applied into the airways by inhalation of aerosolized extract. Pulmonary function tests, usually the FEV_1, are directed to monitor the reactions after inhalation of allergen. Concentration of allergen extracts are administered in exponential increments up to a certain end point (for example a 15% reduction in FEV_1). It is crucial to compare the narrowing of the airways after allergen intake with measurements made on a control day, since diurnal variation in airway caliber is known to occur in patients with asthma.

Bronchial allergen inhalation challenges are used as a model to study respiratory responses to natural allergen exposure. However, provocation tests create a somewhat artificial situation because of a relatively high dose of allergen in a soluble form within a short period of time. Another problem of the clinical relevance of allergen

challenge is what is the so called positive response. Variability of repeated measurements of FEV_1 in individuals may be as high as 5%. Therefore, a 5% change in FEV_1 can not easily be detected with spirometry. A threshold of 15 or 20% decrease in FEV_1 after allergen challenge is generally chosen because this threshold is readily detected by spirometry and does not give severe complaints. This does not imply that less decrease in FEV_1 is irrelevant to airways disease. There are no published data linking clinically significant respiratory tract allergic disease to any particular threshold of antigen-responsiveness.

Non-specific bronchial responsiveness is assessed by histamine or methacholine, both probably directly acting on peribronchial smooth muscle. With a histamine or metacholine challenge test, usually doubling doses of aerosolized histamine or metacholine are inhaled at regular intervals, while the bronchospastic response is measured by changes in FEV_1. Dose-response curves are analysed log-linearly and are characterized by their position and sometimes by plateau. The results of a histamine or metacholine challenge are usually expressed in PC_{20} (the concentration of histamine or metacholine causing a 20% fall in FEV_1). There is a good correlation between the response on histamine and metacholine.

In bronchial hyperresponsiveness, the dose-response curve may shift to the left, have a steeper slope or a higher degree of maximal airway narrowing, or both. Generally, only the position (PC_{20}) of the dose response curve is used as the indicator of bronchial hyperresponsiveness. The value of the plateau as an indicator for the severity of asthma becomes increasingly evident [73].

Although patients with asthma generally have lower PC_{20} histamine or metacholine values than asymptomatic subjects, there is a considerable overlap [74]. Patients with rhinitis have PC_{20} histamine or metacholine values which are between those of asymptomatic subjects and patients with asthma [75,76].

A dissimilarity between skin test and bronchial challenge test is that mast cells in the skin differ from those in the lung. Skin mast cells predominantly contain the enzymes tryptase and chymase, while most mast cells in the lung contain only tryptase [77]. Functionally, lung mast cells differ from skin mast cells by their resistance to activation by non-IgE secretagogues like compound 48-80 and Ca-ionophore A23187 [78,79].

Releasability of skin mast cells is difficult to measure *in vivo*; codeine [80], compound 48—80 [81] and anti-IgE [82] have been used as indirect measures of releasability. Clear differences were found between individuals in reactions on these substances. Unfortunately, the authors were not able to relate the differences to clinical syndromes.

In addition, the target of mast cell mediators is completely different in skin tests from that of bronchial challenge tests. The skin test measures only quantitative changes in vasopermeability and vasodilatation. In a bronchial challenge test, contraction of peribronchial smooth muscle has a more prominent effect than mucosal vasopermeability and vasodilatation on lung function parameters.

Permeability of the epithelium is essential in bronchial challenge because allergen is deposited above the epithelium. With a skin test, allergen is brought directly into the compartment where mast cells are to be found.

3. Identification and detection of allergens

Prolonged allergen avoidance can reduce the symptoms of asthma and can decrease bronchial hyperresponsiveness [30,31,83—85]. For house dust mite, threshold levels of exposure have been proposed for sensitization (>2 μg Der p 1/gram dust) [86—88] and for allergic symptoms (>10 μg Der p 1/ gram dust) [89—91]. An important aspect of treatment is to minimize exposure to allergens by appropriate measures. To evaluate the effect of these measures a reliable method is needed for the detection of allergens. Particular indoor allergens play a role in asthma [40]. House dust contains a wide range of glycoproteins, many can manifest as allergens. Specific immuno-assays have been developed to measure various major allergens in house dust. Ideally, the actual exposure of patients, i.e. the quantity of inhaled allergens, should be measured. However, measuring of airborne allergens is technically difficult. In addition, the quantity of airborne allergens critically depends on disturbance, which is difficult to standardize. Therefore, exposure is mostly measured by determining the amount of allergens in house dust that is sampled with a vacuum cleaner.

The dust sample is extracted and the supernatant fluid is assayed by a specific immuno-assay. Results are generally expressed as the concentration of allergen in μg per gram of dust. A drawback of this method is that dust contains variable amounts of relatively heavy material, which influences the calculated allergen fraction. Because of these differences between dust samples from floors (more sand) and from bedding and furnishings (fibres and skin flakes), it is probably more appropriate to compare allergen levels in samples obtained from similar collection sites, or to relate the results to an indicator protein.

Insect allergens

Insects have been recognized as inhalant allergens for many years. The earliest reports on allergic respiratory disease from inhaling insect allergens involved caddis flies [92]. Insects constitute about 70% of the known animal species. There is a complex variety of insect-derived allergens in the environment. Some of these allergens occur in a particle size appropriate for entering the respiratory tract, where they may induce sensitization similar to other animal-derived allergens. Insects are indoors, outdoors, at home and at work. The symptoms of patients with respiratory allergy caused by insects are similar to those with inhalant allergies caused by other sources. Little is known about the world-wide incidence, the distribution, and the nature of these allergens. Probably, there is cross-reactivity between house dust mites and insects allergens [93—95], but the extent of these cross-reactivities is still unknown. Based on the natural indoor occurrence, cockroaches and silverfish are a potential source of environmental allergens in The Netherlands. Chironomids are used to investigate the cross-reactivity between insects and house dust mite.

• Cockroach allergens

The potential role of cockroach allergy in human disease receives more and more attention over the past years. Positive skin tests, RAST and bronchoprovocation test have been carried out [96]. Cockroaches belong to the order of *Orthophera* and the family of *Blattidae*, most common are *Blatella germanica* and *Periplaneta americana*. Two major allergens (Bla g 1 and Bla g 2) have been identified [97—99]: Bla g 1 is a 25 kD, heat stable acidic protein; Bla g 2 a 36 kD heat sensitive protein. Probably, both major allergens are present in body and faeces of the insects. Humans are exposed by infestation at home. The rate of infestation depends on geographic locations and socioeconomic conditions. Although cockroach infestation is a world-wide phenomenon, it is rampant in crowded urban areas. A high incidence of allergy to cockroaches in patients with asthma (30—48%) has been reported in The United States [100,101].

• Chironomid allergens

Chironomidae (red mosquito larvae) represent a family of non-biting midges (order: *Diptera*). In areas abounding in water, such as Sudan [102] and Japan [95,103] Chironomids are important sources of environmental allergens. In Europe, allergy against Chironomids has been reported in Sweden [104,105]. In Germany, Chironomid allergy is common among fish breeders since the larvae of Chironomids are frequently

used as fish food [106]. Lyophilized products of these larvae generate airborne dust. People engaged in the production of fish food are frequently affected due to high exposure, about 20% of them develop an immediate type of hypersensitivity with significant levels of specific IgE antibodies. A major allergen (Chi t 1) could be identified [107], a haemoglobin with a molecular weight of 16 or 32 kD for the monomeric or dimeric form.

• Silverfish allergens

Silverfish (*Lepisma saccharina*), primitive insects, belong to the order of *Thysanura*. They measure 3—12 mm, have 3 tail feelers and are covered with shiny scales. Silverfish avoid light, and need a humid environment, and are mostly found in kitchens and bathrooms. They live amongst others from crumbs of bread, flour and paper fragments. In The Netherlands silverfish are common in households; the incidence of allergy is hardly known. Baldo et al. tested 41 allergic patients who had a history suspect of allergy to insects and found in 25 of them IgE antibodies against silverfish [93].

4. Aim of this thesis

Different tests are used to study the quantitative aspects of the IgE-mediated reactions in patients with asthma and rhinitis.

The symptoms of inhalant allergy are schematically determined by a combination of allergen-exposure, IgE antibody level, mediator release and organ sensitivity for these mediators. Most tests measure an overall effect of several of these factors. By using a combination of different tests, inferences can be made concerning the relative importance of the separate factors of the allergic cascade.

Another approach is to vary one specific factor within one and the same patient, keeping the other factors constant. For instance, it is possible to compare allergic reactions against two different allergens with similar biological activity in a patient with different IgE titers against these allergens. In this system, the difference in test results is solely determined by the difference in specific IgE-antibody level.

Allergens from sources like house dust mite cultures are complex mixtures of glycoproteins. There are significant differences in recognition patterns of the IgE response to these glycoproteins between patients. Major allergens are quantitative important glycoproteins to which the majority of allergic patients has a significant IgE-antibody response. In the past decade, monoclonal antibodies against major allergens have become available [108—110]. The development of these monoclonal

antibodies has facilitated characterization and isolation of major allergens. By using isolated major allergens, instead of complex allergen mixtures, in the skin test and measuring the corresponding concentration of specific IgE, quantification of the relation between these parameters is possible.

The second aim is identification and detection of allergens. Besides the well-known indoor allergens like house dust mite and animal dander, the role of indoor allergens like insects is, in The Netherlands, not established. The cross-reactivity between insects and *D.pteronyssinus* is known [93—95], but the extent of these cross-reactions in not known.

In the second part of this thesis the prevalence of IgE antibodies against insects and cross-reactivity with *D.pteronyssinus* was investigated in patients with inhalant allergy. Immuno-assays for insect allergens were developed to study exposure levels to insects.

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Chapter 2

The relationship between RAST and skin test results in patients with asthma or rhinitis: A quantitative study with purified major allergens

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ABSTRACT

Background: Study of the relationship between skin test results and IgE antibody levels is seriously hampered by the use of conventional allergen extracts because the precise amount of relevant allergen for each patient is unknown.

Objective: This study was designed to investigate skin reactivity with purified major allergens and to assess the relation with serum levels of IgE antibodies and to determine which additional factors contribute to the skin test result.

Methods: We used five purified major allergens (Der p 1, Der p 2, Fel d 1, Lol p 1 and Lol p 5) in skin tests, RASTs, and histamine release tests in 43 multisensitized patients with asthma or rhinitis.

Results: The differences in biologic activity of the five major allergens at a given level of specific IgE are within one order of magnitude. A significant residual variation remains in the correlation between skin test results and levels of IgE antibodies, which cannot be explained by imprecision of both tests (Pearson log skin test vs log specific IgE: r=-0.46—0.92). With similar levels of specific IgE, the amount of allergen that is required for a positive skin test result may differ as much as a factor of 100 between patients. The amount of total IgE in serum contributes significantly to the skin test result. High values of total IgE are accompanied by a lower skin reactivity for allergen.

Within individuals, allergens that cause skin test results that deviate from the prediction based on IgE antibody level often show a similar deviation in the histamine release test. This indicates that the type of IgE response (i.e., affinity or epitope recognition pattern) contributes significantly to the skin test result. Skin reactivity for histamine does not significantly influence the skin reactions expressed as allergen threshold. However, increased skin reactions with higher allergen dosages depend on histamine reactivity.

Conclusion: The major allergens tested show similar biologic activities. In addition to IgE antibody level, total serum IgE and type of IgE antibody response contribute significantly to the skin test threshold for allergens. Even in a system with purified allergens, IgE antibody levels and skin test results are not interchangeable as an indicator of the degree of allergic sensitization.

INTRODUCTION

The radioallergosorbent test (RAST) and the skin test are frequently used to diagnose allergy [1]. A significant association has been reported between the presence of allergen-specific IgE and a positive skin test response to the allergen [2—5]. However, when false-positive results are disregarded, the relation is rather weak. A higher correlation was obtained when using the threshold end-point dilution of the skin instead of the diameter of the skin reaction to full-strength extract to assess the cutaneous allergic reactivity [2,3,6].

One of the reasons for this weak quantitative relation might be the use of allergenic extracts. Extracts, complex mixtures of various major and minor allergens, also contain nonallergenic components. These mixtures make comparison of RAST and skin test results difficult. An excess of minor allergens in the solid phase in the RAST, which is necessary for efficient detection of IgE antibodies, can be difficult to obtain. It is not yet known how the dose-response curves of the different allergenic components influence each other in the skin test.

The aim of our study was to investigate the relationship between skin test results and specific IgE and to determine which factors, other than specific IgE, contribute to the skin test result.

Theoretically, intraindividual and interindividual variation of the skin reaction for a given level of allergen-specific IgE might be explained by several factors. We distinguish allergen-dependent factors and patient-dependent factors. The allergen-dependent factors or the biologic activity of allergens (potency) may be determined by the capability of the allergen to cross-link membrane-bound IgE. The number and location of the IgE-binding epitopes on the allergen molecule are probably relevant in this respect. Alternatively, the rate of degradation of the allergen in the skin may play a role.

Patient-dependent factors may be non-specific and allergen-specific. Non-specific factors concern the number of mast cells at the test site, the capability of mast cells to release mediators, and the sensitivity of the skin for mast cell mediators, predominantly histamine.

Allergen-specific, patient-dependent factors may be determined by the type of IgE response against the allergen, that is, heterogeneity of the antibody response (IgE antibodies against different epitopes on the major allergen) and affinity of the antibodies.

In patients with multiple sensitivities, we compared RAST and skin test results under optimal conditions by using purified Der p 1 and Der p 2 from *Dermatophagoides pteronyssinus*, Lol p 1 and Lol p 5 from grass pollen, and Fel d 1 from cat dander. The

contribution of various patient-dependent factors was examined after correction was made for the effect of the amount of specific IgE. Furthermore, the biologic activity of the five major allergens was compared. We used histamine release from peripheral blood basophils as an in vitro model for allergic reactions [7] to verify the results of the skin test regarding allergen-dependent factors, as well as allergen-specific, patient-dependent factors.

PATIENTS AND METHODS

Patients

The study included 43 patients with allergic rhinitis, allergic asthma, or both who were recruited from the Departments of Otorhinolaryngology and Pulmonology of the Academic Medical Center. Patients with allergic rhinitis had a history of sneezing, nasal itching, runny nose, or nasal conjestion. Asthma was defined according the criteria of the American Thoracic Society [8]. Patients with asthma had a history of paroxysms of dyspnea, wheezing, and coughing. The median forced expiratory volume in 1 second (FEV_1) was 85% of predicted value (range, 62—114%). The median provocative concentration causing a 20% fall in FEV_1 (PC_{20}) was 2 mg histamine/ml (range, 0.07—22.6 mg histamine/ml). The median age of the patient group was 32 years (range, 16—72 years). Patient selection was based on a positive RAST score for at least two allergen extracts (house dust mite, grass pollen, or cat dander). Excluded were patients with eczema in the test area, patients treated with oral steroids or antihistamines, and patients who had received immunotherapy. The study was approved by the local medical ethical committee, and written informed consent was given by all patients.

Allergens

Dried *D.pteronyssinus* mites were obtained from the Commonwealth Serum Laboratories (CSL; Melbourne, Australia). Mites were extracted for 12 h in phosphate-buffered saline (PBS) at 4°C (2% wt/vol extract). Cat extract was obtained from HAL Allergenen Laboratories (Haarlem, The Netherlands). Ryegrass pollen, obtained from Diephuis Laboratories (Groningen, The Netherlands), was extracted with distilled water (5% wt/vol) for 2 h. The pH was maintained at 8.5 by readjustment with NaOH (0.1 mol/L) every 15 min. After centrifugation, the supernatants were defatted, dialyzed against distilled water, and freeze-dried [9].

Monoclonal antibodies and rabbit antibodies

Monoclonal antibodies were prepared as previously described [9—11]. Rabbit monospecific polyclonal antibodies were prepared by immunization with monoclonal antibody-purified major allergens [9—11]. Monospecificity was confirmed by a single precipitation line with allergen extracts in the agar gel double-diffusion technique.

Preparation of purified allergens

For skin and histamine release tests, allergens were affinity-purified with monospecific rabbit polyclonal antibodies. Mite-body extract (2% wt/vol, 1 ml/100 mg Sepharose), ryegrass extract, and cat extract (1 mg protein/100 mg Sepharose) were coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The Sepharose was incubated with rabbit antibodies for 12 h at 4°C (500 µl serum/100 mg Sepharose) and washed five times with PBS containing 0.01% Tween-20 and five times with PBS. Rabbit antibodies were eluted with a buffer containing 10% dioxane (vol/vol) in 0.1 mol/L glycine at pH 2.5. After dialysis, the eluate was coupled to CNBr-activated Sepharose (1 mg protein/100 mg Sepharose). Next, the Sepharose was incubated with 2% wt/vol mite body extract (5 ml/100 mg Sepharose), grass pollen extract, or cat dander extract (5 mg protein/100 mg Sepharose) for 12 h at 4°C. The Sepharose was washed five times with PBS-Tween and five times with PBS. Der p 1 was eluted with a buffer containing 50% ethyleneglycol (vol/vol) in 10 mmol/L lysine at pH 11.0. Der p 2, Lol p 1, Lol p 5, and Fel d 1 were eluted with a buffer containing 10% dioxane (vol/vol) in 0.1 mol/L glycine at pH 2.5. The eluates were dialyzed against PBS.

To ensure purity of the major allergen eluate, we measured, in each eluate, the amount of the other major allergen. Cat albumin was measured as an indicator for purity of Fel d 1. Contamination with the other major allergen was less than 1%.

For the skin test, the allergens were diluted in PBS containing 0.03% human serum albumin and 0.5% phenol and were stored at 2—8°C. Working dilutions were prepared on the day of the test, and testing was completed within a 4-month period. The allergens were stored at -20°C for the histamine release test. For the RAST, the allergens were affinity-purified with monoclonal antibodies [9—11].

Der p 1, Der p 2, Lol p 1, Lol p 5, and Fel d 1 assays

An inhibition assay, as described by Chapman et al. [12], was performed to measure the major allergen concentrations in the extracts. To 0.5 ml protein A-Sepharose (1 mg/ml), 50 µl of diluted rabbit antibodies, 50 µl of iodine 125-labeled major allergen, and 50 µl of the test extract were added. The mixture was incubated overnight under vertical rotation at room temperature. After centrifugation and washing away of non-bound components, the radioactivity bound to the solid phase was counted. Quantification of Der p 2 was performed by means of a binding assay [10]. For Der p 1 (100 U = 12.5 ng) and Der p 2 (100 U = 0.5 ng), the concentration was read from the World Health Organization standard curve [13,14]. For Lol p 1 and Lol p 5, the concentration was read from a reference extract curve [9]. For Fel d 1, the concentration was read from a Food and Drug Administration (FDA) standard curve (1 FDA unit = 4 µg Fel d 1) [15].

RAST

The RAST was performed as described previously [16]. For Der p 1, Lol p 1, Lol p 5, and Fel d 1, monoclonal antibody affinity-purified allergen was coupled to CNBr-activated Sepharose 4B. To 100 mg Sepharose, 100—200 µg of major allergen was coupled. For the Der p 2 RAST, recombinant allergen was used. The complementary DNA coding for Der p 2 [17] was cloned into the bacterial expression plasmid pDS56/RBS II, 6* His. The recombinant allergen was purified by means of nickel-chelate affinity chromatography according to the method of Stuber et al. [18]. For this allergen 10 µg was coupled to 100 mg Sepharose. Per test, 50 µl of serum was added to 0.25—1.5 mg of Sepharose in a final volume of 300 µl (PBS-AT: PBS/0.3% (wt/vol) bovine serum albumin/ 0.1% (vol/vol) Tween 20). Each serum sample was tested in duplicate and in threefold dilutions of the Sepharose. Immunodetection of IgE was done with ^{125}I-sheep anti-human IgE. Sera were tested in such a dilution that the binding of radioactivity was less than 20% of the added radioactivity. The amount of specific IgE was calculated as described by Aalberse et al. [19]. The results were read from a total IgE standard curve with

anti-IgE Sepharose. Therefore the results were expressed in international units. The threshold sensitivity of the RAST (0.1 IU/ml) was established as a percentage binding of added radioactivity of more than two times the background values. The coefficient of variation of the RAST was 10%.

Purity of monoclonal antibody-purified major allergens used in the IgE antibody assay was similar to the purity of the preparations used in skin test and histamine release test (<1% contaminating major allergens).

Previously, we showed similarity of IgE antibody measurements by using major allergens isolated with either monoclonal antibodies or monospecific polyclonal antibodies from the same allergen extract [10,11]. This was affirmed by a high correlation between IgE antibody levels as detected by monoclonal purified major allergen (or recombinant allergen in the case of Der p 2) and polyclonal purified major allergen as used in the skin test with the sera investigated in this study (Der p 1, n=28, r=0.95, p<0.0001; Der p 2, n=28, r=0.87, p<0.0001; Lol p 5, n=20, r=0.95, p<0.0001).

Skin test

Standard intracutaneous skin tests were performed by injection of 0.02 ml of 10-fold dilutions in the forearm. After 15 min, the longest (A) and orthogonal (B) erythemal diameters were measured; mean diameters were calculated as (A+B)/2. The allergen concentration eliciting an erythemal diameter of 10 mm was calculated by log-linear interpolation. The negative control wheal with dilution buffer had to be smaller than 2 mm without erythema. All skin tests were done by the same investigator. We did quadruplicate skin tests in three persons in random order to investigate reproducibility. The coefficient of variation (CV) of the skin test threshold for these three persons was less than 15%. To investigate the potency of the extracts in time, we tested three persons at the beginning and at the end of the test period. The change in skin test threshold concentration was not significant (t test, δlog skin test = -0.02, -0.66 <95% confidence interval <0.25).

Histamine release

The histamine release test was performed in 31 of the 43 patients. The isolation of human leukocytes and incubation of the leukocytes with allergen or anti-IgE were performed with a method modified slightly from that of Lichtenstein and Osler [20]. The anti-IgE was a polyclonal sheep antiserum (CLB no. SH25P01). The concentration used was 100 ng/ml. The erythrocytes were lysed with isotonic ammonium chloride at 4°C [21]. The cells were separated by centrifugation and washed in medium containing 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 1.2 mmol/L potassium phosphate, 20 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Sigma Chemicals Co., St. Louis, MO.), 5.5 mmol/L glucose, and 0.5% (wt/vol) human albumin, pH 7.4. A 250 µl cell suspension was incubated with 50 µl of the stimulus soluton for release at 37°C for 45 min. Histamine content in the supernatant of the samples was measured with an automated fluorometric method [22]. The release was calculated as a percentage of the total histamine release minus the spontaneous release. The allergen concentration with a 10% release was calculated by log linear interpolation. Twenty-nine of 31 patients had a positive histamine release test response to a specific allergen. Two patients were nonresponders: they did not react to anti-IgE or to a specific antigen. We excluded these two patients from the analysis of the histamine release test results.

Statistical methods

Parametric correlations between variables were expressed as Pearson's r and nonparametric correlations as Spearman's rho. Differences between groups were analyzed with Student's t test. To compare the biologic activities of the major allergens, a repeated-measures analysis of variance (ANOVA) [23] was performed for the results of the skin and histamine release tests. The repeated-measures ANOVA was done to account for the fact that different allergens were tested on the same person. The compound symmetry model was used for the intraindividual covariance matrix of the skin test and histamine release measurements, the type of allergen as within-factor and IgE values for the allergens as within-person varying covariate. Estimation was done with the restricted maximum likelihood approach, and the Wald test was used to calculate p values. Equality of slopes was tested by the introduction of allergen*IgE interaction terms. The 10 p values for interallergen differences in potency were adjusted for multiple comparisons using Hommel's procedure [24]. In the skin test there is some indication that the slopes were different (p=0.065). The equation log y = alogx + b is used. Estimated slopes of log-transformed specific IgE (x), versus log-transformed skin test threshold concentrations (y) were -1.31 (Der p 1), -1.05 (Der p 2), -0.64 (Fel d 1), -1.08 (Lol p 1) and -1.28 (Lol p 5). However, for simplicity we assumed that the slopes were equal (estimate, -1.05). In the histamine release test the estimated slopes are -1.09 (Der p 1), -1.37 (Der p 2), -0.91 (Fel d 1), -0.83 (Lol p 1) and -0.91 (Lol p 5). The differences between slopes are not significant (p = 0.33). Assuming that slopes were equal, we find an overall estimate of -0.959 (SEM = 0.075).

To investigate the influence of factors other than specific IgE on the skin test result, a parallel-line linear regression, assuming equal slopes for the five allergens, was performed (log skin test threshold = alog specific IgE + b; a = common slope, b = intercept dependent on allergen). Subsequently, observed/predicted (O/P) ratios were calculated with this formula.

The analyses were performed with the SPSS/PC+ statistical package (Statistical Program for the Social Sciences, Chicago, Ill).

Results

The relationship between specific IgE and skin test threshold for different major allergens is shown in figure 1. Each of the allergens showed a significant log-log-transformed correlation. The residual variance per major allergen is still large. The correlation coefficients for log-specific IgE and log histamine release threshold were: Der p 1: r=-0.60, p=0.005, n=20, Der p 2: r=-0.61, p=0.003, n=21; Fel d 1: r=-0.58, p=0.02, n=16; Lol p 1: r=-0.40, p=0.09, n=19; Lol p 5: r=-0.71, p=0.003, n=15.

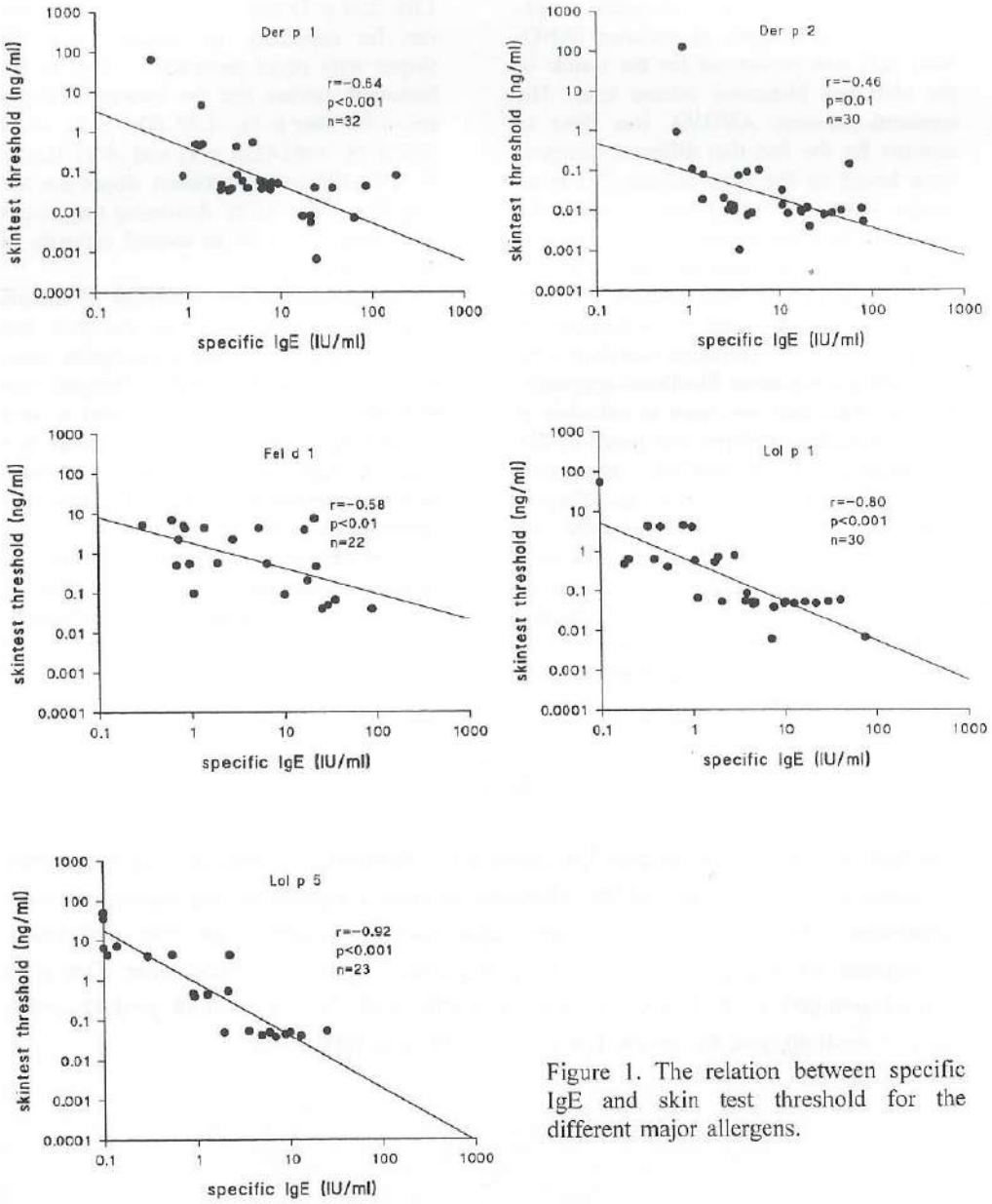

Figure 1. The relation between specific IgE and skin test threshold for the different major allergens.

Allergen-dependent factors

To compare the biologic activities of the major allergens a repeated-measures ANOVA [23] was performed for the skin and histamine release test results. The between-allergen differences in potency are given in table I, together with their geometric standard error of the mean (GSEM) and adjusted *p* values. Differences between allergens are expressed as ratios of allergen concentrations (on dry weight basis and on molar basis) with equal activity.

Table I. Estimated between-allergens differences

allergens	ratio $\frac{g/l\ M^*}{g/l\ M}$	GSEM	*p* value (adjusted)
Skin test			
Dp2/Dp1	0.40 (0.71)	1.39	0.024
Fd1/Dp1	7.14 (4.96)	1.47	<0.001
Lp1/Dp1	1.10 (0.86)	1.42	0.80
Lp5/Dp1	2.30 (2.05)	1.49	0.073
Fd1/Dp2	17.70 (6.88)	1.48	<0.001
Lp1/Dp2	2.72 (1.19)	1.43	0.022
Lp5/Dp2	5.69 (2.84)	1.50	<0.001
Lp1/Fd1	0.15 (0.17)	1.48	<0.001
Lp5/Fd1	0.32 (0.41)	1.53	0.029
Lp5/Lp1	2.09 (2.39)	1.45	0.090
Histamine release test			
Dp2/Dp1	0.15 (0.27)	1.36	<0.001
Fd1/Dp1	0.63 (0.43)	1.45	0.61
Lp1/Dp1	0.17 (0.13)	1.38	<0.001
Lp5/Dp1	0.59 (0.52)	1.42	0.41
Fd1/Dp2	4.30 (1.67)	1.44	<0.001
Lp1/Dp2	1.14 (0.50)	1.39	0.88
Lp5/Dp2	4.06 (2.03)	1.43	<0.001
Lp1/Fd1	0.27 (0.30)	1.43	0.001
Lp5/Fd1	0.94 (1.21)	1.45	0.88
Lp5/Lp1	3.55 (4.06)	1.37	<0.001

Differences are expressed as ratios of allergen with equal activity.
Dp, Der p; Fd, Fel d; Lp, Lol p.
*Molecular weights used for the calculations: Der p 1, 25 kD; Der p 2, 14 kD; Fel d 1, 36 kD; Lol p 1, 32 kD; Lol p 5, 28 kD.

The same procedure was performed for the histamine release test. The rank order for the potency of the allergens on weight basis in the skin test is: Der p 2 > Der p 1 = Lol p 1 = Lol p 5 > Fel d 1. The rank order for the histamine release test is Lol p 1 = Der p 2 > Fel d 1 = Lol p 5 = Der p 1. On a molar basis, the differences in potency between the major allergens in both the skin test and the histamine release test are within 1 order of magnitude.

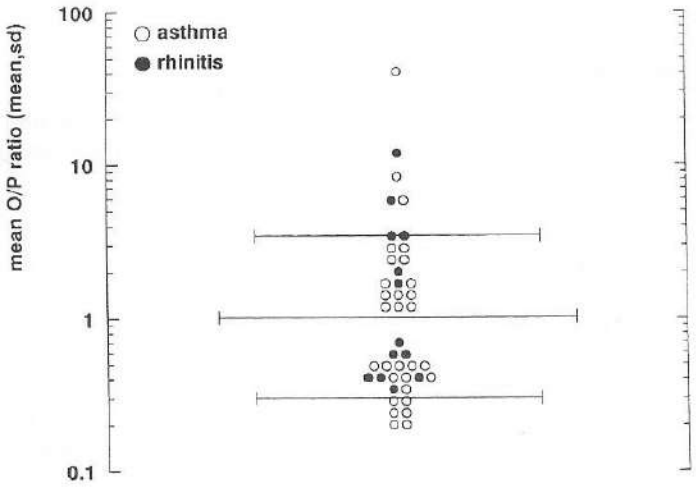

Figure 2. The geometric mean O/P ratios of the patients. The O/P ratio was calculated to quantify deviations of individual skin test results from the results given by the regression line (IgE vs skin test). The GM ratio was calculated per patient from the O/P ratios for the different allergens (median geometric standard deviation of mean O/P ratios: 2.3; interquartile range, 1.9).

Patient-dependent factors

To investigate the influence of factors other than specific IgE on the skin test, the skin test result (y) is predicted on the basis of specific IgE values (x) with a parallel-line linear regression method (log y = alogx + b). The predicted relation between skin test and specific IgE for the major allergens is based on a common slope with different intercepts caused by differences in biologic activities of the major allergens (as reported above).

To quantify deviations of the individual skin test results (the observed results) from the results given by the regression line (the predicted results), we used the O/P ratios. The

O/P ratio is a measure of the residual variation in skin test results after prediction based on specific IgE in serum. Therefore this measure includes variation caused by factors other than specific IgE, as well as random variation. A high O/P ratio means that more allergen is required for a positive skin test result than predicted on the basis of specific IgE, indicating a relatively low sensitivity in the skin test. A low ratio means a high sensitivity in the skin test. The geometric mean (GM) of the O/P ratios of the different allergens was calculated per patient (mean O/P ratio). The mean O/P ratio is considered to be an indicator for non-specific (allergen-independent) patient-dependent factors. In figure 2 the mean O/P ratios of the skin test are shown for all the patients.

Patient-dependent, non-specific factors

To investigate the influence of patient-dependent factors that are independent of the allergen applied, we performed an one-way ANOVA. Dividing the variance of the O/P ratios in within- and between-patient components, 36% is estimated to be attributable to systemic differences between patients ($p<0.001$). We found no differences in mean O/P ratios between asthma (GM = 0.94, GSEM = 1.25) and rhinitis (GM = 1.26, GSEM = 1.39). Also, in the histamine release test no differences were observed in mean O/P ratios between patients with asthma (GM = 0.86, GSEM = 1.34) and those with rhinitis (GM = 1.18, GSEM = 1.54). No influence of age on the mean O/P ratio was found ($n=43$, $r=-0.09$, $p=0.57$). There was no significant correlation between mean O/P ratios in skin test and histamine release test.

Histamine sensitivity of the skin

No correlation was found between skin sensitivity for histamine (measured as threshold concentration) and the mean O/P ratio (Pearson $r=0.15$, $p=0.3$). The skin reaction to a single high concentration of allergen, independent of amount of specific IgE, correlates with the skin reaction to histamine (for 0.1 ng/ml Der p 2 $r=0.65$, $p<0.0005$).

Total IgE

Figure 3 shows a significant positive correlation (Pearson $r=0.37$, $p<0.05$, Spearman's rho=0.30, $p<0.05$) between log total serum IgE and log mean O/P ratio, indicating that a high serum IgE is accompanied by a lower sensitivity for allergen in the skin test. In the histamine release test a significant correlation between log total serum IgE and log mean O/P ratio (Pearson $r=0.49$, $p<0.01$, Spearman's rho=0,47, $p<0,01$) is also found.

Releasability

In the histamine release test an inverse correlation (Pearson r=-0.49, p=0.005, Spearman's rho=-0.46, p<0.01) between log histamine release activity for anti-IgE, as an indicator for releasability, and the log mean O/P ratio was found (fig. 4). This indicates that a higher anti-IgE release is accompanied by a higher allergen sensitivity in the histamine release test.

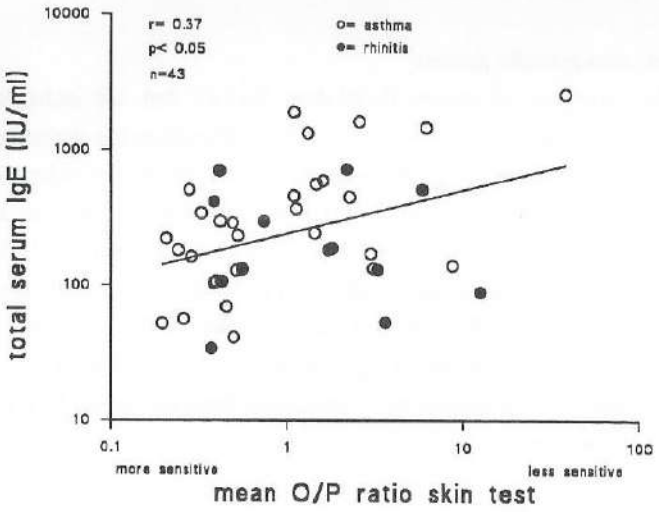

Figure 3. The relation between the mean O/P ratio in the skin test and total serum IgE. The O/P ratio was calculated to quantify deviations of individual skin test results from the results given by the regression line (IgE vs skin test). A high O/P ratio means that more allergen is required for a positive skin test results than predicted on the basis of specific IgE, indicating a relatively low sensitivity in the skin test. A low ratio means a high sensitivity in the skin test.

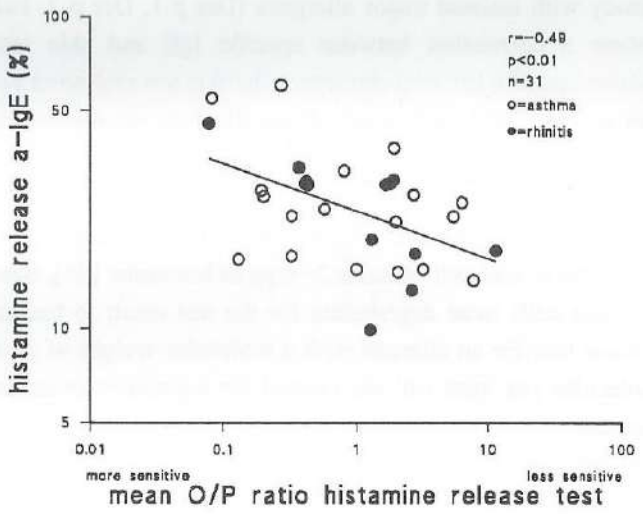

Figure 4. The relation between the mean O/P ratio in the histamine release test and the histamine release of anti-IgE.

Patient-dependent, allergen-dependent factors

Within patients there is a significant variation in the O/P ratios for different allergens. These differences might be caused by random variation or by the specific interaction between IgE and allergen. We performed a rank correlation of the O/P ratios in skin test and histamine release test in each patient. The individual Kendall Tau values were pooled after weighing for the number of allergens tested per patient. There was a significant rank correlation for O/P ratios in both tests (pooled Kendall-Tau z=2.74, p=0.007). This is most likely explained by differences in the affinity or epitope recognition pattern of the IgE antibody response because such differences will similarly affect results of skin test and histamine release test.

Discussion

The results of our study with isolated major allergens (Der p 1, Der p 2, Fel d 1, Lol p 1, and Lol p 5) show a correlation between specific IgE and skin test threshold concentrations. At higher specific IgE concentrations, the skin test end-point concentration tends to level off. In spite of very high levels of specific IgE, we did not find positive intracutaneous skin test results when less than 0.01 pg of allergen was used per test. Apparently, a minimum allergen dose is needed for a positive skin reaction. The minimal histamine dose for an erythemal diameter of 10 mm in the intracutaneous skin test is about 30 ng. Assuming that a mast cell contains 3—6 pg of histamine [25], this means that about 5,000—10,000 mast cells must degranulate for the test result to become positive. The 0.01 pg limit means that for an allergen with a molecular weight of 25 kD, at least 50—100 allergen molecules per mast cell are needed for a positive intracutaneous skin test. This is in agreement with in vitro studies on basophil histamine release. Histamine release was initiated with 100—200 molecules of antigen per basophil [26,27].

According to Turkeltaub et al. [6], we have expressed the skin test threshold as allergen concentration eliciting erythema. This is because the dose-response curve of the erythema is steeper than that of the wheal. We found a high correlation between the allergen threshold as detected by wheal (5 mm) and flare (10 mm) reaction (r=0.98, p<0.001). The choice for wheal or flare did not significantly influence the results of this study.

One of the methods to estimate the biologic activity of an extract is to measure the major allergen content in the extract. For allergen standardization, whether major allergen determination can replace biologic standardization has been discussed [28]. A prerequisite for such an approximation is a similar biologic activity of different major allergens. We found only small differences in biologic activity (< factor of 10) for the different major allergens. Still, the question is, which major allergen should be used for standardization? Especially for house dust mites, the patients show a clear variation in IgE response to the different allergens. We wonder whether it is feasible to replace biologic standardization with major allergen determination because it is difficult to define and measure all important allergens in an extract.

Despite the use of purified major allergens, only 21—85% of the variation in skin test results can be accounted for by specific IgE level. This might suggest that either test or both the skin test and the RAST are imprecise, but the reproducibility of the skin test (CV <15%) and the RAST (CV <10%) does not support this. Other reasons could be additional factors that contribute to the skin reaction. To further elucidate the relationship

between specific IgE levels and skin test results, we investigated the influence of patient-dependent factors in the skin test.

We applied highly purified major allergen preparations contaminated by less than 1% of other allergenic components. Minor contamination of the major allergen preparation with other allergenic components is more likely to influence the skin and histamine release test results than the IgE antibody assays. In the latter assay an excess amount of antigen is required for efficient antibody detection. The possibility that contaminants will influence the skin test result is greatest in patients with a low IgE antibody response to the relevant major allergen compared with the IgE response to the native extract. However, we found no relation between O/P ratios and contribution of IgE anti-major allergen to the IgE response to the native extract, rendering a significant role of contaminants in skin reactivity unlikely.

We found no correlation between the skin threshold for histamine and the mean O/P ratio. However, larger skin reactions for higher allergen concentrations correlate with the skin reaction for histamine, independent of the amount of specific IgE. Therefore in our view, correction for the skin sensitivity to histamine is important for the size of the skin reaction to allergen concentrations above threshold but less so for end-point concentrations.

In the skin test, as well as in the histamine release test, we found a significant inhibitory effect of total serum IgE. A likely explanation is competition of the irrelevant IgE with specific IgE for binding to the IgE receptors on basophils and mast cells. This in vivo finding is in accordance with earlier studies in vivo and in vitro. The mechanism of inhibition through saturation of mast cell Fcε receptors is established in vitro. Already in 1976, it was shown that lung fragments first exposed to IgE-rich serum become resistant to further passive sensitization with serum containing grass pollen-specific IgE [29]. In another study the Prausnitz-Kustner titre of a donor serum sample in different recipients was found to be inversely related to the serum IgE level of the recipients [30]. Some reports about lower allergic reactivity in a tropical environment with helminthic infections and high total serum IgE levels have been published [31—33]. An inhibitory effect of total IgE in the skin test was suggested by Eriksson [34]. He tested 593 patients with hay fever and found that, at each level of atopy (defined by skin test), patients with high total IgE had higher RAST values than those with low total IgE.

Another patient-dependent factor, influencing the skin test, might be mast cell releasability, which is difficult to measure in vivo. Codeine [35], compound 48-80 [36], and anti-IgE [37] have been used in skin tests as indirect measures of releasability. In vitro histamine release with anti-IgE from peripheral blood basophils can be used as a

model for releasability of basophils, although activation by anti-IgE differs in some important aspects from activation by allergen [38]. Releasability of the basophil might play a role in the pathogenesis of asthma [39,40]. Little is known about the releasability of mast cells. We did not measure mast cell releasability through direct activation of the Fcε receptor but showed that releasability of the basophil, measured by anti-IgE response, has a significant influence on the relation between specific IgE and histamine release with allergen. Probably, releasability of mast cells is also of importance for the skin test. The lack of correlation between mean O/P ratios in skin test and histamine release test indicates that releasibility differs between mast cells and basophils.

As yet, we have not measured antibody affinity. The significant correlation between skin and histamine release test results in the within-patients ranks of the O/P ratios of different allergens supports the view that the type of IgE response to a major allergen, as determined by the number of epitopes recognized or by the affinity of the antibodies, similarly influences the results of both tests. Affinity is less likely to influence the results of the RAST because of the excess amount of allergen applied to the solid phase.

Our results could be of interest for the allergen inhalation challenge test. Airway responsiveness to allergen is usually predicted by the skin threshold to allergen and airway responsiveness to histamine [41]. The skin sensitivity to allergen is defined as end-point concentration in the skin prick test. IgE antibody determination will produce results different from those of skin testing. The question to be answered is whether the factors measured in the skin test, in addition to specific IgE (i.e., type of IgE response, total IgE, and releasability of the mast cell), contribute to the prediction of airway responsiveness to allergen.

In conclusion, despite the use of purified allergens, the quantitative relation between skin test and specific IgE is weak. Skin test allergen threshold may differ by as much as a factor 100 between patients with similar levels of specific IgE. This is not caused by imprecision of either RAST or skin test. In addition to specific IgE, there are patient-dependent factors that contribute significantly to the outcome of the skin test. A higher total serum IgE is associated with a lower sensitivity in the skin test. Presumably, releasability of the mast cell and the affinity or heterogeneity of the IgE response are other important factors. Histamine sensitivity of the skin did not influence allergen threshold of the skin. The differences in biologic activity of the different major allergens were less than a factor of 10. At least about 50—100 allergen molecules per mast cell are needed for a positive intracutaneous skin test result.

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Chapter 3

Fel d 1 specific IgG antibodies induced by natural exposure, have blocking activity in skin tests

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Submitted

Abstract

Cat-allergic patients frequently have IgG antibodies directed against Fel d 1. The aim of this study was to investigate whether these IgG antibodies influence the results of the skin test.

Titrated skin tests were performed with Fel d 1 and IgE- and IgG-antibody levels were measured in 59 patients with cat allergy.

Levels of specific IgG against Fel d 1 ranged from less than 0.25 to 3.5 μg/ml. By means of a multiple regression analysis it was shown that the amount of specific IgG antibodies contributes significantly to the results of the skin test. Presence of specific IgG against Fel d 1 was accompanied by higher skin thresholds for Fel d 1.

In conclusion, this study indicates that even low levels of specific IgG, induced by natural exposure to cat allergens, have a blocking effect on the early phase skin reaction.

Introduction

The role of IgG antibodies in skin tests is not established as yet. Already before 1940 it was shown that in sera of patients that had received a course of allergen-specific immunotherapy a heat-stable antibody had developed [1,2]. This antibody was called blocking antibody because of its allergen neutralizing capacity in skin tests [3—5]. Allergen extracts pre-incubated with serum of desensitized patients, showed significantly decreased diameters of wheals and flares in the skin test compared to allergen extracts incubated with serum of the same individuals before immunotherapy or with saline. The question arises whether allergen specific IgG antibodies also act as blocking antibodies without preincubation, an approach that resembles more closely the physiological situation.

In vitro, the inhibitory effect of IgG antibodies on basophil histamine release was demonstrated by several investigators [6—10].

In several studies on immunotherapy a decrease in early phase skin reactivity was observed [11—15]. However, those studies frequently failed to show a correlation between the decrease in skin reactivity and the concentration of IgG antibodies. This might have been due to the fact that in these studies IgG antibodies against total allergen extracts were measured. The results may than be confounded by the inclusion of IgG antibodies with irrelevant specificities. IgG antibodies to irrelevant, i.e. non-allergenic material in allergen extracts or to allergens against which no IgE antibodies

are directed, will not contribute to the blocking of the binding of specific IgE antibodies. IgG antibodies that are directed against the same molecules as the IgE antibodies and probably even the same or adjacent epitopes, should be measured. For these reasons purified major allergens offer a better opportunity to investigate the influence of specific IgG on the skin test.

IgG antibodies against allergens are commonly found in allergic patients. It has been shown that atopic patients have increased levels of IgG to the grass pollen allergen Lol p 1 [16] and to the house dust mite allergen Der p 1 [17]. The amount of specific IgG is related to the exposure-level [18,19]. Higher levels of IgG antibodies are found to the cat allergen Fel d 1, when a cat is in the house [20]. In case of occupational exposure to experimental animals, with very high exposure levels, not only allergic but also nonallergic individuals develop IgG antibodies [21]. The aim of this study was to investigate whether IgG antibodies induced by natural exposure to cats have an inhibitory effect on the skin test results.

Patients and methods

Patients

The studies were performed in 59 cat-allergic patients attending the Department of Otorhinolaryngology and the Department of Pulmonology. Patients with allergic rhinitis had a history of sneezing, itching in the nose, a running nose or a blocked nose. Asthma was defined according the criteria of the American Thoracic Society [22]. Patients with asthma had a history of paroxysms of dyspnea, wheezing and coughing. The median age of the patients was 30 years (range, 16—36 years). Patient characteristics are given in table 1. Patients were selected on basis of a positive RAST for cat dander. Excluded from the study were patients with eczema in the test area, patients treated with oral steroids or antihistamines and patients treated with immunotherapy. The study was approved by the local medical ethical committee and informed consent was given by all patients.

Preparation of purified Fel d 1

The isolation of major allergen with monospecific polyclonal antibodies for application in vivo was performed as described previously [23]. Briefly, Fel d 1 for immunization of rabbits was isolated from cat dander extract (HAL Allergen Laboratories, Haarlem, The Netherlands) by affinity chromatography with monoclonal antibodies against Fel d 1 [24,25]. Rabbit polyclonal antibodies were purified by affinity chromatography with cat dander extract immunosorbent: the cat extract was coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) (1 mg protein/100 mg Sepharose). This Sepharose was incubated with rabbit serum containing anti-Fel d 1 for 12 hours at 4°C (500 μl serum/100 mg Sepharose). The Sepharose was washed 5 times with PBS (Phosphate buffered saline), containing 0.01 % Tween-20, and 5 times with PBS.

Table I. Patient characteristics

	age *year*	gender	disease	skin test *ng/ml*	sp IgE *IU/ml*	sp IgG *µg/ml*	total IgE *IU/ml*	cat at home
1	33	M	AR	4.46	1.39	<0.26	1894	no
2	38	F	A	0.04	89.35	0.46	455	no
3	35	F	R	4.33	0.86	<0.26	130	no
4	29	F	AR	0.46	22.21	3.46	181	yes
5	37	F	R	7.00	0.62	0.39	718	no
6	31	M	A	0.55	0.95	0.38	56	yes
7	31	M	R	0.04	25.51	0.39	130	no
8	34	M	A	7.43	21.31	2.12	2587	yes
9	52	M	AR	2.29	2.77	<0.26	171	no
10	36	M	AR	0.55	6.40	<0.26	1323	no
11	46	M	A	0.51	0.69	<0.26	340	no
12	43	M	A	0.10	1.05	<0.26	52	no
13	18	F	AR	4.33	5.25	0.91	1467	yes
14	22	F	AR	0.57	1.90	<0.26	288	no
15	36	M	AR	3.90	16.54	2.84	140	yes
16	33	M	A	0.21	17.75	3.22	232	yes
17	29	F	A	0.09	9.98	1.44	106	yes
18	36	M	R	2.29	0.73	<0.26	34	no
19	18	M	R	5.09	0.82	<0.26	188	no
20	25	F	R	5.29	0.30	0.29	699	yes
21	25	F	R	0.05	29.60	0.65	106	no
22	36	M	AR	0.07	35.78	0.54	506	no
23	40	M	A	4.00	2.01	<0.26	1200	no
24	28	M	AR	0.39	4.51	<0.26	3400	no
25	20	F	AR	0.55	0.65	0.31	1100	no
26	29	F	R	4.60	0.99	<0.26	456	no
27	32	M	R	5.09	0.36	<0.26	355	no
28	22	F	AR	0.41	59.87	3.31	571	yes
29	53	M	A	0.31	12.71	0.34	1600	no
30	15	F	R	0.53	0.65	<0.26	257	no
31	23	F	R	6.00	0.26	0.52	694	no
32	28	M	AR	0.14	23.88	1.13	824	no
33	21	M	R	0.05	3.56	<0.26	84	no
34	44	F	AR	0.55	1.78	0.56	293	no
35	41	F	AR	0.009	49.09	<0.26	1300	no
36	37	M	AR	0.79	0.10	<0.26	2200	no
37	42	M	R	0.04	13.71	<0.26	300	no
38	29	M	AR	0.006	13.23	0.81	215	no
39	21	M	AR	0.05	359.69	0.74	3000	no
40	24	F	A	4.0	1.16	<0.26	1500	no
41	25	F	AR	0.04	2.11	<0.26	461	no
42	30	F	AR	0.15	15.70	1.48	100	yes
43	26	F	A	0.06	11.07	<0.26	35	no
44	24	M	R	6.62	0.14	0.83	344	no
45	22	F	AR	0.18	2.42	<0.26	308	no
46	44	M	AR	5.09	1.68	<0.26	607	no
47	35	M	AR	0.63	1.52	<0.26	761	no
48	29	M	R	0.06	15.32	<0.26	421	no
49	26	F	R	4.21	0.41	<0.26	627	no
50	25	F	R	0.06	2.01	0.97	78	no
51	27	M	AR	5.50	0.62	<0.26	171	no
52	40	M	R	0.09	1.86	0.48	145	no
53	35	F	R	0.10	0.31	0.27	63	no
54	31	M	AR	0.41	0.84	0.32	836	no
55	24	F	R	13.91	0.22	0.89	125	yes
56	23	F	R	4.21	0.21	<0.26	75	no
57	18	M	R	4.46	0.60	0.60	57	yes
58	26	F	R	0.66	0.52	<0.26	96	no
59	43	F	A	1.00	0.52	0.68	383	yes

1. A = asthma; 2. R = rhinitis

Rabbit anti-Fel d 1 was eluted with a buffer containing 10% dioxane (v/v) in 0.1 M glycine at pH 2.5. After dialysis, the eluate was coupled to CNBr-activated Sepharose (1 mg protein/100 mg Sepharose). Subsequently, this anti-Fel d 1 allergosorbent was incubated with cat extract for 12 hours at 4°C (5 mg protein/100 mg Sepharose). The allergosorbent was washed 5 times with PBS-Tween and 5 times with PBS. Fel d 1 was eluted with a buffer containing 10% dioxane (v/v) in 0.1 M glycine at pH 2.5. The eluate was dialyzed against PBS. Specificity of the monoclonal and polyclonal anti-Fel d 1 and characterisation of Fel d 1 purified by affinity chromatography were described previously [24,25].

For the skin test the Fel d 1 was diluted in PBS, containing 0.03% human albumin and 0.5% phenol and stored at 2-8°C.

Fel d 1 assay

An inhibition assay analogous to the assay described by Chapman et al. [26] was applied to measure the Fel d 1 concentration in the extract. To 0.5 ml protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) (1 mg/ml), 0.05 ml diluted rabbit antibodies, 0.05 ml ^{125}I-labeled Fel d 1 and 0.05 ml of the test extract was added. The mixture was incubated overnight under vertical rotation at room temperature. After centrifugation and washing of the Sepharose, radioactivity bound to the solid phase was counted. The Fel d 1 levels were read from the FDA standard curve (1 FDA unit= 4µg Fel d 1) [27].

RAST (Radioallergosorbent test)

RAST was performed as described previously [28]. Monoclonal antibody affinity purified Fel d 1 was coupled to CNBr-activated Sepharose 4B (100 µg to 100 mg Sepharose). Per test 50 µl of serum was added to 0.5 mg Sepharose in a final volume of 300 µl (PBS-AT: PBS/0.3% (w/v) BSA/0.1% (v/v) Tween 20). Each serum was tested in duplicate, in threefold dilutions of the Fel d 1-Sepharose. Immune detection of IgE was performed with ^{125}I-sheep anti-human IgE. Each serum was tested in such a dilution that the percentage binding of radioactivity was less than 20% radioactivity added. The amount of specific IgE was calculated as described by Aalberse [29]. The results were read from a standard dilution curve with a reference serum with anti-IgE Sepharose. Therefore, the results were expressed in IU (International Units)/ml specific IgE. Total serum IgE was quantified by an inhibition assay as described earlier [30].

Assay of IgG against Fel d 1

One µl serum was incubated with 250 µl protein A-Sepharose (2 mg/ml) and 50 µl radiolabeled Fel d 1. IgG4 antibodies were detected with anti-IgG4 Sepharose as described previously [31]. After overnight incubation under vertical rotation the Sepharose was washed with PBS containing 0.1% Tween, and bound radioactivity was measured. Results were expressed in Arbitrary Units (AU), read from a rabbit reference serum. The amount of specific IgG antibodies in serum was calculated according to Steward and Petty [32]. Binding of radiolabeled Fel d 1 to insolubilized rabbit IgG was measured after addition of increasing amounts of unlabeled Fel d 1. The antigen binding capacity of the IgG was determined by extrapolation of the line resulting from the plot 1/bound antigen versus 1/free antigen. It was calculated that 1 AU is 0.35 µg IgG. Positive sera showed more than 2% binding of total radioactivity, corresponding to more than 0.25 µg/ml. After immunotherapy with cat dander extract patients on average have IgG levels against Fel d 1 of 35 µg/ml.

Skin test

Standard intracutaneous skin tests were performed by injection of 0.02 ml of ten-fold dilutions in the fore-arm. The highest concentration of Fel d 1 was 100 ng/ml, the lowest concentration was 0.001 ng/ml. After 15 min the longest (A) and orthogonal (B) erythema and wheal diameters were measured. Mean diameters were calculated as (A+B)/2. The Fel d 1 concentrations eliciting an erythema of 10 mm and a wheal of 5 mm were calculated by log linear interpolation. Histamine sensitivity was expressed as mean diameter of erythema (mm) for 10 µg/ml histamine-phosphate. The negative control wheal with dilution buffer had to be smaller than 2 mm without erythema. All skin tests were performed by the same investigator. A high correlation between the allergen threshold as detected by wheal (5 mm) and flare (10 mm) reaction (r=0.98, p<0.001) was found.

To investigate the reproducibility of the endpoint concentration, we performed quadruplicate skin tests with serial dilutions in 4 persons. The coefficient of variation of all erythema diameters was 14.2%. The within-patient reproducibility of the 10 mm erythema threshold was +/- 0.065 of a ten-fold dilution.

Working dilutions were made on the day of the test and the testing was completed within a period of 4 months.

During the study no significant change in the Fel d 1 concentration of the skin test material was detected with the Fel d 1 assay. Moreover, repeated skin tests in one patient before and after the study did not show loss of biologic activity.

Statistical methods

Parametric correlations between variables were expressed as Pearson's r. Differences between groups were analyzed with Mann-Whitney U-test. Stepwise multiple linear regression was performed with p-value limits of <0.05 (in) and >0.1 (out). The regression equation was used to calculate the predicted skin test thresholds. Sera with IgG levels below the detection limit were allotted half the detection limit. The analyses were performed with the SPSS/PC+ statistical package (version 5.0). P-values of less than 0.05 were considered significant.

Results

The relation between specific IgE anti-Fel d 1 and skin test threshold for Fel d 1 (expressed as Fel d 1 concentration eliciting an erythema of 10 mm) is shown in figure 1. There is a significant correlation (Pearson r=-0.61, p<0.001) between log skin test threshold and log specific IgE. However, there are striking differences (sometimes > factor 1000) in skin test threshold between patients with a similar amount of specific IgE.

Thirty patients had detectable amounts of specific IgG antibodies. In only 20 patients IgG4 anti-Fel d 1 was detected. The average contribution of IgG4 anti-Fel d 1 to the protein A-binding isotypes in these patients was 55% (SD 33).

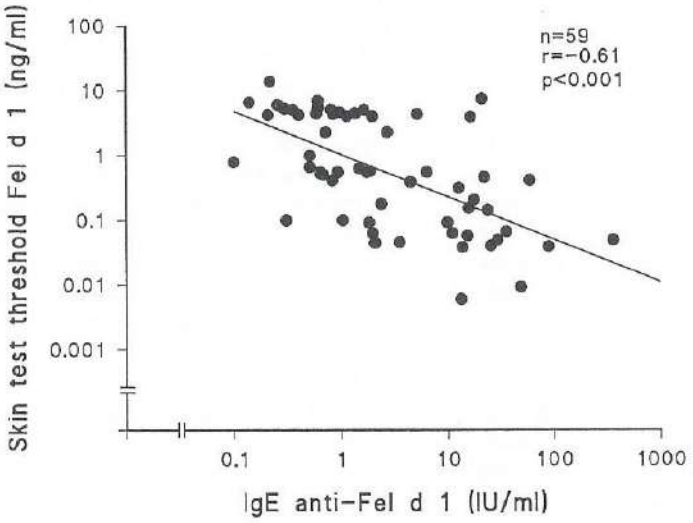

Figure 1. Relation between skin test threshold and specific IgE.

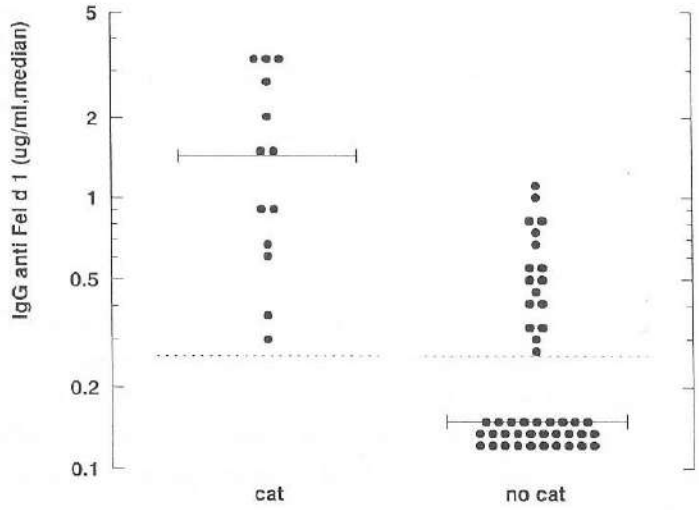

Figure 2. Difference in level of specific IgG between patients with and without a cat at home. Patients with a cat at home had higher levels (Mann-Whitney U-test, $p<0.001$) of specific IgG than patients without a cat at home.

To estimate exposure, patients were asked about a cat at home. The maximum number of cats at home was three. Patients with a cat at home had higher levels (Mann-Whitney U-test, $p<0.001$) of specific IgG than patients without a cat at home (fig. 2). There was no significant difference in levels of specific IgE between patients with or without a cat.

To investigate the influence of IgG antibodies on the skin test a stepwise multiple regression analysis was performed including other independent variables known or suspected to act upon the skin reaction. In the multiple linear regression analysis with log allergen threshold as dependent variable only specific IgE, specific IgG, total IgE and histamine erythema contribute significantly to the prediction of the results of the skin test (table 2). When the same procedure was performed only for the patients with detectable amounts of IgG the influence of specific IgG on the skin test result was similar to the result obtained in the whole group.

Table II. Multiple linear regression analysis. Regression coefficients and p-values for predicting the skin test threshold (log ng/ml). Complete model; $r^2=0.57$, $p < 0.0001$

	B[1]	SEB[2]	Beta[3]	p-value
intercept	-0.59	0.53		0.27
specific IgE (log IU/ml)	-0.88	0.11	-0.82	<0.001
specific IgG (AU/ml)	0.13	0.04	0.37	<0.001
total IgE (log IU/ml)	0.51	0.15	0.30	0.001
histamine erythema (mm)	-0.03	0.01	-0.19	0.03

1. B = the regression coefficient; 2. SE B = the standard error of B; 3. Beta = the standardized regression coefficient

The kind of disease (asthma or rhinitis), time between isolation of Fel d 1 and skin testing, subclass distribution of the IgG antibodies, age and gender of the patients did not contribute to the results of the skin test. Figure 3 shows the relation between specific IgG and ratio of observed skin test threshold and threshold predicted on the basis of specific IgE, total IgE and histamine sensitivity.

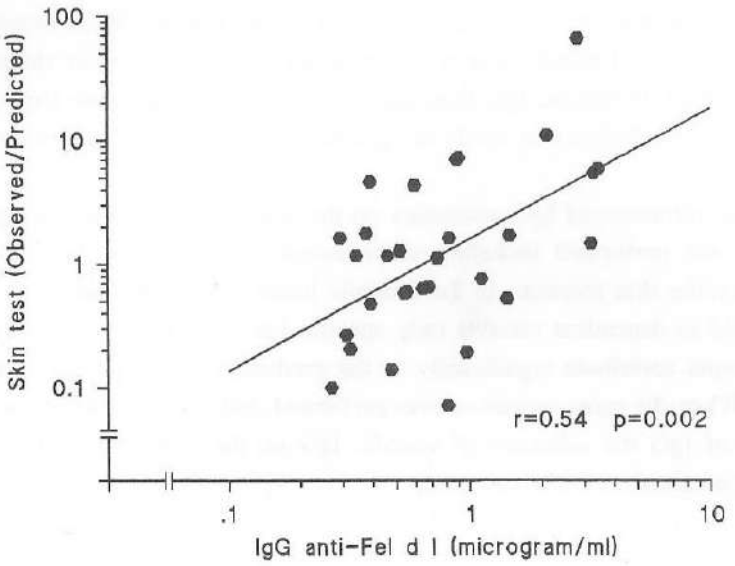

Figure 3. Relation between specific IgG and the ratio of observed skin test threshold and predicted skin test threshold. The predicted skin test threshold was calculated from the regression equation with log allergen threshold as dependent variable and log specific IgE, log total IgE and histamine erythema as independent variables.

Discussion

Our study indicates that levels of specific IgG (between 0.25 and 3.5 μg/ml), induced by natural exposure, have an inhibitory effect on the early phase skin reaction. We have no indication that these low IgG levels had an inhibitory effect on the detection of IgE antibodies. Previously we have confirmed that when an excess of solid phase-coupled allergen is used, the RAST score is independent of the amount of IgG in the serum [29,33]. Even if there was some inhibitory effect of specific IgG in the RAST, the IgE levels of the patients with specific IgG would have been underestimated. In that case the inhibitory effect of IgG in the skin test would have been underestimated as well.

We have expressed the skin test threshold as allergen concentration eliciting an erythema because the dose-response curve of the erythema is steeper than that of the wheal [34]. However, we found a high correlation between the allergen threshold as detected by wheal and as detected by flare reaction. Similar results with regard to the

effect of IgG were found with wheal diameters.

In our assay for IgG against Fel d 1 we measured protein A binding isotypes (IgG1, IgG2 and IgG4). IgG3 antibodies, not measured in this study, are less likely to be relevant as allergen binding antibodies. It has been shown that the IgG response against allergens is dominated by IgG1 and IgG4 [4,35].

In sixteen patients in vitro histamine release from blood basophils was performed with Fel d 1. In these patients no effect of IgG against Fel d 1 was detected on the histamine release with Fel d 1. The histamine release experiments were performed with washed basophils, so no serum factors were able to interfere with the results of this assay. This finding supports a causal relationship between specific IgG in serum and the decreased skin reactivity.

The results of the stepwise multiple regression analysis confirm that different factors, besides specific IgE, like total IgE influence the results of the skin test. In another study we also found that a high total IgE is accompanied by a lower allergen sensitivity in the skin test [36]. The finding that inclusion of the data on the subclass distribution of the IgG anti-Fel d 1 did not contribute to the prediction of the skin test results renders it unlikely that significant differences in skin test blocking activity exist between the IgG isotypes.

An interesting question is how relevant the influence of specific IgG on the skin test results is. The idea behind the inhibitory effects of IgG antibodies is that the IgG antibodies catch the allergen molecules before binding to the IgE antibodies on the mast cell surface. This means that the effect of IgG antibodies on the skin test will be most pronounced when limiting amounts of allergen are present. In most instances of natural exposure of allergic patients the model of limiting amounts of allergen is probably valid. This study shows that for these endpoint titrations there is a clear influence of specific IgG. Using the regression coefficients from table 2, it can be calculated that in the presence of 1.7 or 3.5 μg/ml IgG anti-Fel d 1, 4.5 or 20 times more allergen is required for the same reaction than as in the absence of specific IgG.

In routine diagnostic skin tests, usually, the size of the skin reaction to a full strength allergen extract is determined. We also investigated the influence of specific IgG antibodies on the size of the skin reaction to a fixed allergen concentration above threshold. By means of a stepwise multiple linear regression, we found for a Fel d 1 concentration of 0.1 ng/ml, a significant effect of specific IgG on the size of the erythema as well as on the size of the wheal.

We conclude that even low levels of specific IgG have a blocking effect on the early phase skin reaction.

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Chapter 4

Subjects with low levels of allergic sensitization to indoor allergens show significant reactions after bronchial allergen challenge

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SUMMARY

Most studies on bronchial allergen challenges concern patients with high levels of allergic sensitization. The present study was directed to bronchial reactions after allergen challenge in 20 subjects with low skin reactivity to *Dermatophagoides pteronyssinus* or cat dander.

Titrated intracutaneous skin tests, skin-prick tests, specific IgE assays, histamine release on washed leucocytes, bronchial histamine and allergen challenge tests, were performed in 20 subjects with an intracutaneous skin test threshold for cat dander or *D. pteronyssinus* above 0.1 BU/ml. Ten of the 20 patients had specific IgE below the detection limit in at least one of the 3 IgE-assays which we performed. Fifteen patients had a specific IgE level below 2 RAST units in all 3 tests.

As a positive control group, the same parameters were studied in 7 moderately allergic patients with skin test threshold between 0.1 and 0.01 BU/ml.

The group of 20 subjects with low levels of allergic sensitization showed significant early ($p<0.01$) and late reactions ($p<0.05$). Furthermore, there were indications for a decrease in PC_{20} histamine 24 h after allergen challenge (paired Student's t-test, $p=0.049$, one tailed).

In conclusion, subjects with low levels of allergic sensitization showed significant early and late reactions; induction of bronchial hyperresponsiveness occurred after bronchial allergen challenge.

INTRODUCTION

Bronchial allergen challenges were used as a model for bronchial reactions under natural allergen exposition. Allergen challenge often leads to an early allergic reaction, induction of a non-specific bronchial hyperresponsiveness and a late allergic reaction in allergic individuals [1].

The early allergic reaction in the airways is an episode of airflow obstruction immediately after allergen exposure and reaches its maximum within one hour. The early allergic reaction is mainly caused by IgE dependent mast cell degranulation [2]. Allergen-induced increase in non-specific bronchial responsiveness is associated with the late reaction [3,4]. Bronchial responsiveness increases between 2 and 3 hours after exposure, is maximal after 24 h and may persist for several days [5,6].

The late allergic reaction is an episode of airflow obstruction occurring between 3 and 10 hours after allergen exposure [7]. The pathogenesis of the late allergic reaction is, as yet, not fully known. Studies in humans have shown that induction of bronchial hyperresponsiveness and late allergic reactions are associated with an increase in bronchial inflammation, particularly by influx of eosinophils [8].

It has been claimed that the allergen concentration which causes a 20% fall in FEV_1 in the first hour after allergen challenge (PD_{20} early), can be predicted from the level of allergy and the bronchial responsiveness to inhaled histamine or metacholine [9,10]. The level of allergy is determined by the skin test or by the level of specific IgE antibodies. Less is known about the prediction of the late allergic reaction and the relation with induction of bronchial hyperresponsiveness. Important determinants appear to be high concentrations of specific IgE, magnitude of the early allergic reaction and the initial level of bronchial responsiveness [11,12]. In this respect, the role of the allergen dose is controversial. Some authors showed that inhalation of high doses of allergen resulted in an increase of late reactions [13], whereas others indicated that isolated late allergic reactions and induction of bronchial hyperresponsiveness may occur after repeated inhalation of low doses of allergen [14].

Skin test and RAST are mostly used to diagnose allergy. The agreement between the tests is 80—95% [15—17]. Discrepancies between skin test and RAST are usually a positive skin test without detectable IgE antibodies in serum. The clinical relevance of these discrepancies is not clear. In an earlier study we found the majority of discrepancies between the two tests in patients with an intracutaneous skin test threshold above 0.1 BU/ml [17]. To learn more about the relevance of such a low degree of allergic sensitization, we measured bronchial reactions after allergen challenge in a group of 20 subjects with an intracutaneous skin test threshold for *Dermatophagoides pteronyssinus* or cat dander above 0.1 BU/ml. Seven moderately allergic patients with skin test thresholds between 0.1 and 0.01 BU/ml served as positive control group.

In addition, we investigated which parameters are decisive for predicting the early reaction, the late reaction and the induction of bronchial hyperresponsiveness.

Table I. Subject characteristics

	gender	age	disease	smoking	FEV_1	PC_{20} histamine
		years			*(% predicted)*	*(mg/ml)*
subjects with skin test thresholds above 0.1 BU/ml						
1	M	27	R[1]	ex	120	22.0
2	F	57	A[2]	yes	65	2.2
3	F	26	AR	no	97	4.9
4	F	42	A	ex	109	7.0
5	M	35	AR	no	73	12.8
6	F	27	-	no	93	9.5
7	F	29	R	no	119	13.1
8	M	22	AR	no	104	2.4
9	M	19	-	no	99	21.8
10	F	20	-	no	96	6.0
11	M	42	-	no	115	>32.0
12	F	30	A	no	94	5.6
13	F	32	-	yes	115	>32.0
14	F	28	AR	ex	123	1.0
15	M	42	A	ex	98	1.4
16	M	42	A	ex	115	6.6
17	M	33	R	no	85	22.6
18	F	29	R	no	118	>32.0
19	F	23	R	no	89	>32.0
20	F	29	AR	ex	82	0.9
positive controls with skin test thresholds between 0.1-0.01 BU/ml						
1	M	24	AR	ex	101	6.4
2	F	21	A	no	105	7.8
3	M	39	AR	no	102	6.7
4	F	21	AR	no	96	4.3
5	M	22	AR	no	89	1.1
6	F	26	R	ex	92	1.1
7	F	25	R	ex	106	13.4

1. R = rhinitis; 2. A = asthma

PATIENTS AND METHODS

Patients

Low levels of sensitization to allergen (n=20) were defined as intracutaneous skin test thresholds (allergen concentrations evoking an erythema of 10 mm) for *D.pteronyssinus* or cat dander above 0.1 BU/ml. The group consisted of patients with asthma (n=10), rhinitis (n=5) and asymptomatic subjects (n=5). The group with allergic asthma was selected from the new patients of the Outpatient Department of Pulmonology. They had a history of asthma as defined by the criteria of the American Thoracic Society [18]: episodes of dyspnoea and/or wheezing. The subjects with rhinitis had symptoms of watery rhinorrhoea, nasal blockage, sneezing attacks or nasal pruritus; without current or past symptoms of dyspnoea or wheezing. The asymptomatic subjects were volunteers with positive skin tests, without symptoms of the lower airways.

Moderate allergy, in 7 patients was defined as an intracutaneous skin test threshold between 0.1 and 0.01 BU/ml. Five of them had asthma and 2 rhinitis.

Medications for asthma were short-acting β_2-agonists and inhalation corticosteroids. Inhalation corticosteroids were stopped 2 weeks before the bronchial challenge test. No short- acting β_2-agonists were taken at least 8 h before the bronchial challenge test. The patients with rhinitis and the asymptomatic subjects did not use medication.

The study was approved by the AMC Medical Ethical Committee and written informed consent was given. The subject characteristics are given in table I.

RAST and total IgE

Three different RAST assays were performed. In the in vivo reagents RAST (IVR-RAST) allergens from the same production batch were used as in the intracutaneous skin test and bronchial challenge test (ALK-Benelux, Groningen, The Netherlands). After dialysis, *D.pteronyssinus* and cat dander were insolubilized with CNBr-activated Sepharose (2 mg/100 mg Sepharose). The RAST was performed as described earlier [19]. For routine detection of IgE antibodies (routine-RAST), we used the same procedure, but allergen extracts were obtained from another manufacturer (HAL, Haarlem, The Netherlands). Results were expressed in arbitrary RAST units (RU). A standard curve was made by measuring binding of a reference serum in a two-site assay with Sepharose-coupled sheep-antihuman IgE and ^{125}I-labeled anti-IgE. The detection limit was 0.1 RU/ml.

In addition, Pharmacia-RAST (CAP) (Pharmacia, Uppsala, Sweden) was performed according to the manufacturer's instructions. The sensitivity of the assay was 0.35 kU/l. All RAST tests were performed in duplicate. Total serum IgE was quantified by an inhibition assay as described earlier [20].

Histamine release

The isolation of human leucocytes and incubation of the leucocytes with allergen (highest concentration 1000 BU/ml) and anti-IgE (20 ng/ml) were performed with the method according to Lichtenstein and Osler [21] with slight modifications. The anti-IgE was a polyclonal sheep anti-serum (CLB no. SH25P01). The erythrocytes were lysed with isotonic ammonium chloride at 4°C [22]. The cells were separated by centrifugation and washed in medium containing 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM HEPES (Sigma Chemicals Co. St. Louis, MO, USA), 5.5 mM glucose and 0.5% (w/v) human al-

bumin, pH 7.4). A 250 µl cell suspension was incubated with 50 µl of the stimulus at 37°C for 1 h. Histamine in the supernatant of the samples was measured with an automated fluorometric method [23]. The results were expressed as the percentage of the totally released histamine, minus the spontaneous release. By interpolation of the log-linear dose-response curve, the allergen concentration causing a 10% release was calculated. Patients with a histamine release of less than 10% for allergens and anti-IgE were considered non-responders.

Skin tests

Skin prick tests and intracutaneous tests with cat dander and house dust mite (ALK-Benelux, Groningen, The Netherlands) were done in the forearm. For the intracutaneous test, an end-point titration was performed by injecting 0.02 ml of 10-fold dilutions, starting with 30 BU/ml. The reactions were read after 15 minutes. The wheal size was calculated as the mean of the largest wheal diameter and that perpendicular to it. A threshold concentration eliciting an erythema of 10 mm was calculated by log-linear interpolation. An earlier study showed a high correlation (r=0.98, p=0.001, n=59) between the thresholds measured by wheal (5 mm) and flare (10 mm) [24]. The control test with dilution buffer had to be negative. Histamine sensitivity was expressed as mean diameter of erythema in the prick test with 10 mg/ml histamine diphosphate. The skin-prick test with allergen was done with 10000 BU/ml.

Bronchial challenge tests

A histamine-challenge test was performed according to the method described by Cockcroft et al [25]. Histamine aerosol was inhaled by quiet tidal breathing by means of a De Vilbiss Nebulizer (output 0.13 ml/min) during 2 minutes. The first aerosol inhaled was the control diluent (phosphate buffered saline) and followed by doubling concentrations of histamine from 0.015-32 mg/ml at 5 minutes. The FEV_1 was measured with a wet spirometer (Sensor Medics BV, Bilthoven, The Netherlands) before the test and 30 and 90 seconds after each inhalation. The test was stopped if the FEV_1 had fallen 20% or more from the baseline value. The results were expressed as the concentration of histamine causing a 20% fall in FEV_1 (PC_{20}). The PC_{20} was calculated by interpolation of the logarithmic dose-response curve. The log ratio PC_{20} 48 h before and 24 h after allergen challenge was used as measure for the induction of bronchial hyperresponsiveness.

The allergen challenge procedure was completed in 4 consecutive days. On day 1, spirometry and histamine threshold was measured; on day 2, the challenge procedure with control solution for establishing diurnal variation; on day 3, the allergen challenge; on the morning of day 4, spirometry and histamine threshold were repeated.

Allergen was inhaled by tidal breathing (1 minute at 15-minutes intervals) with 5-fold step-wise increasing concentrations (80-10000 BU/ml) of cat dander or *D.pteronyssinus* extract (ALK-Benelux, Groningen, The Netherlands), using a De Vilbiss Nebulizer (output 0.13 ml/min). The extracts were diluted in phosphate-buffered saline with 0.03% human serum albumin and 0.5% phenol (ALK-Benelux, Groningen, The Netherlands). If a drop of more than 15% from initial value of FEV_1 occurred, no additional allergen inhalations were administered.

Before and during the first hour after provocation, the FEV_1 was measured with the wet spirometer as described for the histamine provocation procedure as well as with a portable dry spirometer (micro-

medical diarycard spirometer, Sensor Medics BV, Bilthoven, The Netherlands). Thereafter, the subjects went home on the control day and the FEV_1 was measured every hour with the portable spirometer till the subject went to bed. The next day when the allergen was inhaled, the same procedure was repeated, but the patients stayed in the hospital for at least 12 h.

All FEV_1 values were expressed as percentage change from the baseline FEV_1 value (mean of 3 measurements within 5%), measured at the start of every study day. For each individual at each time point, the FEV_1 values were corrected for diurnal variation according to the formula: corrected ΔFEV_1 value (% baseline) = FEV_1 after allergen challenge (% baseline) - FEV_1 after control challenge (% baseline).

Because the dose of allergen administered to reach the bronchial threshold differed between the subjects in the control group of moderately allergic patients, the provocation cumulated dose of allergen necessary to induce a fall in FEV_1 value of 20% from baseline was also calculated to characterize the early and late allergic reaction (PD_{20}-early and PD_{20}-late) [26].

Statistical methods

Statistical methods included simple correlation, Student's t-test, analysis of variance with linear trend analysis and backward multiple linear regression. For statistical analysis, negative test results were allotted half of the detection limit. Analyses were performed with the SPSS/PC+ statistical package (version 5.0) (Chicago, Ill).

Results

The results of the different allergy tests to characterize the subjects are given in table II and III. Four subjects had a negative RAST in the three specific IgE assays. Two of these 4 subjects, had a positive histamine release from basophils, 1 subject was a non-responder and in 1 subject histamine release was not performed.

The IVR-RAST correlated well with the routine-RAST (r=0.73, p<0.0001). Two subjects (16 and 18) had striking differences between the routine- and the IVR-RAST. Both subjects had a low or negative cat dander routine-RAST, while the IVR-RAST showed a high RAST-score.

Figure 1a shows the results of the allergen challenge in the moderately allergic patients. All showed more than 15% decrease in FEV_1 in the first hour after challenge. The threshold was reached with less than 10000 BU/ml in 4 of the 7 patients. Five of the 7 patients had more than 15% decrease in FEV_1 3 to 12 hours after challenge.

In contrast to the moderately allergic patients, all subjects with low levels of allergic sensitization tolerated the highest allergen concentration (10000 BU/ml). Three of them showed a decrease of more than 15% in FEV_1 in the first hour after allergen challenge. None showed more than 15% decrease after 3 to 12 hours.

Table II. Skin test results

	allergen	i.c. skin threshold	i.c. 30 BU/ml		prick test 10000 BU/ml		prick test histamine 10 mg/ml	
			wheal	flare	wheal	flare	wheal	flare
		BU/ml	*mm*	*mm*	*mm*	*mm*	*mm*	*mm*
1	mite	13	9	27	3	11	5	28
2	mite	13	9	27	3	11	6	37
3	mite	11	9	33	9	24	9	37
4	mite	1.8	9	34	2	14	6	28
5	mite	1.6	15	30	5	10	7	11
6	mite	1.2	11	41	6	26	6	28
7	mite	1.2	10	33	4	18	5	18
8	mite	1.1	11	31	7	28	5	22
9	mite	1.1	9	42	2	16	4	21
10	mite	0.96	na	na	5	28	6	28
11	mite	0.13	12	37	9	27	8	37
12	mite	0.11	6	18	3	11	8	30
13	mite	0.11	12	38	7	30	9	23
14	cat	12	9	29	3	11	11	35
15	cat	3.0	8	15	2	4	6	26
16	cat	1.0	16	42	4	10	7	30
17	cat	0.94	14	47	na	na	9	35
18	cat	0.17	11	39	3	10	6	20
19	cat	0.14	na	na	8	32	5	20
20	cat	0.13	13	35	4	28	9	22
Mean			10.7	33.2	4.4	17.4	6.8	26.8
Geometric Mean		1.0						
1	mite	0.099	na	na	7	35	9	35
2	mite	0.013	na	na	8	33	4	20
3	mite	0.012	na	na	na	na	na	na
4	mite	0.012	na	na	7	25	5	15
5	mite	0.011	na	na	7	35	6	39
6	cat	0.020	na	na	4	24	5	26
7	cat	0.011	na	na	10	35	8	34
Mean					7.2	31.2	6.2	28.2
Geometric Mean		0.025						

Table III. In vitro allergy test results

	allergen	RAST IVR	RAST routine	RAST Pharmacia	total IgE	histamine release threshold
		RU/ml	*RU/ml*	*kU/l*	*IU/ml*	*BU/ml*
1	mite	neg	neg	neg	58	30.7
2	mite	neg	1.1	0.5	173	nr[1]
3	mite	0.2	0.3	1.0	99	nr
4	mite	neg	neg	neg	14	50.0
5	mite	0.9	neg	0.6	101	nr
6	mite	0.3	1.0	0.8	3	0.6
7	mite	1.3	1.0	1.3	466	500.0
8	mite	0.9	neg	0.6	298	293.2
9	mite	1.1	1.7	2.2	139	307.1
10	mite	2.4	2.4	4.1	63	4.6
11	mite	3.0	2.3	5.6	150	7.0
12	mite	neg	0.3	neg	14	nr
13	cat	0.7	0.2	0.9	55	7.8
14	cat	0.6	neg	neg	803	354.3
15	cat	neg	neg	neg	94	nr
16	cat	10.0	0.3	1.4	1000	5.9
17	cat	neg	neg	neg	128	na[2]
18	cat	17.8	neg	3.6	233	0.8
19	cat	0.6	0.7	0.7	694	nr
20	cat	0.3	0.3	0.5	396	517.6
Mean		2.0[3]	0.6			
Geometric Mean					119.7	28.7
1	mite	14.7	9.4	na	1700	0.8
2	mite	16.0	16.7	na	378	0.8
3	mite	8.2	18.9	na	86	na
4	mite	10.8	23.1	na	308	na
5	mite	18.4	18.4	na	170	na
6	cat	1.3	0.8	1.4	627	2.0
7	cat	14.7	2.6	na	461	0.1
Mean		12.1	12.8			
Geometric Mean					363.4	0.6

neg = negative; 1. nr = non releaser; 2. na = not available; 3. negative results were allotted half of the detection limit (0.05 RU) for statistical analysis

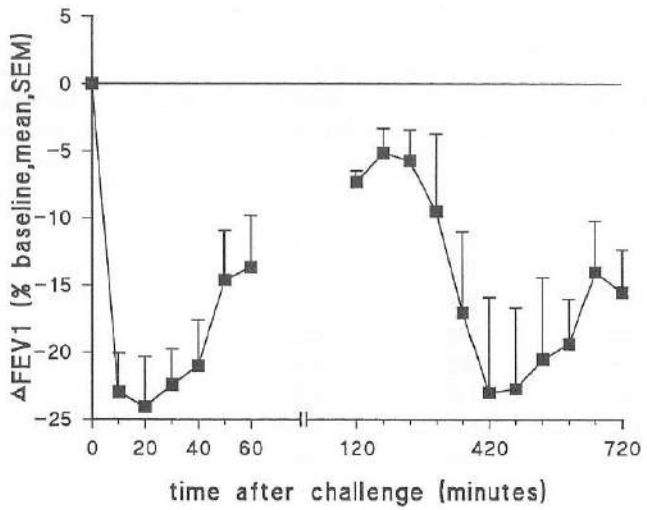

Figure 1a. Results of bronchial allergen challenge for the group (n=7) of moderately allergic patients.

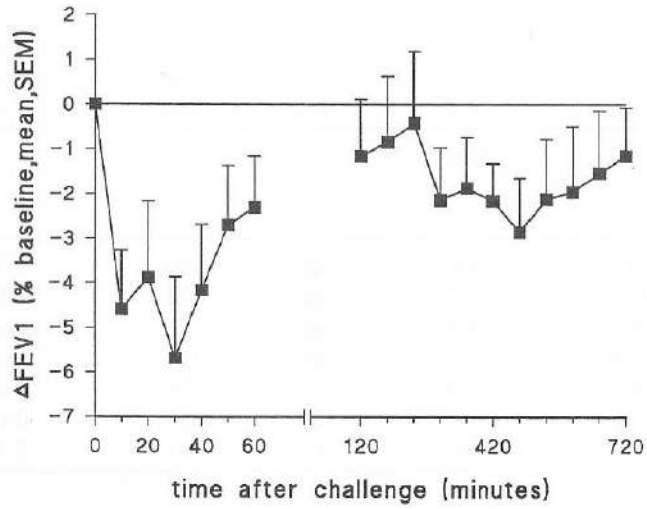

Figure 1b. Results of bronchial allergen challenge for the group (n=20) of subjects with low levels of allergic sensitization.

Figure 1b shows the results of the allergen challenge of the subjects with low levels of allergic sensitization. In this group there is a statistically significant decrease (p<0.005) in FEV_1 in the first hour after challenge, on the time points of maximal drop in FEV_1 in the moderately allergic patients. The decrease remained significant after exclusion of the 3 subjects with a more than 15% decrease in the first hour (p<0.005). Concerning the late-phase reaction in this group, there was also a significant decrease (p<0.05) in FEV_1 at 7 and 8 h after allergen challenge.

The 20 subjects with an intracutaneous skin test threshold above 0.1 BU/ml, showed a relation between the skin test threshold and the early decrease in FEV_1 (linear trend, p=0.046) (figure 2).

Figure 3a shows the change in PC_{20} after allergen challenge for the moderately allergic patients: a clear increase in bronchial responsiveness after allergen challenge from 4.2 to 0.9 mg/ml histamine (paired Student's t-test, p=0.008, two-tailed). Change in bronchial responsiveness in the 20 subjects with an intracutaneous skin test threshold above 0.1 BU/ml is shown in figure 3b. In this group the results also indicated a fall in PC_{20} histamine after allergen challenge, from 9.1 to 6.7 mg/ml histamine (paired Student's t-test, p=0.49, one tailed).

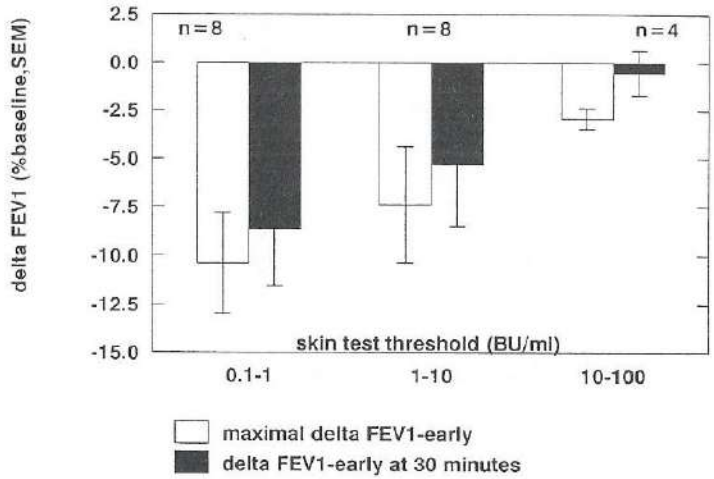

Figure 2. Relation between skin test threshold and early decrease in FEV_1 in the subjects with low levels of allergic sensitization. The early decrease in FEV_1 is shown as maximal ΔFEV_1 in the first hour and as ΔFEV_1 at 30 min.

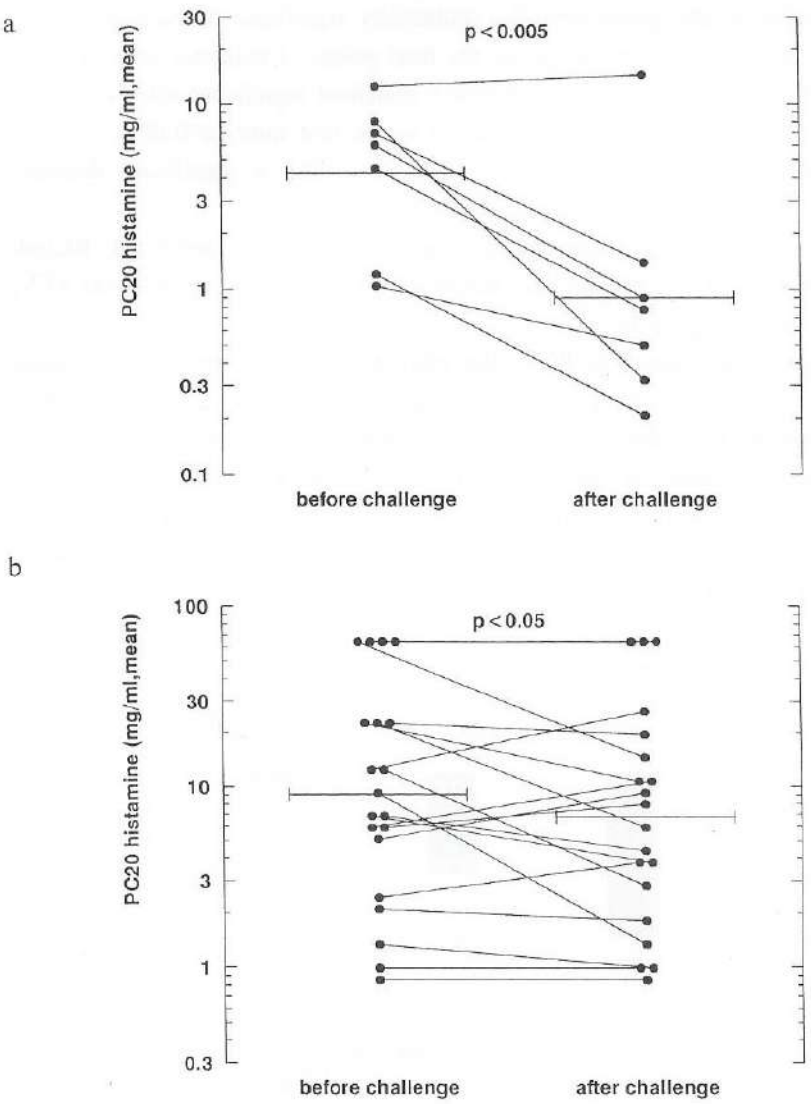

Figure 3.
a. Change in PC_{20} after allergen challenge for the moderately allergic patients.
b. Change in PC_{20} after allergen challenge for the subjects with low levels of allergic sensitization.

In the combined groups (n=27), we calculated the variables which correlated with the log PD_{20}-early, the log PD_{20}-late, and the change in PC_{20} after challenge. Table IV shows that the log PD_{20}-early correlates with RAST, skin test and basophil histamine release threshold. For the log PD_{20}-late, there is a correlation with RAST, skin test, maximal ΔFEV_1-early and smoking. For the change in PC_{20} after challenge there is a correlation with the maximal ΔFEV_1-late, the maximal ΔFEV_1-early and the RAST.

Table IV. Pearson correlations

		log PD_{20} early		**log PD_{20} late**		**difference in PC_{20} before and after challenge**	
	n	*r*	*p*	*r*	*p*	*r*	*p*
gender	27	0.02	ns	-0.09	ns	0.01	ns
age	27	0.20	ns	0.45	0.02	-0.18	ns
disease	27	-0.07	ns	-0.22	ns	0.05	ns
smoking	27	0.28	ns	0.49	<0.01	-0.19	ns
routine-RAST	27	-0.71	<0.001	-0.78	<0.001	0.63	<0.001
IVR-RAST	27	-0.56	<0.01	-0.58	<0.01	0.64	<0.001
log total IgE	27	0.05	ns	0.02	ns	-0.02	ns
log i.c. skin test (threshold)	27	0.74	<0.001	0.62	<0.001	-0.09	ns
prick test (diameter)	25	-0.58	<0.01	-0.43	0.03	0.31	ns
skin sensitivity to histamine	27	-0.06	ns	-0.03	ns	-0.03	ns
log basophil histamine release	18	0.68	<0.01	0.45	ns	-0.45	ns
FEV_1 % predicted	27	0.12	ns	-0.03	ns	0.15	ns
log PC_{20} histamine	27	0.10	ns	0.13	ns	0.02	ns
max. ΔFEV_1-early	27			-0.77	<0.001	0.61	<0.001
max. ΔFEV_1-late	27					0.74	<0.001

Table V. Multiple linear regression

	B[1]	Beta[2]	p
log PD_{20}-early as dependent variable, model: r^2=0.69, p<0.0001			
Independent variables:			
constant	4.23		
total IgE (log IU/ml)	0.35	0.24	0.06
skin test threshold (log BU/ml)	0.46	0.53	<0.005
RAST-routine (RU/ml)	-0.03	-0.39	<0.05
log PD_{20}-late as dependent variable, model: r^2=0.68, p<0.0001			
Independent variables:			
constant	4.96		
maximal ΔFEV_1-early (%)	-0.03	-0.42	<0.05
RAST-routine (RU/ml)	-0.03	-0.46	<0.05
change in PC_{20} after challenge as dependent variable, model: r^2=0.52, p<0.0001			
Independent variables:			
constant	-0.05		
maximal ΔFEV_1-late (%)	0.02	0.72	<0.0001

1. B = regression coefficient; 2. Beta = standardized regression coefficient

Backward multiple linear regression was performed with log PD_{20}-early as dependent variable, and RAST, FEV_1 (% predicted), log total IgE, log PC_{20} and log skin test threshold as independent variables. Only log total IgE, log skin test threshold and RAST contributed to the prediction of the PD_{20}-early (table V). Using the same independent variables together with the maximal ΔFEV_1-early, only the maximal ΔFEV_1-early and the RAST contributed to the prediction of the PD_{20}-late (table V). Using the same independent variables together with the maximal ΔFEV_1-late, only the maximal ΔFEV_1-late contributed to the change in PC_{20} (table V).

Discussion

We found significant early and late reactions, the mean early decrease in FEV_1 being 5.7%, in the group of subjects with low levels of allergic sensitization, as well as a relation between skin test threshold and decrease in FEV_1. This indicates that even in patients with a low skin reactivity, pathophysiologic reactions leading to airway obstruction and bronchial hyperresponsiveness will occur after inhalation of allergen. Most patients will not be aware of a 5% reduction in FEV_1. Moreover, a 5% change in FEV_1 can not be easily detected by spirometry in an individual patient, due to the variability of the test. This does not necessarily imply that small changes in lung function parameters after allergen exposure are irrelevant to airways disease. This study has shown that even a 5% reduction in FEV_1 is accompanied by a late-phase reaction and by induction of non-specific hyperresponsiveness. There is overwhelming evidence for a relation between late-phase reactions, non-specific hyperresponsiveness and airway inflammation [27–30]. Holgate and co-workers took bronchial biopsies of patients with mild clinical and subclinical asthma and found clear indications for inflammatory changes [31]. Therefore, it is likely that induction of bronchial hyperresponsiveness in our patients with low levels of allergic sensitization was accompanied by an increase of bronchial inflammation.

Prediction of the early reaction was influenced by RAST-score, skin test and total IgE, but not by PC_{20} histamine. A higher total IgE means that more allergen is required for an early decrease in FEV_1, this indicates an inhibitory effect of total serum IgE. In an earlier study, we found a similar inhibitory effect of total IgE in the skin test as well as in the histamine release from basophils [24]. A likely explanation is competition of irrelevant IgE with specific IgE for binding to the IgE receptor on basophils and mast cells.

The finding that the skin test and the specific IgE contribute independently to the prediction of the early reaction, indicates that skin test results and IgE antibody levels are not interchangeable as indicator of the degree of allergic sensitization.

In contrast with the findings of Cockcroft et al. [9,10], we found no influence of PC_{20} histamine on the prediction of the early reaction. There was no difference between our study and that of Cockcroft et al. concerning the variance in PC_{20} histamine of the subjects studied. However, the patients reported by Cockcroft et al. had lower PC_{20} histamine values (2.5 doubling doses lower) than our subjects. A recent study of Muller et al. [32] showed that the influence of non-specific bronchial hyperresponsiveness on the prediction of the early response depends on the level of

hyperresponsiveness.

Prediction of the late reaction was influenced by the magnitude of the early reaction and the routine-RAST. This is in agreement with other studies [7,9].

In the present study, the induction of non-specific bronchial hyperresponsiveness is only predicted by the magnitude of the late reaction. Also other authors found that the magnitude of the late- phase response is pivotal to a decrease of PC_{20} histamine [3,6].

In the patients with low levels of IgE antibodies there is a considerable variation between the results of the different RAST assays. Only 10 of the 20 subjects with an intracutaneous skin test threshold above 0.1 BU/ml were positive in all 3 RAST-systems.

Four of the 20 patients with a positive skin test had a reproducible negative RAST in all 3 systems. The skin test in these patients was repeated and the same positive results were obtained. At least 2 of these patients had a positive histamine release from washed leucocytes with the allergen. This is in agreement with our earlier study on histamine release tests in 35 patients with a positive skin test but without detectable circulating IgE antibodies. For 80% of the patients a positive histamine release test was obtained, strongly suggesting an IgE-mediated reaction and false-negative RAST results [17].

Two subjects had a negative cat routine-RAST and a highly positive IVR-RAST. Although the IVR-RAST score was similar to the RAST-scores of the positive controls, both subjects had skin test thresholds above 0.1 BU/ml and less than 15% decrease in FEV_1 after allergen challenge. All positive controls with such RAST-scores showed a more than 15% decrease in FEV_1. The IgE-antibodies of these two patients were not directed against the major allergen of cat cander, Fel d 1 (results not shown). The identity of the allergenic component with an apparent low biologic activity is currently under study.

In conclusion, we found in subjects with low levels of allergic sensitization, a significant early decrease in FEV_1, a significant late decrease in FEV_1 and indications for a decrease in PC_{20} after allergen challenge. This indicates that patients with low-grade IgE responses against inhalation allergens develop asymptomatic early and late bronchial reactions upon allergen exposure. This probably leads to enhanced airway inflammation and non-specific bronchial hyperresponsiveness.

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Chapter 5

Differences in non-specific bronchial responsiveness between patients with asthma and patients with rhinitis are not explained by type and degree of inhalant allergy

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Submitted

ABSTRACT

Patients with allergic asthma have higher levels of non-specific bronchial responsiveness than patients with allergic rhinitis. Aim of the study was to investigate the influence of the degree of inhalant allergy on the difference in bronchial responsiveness between asthma and rhinitis.

In patients with allergic asthma, a correlation was found between non-specific bronchial responsiveness and IgE against indoor allergens (n=136, r=0.34, p<0.001) and % predicted FEV_1 (n=136, r=0.37, p<0.001). Patients with allergic asthma and patients with allergic rhinitis differed with respect to the level of bronchial responsiveness (p<0.001), the amount of specific IgE antibodies against indoor allergens (p=0.01) and possibly the % predicted FEV_1 (p=0.09).

The difference in level of bronchial responsiveness remained (p<0.001) after correction with % predicted FEV_1 and specific IgE against indoor allergens as confounding variables. Similarly, after matching of patients with allergic rhinitis (n=25) with patients with allergic asthma (n=25) regarding the specific IgE, total IgE and age of the patients, the difference in level of bronchial responsiveness remained (p<0.001).

Furthermore, 25 patients with non-allergic rhinitis and 18 healthy individuals were evaluated as non-allergic controls. Patients with non-allergic rhinitis had higher levels of non-specific bronchial responsiveness than healthy controls and did not differ from patients with allergic rhinitis.

In conclusion, the results indicate that the level of IgE against common indoor allergens cannot account for the difference in bronchial responsiveness between patients with asthma and patients with rhinitis.

INTRODUCTION

Both asthma and rhinitis are clinical manifestations of inhalant allergy. So far, there is no satisfactory explanation why inhalant allergy manifests itself in either way. There is a strong correlation between both syndromes since many asthma patients (up to 80%) have symptoms of rhinitis. Less patients with rhinitis (5—15%) develop asthma [1].

Inhalant allergy is associated with non-specific bronchial hyperresponsiveness [2—5]. Recently, Burrows et al. [6] found a significant relationship between the degree of allergy, measured by the size of skin-prick tests for indoor allergens (mite, cat, dog and Aspergillus), and bronchial responsiveness for histamine in 662 13-year-old children from New Zealand. Prolonged allergen avoidance can decrease bronchial responsiveness [7,8]. Natural [3,9,10] and experimental [11—13] exposure to allergens in sensitized subjects may result in a long-lasting increase in bronchial responsiveness. The increase in bronchial responsiveness is associated with the allergen-induced late asthmatic response [12,14].

There are quantitative and qualitative differences in IgE-antibody response between asthma and rhinitis. In an earlier study, we compared 1281 patients who had predominant upper airways symptoms with 1054 patients with lower airways symptoms. Cat dander and house dust mite allergy was more closely related to lower airways symptoms, whereas pollen allergy was more closely related to upper airways symptoms [15].

Patients with allergic rhinitis appear to be more susceptible for developing asthma than normal subjects [16]. Several authors [17,18] showed that patients with allergic rhinitis have a level of non-specific bronchial responsiveness between that of normal subjects and patients with asthma. However, it is not clear whether the difference in bronchial responsiveness was related to quantitative or qualitative differences in IgE-antibody response.

Aim of the present study was to investigate whether the difference in bronchial responsiveness between patients with allergic asthma and patients with allergic rhinitis was associated with the type and degree of inhalant allergy.

For this purpose, bronchial responsiveness to histamine was measured in 25 allergic patients with isolated upper airways symptoms. Non-specific bronchial responsiveness in this group was compared with non-specific bronchial responsiveness in a group of 136 patients with allergic asthma, with allergy and % predicted FEV_1 as confounding variables. In addition, a matched pair analysis was performed.

Twenty-five patients with non-allergic rhinitis served as controls to evaluate the influence of an IgE-independent inflammatory reaction in the upper respiratory tract on the level of bronchial responsiveness. Furthermore we investigated the level of non-specific responsiveness in 18 healthy controls.

Table I. Patient characteristics

	gender	age	IgE	IgE	IgE	smoking	FEV_1	PC_{20}
		years	I[1]	O[2]	*IU/ml*		*%pred*	*mg/ml*
Controls								
1	M	26	0	0	71	ex	105	64
2	F	22	0	0	50	—	107	64
3	M	28	0	0	15	—	113	16.8
4	F	28	0	0	38	—	96	11.2
5	M	28	0	0	16	ex	110	64
6	F	27	0	0	61	—	111	13.4
7	M	39	0	0	13	+	115	64
8	M	28	0	0	35	+	90	14.4
9	F	33	0	0	43	ex	107	64
10	F	32	0	0	60	—	98	64
11	F	30	0	0	4	ex	97	24
12	F	21	0	0	13	—	87	27.5
13	F	26	0	0	69	+	90	64
14	F	29	0	0	25	—	101	29.7
15	M	47	0	0	55	ex	126	64
16	M	28	0	0	0	+	115	30.5
17	F	31	0	0	35	+	101	15.1
18	F	23	0	0	24	+	105	64
							104	
Mean		29						
(SEM)							(2.4)	
GM[3]					24.5			34.7
(GSE)					(1.3)			(1.2)

	gender	age	IgE	IgE	IgE	smoking	FEV_1	PC_{20}
		years	I[1]	O[2]	*IU/ml*		*%pred*	*mg/ml*
Patients with non-allergic rhinitis								
1	F	47	0	0	14	+	124	64
2	F	34	0	0	37	+	104	2.7
3	M	37	0	0	33	ex	128	13.8
4	M	43	0	0	18	—	100	64
5	F	19	0	0	44	—	97	3.9
6	F	28	0	0	57	+	108	4.5
7	F	20	0	0	5	—	93	2.3
8	F	39	0	0	17	ex	85	2.1
9	F	33	0	0	99	—	93	22.2
10	F	29	0	0	43	+	104	64
11	F	26	0	0	19	—	86	3.7
12	M	24	0	0	34	+	103	64
13	F	26	0	0	12	+	95	6.6
14	F	53	0	0	67	ex	125	64
15	F	32	0	0	12	ex	81	12.5
16	F	25	0	0	68	—	111	64
17	M	18	0	0	85	—	107	28
18	M	29	0	0	24	—	110	22.4
19	M	46	0	0	16	ex	108	11.8
20	M	33	0	0	10	—	116	23.2
21	M	35	0	0	50	ex	104	64
22	F	27	0	0	56	—	87	7.8
23	F	18	0	0	58	ex	112	7.5
24	F	29	0	0	51	—	118	8.3
25	M	34	0	0	4	ex	105	6.4
Mean		31					104	
(SEM)							(2.5)	
GM[3]					27			14.1
(GSE)					(1.2)			(1.2)

	gender	age	IgE	IgE	IgE	smoking	FEV_1	PC_{20}
		years	I[1]	O[2]	*IU/ml*		*%pred*	*mg/ml*
Patients with allergic rhinitis								
1	M	31	2	0	141	+	109	64
2	M	27	4	0	114	ex	91	2.1
3	M	28	0	7	332	+	115	64
4	M	28	4	0	1400	+	127	1
5	M	30	10	0	1700	—	121	19.2
6	F	39	0	7	172	ex	107	4.9
7	M	29	1	7	902	ex	88	21.6
8	M	20	11	1	199	—	99	1.2
9	M	30	0	4	170	—	102	16
10	F	27	4	0	224	—	81	5
11	F	30	4	9	1300	—	78	2.1
12	F	20	5	1	449	ex	81	10.4
13	F	27	6	3	89	—	89	2.5
14	M	18	1	6	334	—	115	64
15	F	28	1	4	67	+	87	7.8
16	M	36	4	5	34	ex	106	12.2
17	F	32	0	2	98	—	97	64
18	F	21	4	1	64	—	97	8.8
19	F	21	4	10	729	—	95	3.8
20	F	42	0	9	54	ex	108	64
21	M	40	6	0	145	—	119	30.8
22	M	24	0	2	35	—	75	32
23	M	29	3	0	54	—	97	18
24	M	42	9	1	300	+	93	6.4
25	F	35	4	0	303	ex	97	10.2
Mean		29	3.5	3.2			99	
(SEM)			(0.6)	(0.7)			(2.8)	
GM[3]					199			11.0
(GSE)					(1.2)			(1.3)

	gender	age	IgE	IgE	IgE	smoking	FEV_1	PC_{20}
		years	I[1]	O[2]	*IU/ml*		*%pred*	*mg/ml*
Patients with allergic asthma								
1	F	21	2	0	348	—	83	9.6
2	F	26	4	0	190	—	95	3.1
3	F	33	0	6	383	—	62	0.3
4	F	25	4	0	465	—	76	0.5
5	F	22	10	0	571	+	101	0.5
6	F	41	0	6	224	—	79	5.5
7	F	31	1	7	339	—	97	15.6
8	F	31	10	0	108	ex	94	0.4
9	M	27	0	5	171	—	91	2.4
10	M	21	4	0	258	+	107	11.3
11	M	27	3	9	1100	+	89	2
12	M	17	5	0	574	—	82	1
13	M	28	5	3	326	—	100	1
14	F	20	1	8	520	—	88	3.9
15	M	15	1	4	160	—	100	17.1
16	F	20	4	6	207	—	91	2.1
17	M	25	0	2	81	+	66	0.02
18	M	22	4	1	118	ex	93	5.8
19	M	17	5	8	274	—	91	1.2
20	F	34	2	7	143	—	121	4.88
21	M	35	6	0	279	ex	112	2
22	M	48	0	3	36	—	98	32
23	M	39	3	0	6	—	79	0.3
24	M	41	9	0	297	—	65	0.5
25	M	32	4	0	228	—	85	0.4
Mean		28	3.5	3.0			90	
(SEM)			(0.6)	(0.6)			(2.8)	
GM[3]					209			1.7
(GSE)					(1.2)			(1.4)

1. I = IgE to indoor allergens; 2. O = IgE to outdoor allergens; 3. GM = Geometric Mean

PATIENTS AND METHODS

Allergic rhinitis

The group with allergic rhinitis (n=25) was selected from the new patients of the Outpatient Department of Otorhinolaryngology. Allergic rhinitis was characterized by at least 2 of the following symptoms: sneezing (>5 times in a row); pruritus in the nose; rhinorrhoea (not purulent) or nasal blockage. A positive RAST-score (≥class 2, see RAST) and skin test for common inhalant allergens. Patients with current or past episodes of dyspnoea and/or wheezing were excluded.

Allergic asthma

The group with allergic asthma (n=136) comprised new patients of the Outpatient Department of Pulmonology in the same study period (1992-1994). They had a history of asthma as defined by the criteria of the American Thoracic Society [19]: episodes of dyspnoea and/or wheezing. They had a positive RAST-score (≥class 2, see RAST) and skin test for common inhalant allergens. The FEV_1 was at least once during the study period above 70% of the predicted value. The age of the patients ranged from 16 to 55 years.

Controls

The group with non-allergic rhinitis (n=25) was selected from the new patients of the Outpatient Department of Otorhinolaryngology. They had similar symptoms as those with allergic rhinitis. The normal controls (n=18) were volunteers who never had symptoms of dyspnoea or wheezing and had no symptoms of watery rhinorrhoe, nasal blockage, sneezing attacks or nasal pruritus. The patients with non-allergic rhinitis and the normal controls had a negative RAST score and skin test for house dust mite, cat dander, grass pollen, birch pollen, dog dander, guinea-pig dander, rabbit dander, bird feathers and moulds. Their total IgE was less than 100 IU/ml.

The study was approved by the AMC Medical Ethical Committee and written informed consent was given by the patients upon entering the study. The patients characteristics are given in table I.

RAST

For the detection of IgE antibodies to grass pollen (mixture of *Dactylus glomerata* and *Phleum pratense*), cat dander, dog dander, guinea-pig and rabbit dander, we used an indirect RAST system with hapten-modified allergens as described previously [20]. For house-dust mite, birds (mixture of budgerigar, canary, and parrot feathers), moulds (*Aspergillus fumigatus*, *Alternaria alternata*) and birch pollen, the allergens were insolubilized by coupling to CNBr-activated Sepharose [21]. Results were expressed in arbitrary RAST units (RU). A standard curve was made by measuring binding of a reference serum in a 2-site assay with Sepharose-coupled sheep-anti-human IgE and ^{125}I-labeled anti-IgE. Specific IgE was classified as: <0.1 RU was negative (class 0), 0.1—1 RU weakly positive (class 1) and > 1 RU strong positive; 1—2.5 RU (class 2), 2.5—6 RU (class 3), 6—27 RU (class 4), >27 RU (class 5).

Total IgE

Total serum IgE was quantitated by an inhibition assay [22], using Sepharose-coupled IgE: 100 µl of serum with a high IgE level was added to 200 mg CNBr-activated Sepharose (Pharmacia). After coupling and washing, the Sepharose particles were resuspended in an incubation medium containing 0.2% Tween 20 and 0.1 ml/ml normal sheep serum. To 250 µl of this suspension, 10 µl serum and 200 µl ^{125}I-anti-IgE were added. The mixture was incubated overnight and the Sepharose-bound radioactivity was measured. The amount of IgE was read from a dilution curve of a reference serum and expressed in IU/ml. A binding assay [22] was performed with the sera in which total IgE was less than 100 IU/ml.

Skin test

Skin-prick tests were performed with grass-pollen mixture, tree-pollen mixture, mugwort, *D.pteronyssinus*, cat dander, dog dander, guinea-pig and rabbit dander, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Alternaria alternata* (ALK-Benelux, Groningen, The Netherlands). The reactions were read after 15 min. The wheal size was calculated as the mean of the largest wheal diameter and that perpendicular to it. The control test with dilution buffer had to be negative. The positive control test was histamine diphosphate (10 mg/ml). The score of the result of the skin test for indoor- and outdoor allergens was based on addition of wheal diameters [6].

Bronchial challenge test

A histamine-challenge test was performed according to the method described by Cockcroft et al. [2]. Histamine aerosol was inhaled by quiet tidal breathing during 2 minutes using a De Vilbiss Nebulizer (output 0.13 ml/minute). The first aerosol inhaled was the control diluent (phosphate buffered saline) followed at 5 minutes intervals by doubling concentrations of histamine ranging from 0.03 to 32 mg/ml.

The FEV_1 was measured before the test and at 30 and 90 seconds after each inhalation. The test was stopped if the FEV_1 decreased by 20% or more from the baseline value. The results were expressed as the concentration of histamine causing a 20% fall in FEV_1 (PC_{20}). The PC_{20} was calculated by interpolating the logarithmic dose-response curve. All patients with allergic rhinitis were tested at the end of the grass-pollen season.

Statistics

Matching of the patients with allergic rhinitis and allergic asthma was done with respect to specific IgE, total IgE and age. Specific IgE was scored in RAST classes (from 0 to 5) for each allergen. Two groups of allergens were distinguished: indoor allergens (house-dust mite, cat-, dog-, guinea-pig- and rabbit- dander, birds and moulds) and outdoor allergens (grass pollen and birch pollen). Scoring of sensitization was based on the sum of RAST-classes in the two groups.

Statistical analysis included simple correlation (Pearson), analysis of co-variance, and step-wise multiple linear regression (p-limits in: <0.05, out: >0.1). Paired groups were compared with Student's t-test and the Wilcoxon test. Unpaired groups were compared by Student's t-test and the Mann Whitney U-test.

Analyses were done with SPSS/PC+ statistical package (version 5.0). All reported p-values were two tailed, and p-values of less than 0.05 were considered significant.

RESULTS

In the patients with allergic asthma (n=136) it was calculated which variables correlated with the log PC_{20}. There was no significant correlation between log PC_{20} and gender, age of the patients and the amount of specific IgE against grass pollen and birch pollen. A significant correlation was found between log PC_{20} and % predicted FEV_1, log total IgE, level of IgE against house dust mite, cat, dog and sum of indoor allergens scores (table II). Step-wise multiple linear regression based on the independent variables from table II and log PC_{20} as dependent variable, showed that only the amount of IgE to indoor allergens and the % predicted FEV_1 contributed significantly to the level of bronchial responsiveness (table II).

Table II. Correlations of different variables with log PC_{20}

	***r* (bivariate)**	*p*	
gender	0.01	>0.05	
age	−0.01	>0.05	
FEV_1 (% predicted)	0.37	<0.001	*
log total IgE	−0.24	<0.01	*
IgE grass pollen	−0.04	>0.05	
IgE birch	0.02	>0.05	
IgE house dust mite	−0.20	<0.01	*
IgE cat	−0.27	<0.01	*
IgE dog	−0.26	<0.01	*
IgE indoor allergens	−0.34	<0.001	*
	***r* (multiple linear regression)**	*p*	
IgE indoor allergens and FEV_1 (% predicted)	0.52	<0.001	*

There was a difference in PC_{20} (Student's t-test, $p<0.0001$) between the 136 patients with allergic asthma (GM=1.7 mg/ml histamine, GSE=1.1) and the 25 patients with allergic rhinitis (GM=11.0 mg/ml histamine, GSE=1.3). Furthermore, there was a difference in IgE antibody level against indoor allergens (Student's t-test, $p=0.01$) between patients with allergic asthma (mean score=5.8, SEM=0.4) and patients with allergic rhinitis (mean score=3.5, SEM=0.6). There were indications for a difference in % predicted FEV_1 (Student's t-test, $p=0.09$) between allergic asthma (mean=92.9%, SEM=1.4) and allergic rhinitis (mean=99.0%, SEM=2.8). No statistically significant difference in total IgE (Student's t-test, $p>0.1$) was found between allergic asthma (GM=303 IU/ml, GSE=1.1) and allergic rhinitis (GM=201 IU/ml, GSE=1.2).

To adjust for the effects of % predicted FEV_1 and specific IgE against indoor allergens in patients with allergic rhinitis and those with allergic asthma, an analysis of co-variance was performed. The difference in level of bronchial responsiveness between allergic asthma and allergic rhinitis remained ($p<0.001$) with % predicted FEV_1 and specific IgE against indoor allergens as confounding variables.

Matching between patients with allergic rhinitis and patients with allergic asthma appeared to be effective for IgE against indoor allergens ($r=0.98$, $p<0.0001$), IgE against outdoor allergens ($r=0.96$, $p<0.0001$), total IgE ($r=0.68$, $p=0.0001$) and age of the patients ($r=0.45$, $p=0.01$).

There was no difference between the matched patients with asthma and those with rhinitis with regard to the skin test reaction to histamine (paired t-test, $p>0.1$), the skin reaction to indoor allergens (paired t-test, $p>0.1$) or outdoor allergens (paired t-test, $p>0.1$). The patients with asthma had slightly lower % predicted FEV_1 values (paired t-test, $p=0.04$) than those with allergic rhinitis.

The PC_{20} values of the matched groups and the controls are shown in figure 1. Non-specific bronchial responsiveness was less pronounced in patients with allergic rhinitis than in allergy-matched patients with allergic asthma (Student's t-test, $p=0.0003$).

Patients with non-allergic rhinitis had lower levels of bronchial responsiveness than healthy individuals (Mann-Whitney U-test, $p=0.02$) and did, in this respect, not differ from the patients with allergic rhinitis (Mann-Whitney U-test, $p>0.1$).

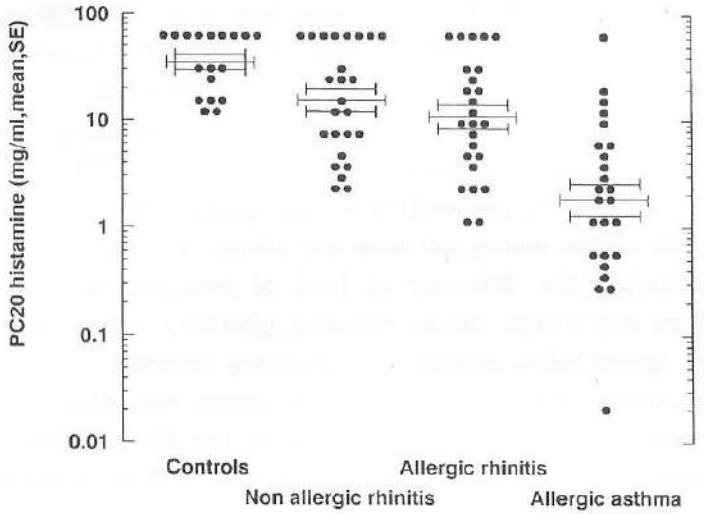

Figure 1. Level of PC_{20} histamine in allergy-matched patients with allergic asthma and allergic rhinitis, in non-allergic rhinitis and in healthy controls.

Discussion

In agreement with other authors, we showed a correlation between the level of non-specific bronchial responsiveness and the degree of allergy, measured by specific IgE for indoor allergens. Furthermore, patients with allergic asthma and similar levels of specific IgE antibodies against common inhalant allergens as those with allergic rhinitis, had a higher degree of bronchial responsiveness to histamine. Just like patients with allergic rhinitis, patients with non-allergic rhinitis, appeared to have a higher bronchial responsiveness to histamine than controls.

Allergy and non-specific bronchial hyperresponsiveness

An association has been shown between allergy and non-specific bronchial responsiveness [2—6]. Exposure to allergens induces an increase of non-specific bronchial responsiveness [11], avoidance of allergen exposure leads to a decrease [7].

In our study, allergy was established as the level of specific IgE antibodies against common inhalant allergens. Possibly this does not include all the relevant IgE since 'hidden allergens' might be involved. It has been speculated that in allergic patients,

IgE-dependent histamine releasing factor (HRF) is the missing link between the clinical symptoms and the level of specific IgE [23]. In another study, we found indications for a correlation between the levels of non-specific bronchial responsiveness and the response to IgE-dependent HRF [24]. Patients, responsive to HRF, had lower histamine thresholds than those that were not responsive to HRF.

There are conflicting opinions whether total serum IgE or specific IgE (measured by skin test or RAST) is a better predictor of bronchial responsiveness [6,25,26]. The total serum IgE level may reflect a continuous index of overall allergy, as yet unknown but relevant specificities included. However, not all specificities of IgE are of relevance to bronchial hyperresponsiveness. Our findings are in agreemeent with Burrows et al.[6], who found only an association between the level of bronchial responsiveness and IgE to indoor allergens and not with IgE to outdoor allergens. We found no additional effect of total IgE for predicting the level of non-specific bronchial responsiveness. Furthermore, the difference in bronchial responsiveness in the matched groups remained without a difference in specific IgE and total IgE.

Allergen exposure may play a role, as suggested by studies on the effect of chronic exposure to house dust mites on bronchial responsiveness [7,8,27—30]. However, it is not clear, whether exposure also influences the clinical manifestation (allergic asthma or allergic rhinitis) of inhalant allergy. The total allergen load of subjects is difficult to measure. Interpretation of current exposure levels of patients compared to controls is hampered since patients with allergic diseases are probably more prone to improve their indoor environment. We were not informed about the levels of indoor allergens. If the allergen exposure had been higher in the patients with asthma, we would also have expected to find more symptoms of the upper airways in the patients with asthma than those with rhinitis. This was not confirmed by the analysis of standardized questionnaires. Recent exposure to outdoor allergens was maximal in allergic rhinitis because the patients were tested at the end of the grass pollen season (August to October). At that time, the exposure to house dust mite is maximal in The Netherlands [31,32]. This means that the difference we found between asthma and rhinitis is possibly underestimated.

Non-allergic factors and non-specific bronchial hyperresponsiveness

The possibility remains that there are IgE-independent differences between asthma and rhinitis as far as local factors in the lower airways are concerned, which influence non-specific bronchial hyperresponsiveness. The number of mast cells and basophils in the bronchial lumen [33,34], the releasability of these cells [35—37] and local

inflammatory cell responses may differ between the two patient groups.

Non-specific bronchial hyperresponsiveness can occur without presence of allergy, intrinsic asthma being the best example. There is compelling evidence that inflammation underlies the phenomenon of non-specific bronchial responsiveness [38]. Recently authors have highlighted that the inflammatory process in asthmatic airways appears to have common features, irrespective whether asthma is allergic in origin or apparently non-allergic [39].

Our finding that patients with non-allergic rhinitis have higher levels of bronchial hyperresponsiveness than normal subjects is compatible with the view that IgE-independent mechanisms are involved in non-specific airways hyperresponsiveness.

Association between upper and lower airways

An association between upper and lower airway inflammation has been suggested because local treatment with nasal corticosteroids in patients with allergic rhinitis resulted in a significant reduction of asthma symptoms and bronchial hyperresponsiveness [40—42].

Various non-allergic mechanisms have been suggested for the association between upper and lower airways. A reflex pathway, a nasobronchial reflex, has been suggested [43]. Propagation of inflammation from the nose to the lungs may be another possibility. Whether postnasal drainage of inflammatory material into the lower airways occurs is still controversial [44,45]. Little is known about systemic absorption of mediators or chemotactic factors.

We found only one study about the bronchial responsiveness in patients with non-allergic rhinitis [46]. The authors measured normal methacholine bronchial thresholds in 37 patients with non-allergic rhinitis. We can not explain why our results regarding the bronchial responsiveness of patients with non-allergic rhinitis differ from that study.

In conclusion, we confirmed the relation between specific IgE for indoor allergens and non-specific bronchial hyperresponsiveness. Our results indicate that the level of IgE against common indoor allergens cannot account for the difference in bronchial responsiveness between patients with asthma and patients with rhinitis. The importance of non-allergic mechanisms is suggested by the increased non-specific bronchial responsiveness in non-allergic rhinitis.

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Chapter 6

IgE antibodies reactive with silverfish, cockroach and chironomid are frequently found in mite-positive allergic patients

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ABSTRACT

Approximately 30% of the house dust mite allergic patients in The Netherlands have IgE antibodies reactive with silverfish, cockroach and/or chironomid. In allergic patients without IgE antibodies against *Dermatophagoides pteronyssinus* less than 5% have IgE antibodies reactive with these insects. By means of RAST inhibition studies it is shown that cross-reactivity exits between *D.pteronyssinus* and silverfish, cockroach or chironomid. This means that a positive RAST for silverfish, cockroach, chironomid or *D.pteronyssinus* cannot be taken as evidence for exposure.

INTRODUCTION

Allergy to insects is common in most parts of the world. Asthma caused by the caddisfly was described some 60 years ago [1]. Caddisfly allergy has been reported from Japan [2] and the United States [3,4]. In the United States cockroach allergens have also been established as sensitizing agents that are important in the induction of asthma [5—9]. Widespread allergy for chironomids (popularly known as nonbiting midges or red mosquito larves) has been described for example from the Sudan [10], Japan [2] and Sweden [11]. It has been reported that sera with IgE antibodies reactive with insects also contain IgE antibodies reactive with *D.pteronyssinus* [12—14]. A possible explanation is cosensitization, i.e. the exposure to insects is associated with the exposure to house dust mites. Both mites and insects need a humid environment. An alternative explanation is the cross-reactivity between insects and *D.pteronyssinus*. We have identified tropomyosin, the major shrimp allergen [15,16], as a cross-reacting allergen in shrimp, mite and insects [17]. A high rate of cockroach infestations is present in some suburbs in The Netherlands [18]. Also the presence of silverfish in households is common.

The aim of this study was to estimate the prevalence and relevance of IgE antibodies reactive with silverfish, cockroach and chironomid in patients with asthma and rhinitis in The Netherlands, and to study cross-reactivity with *D.pteronyssinus*.

MATERIALS AND METHODS

Patients

The RAST and RAST inhibition were performed on sera from patients attending the Departments of Otorhinolaryngology and Pulmonology of the Academic Medical Center. The patients suffered from rhinitis and/or asthma. The patients were divided in two groups. The first group (n=82) was selected on the basis of IgE antibodies against house dust mite. The second group (n=67) was selected on the basis of the absence of IgE antibodies against mites but presence of IgE antibodies against at least one other inhalant allergen. The groups did not differ in the RAST results for cat dander or grass pollen. From the mite-positive group we selected one serum (no. 3) because of a high RAST score for insects. It was the serum of a 18-year-old man with wheezing in the morning, a blocked nose and itching in his throat. He was not aware of insect exposure at home and said he had never eaten shrimp. His RAST scores were (anti-IgE bound, % of added): mite 30.5, chironomid 24.6, silverfish 19.2, cockroach 18.5, shrimp 15.6, cat dander-negative, and grass pollen-negative.

None of the patients had received immunotherapy in the last 10 years. Patients with IgE antibodies against silverfish who also had IgE antibodies against flour (n=4) were excluded because the silverfish were cultured on flour and therefore the extract was contaminated with flour.

Allergens

Dried *D.pteronyssinus* mites were obtained from the Commonwealth Serum Laboratories (CSL; Melbourne, Australia). Mites were extracted for 12 h in phosphate-buffered saline (PBS), containing 0.1% Tween 20, at 4°C (2% w/v).

Frozen chironomids (genus Chironomus, species unknown, obtained from the local pet shop) were extracted (2% w/v) at room temperature in PBS, containing 0.1% Tween 20 and 0.01% NaN_3 for 4 h. Silverfish (*Lepisma saccharina*) were obtained from our own culture (fed with flour). Cockroaches (*Blattella germanica*) were collected at a house with a cockroach infestation. Silverfish and cockroaches were killed by freezing, sonicated and extracted (2% w/v) either at room temperature in PBS, containing 0.1% Tween 20 and 0.01% NaN_3 for 12 h, or at 100°C in PBS for 15 min (to prevent the proteases in the extracts from destroying the tropomyosin, a heat-stable [19,20] cross-reactive allergen in shrimp, mite and insects). After extraction the solution was defatted with Freon and filtered.

RAST and RAST inhibition

RAST was performed as described previously [21]. For the *D.pteronyssinus* RAST, mite body extract, 1.2 mg protein, was coupled to 100 mg of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). For the insects, 2 mg protein (silverfish 2 ml, cockroach 1.4 ml) were coupled to 100 mg of CNBr-activated Sepharose. For the silverfish and cockroach RAST, the allergosorbent with extracts prepared at room temperature was mixed (1:1) with an allergosorbent with extracts prepared at 100°C. Tests were performed by incubating 50 µl serum with 0.5—3 mg allergosorbent. Immunodetection of IgE was performed with ^{125}I-labeled sheep-antihuman IgE (CLB, Amsterdam). The results are expressed as percentage of total counts added without correction of background values (<3% of added radioactivity). The cutoff for a positive RAST is a binding of >5% of added radioactivity. RAST inhibition assays were performed as follows: 50 µl allergen solution (1 mg/ml) was incubated with 50 µl serum for 2 h at room tempera-

ture; this mixture was incubated overnight with the allergosorbent, followed by the normal RAST procedure. In the detailed inhibition experiment, the amount of allergosorbent was adjusted to give optimal inhibition (0.375 mg silverfish Sepharose, 0.75 mg cockroach and mite Sepharose).

RESULTS

The prevalence of IgE antibodies (RAST result: >5% of total counts added) against insects is 26 of 82 (32%) in the mite-positive group and 1 of 67 (1.5%) in the mite-negative group (fig. 1). For all three insect species the differences between the groups are significant (Chi-square, cutoff 5%, $p<0.01$). Table I shows, for the patients with a percentage binding of >10% to one of the insect species, the RAST results for the different insect species. It is clear that most sera react with more than one insect species.

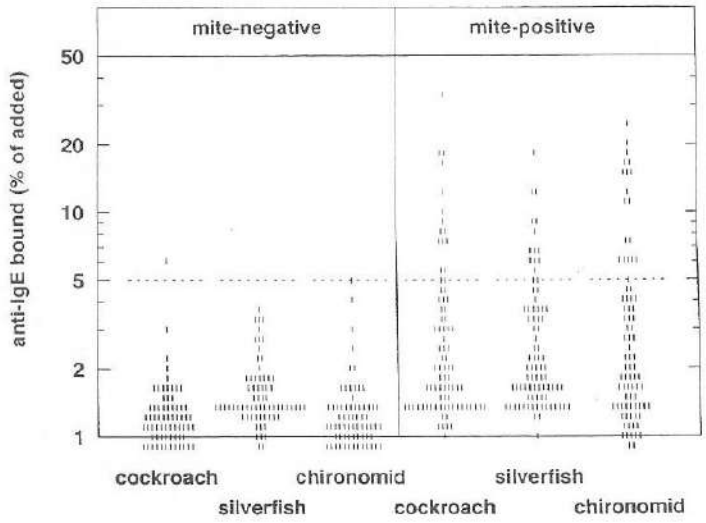


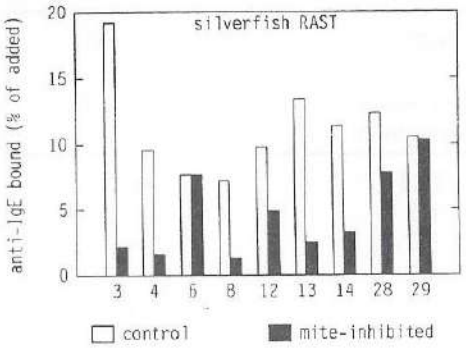
Figure 1. RAST results in the mite-positive and mite-negative group. Results are expressed as percentages of added radioactivity without background correction.

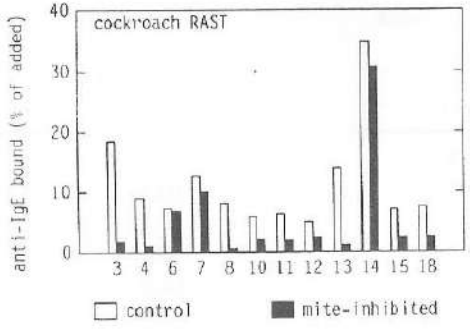
Table I. IgE reactivity to the different insects of the patients with an percentage of >10% to one of the insect species

patient	cockroach	silverfish	chironomid
	% of added radioactivity	*% of added radioactivity*	*% of added radioactivity*
3	18.5	19.2	24.6
4	10.5	6.3	5.8
7	12.3	3.7	12.0
12	8.1	8.3	18.3
13	16.9	11.7	15.4
14	32.9	8.7	6.0
16	7.1	9.5	11.1
28	3.8	6.4	20.0
29	3.1	6.0	16.9
31	18.4	12.1	15.2
32	3.4	3.7	15.0
35	1.8	5.0	10.9
36	2.0	3.1	17.0

To investigate the possibility of cross-reactivity between insects and mites a RAST inhibition test was performed. The silverfish, cockroach, and chironomid RAST were inhibited to a variable degree by mite extract. The results are shown in figures 2a—c. In the three insect RASTs many sera (40—78%) show significant inhibition (>25% inhibition) by mite extract. There are also sera (8% for cockroach, 50% for chironomid) that are not inhibited (<10% inhibition) by mite extract.

We also investigated the role of shrimp allergens in cross-reactivity between mites and insects. From the 26 sera that had a positive RAST score for insects, 15 sera were available for a shrimp RAST. From these 15 sera 4 sera had a positive RAST score for shrimp. In serum 3 (selected because of a high RAST score for silverfish and cockroach) we performed more detailed inhibition experiments. In figure 3 the results are shown for serum 3. The maximal inhibition of the silverfish and cockroach RAST was >90% with shrimp, mite and insect extract. The maximal inhibition of the mite RAST was 50% with shrimp and insect extract.





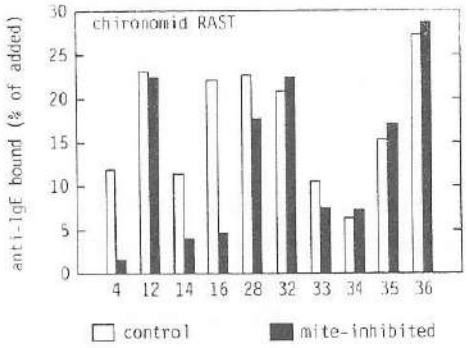
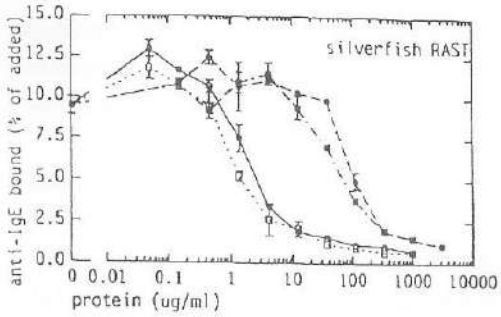
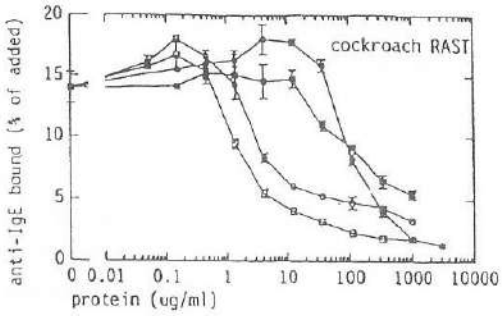
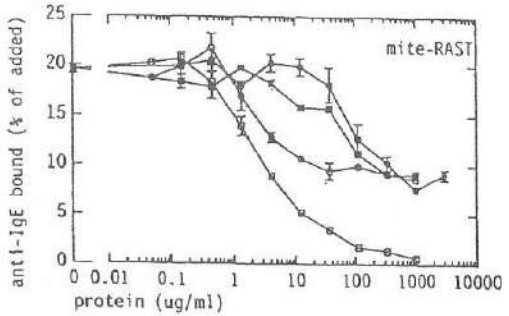


Figure 2a-c. Mite inhibition of the insect RAST.







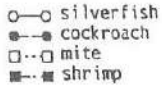


Figure 3. RAST inhibition data of serum 3.

DISCUSSION

The results outlined here show that IgE antibodies to insects are very common (approximately 30%) in patients with IgE antibodies to mite. In Dutch allergic patients without IgE antibodies to mites, however, the incidence of IgE antibodies to insects is low (<5%).

This is in agreement with other studies in which it was found that IgE antibodies reactive with insects were frequently found in sera containing IgE antibodies to mite. Baldo et al. [12] selected Australian patients with inhalant allergy to insects on the basis of case histories and skin test reactions to insect extracts. In 38 patients with IgE antibodies to one or more insect species, they found only 6 patients with negative reactions to *Dermatophagoides farinae*. Eriksson et al. [22] found that 23% of atopic patients with asthma or rhinitis in Denmark had a positive skin prick test with chironomid. Of this group 47% also had a positive prick test to *D.pteronyssinus*. A study in Japan [14] showed that 38% (115 out 303) of the patients with asthma had a positive prick test with chironomid extract, 80% of this group also had a positive prick test with mites.

The results of our RAST inhibition studies show that many of the cockroach-, silverfish- and chironomid-positive sera were inhibited by mite extract. This indicates cross-reactivity between silverfish, cockroach or chironomid and mite. However, there are also sera that are not inhibited by mite extract, indicating that 'specific' reactions, i.e. IgE not cross-reactive with mites, also occur.

Tropomyosin, the major shrimp allergen and a cross-reactive allergen in shrimp, mite and insects, is a heat-stable allergen [19,20]. For that reason insects were partly extracted at 100°C, to prevent the proteases in the extracts from destroying the tropomyosin. In the detailed reciprocal inhibition experiment with serum 3, it is clear that shrimp extract inhibits the cockroach and silverfish RAST, but at a more than 10-fold higher protein concentration than the mite extract. Serum 3 probably has cross-reactive IgE to tropomyosin.

Only 4 of 15 insect-positive patients also had IgE antibodies against shrimp, indicating that other cross-reactive allergens are operative as well. In reciprocal RAST inhibition experiments with other sera we also found cross-reactivity between mites and different insect species but not always with shrimp. The overall pattern was strikingly heterogeneous and suggested the presence of different cross-reactive epitopes.

The finding that in several reciprocal inhibition experiments the insect RAST was for >90% inhibited by mite extract, whereas the mite RAST was only partially inhibited by

insect extracts, suggests that mites are often the primary sensitizing agent. Therefore a positive RAST for insects cannot be taken as evidence for exposure to these insects. In future studies we want to focus on exposure levels to these insects in The Netherlands, and to investigate whether patients with high levels of IgE antibodies to insects are frequently exposed to insects.

The clinical relevance of IgE antibodies against the different insect species is not always clear. Several studies have shown positive provocation tests in patients with IgE antibodies to cockroaches [7,23,24]. Eriksson et al. performed nasal and conjunctival provocation tests with chironomids on 23 patients with positive skin prick tests to chironomids [22]. They were positive in 7 cases (30%). We are not aware of studies with provocation tests with silverfish extract. Further studies on exposure levels to insects and the clinical relevance of the IgE antibodies reactive with insects need to be done.

Acknowledgements

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Chapter 7

Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects

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ABSTRACT

A monoclonal antibody to *Dermatophagoides pteronyssinus* is described that cross-reacts with an IgE-binding antigen present in insects, *Crustacea* (e.g. shrimp) and other invertebrates. By means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration and immunofluorescence it was shown that this monoclonal antibody presumably recognizes tropomyosin. Tropomyosin was shown to be involved in cross-reactivity between mite, shrimp and insects in shrimp-allergic patients.

INTRODUCTION

In the course of our study on allergens of the house dust mite we obtained a monoclonal antibody (mAb) that reacted not only with mite extracts, but also with extracts of insects and other invertebrates (e.g. shrimp). This observation indicated the presence of a cross-reactive antigen in mite, shrimp and insects. In the literature it has been suggested that there are cross-reacting IgE antibodies against mite, insects and *Crustacea* [1-6].

Invertebrates are known to be strong sensitizers for IgE-mediated immune responses. The most important species in many parts of the world are mites. However, insects have also been found to contribute to inhalant allergies, e.g. cockroaches [7-9], moth [10], non-biting midges [5] and caddis fly [11].

Several patient studies have shown that high reactivity against insects is frequently accompanied by high reactivity against mites [1,2].

Crustacea are a common cause of ingestant allergic disease [12]. The characterization of shrimp allergens is discussed in a number of studies [13-15]. Very recently, Pen a 1 or Sa 2, a major shrimp allergen, has been identified as tropomyosin [16,17].

There have been some reports in the literature about asthma attacks after eating snails, *Crustacea* or other nonfish seafoods in patients with allergy to mites and/or insects [1,4]. Cross-reactivity between *Arthropoda* and *Crustacea* has been observed; for instance, Eriksson et al. [5] showed cross-reactivity between chironomids and shrimp in 1988. Cross-reactivity between snails and *Dermatophagoides pteronyssinus* was described by Ardito et al. [6] in 1990.

In this study we investigate cross-reactivity between *D.pteronyssinus*, *Crustacea* and insects. Tropomyosin was found to be a cross-reactive allergen. A cross-reactive mAb against tropomyosin is described.

MATERIALS AND METHODS

Patients

Two groups of sera were obtained from our diagnostic department. Group A (n=10) was selected on the basis of IgE antibodies against mite and did not have IgE antibodies against shrimp. Group B (n=3) was selected on the basis of allergy symptoms after eating shrimp and had IgE antibodies against shrimp and mite. IgE antibodies were determined as described in the section 'RAST'.

Allergens

Dried *D.pteronyssinus* mites were obtained from the Commonwealth Serum Laboratories (CSL Melbourne, Australia). Mites were extracted for 12 h in phosphate-buffered saline (PBS) (10 m*M* phosphate, 140 m*M* saline), containing 0.1% Tween 20, at pH 7.4 at 4°C (2% w/v). Mite spent medium from the same source was extracted in the same way.

The shrimps, oysters, crabs and mussels were obtained locally (Amsterdam). They were cleaned, crushed and extracted at room temperature (10% w/v) in water, maintained at pH 8, for 4 h, dialyzed against distilled water and lyophilized. The shrimps (*Crangon crangon*) were extracted at 100°C (10% w/v) for 15 min, defatted by Freon extraction and dialyzed against distilled water.

Chironomids (aquarium fish food) were obtained locally (Amsterdam). Mosquito extract was obtained from Diephuis Laboratories (Diephuis, The Netherlands). Cockroaches (*Blattella germanica*) were collected from a household in Amsterdam. The insects were extracted at room temperature (10% w/v) in PBS, containing 0.1% Tween 20 for 4 h at pH 7.4.

Gel filtration

Gel filtration was performed with 2 ml 2% (w/v) *D.pteronyssinus* extract on a 200 ml column of ACA-54 (Sepracor, France). Fractions were monitored for Der p 1 and Der p 2 by radioimmunoassay (RIA) [18,19] and for amylase using the iodine starch reaction.

Monoclonal antibody

BALB/c mice were immunized subcutaneously with 0.05 mg of the 25—80 kD molecular weight fraction of *D.pteronyssinus* extract in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., USA) and boosted after 6 weeks with the same dose in incomplete Freund's adjuvant (Difco). Three days before removal of the spleen, a final booster injection was administered intravenously with the same dose. Cell fusion was performed as described by Astaldi et al. [20]. Antibody-producing (IgG1 κ light chain) hybridomas were selected and cloned by limiting dilution [21]. Monoclonal 1A6 antibodies were purified from culture supernatant by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Antibody 1A6 was selected based on radioallergosorbent test (RAST) and indirect RAST with *D.pteronyssinus* extract or insect extract coupled to the Sepharose (see RAST). The antibody was coupled to CNBr-activated Sepharose (4 mg antibody/100 mg Sepharose).

RAST

RAST was performed as described previously [22]. For the *D.pteronyssinus* RAST, mite-body extract, 3.6 mg protein, was coupled to 300 mg (equivalent to 1 ml packed gel) of CNBr-activated Sepharose 4B (Pharmacia). For the oyster, crab, mussel and shrimp, 15 mg of the lyophilized extract was coupled to 300 mg of CNBr-activated Sepharose. For the insects, 6 mg protein was coupled to 300 mg of CNBr-activated

Sepharose. Tests were performed by incubating 50 µl serum or 50 µl 1A6 supernatant (30 µg IgG/ml) with 0.5—1.5 mg allergen-coupled Sepharose. Immunodetection of IgE was performed with 1 ng ^{125}I-labeled sheep anti-human IgE (MH25-01, CLB, Amsterdam, The Netherlands) in 200 µl PBS, containing 0.1% Tween 20 and 0.3% bovine serum albumin (BSA). Mouse IgG was detected with 1 ng ^{125}I-labeled rat anti-mouse IgG (CLB). The results were expressed as percentage of total counts added.

For the indirect RAST the Sepharose-coupled 1A6 monoclonal was incubated for 24 h with *D.pteronyssinus* extract or shrimp extract. After removal of the nonabsorbed components by washing, serum of a shrimp-allergic patient was added, and binding of IgE was detected with radiolabeled anti-IgE.

RAST inhibition assays were performed as follows: 50 µl allergen solution was incubated with 50 µl serum for 2 h at room temperature; this mixture was then incubated overnight with the Sepharose-coupled allergen extract and followed by the normal RAST procedure. The amount of allergosorbent was adjusted to give 10—20% binding of radioactivity in absence of inhibitor.

Solid-phase absorption and depletion of extracts for tropomyosin

Tropomyosin-depleted shrimp extract was prepared by taking the supernatant after incubation of 0.1 ml shrimp extract (0.85 mg/ml) with 100 mg 1A6-Sepharose for 12 h at 4°C. As a control experiment the same amount of shrimp extract was incubated with 100 mg glycine-Sepharose. The result of the depletion was measured in the indirect RAST, with 1A6 coupled to Sepharose, 1A6-depleted shrimp extract or glycine-depleted shrimp extract and serum of a shrimp allergic patient (see RAST). The amount of tropomyosin in the depleted extract was reduced by a factor of 1,000 compared with the glycine-depleted extract.

Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Laemmli [23]. Allergen extracts were applied to a polyacrylamide gradient gel (10—20%) under reducing conditions. Subsequently gel proteins were electrophoretically transferred to a nitrocellulose sheet by the Western blotting technique according to Burnette [24]. The blots were incubated for 2 h in 100 ml of PBS, contai-ning 10 m*M* EDTA, 0.1% (w/v) Tween 20, 0.45% (w/v) BSA and 0.05% (w/v) NaN_3 at room temperature. Blots were incubated in a blotting incubation apparatus as developed by Westgeest et al. [25]. After incubation with mAb 1A6, a second antibody (1 ng ^{125}I-goat anti-mouse IgG) was used. Both incubations were performed overnight in 4 ml of PBS, containing 10 m*M* EDTA, 0.1% Tween 20, 0.3% BSA and 0.05% NaN_3. Between and after the incubations the blots were washed 5 times with PBS, containing 0.1% Tween 20. Autoradiography was performed by exposing immunoblots to X-ray films at —70°C for 48 h with an intensifying screen.

Agar electrophoresis

Agar electrophoresis was performed according to Johansson [26]. 3 µl of 2% (w/v) *D.pteronyssinus* extract (1.2 mg/ml protein, determined with Pierce BCA Protein Assay Reagent according to the manufacturer's instructions) was applied to agar gel. After electrophoresis, the proteins were transferred to a nitrocellulose sheet. After incubation with mAb 1A6 a second antibody (1 ng ^{125}I-goat anti-mouse IgG) was used. Autoradiography was performed by exposing immunoblots to X-ray films at —70°C for 48 h with an intensifying screen.

Immunohistochemical methods

Shrimps were snap-frozen in liquid nitrogen. 4-µm cryostat sections were cut, air-dried and fixed in acetone for 10 min. Fixation and all subsequent washes and incubations were performed at room temperature. After fixation, sections were washed for 5 min in three changes of PBS, incubated for 30 min with protein A-purified mAb 1A6, 0.1 mg/ml, washed again in three changes of PBS and incubated for 30 min with FITC-labeled goat anti-mouse (5 mg/ml, 1:100; CLB). After these incubations the sections were again washed in three changes of PBS. As a negative control we used another mAb anti- mite (anti-Der p 2; CLB).

Isoelectric focusing

Isoelectric focusing was performed with a Pharmacia Kit according to the manufacturer's directions. 1 µg of affinity purified tropomyosin was applied to the Ampholine PAG plate (pI 4.0—6.5). Detection was performed by immunoblot with mAb 1A6 as described above.

Results

Specificity of mAb 1A6

The antibody reacted with a mite component of 34—36 kD in SDS-PAGE immunoblot under reducing conditions (fig. 1). It reacted with a component with an apparent molecular weight (MW) of >80 kD in gel filtration and an electrophoretic mobility close to human serum albumin in agar electrophoresis. A pI of 5.0 was measured. The 1A6 antibody reacted not only with mite extract, but also with extracts of various insects (chironomid, mosquito and cockroach) and *Crustacea*, e.g. shrimp (fig. 2). By immunofluorescence microscopy on shrimp, muscle staining was observed (fig. 3). On the basis of these results we assume that the mAb 1A6 recognizes tropomyosin.

Identification of IgE antibodies to tropomyosin

To investigate the role of tropomyosin as a cross reactive allergen, we performed an indirect RAST. In the indirect RAST, Sepharose-coupled 1A6 monoclonal was incubated with *D.pteronyssinus* extract. None of the ten sera of group A (mite-positive, shrimp-negative sera) reacted in the indirect RAST, but all three sera of group B (mite- and shrimp-positive) were strongly positive (fig. 4). This suggests that only the mite-positive sera that also react with shrimp, have IgE antibodies against tropomyosin. The control experiment with an irrelevant Sepharose-coupled mAb was negative.

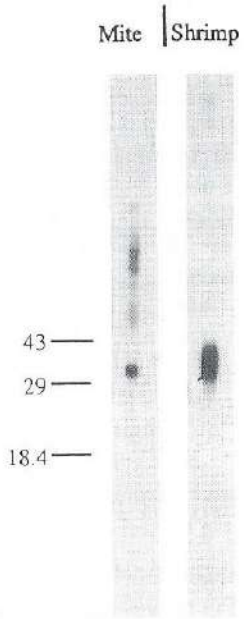


Figure 1. Autoradiography of SDS-PAGE on shrimp and mite extract after incubation with mAb 1A6.

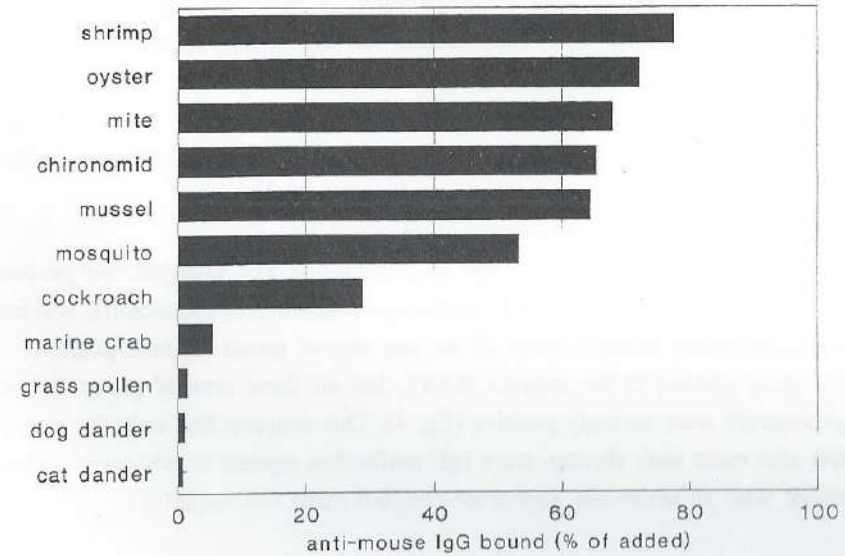


Figure 2. Reactivity of mAb 1A6 to mite, various insects and *Crustacea*. The mAb 1A6 was incubated with the allergen Sepharoses. Mouse IgG was detected with ^{125}I-labeled rat anti-mouse IgG.

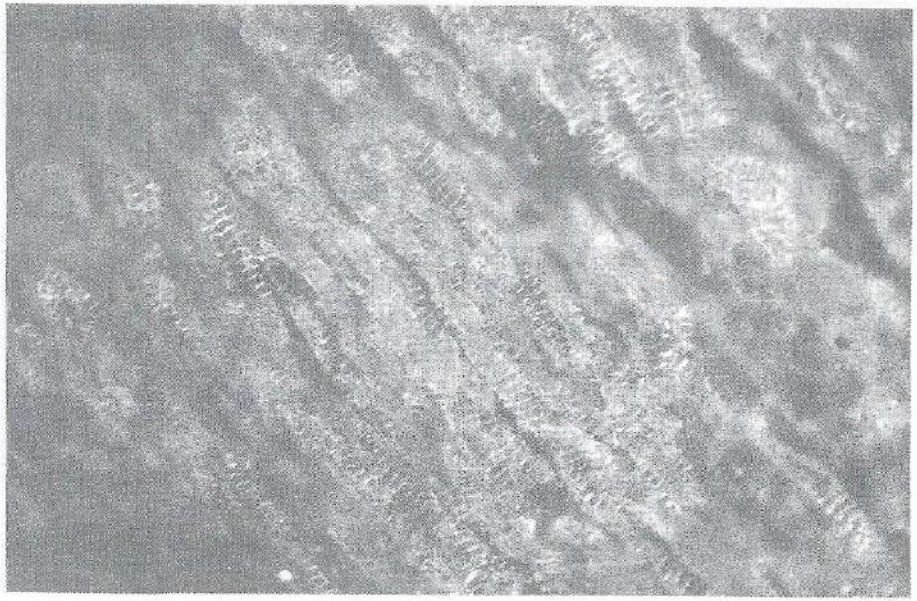


Figure 3. Shrimp incubated with mAb 1A6. A clear muscle staining was observed.

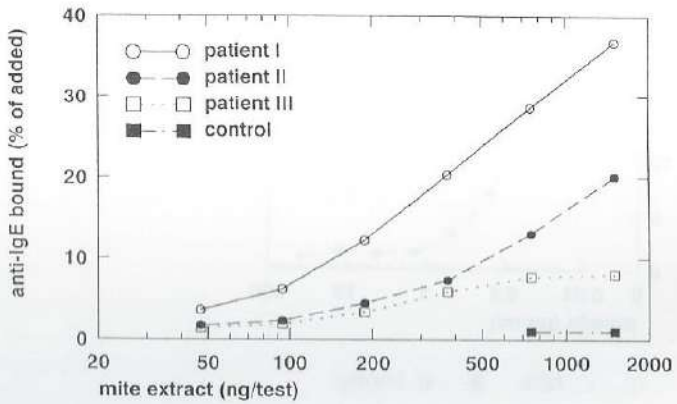
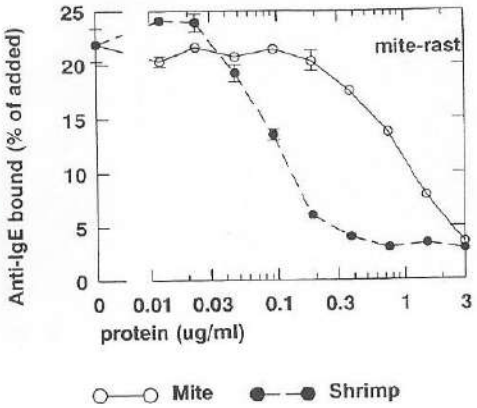


Figure 4. Results of the indirect RAST: mAb 1A6 coupled to Sepharose catches the IgE binding mite component in three sera of shrimp-allergic patients and binding of IgE was detected with radiolabeled anti-IgE. The control shows the result with an irrelevant mAb.

To determine whether the observed co-occurrence of IgE antibodies against shrimp and mite is based on cross-reactivity of these antibodies, the sera of group B were tested in a RAST inhibition assay. In the case of serum I and II, the RAST for mite was completely inhibited by shrimp extract and the RAST for shrimp partly by mite extract (fig. 5 shows the result with serum II). In case of serum III, the RAST for mite was only partially inhibited by shrimp.



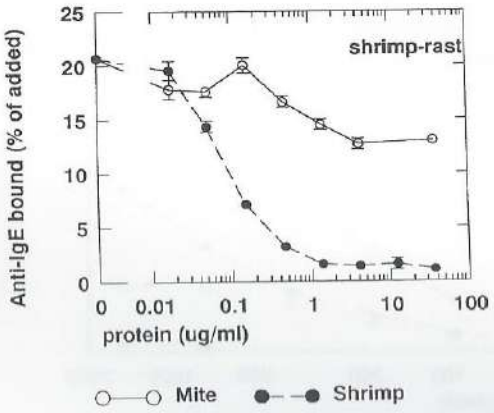


Figure 5. RAST inhibition data of serum II. The mite RAST is completely inhibited by shrimp and the shrimp RAST partly by mite.

To investigate if tropomyosin is responsible for this cross-reactivity, we performed a mite RAST inhibition with supernatants of 1A6 solid phase absorbed extracts of shrimp. The amount of tropomyosin in the depleted extract was reduced by a factor of 1,000 as described above. Tropomyosin-depleted shrimp extract was a 100-fold less potent inhibitor of the mite RAST compared to the control (glycine-depleted shrimp extract) (fig. 6).

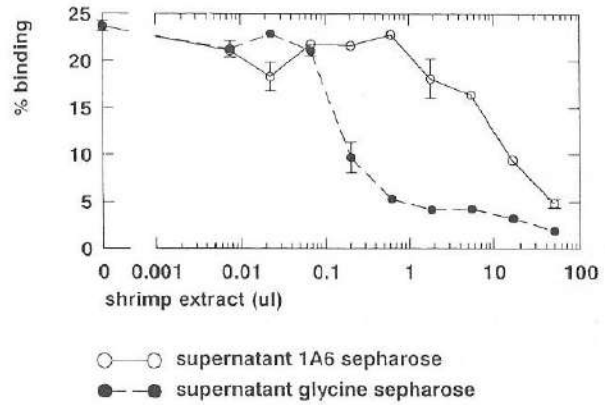


Figure 6. Mite RAST inhibition of serum II with mAb 1A6 depleted and undepleted shrimp extract.

Discussion

Tropomyosin has recently been identified as major shrimp allergen Pen a 1 or Sa 2 [16,17,27,28]. It is a highly conserved protein and 1 out of 3 mAbs to shrimp tropomyosin was found to cross-react with insect tropomyosin [29]. We assume that our mAb 1A6 reacts with tropomyosin, because it reacts with a major shrimp allergen with the physicochemical properties described for tropomyosin: a homodimer of a 36-kD filamentous monomer with a pI of 4.8—5.4. The high apparent MW on gel filtration indicates an anisomorphic structure. Furthermore, by immunofluorescence microscopy on shrimp, muscle staining was observed.

The finding, in several studies, that many mite-positive sera reacted with insects [1] or *Crustacea* [6], suggested that the sera contained cross-reacting antibodies. In this study we identified tropomyosin as a cross-reactive allergen. In our laboratory we have indications for additional mite allergens that cross-react with insects.

An interesting question is which allergen induces the immune response. In case of sera I and II it seems to be shrimp tropomyosin because the IgE binding to mite can be completely blocked by shrimp. However, in serum III we found only a partial inhibition of the mite RAST by shrimp. The initial sensitization route may be different for different patients.

The clinical relevance of tropomyosin as a cross reactive allergen is still unclear. In order to quantify the cross-reactivity between mite and shrimp, we tested 139 mite-positive sera for the presence of IgE antibodies to shrimp. From these sera, 15 (11%) were positive in the shrimp RAST (% binding more than 5% of total radioactivity). We do not know whether these sera were from people who had complaints of shrimp allergy. Skin tests and histamine release tests need to be done to clarify the clinical relevance.

In the literature there are indications for clinical relevance of the cross-reactivity between mite and shrimp. Banzet et al. [3] observed serious clinical manifestations after eating snails in a few patients who had received immunotherapy against mite. These patients developed also an erythematous and indurated reaction of the skin and subcutaneous tissue of the region of the arm in which the mite desensitization injections had been administered during previous years.

We found that mite body extract contains more tropomyosin than spent medium extract. Therefore, in case of mite immunotherapy in a patient with concomitant allergy to *Crustacea* the use of an extract which contains relatively more mite excreta than mite body components might be preferable.

The clinical implications of the cross-reactivity between shrimp and mites requires further study.

Acknowledgements

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Chapter 8

Silverfish proteins in house dust in relation to mite- and total arthropod-level

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Submitted

Abstract

Two assays have been developed to measure arthropod-levels in house dust. The first assay measures silverfish-antigens. The second assay measures invertebrate tropomyosin and gives a global assessment of the level of arthropod-derived material.

These assays and a Der p 1 and Der p 2 assay were used to analyze 53 dust samples. In most dust samples the ratio tropomyosin/Der p 2 was higher than in mite body extract, indicating that the assay measures other arthropods besides mites. Silverfish-antigen was detectable in most of the dust samples. In many homes in which the inhabitants where unaware of the presence of silverfish, silverfish antigen was detectable. Therefore for information on exposure an immunochemical analysis is superior to a questionnaire.

Introduction

The presence of specific IgE antibodies to arthropods is common among patients with rhinitis and asthma [1-4]. However, the presence of specific IgE alone is not sufficient to conclude that these IgE antibodies are related to the symptoms, since exposure to arthropods is also necessary for the development of symptoms.

Arthropods are present in many households. Several studies have investigated the levels of *D.pteronyssinus* in house dust [5-7]. For *D.pteronyssinus* a threshold level of exposure that increases the risk of sensitization and symptomatic asthma has been proposed [8-11]. Much less is known about exposure levels to insects due to the lack of quantitative detection methods. Only for cockroach, immunoassays have been described to measure exposure levels [12-14].

In The Netherlands the presence of silverfish in households, besides cockroaches, is common. Silverfish are very primitive wingless insects. They are 3—12 mm long, have three tail feelers and are covered with shiny scales. They shun light and need a humid environment, therefore they prefer kitchens and bathrooms. They live a.o. from paper, crumbs of bread and flour.

In this report we describe two immunoassays we have developed to monitor insects in house dust. The first assay measures silverfish-antigens. The second assay, with a monoclonal antibody [15] that reacts both with mite and with insects, gives a global assessment of the level of arthropod-derived material in dust samples.

Materials and methods

Allergens

Dried *D.pteronyssinus* mites were obtained from the Commonwealth Serum Laboratories (CSL; Melbourne, Australia). Mites were extracted for 12 h in PBS, containing 0.1% Tween 20 at pH 7.4 at 4°C (2% w/v). Frozen chironomids (genus Chironomus, species unknown, obtained from the local pet shop) were extracted (2% w/v) at room temperature in PBS, containing 0.1% Tween-20 and 0.01% NaN_3 for 4 h. Silverfish (*Lepisma saccharina*) were obtained from our own culture. Cockroaches (*Blattella germanica*) were lured from a house with a cockroach infestation. Silverfish and cockroaches were killed by freezing, sonicated and extracted (2% w/v) either at room temperature in PBS, containing 0.1% Tween-20 and 0.01% NaN_3 for 12 h. After extraction the solution was defatted with freon and filtered.

Rabbit antibodies and monoclonal antibodies

New Zealand white rabbits were immunized with silverfish extract, 100 µg protein, in Freund's complete adjuvant on day 0, followed by three injections with the same amount in Freund's incomplete adjuvant at monthly intervals. Solid-phase adsorption was used to remove the antibodies directed against flour (on which the silverfishes were cultivated). Ten ml rabbit antiserum was incubated with 300 mg flour-Sepharose for 4 h at room temperature. The supernatant was affinity-purified by incubation for 4 h with 300 mg Sepharose-coupled silverfish extract. Bound antibodies were eluted with 0.1 M glycine HCl, 0.15 M NaCl, pH 2.5. The antibodies were radiolabeled by the chloramine-T method; 30 µg of protein was iodinated with 37 MBequerel.

A monoclonal antibody to tropomyosin (1A6 antibodies) was obtained as described earlier [15]. The antibody was coupled to CNBr-activated Sepharose (4 mg antibody/100 mg Sepharose).

Purification of tropomyosin

Shrimp-extract was prepared by extraction in water at 100°C for 15 min, filtered, concentrated and dialyzed against distilled water. The shrimp extract (30 ml 850 µg/ml protein) was incubated for 4 h with 600 mg 1A6 Sepharose. Bound components were eluted with 0.1 M glycine HCl, 0.15 M NaCl, pH 2.5. In this way we collected 3 ml tropomyosin, with 180 µg protein per ml by Pierce protein assay. Tropomyosin was radiolabeled by the chloramine-T method; 45 µg of protein was iodinated with 37 MBequerel.

House dust

In 53 households dust was collected by vacuum cleaning of the entire house. Unsieved dust was extracted (20% w/v) for 4 h in 0.35 M NaBenzoate.

Silverfish assay

To 1 ml silverfish Sepharose (0.33 mg/ml), 50 µl ^{125}I-labeled rabbit-anti-silverfish and 50 µl of the test extract was added. The mixture was incubated overnight under vertical rotation at room temperature. After centrifugation and removal of non-bound components, the radioactivity bound to the solid phase was counted. The concentration was read from a standard curve of silverfish extract. The detection limit was 230 ng total silverfish protein/ml.

Tropomyosin assay

To 0.5 ml goat-anti-mouse Sepharose (1 mg/ml), 50 µl diluted 1A6 antibodies, 50 µl ^{125}I-labeled tropomyosin and 50 µl of the test extract was added. The mixture was incubated overnight under vertical rotation at room temperature. After centrifugation and

removal of non-bound components, the radioactivity bound to the solid phase was counted. The concentration was read from a standard curve. The detection limit was 20 ng/ml.

Der p 2 ELISA

Purified mAb against Der p 2 (mAb 10E11) was coated overnight at room temperature (1 µg/ml in PBS, 100 µl/well) on microtiter plates (Nunc, Maxisorb). All subsequent incubations were in 100 µl volumes at room temperature.

The plates were washed (3x) with PBS, 0.02% Tween and test samples were added to the plates for 1 hr. Again the plates were washed 3 times with PBS-Tween and biotinylated purified monoclonal antibody against Der p 2 (mAb αDpx [16]), diluted in PBS-Tween, 0.2% gelatin (final concentration 3 µg/ml) was added for 1 hr. After washing (3 times with PBS-Tween), the plates were incubated with streptavidin-horseradish peroxidase (strep-HRP, Amersham, UK), 1/1000 diluted in PBS-Tween, for 0.5 hr, washed and developed with a solution of 100 µg/ml of 3, 5, 3', 5'-tetramethylbenzidine (Merck, FRG) with 0.003% H_2O_2 in 0.11 M sodium acetate, pH 5.5. The reaction was stopped by the addition of 2 M H_2SO_4 to the wells. Plates were read at 450 nm in a Titertek Multiskan reader Control values read at 450 nm were subtracted. The concentration was read from a WHO standard curve, assuming that the WHO standard of 100.000 kU contains 500 ng Der p 2 [8,17]. The detection limit was 1 ng/ml.

Der p 1 assay

An inhibition assay analogous to the assay described by Chapman [18] was applied to measure the Der p 1 concentration in the samples. To 0.5 ml protein A-Sepharose 0.05 ml diluted rabbit antibodies, 0.05 ml ^{125}I-labeled Der p 1 [16] and 0.05 of the test extract was added. After centrifugation and washing of the Sepharose, radioactivity bound to the solid phase was counted. The concentration was read from a WHO standard curve, assuming that the WHO standard of 100.000 kU contains 12.5 µg Der p 1 [8,17]. The detection limit was 1.6 ng/ml.

Results

The silverfish assay is shown in figure 1. As can be seen there is no inhibition with house dust mite-, cockroach- or shrimp extract (a particularly rich source of tropomyosin).

We tested the recovery of silverfish protein in house dust. Different numbers of silverfish (1 silverfish is approximately 20 mg) were added to 25 g house dust (fig. 2). The slope of the regression line indicates that a single silverfish corresponds to 450 µg extractable protein.

We were able to detect tropomyosin and silverfish-proteins in house dust. For tropomyosin only two, and for silverfish eleven of the 53 dust samples were below the detection limit. Figure 3 shows the concentration of the antigens in house dust. The correlations between Der p 2 and tropomyosin (Spearman r=0.5, p<0.001) or silverfish (Spearman r=0.28, p=0.02) are shown in figure 4a and b.

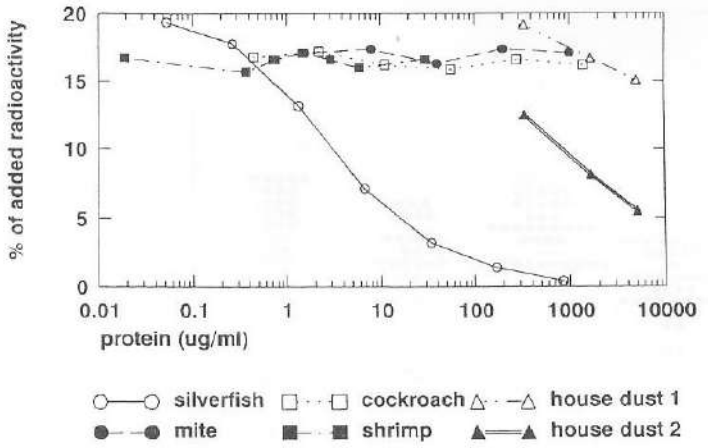

Figure 1. The reference-curve of the silverfish assay. No inhibition occurred with mite-, cockroach- or shrimp extract. House dust 1 is a sample with a low level of silverfish antigen, house dust 2 contains a high level of silverfish antigens.

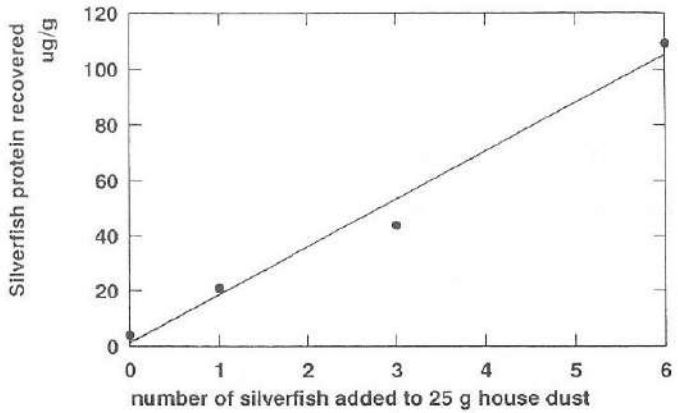

Figure 2. The recovery of silverfish added to house dust.

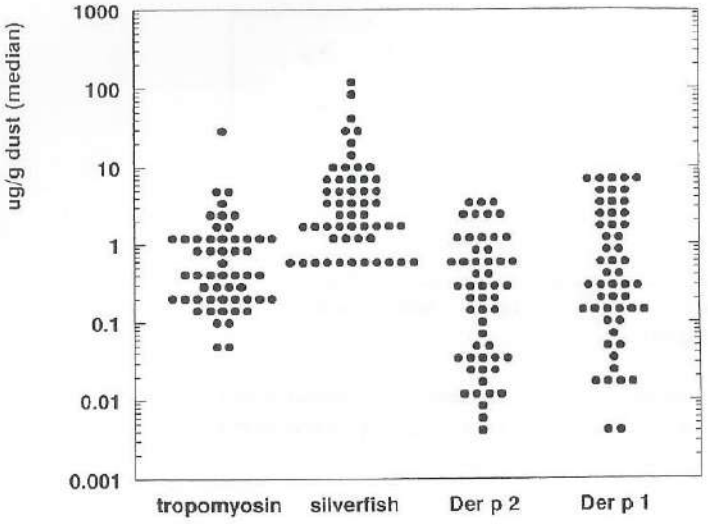

Figure 3. The levels of the different arthropod-antigens in 53 dust samples. The silverfish level is expressed as total silverfish protein.

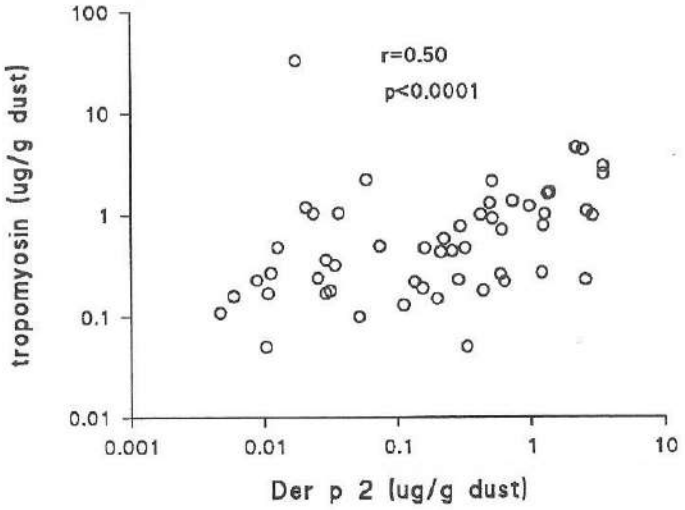

Figure 4a. The relation between Der p 2 and tropomyosin in 53 dust samples. The line represents the ratio tropomyosin/Der p 2 in *D.pteronyssinus* body extract (0.7).

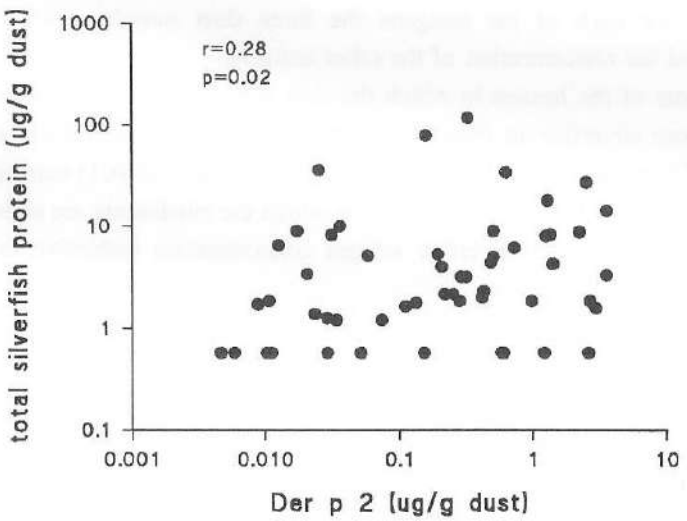

Figure 4b. The relation between Der p 2 and silverfish protein in 53 dust samples.

Table I. Amount of arthropod-antigens in the dust samples which had the highest amount for one of the antigens

tropomyosin	**silverfish**	**Der p 2**	**Der p 1**
µg/g dust	*µg/g dust*	*µg/g dust*	*µg/g dust*
32.99	9.00	0.02	0.15
4.56	8.75	2.20	5.62
4.36	27.00	2.48	5.40
0.05	117.21	0.33	0.60
0.47	78.64	0.16	0.33
0.24	35.88	0.02	0.07
2.51	14.18	3.51	7.35
2.98	3.31	3.51	8.15
0.98	1.58	2.92	6.11
1.09	1.86	2.64	8.15

Table 1 shows for each of the antigens the three dust samples with the highest concentration and the concentration of the other antigens.

The inhabitants of the houses in which the dust was sampled were asked (n=50) if they ever had seen silverfish in their homes (fig. 5). Although significant differences (median "yes": 7.4, median "no": 1.86, Mann-Whitney U-test, p<0.001) exist in silverfish content between the two groups, many homes in which the inhabitants are unaware of the presence of silverfish contain silverfish antigen concentrations indicative of silverfish exposure.

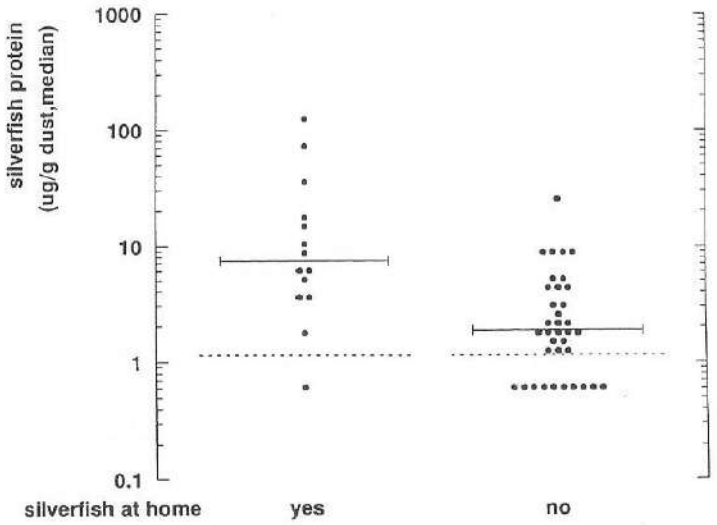

Figure 5. Differences in silverfish protein in house dust from houses in which the inhabitants gave a positive answer to the question: "Do you have silverfish in your home?" and in house dust from houses in which the inhabitants gave a negative answer to that question (Mann-Whitney U-test, p=0.0007).

Discussion

The purpose of this study was to determine levels of insect antigens in house dust. Therefore we developed two immunoassays. In the assay to measure silverfish-antigens we used polyclonal rabbit antibodies against silverfish-extract. This was done because no major allergens of silverfish are known as yet. With the silverfish assay we were able to measure silverfish-antigens in 42 of the 53 dust samples. The result are expressed in µg total silverfish protein. Per adult silverfish we recovered 450 µg protein from dust.

With the tropomyosin-assay it was possible to measure tropomyosin in 51 of the 53 dust samples. In addition to the levels of *D.pteronyssinus* the amount of tropomyosin gives information about exposure to insects. Der p 2 as well as tropomyosin are mite body proteins. The ratio of tropomyosin/Der p 2 in mite body extract (CSL) is 0.7. When house dust only contains mites and no insects, the ratio tropomyosin/Der p 2 is expected to be 0.7. As can be seen in figure 3a this is not the case. Most dust samples contain more tropomyosin than Der p 2 and some dust samples have a high concentration of tropomyosin but low levels of Der p 2, indicating the presence of other arthropods than mites as a source for tropomyosin. The correlation between Der p 2 and silverfish protein (fig. 3b) can probably be explained by the fact that both arthropods need a humid environment.

Although we demonstrated differences in silverfish content between homes with and without "known" presence of silverfish, we also found that in many homes in which the inhabitants were unaware of the presence of silverfish, the dust contained significant silverfish levels. The fact that silverfish avoid light presumably explains why some people are not aware of the presence of silverfish at their homes. Another possibility is that these silverfish levels could be the result of external contact with silverfish allergen as suggested by Wood et al. [19] in case of cat allergens.

In conclusion we have developed two assays to monitor insect exposure. The first assay measures silverfish-antigen. The second assay gives a global assessment of the amount of arthropods in the environment. These assays provide the tools for establishing the clinical significance of insect-exposure and make it possible to evaluate the effectiveness of methods used to reduce allergen levels in households.

Acknowledgements

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Chapter 9

Human proteins in house dust

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Allergy 1993;48:383-384

Allergen analyses of house dust samples are useful for investigations of the role of indoor allergens in population-based studies and for the assessment of allergen exposure of individual patients [3—5]. Allergen levels are generally expressed as amounts per gram dust. A drawback of this method is that dust often contains varying amounts of relatively heavy material, such as sand, which influences the calculated allergen contents. This is especially problematic when small changes in allergen content are to be measured. Sieving cannot separate this irrelevant material from allergen-containing particles.

One possible way to circumvent this problem is to relate the allergen content of a dust sample to the amount of human proteins present in the sample; this relation will provide us with a more stable reference. To investigate the use of human proteins as reference components, we measured human albumin, secretory IgA, and a 12—14-kDa protein from human dander [6] in floor dust from seven bedrooms, collected by vacuum cleaning in a standardized way. After preparation of 10% dust extracts (w/v), we determined albumin and human dander protein by application of a competitive RIA, using radiolabeled albumin or human dander protein and rabbit antibodies to these components [1]. We measured secretory IgA by a sandwich assay, using a polyclonal antibody to the secretory component as capturing agent, and radiolabeled anti-IgA for detection of bound IgA.

Recently, an ELISA for determination of secretory IgA, with a murine-monoclonal antihuman secretory piece has become available [2].

The following results were obtained:

Secretory IgA, median 92 μg per g dust (range 8—353).

14-kDa protein, median 285 μg per g dust (range 2—600).

Albumin, median 170 μg per g dust (range 40—301).

The applicability of a human reference protein is illustrated in figure 1, which shows seasonal influences on the allergen level in house dust. The amount of Der p 2 was determined in monthly dust samples from 13 houses by a noncompetitive sandwich assay [7]. Allergen levels were expressed per g dust as well as per μg secretory IgA. We found no seasonal effect for secretory IgA.

On the basis of these results, we feel that when the aim of the investigation is to measure changes in time in one location, it is better to express the amount of allergen per μg human protein.

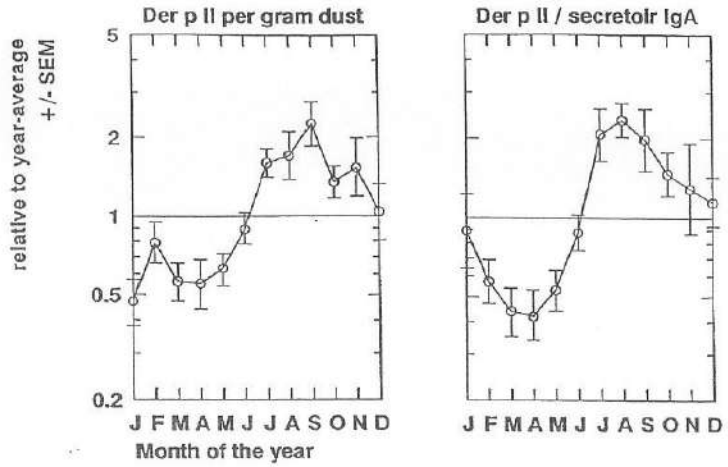

Figure 1. Seasonal influences on the amount of Der p 2, expressed per μg secretory IgA and per g dust.

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Chapter 10

Food allergens in house dust

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Int Arch Allergy Immunol, in press

ABSTRACT

Selected food allergens have been measured in 11 house dust samples. The amount of ovomucoid ranged from 170 to 6,300 ng/g dust. The amount of β-lactoglobulin ranged from <16 to 71 ng/g dust. Ovomucoid levels in some house dust samples are probably sufficiently high to cause sensitization and/or symptoms via inhalation.

INTRODUCTION

Since the establishment of house dust allergy, several studies have investigated the nature and origin of the allergens in house dust [1—3]. It is known that house dust is a complex mixture of multiple allergens. Well-known allergens in house dust are house dust mite, animal danders and moulds. There are however patients with a positive RAST score on house dust without positive RAST scores on *Dermatophagoides pteronyssinus*, dog dander, cat dander or moulds. This suggests the presence of additional, not yet reported, allergens in house dust.

In a recent study on the effect of the season of birth on the risk of developing specific allergic sensitization, we observed that subjects born in late autumn and early winter had an increased risk of developing IgE antibodies to egg white and cow's milk [4]. A similar effect was found for two other allergens: cat dander and dog dander. The level of these dander allergens in house dust did not show a significant seasonal variability, so we assumed that the seasonal effect was a consequence of increased exposure to indoor allergens during winter time.

These findings suggested an analogy between the dander allergens and the food allergens. This raises the question of whether the food allergens should be considered indoor allergens, sensitizing as other indoor allergens via airborne particles.

Food allergens are mostly considered to cause sensitization and allergic symptoms after ingestion. However, in occupational diseases, food allergens are described as inhalant allergens. A well-known example is baker's asthma: an inhalant allergy for flour. It has been estimated that 10—30% of unselected bakers may develop occupational asthma [5,6]. There are also reports of an allergy to inhaled egg material in egg-processing workers [7,8].

The aim of this study was to investigate whether food proteins in dust can cause a positive house dust RAST and if the amount of food proteins is high enough to make it plausible that these proteins can act as inhalant allergens. Therefore ovomucoid levels (as an indicator for egg) and β-lactoglobulin levels (as an indicator for milk) were measured in 11 house dust samples.

MATERIALS AND METHODS

Sera

Two sera were selected from samples submitted for diagnostic IgE tests. The criterion for selection was a positive RAST for chicken egg and milk and a negative RAST for mite, cat, dog and moulds. Serum 1 had a chicken egg RAST of 30% and a milk RAST of 4% (anti-IgE bound, % of added). Serum 2 had a chicken egg RAST of 30% and a milk RAST of 50% (anti-IgE bound, % of added).

House dust extracts

In 11 households dust was collected by vacuum cleaning. The dust was sampled during a month by regular vacuum cleaning (2 times a week) of the entire house. Unsieved dust was extracted (20% w/v) for 4 h in 0.35 *M* Na benzoate.

Radioallergosorbent test

RAST was performed as described previously [9]. For the house dust RAST, the house dust extract was dialyzed against distilled water and lyophilized. Three milligrams of the lyophilized house dust was coupled to 300 mg (equivalent to 1 ml packed gel) of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). RAST inhibition assays were performed as follows: 50 µl allergen solution (1 mg/ml protein) was incubated with 50 µl serum for 2 h at room temperature; this mixture was then incubated overnight with the Sepharose-coupled house dust extract and followed by the normal RAST procedure.

Ovomucoid and β-lactoglobulin assays

New Zealand white rabbits were immunized with ovomucoid purified from egg white [10] or β-lactoglobulin (Sigma Chemical Company, St. Louis, Mo.). An inhibition radioimmunoassay as described by Chapman and Platts-Mills [11] was applied to measure the allergen concentrations in the house dust extracts. Ovomucoid and β-lactoglobulin were iodinated with the chloramine T method. The resulting specific activity was 0.5 MBq/µg ovomucoid and 1 MBq/µg β-lactoglobulin. To 50 µl diluted rabbit antibodies, 50 µl of the test extract, 50 µl ^{125}I-labeled allergen and 0.5 ml protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden, 1 mg/ml) were added. The mixture was incubated overnight under vertical rotation at room temperature. After centrifugation and washing off of nonbound components, the radioactivity bound to the solid phase was counted. The background binding was 0.2% (^{125}I-labeled ovomucoid, % of added) for ovomucoid and 0.7% (^{125}I-labeled β-lactoglobulin, % of added) for β-lactoglobulin. The concentration was read from a reference curve (fig. 1).

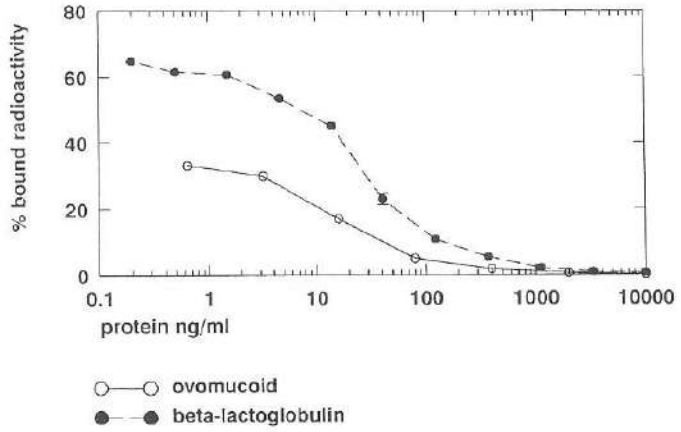

Figure 1. The reference curves for the ovomucoid (0) and β-lactoglobulin (●) assays.

Results

The amount of food proteins found in the floor dust is shown in figure 2. The amount of ovomucoid varies from 170 to 6,280 ng/g dust. The amount of β-lactoglobulin varies from <16 to 71 ng/g dust. One sample was not available for measuring the amount of β-lactoglobulin. The dust samples were collected by vacuum cleaning of the entire house; to be sure that not all the food allergens came from the kitchen floor, we also measured ovomucoid levels in five samples of dust only from the bedroom. Ovomucoid was still detectable and ranged from 100 to 500 ng/g dust. From the household with the highest amount of ovomucoid (6,280 ng/g dust) we collected another dust sample 1 month later. The amount of ovomucoid was then 10,850 ng/g dust. The latter dust extract was coupled to Sepharose and tested in the RAST. Serum 1 had a house dust RAST of 22.6% (anti-IgE bound, % of added). The house dust RAST was inhibited from 22.6 to 8.2% with ovomucoid; this is an inhibition of >60%. Serum 2 had a house dust RAST of 17.1%. The house dust RAST was inhibited from 17.1 to 8.8% with ovomucoid; this is an inhibition of >40%.

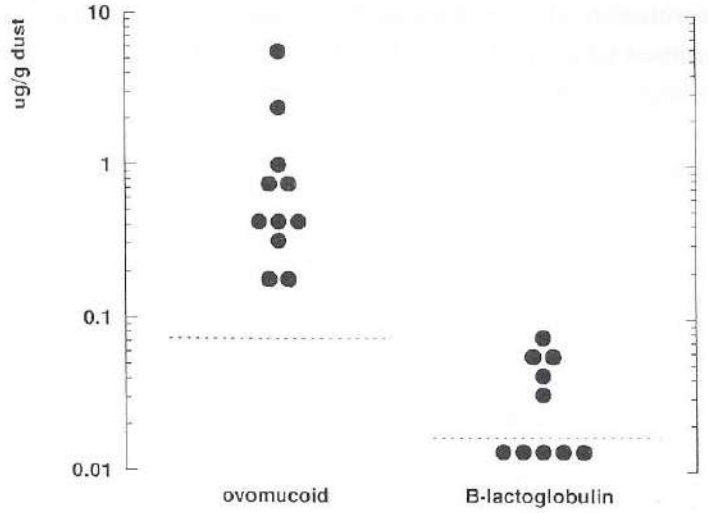

Figure 2. The amount of ovomucoid and β-lactoglobulin in 11 house dust samples.
0.073 μg/g dust = detection limit ovomucoid
0.016 μg/g dust = detection limit β-lactoglobulin

Discussion

The results show that the levels of egg and milk proteins in house dust are high enough to give a positive house dust RAST with egg- and milk-allergic patients. The question arises of whether exposure to these allergens by inhalation of house dust is clinically relevant. In case of house dust mite allergy, it is shown that 2 μg *D.pteronyssinus*/g dust is enough to cause sensibilization [12,13]. We found ovomucoid levels above 2 μg/g dust, so the concentration of food allergens in dust is presumably enough to cause sensibilization. We have not yet established the size of the particles carrying the allergens, so it is unclear whether the allergens become airborne to the same extent as mite or dander allergens do.

One of the possible arguments against the idea of sensitization via the inhalation route is that the time of appearance of IgE antibodies to food allergens is distinctly earlier than that to inhalant allergens. This could be explained by assuming that priming to food allergens occurs already in utero.

In conclusion, sensitisation of food allergens by inhalation of house dust is a distinct possibility. In sensitized subjects the levels of food proteins in house dust are high enough to contribute to allergic symptoms and/or airway inflammation.

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Chapter 11

Summary/Samenvatting

Summary

The symptoms of inhalant allergy are known to be determined by allergen-exposure, IgE antibody level, mediator release and organ sensitivity for these mediators. Allergy test measure an overall effect of several of these factors. Allergen extracts used for diagnostic tests are in general complex mixtures. There are significant differences between patients in recognition patterns of the IgE response to the components of these mixtures. The development of monoclonal antibodies enabled the isolation of major allergens. The use of major allergens, instead of complex allergen mixtures, has made it possible to quantify the interaction of IgE and allergen in allergic reactions. Monoclonal antibodies are also applied for the development of specific and sensitive immuno-assays for the detection of allergens.

In this thesis, the factors that influence the IgE-mediated allergic reaction are studied in two ways. First, by comparing tests in a group of patients, the influence of factors that are involved in some tests but not in others, can be estimated. An alternative method is to vary one factor in an individual patient (e.g. IgE antibody level) while keeping the other factors constant.

In *chapter 2* we studied the relation between RAST, histamine release test and skin test in 43 patients with asthma and/or rhinitis by using purified major allergens (Der p 1, Der p 2, Fel d 1, Lol p 1 and Lol p 5). We found a similar biological activity for the major allergens. With similar levels of specific IgE, the amount of allergen required for a positive skin test differed between the patients as much as a factor 100. In addition to IgE antibody level, total serum IgE and type of IgE antibody response contributed significantly to the skin test threshold for allergens.

The influence of natural occurring specific IgG antibodies in the skin test is discussed in *chapter 3*. The relation between RAST and skin test was studied in 59 cat-allergic patients by using the major allergen Fel d 1. The levels of specific IgG between 0.25 and 3.5 μg/ml, induced by natural exposure, had an inhibitory effect on the early phase skin reaction.

In *chapter 4* we studied bronchial reactions after allergen challenge in 20 subjects with low skin reactions to *Dermatophagoides pteronyssinus* or cat dander. This group of subjects with low levels of allergic sensitization showed significant early and late reactions, as well as induction of non-specific bronchial hyperresponsiveness after bronchial allergen challenge.

Patients with allergic asthma have higher levels of non-specific bronchial responsiveness than patients with allergic rhinitis. In *chapter 5* we investigated whether the difference in non-specific bronchial responsiveness was related to the type and degree of inhalant allergy. In 136 patients with allergic asthma a correlation was found between non-specific bronchial responsiveness and IgE against indoor allergens and percentage of predicted FEV_1. Levels of specific IgE antibodies against indoor allergens differed between allergic asthma and allergic rhinitis. After correction for the level of specific IgE against common indoor allergens and percentage of predicted FEV_1, the difference in non-specific bronchial responsiveness between patients with asthma and patients with rhinitis persisted.

As yet, it is unclear whether allergy to insects plays a role in The Netherlands. Therefore, the prevalence of IgE antibodies against insects in patients with inhalant allergy in The Netherlands was estimated (*chapter 6*). About 30% of the house dust mite allergic patients in The Netherlands had IgE antibodies reactive with silverfish, cockroach and/or chironomid. In allergic patients without IgE antibodies against house dust mite, less than 5% had IgE antibodies reactive with these insects. Cross-reactivity existed between *D.pteronyssinus* and insects.

In *chapter 7* we describe a monoclonal antibody to *D.pteronyssinus* that cross-reacted with an IgE-binding antigen present in insects and *Crustaceae*. This monoclonal antibody presumably recognized tropomyosin which was shown to be involved in cross-reactivity between mite, shrimp and insects.

To be informed about exposure-levels, we developed two immuno-assays for insect allergens and measured insect allergens in house dust (*chapter 8*). We detected silverfish antigen in 42 of the 53 dust samples. A second assay, measuring invertebrate tropomyosin, proved to be useful for global assessment of the level of arthropod-derived material in house dust.

In *chapter 9* a method to express the amount of allergens in house dust per μg human protein instead of per gram dust, was evaluated as an alternative measure to express exposure-levels. In monthly taken dust samples from 13 homes, Der p 2 was expressed per gram dust and per μg secretory IgA. Seasonal variation of the Der p 2 in house dust became more clear if the amount of Der p 2 was expressed per μg secretory IgA.

House dust is a complex mixture of multiple allergens. Well-known allergens in house dust are house dust mite, animal dander and mould components. In *chapter 10* we report the presence of egg- and milk-derived food proteins, ovomucoid and beta-lactoglobulin, as potential airborne allergens in house dust. The amount of ovomucoid in 11 house dust samples ranged from 170 to 6300 ng/g dust. The amount of beta-lactoglobulin ranged from <16 to 71 ng/g dust. Ovomucoid levels in some house dust samples were probably

sufficiently high to cause sensitization and/or symptoms via inhalation.

Samenvatting

De symptomen van inhalatie allergie worden bepaald door de mate van blootstelling aan allergenen, de hoeveelheid IgE antistoffen, de vrijmaking van mediatoren en de gevoeligheid van neus en longen voor deze mediatoren. Diagnostische testen voor allergie meten een of meerdere van deze factoren. Voor deze diagnostische testen wordt meestal gebruik gemaakt van allergeen-extracten. Deze allergeen-extracten zijn complexe mengsels van glycoproteinen. Tussen patiënten zijn er duidelijke verschillen in herkenningspatroon van de IgE respons op de verschillende glycoproteinen. Door de ontwikkeling van monoclonale antilichamen is het mogelijk om zuivere allergenen te verkrijgen. Door deze zuivere allergenen, in plaats van complexe extracten, te gebruiken in diagnostische tests, is het mogelijk de interactie tussen IgE en allergeen in allergische reacties, te quantificeren. Een andere toepassing van monoclonale antistoffen is de ontwikkeling van specifieke en gevoelige immuno-assays voor detectie van allergenen.

De verschillende factoren die de allergisch reactie beïnvloeden worden in dit proefschrift op twee manieren bestudeerd. Door verschillende testen in meerdere patiënten te vergelijken kan de invloed van factoren die in de ene test wel en in de andere test niet betrokken zijn, worden bepaald. Een andere manier om het belang van een enkele factor te bepalen is door binnen een patiënt deze factor te laten verschillen terwijl de andere factoren constant blijven.

In *hoofdstuk 2* bestudeerden we de relatie tussen RAST, histamine release test en huidtest in 43 patiënten met astma of rhinitis met behulp van gezuiverde allergenen (Der p 1, Der p 2, Fel d 1, Lol p 1 en Lol p 5). We vonden een vergelijkbare biologische activiteit voor de verschillende major allergenen. De hoeveelheid allergeen die nodig was voor een positieve huidtest, bij vergelijkbare hoeveelheid specifieke IgE antilichamen, verschilde meer dan een factor 100 tussen patiënten. Behalve de hoeveelheid specifieke IgE antilichamen, droegen ook de hoeveelheid totaal IgE en het type van de antilichaam respons (bijvoorbeeld affiniteit of epitoop herkenningspatroon) significant bij aan de huidtestdrempel voor allergeen.

De invloed van specifieke IgG antistoffen, ontstaan door natuurlijke expositie, op de huidtest, wordt besproken in *hoofdstuk 3*. De relatie tussen huidtest en RAST voor het belangrijkste kat allergeen Fel d 1, werd bestudeerd in 59 kat allergische patiënten. Gevonden werd dat specifiek IgG titers tussen de 0.25 en 3.5 µg/ml, geïnduceerd door natuurlijke expositie, een remmend effect hadden op de vroege huidreactie.

In *hoofdstuk 4* bestudeerden we bronchiale reacties na een allergeen-provocatie in 20 individuen met een lage huid reactiviteit voor *D.pteronyssinus* of kat. In deze groep van

personen met een laag niveau van allergische sensitisatie werden significante vroege en late reacties en een inductie van aspecifieke bronchiale hyperreactiviteit gevonden na bronchiale allergeen provocatie.

Patiënten met astma hebben over het algemeen een sterkere aspecifieke bronchiale hyperreactiviteit dan patiënten met rhinitis. In *hoofdstuk 5* is onderzocht of dit verschil in bronchiale hyperreactiviteit is gerelateerd aan het soort of de ernst van de inhalatie-allergie. In 136 patiënten met allergisch astma werd een correlatie gevonden tussen de aspecifieke hyperreactiviteit en IgE tegen 'binnenshuis' allergenen en FEV_1 (% van voorspeld). De hoeveelheid specifieke IgE antilichamen verschilde tussen de patiënten met allergisch astma en de patiënten met allergische rhinitis. Na correctie voor de hoeveelheid specifiek IgE tegen 'binnenshuis' allergenen en FEV_1 (% van voorspeld), bleef er nog steeds een verschil in aspecifieke hyperreactiviteit tussen patiënten met astma en patiënten met rhinitis aantoonbaar.

Tot nu toe is het onduidelijk of allergie voor insekten van belang is in Nederland. Daarom is het voorkomen van IgE antistoffen tegen insekten onderzocht (*hoofdstuk 6*). Gevonden werd dat ongeveer 30% van de huisstofmijt positieve patiënten in Nederland IgE antistoffen tegen zilvervis, kakkerlak of rode muggelarve heeft. In allergische patiënten zonder IgE antistoffen tegen huisstofmijt heeft minder dan 5% IgE antistoffen tegen deze insekten. Kruisreactiviteit tussen *D.pteronyssinus* en insekten werd aangetoond.

In *hoofdstuk 7* beschrijven we een monoclonaal antilichaam tegen *D.pteronyssinus*, dat kruisreageert met een IgE-bindend antigeen in insekten en *Crustaceae*. Dit monoclonale antilichaam herkent waarschijnlijk tropomyosine waarvan aangetoond werd dat het betrokken is in kruisreactiviteit tussen huisstofmijt, garnaal en insekten.

Om geïnformeerd te zijn over expositie, ontwikkelden we twee immuno-assays voor insekten allergenen en maten insekten allergenen in huisstof (*hoofdstuk 8*). We detecteerden zilvervis antigenen in 42 van de 53 stof-monsters. Een tweede assay, die tropomyosine van invertebraten aantoonde, was geschikt voor een globale weergave van de hoeveelheid overblijfselen van artropoden in huisstof.

In *hoofdstuk 9* wordt een methode geëvalueerd om de hoeveelheid allergeen in huisstof per µg menselijk eiwit uit te drukken, als alternatief voor de gebruikelijke manier om expositie weer te geven als allergeen per gram stof. In maandelijks verzamelde stof-monsters van 13 huizen werd Der p 2 uitgedrukt per gram stof en per µg secretoir IgA. De seizoensvariatie van Der p 2 in huisstof is duidelijker wanneer de hoeveelheid Der p 2 wordt uitgedrukt per µg secretoir IgA.

Huisstof is een complex mengsel van verschillende allergenen. Bekende allergenen in huisstof zijn huisstofmijt, haren en schilvers van huisdieren, en schimmels. In *hoofdstuk 10* beschrijven we de aanwezigheid van de voedseleiwitten, ovomucoid (uit ei) en beta-lactoglobuline (uit melk), als mogelijke inhalatie allergenen, in huisstof. De hoeveelheid ovomucoid in 11 huisstof monsters varieerde van 170 tot 6300 ng/g stof. De hoeveelheid beta-lactoglobuline varieerde van <16 tot 71 ng/g stof. De hoeveelheid ovomucoid in sommige huisstof monsters is waarschijnlijk hoog genoeg om sensibilisatie en/of symptomen via inhalatie te veroorzaken.

Dankwoord

Zonder de hulp van velen was dit proefschrift niet tot stand gekomen. Op deze plaats wil ik iedereen die mij geholpen heeft hartelijk bedanken.

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Op het allergie lab van het CLB heb ik een hele leuke tijd gehad. Jolanda wil ik behalve voor alle praktische hulp vooral ook bedanken voor het scheppen van een goede sfeer. Als ik na een vakantie er tegen op zag weer te beginnen, viel het allemaal weer mee als zij me schreeuwend op de gang verwelkomde. Gerrard heeft mij enorm geholpen met het opstarten van de histamine release metingen. En toen het eenmaal liep heeft hij eindeloos histamine gemeten. Later nam Marcel het van hem over. Zelfs toen ik eigenlijk al weg was, was hij bereid om nog proeven te doen waar de referenten om vroegen. Jaap was er voor de "moeilijke" technieken. Altijd was hij bereid mij te helpen, bijvoorbeeld met het gieten van een gradient-gel. Dankzij Ed konden alle patiënten-gegevens verwerkt worden in een data-base. Zijn computerkennis heeft mij menigmaal geholpen. Ronald volgde op de achtergrond mijn lab werkzaamheden. Steeds als het nodig was had hij goede adviezen. De tijd met Sonja en Ramon was erg produktief. Behalve de gezelligheid van het samen ergens aan werken, hebben zij ook een duidelijke bijdrage geleverd aan de resultaten van dit onderzoek. Verder was er de vrolijkheid van Janine, de kameraadschap van Florine, de nauwkeurigheid van Marja, het doorzettingsvermogen van Suzanne, de grappen van Gert-Jan, de nuchterheid van Wim en natuurlijk het bier en de kunst van Bouke. Maurits, die dit onderzoek voortzet, wens ik veel succes.

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Agnes

Abbreviations

ANOVA	-	analysis of variance
AU	-	arbitary unit
BU	-	biological unit
CI	-	confidence interval
CV	-	coefficient of variation
Der p	-	*dermatophagoides pteronyssinus*
ELISA	-	enzyme-linked immunosorbent assay
Fel d	-	*felis domesticus*
FEV_1	-	forced expiratory volume in 1 second
GM	-	geometric mean
GSE	-	geometric standard error
GSEM	-	geometric standard error of the mean
IQR	-	interquartile range
IU	-	international units
IVR-RAST	-	RAST with in vitro reagents
kD	-	kilodalton
Lol p	-	*lolium perenne*
mAb	-	monoclonal antibody
MW	-	molecular weight
O/P	-	observed/predicted
PBS	-	phosphate-buffered saline
PC_{20}	-	concentration causing a 20% fall in FEV_1
PD_{20}	-	dosis causing a 20% fall in FEV_1
pI	-	isoelectric point
RAST	-	radioallergosorbent test
RIA	-	radioimmuno-assay
RU	-	RAST units
SD	-	standard deviation
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	-	standard error
w/v	-	weight/volume

Stellingen behorende bij het proefschrift

Quantitative aspects of the IgE-mediated reaction in patients with asthma and rhinitis

1. Behalve de hoeveelheid specifiek IgE, dragen ook de hoeveelheid totaal IgE en de aard van de IgE respons bij aan de huidtest drempel voor allergenen.
(dit proefschrift)

2. De lage titers specifiek IgG die ontstaan door natuurlijke blootstelling aan allergeen, hebben een remmend effect op de huidtest.
(dit proefschrift)

3. Niet alleen patiënten met allergische rhinitis, maar ook patiënten met niet-allergische rhinitis, hebben een verhoogde aspecifieke hyperreactiviteit van de lagere luchtwegen ten opzichte van gezonde individuen.
(dit proefschrift)

4. Het belang van IgE antistoffen tegen insekten wordt mogelijk overschat vanwege kruisreactiviteit tussen huisstofmijt en insekten.
(dit proefschrift)

5. Zich nat laten scheren bij de kapper is een mogelijke bron van Hepatitis-C infectie.
(The Lancet 1995;345:658)

6. Het verrichten van wetenschappelijk onderzoek geeft een clinicus een evenwichtiger kijk op zijn vak.

7. Samenwerking leidt tot betere resultaten dan competitie.

8. Een kind groot brengen relativeert het tot stand brengen van een proefschrift.

Amsterdam, 5 september 1995

Agnes Witteman