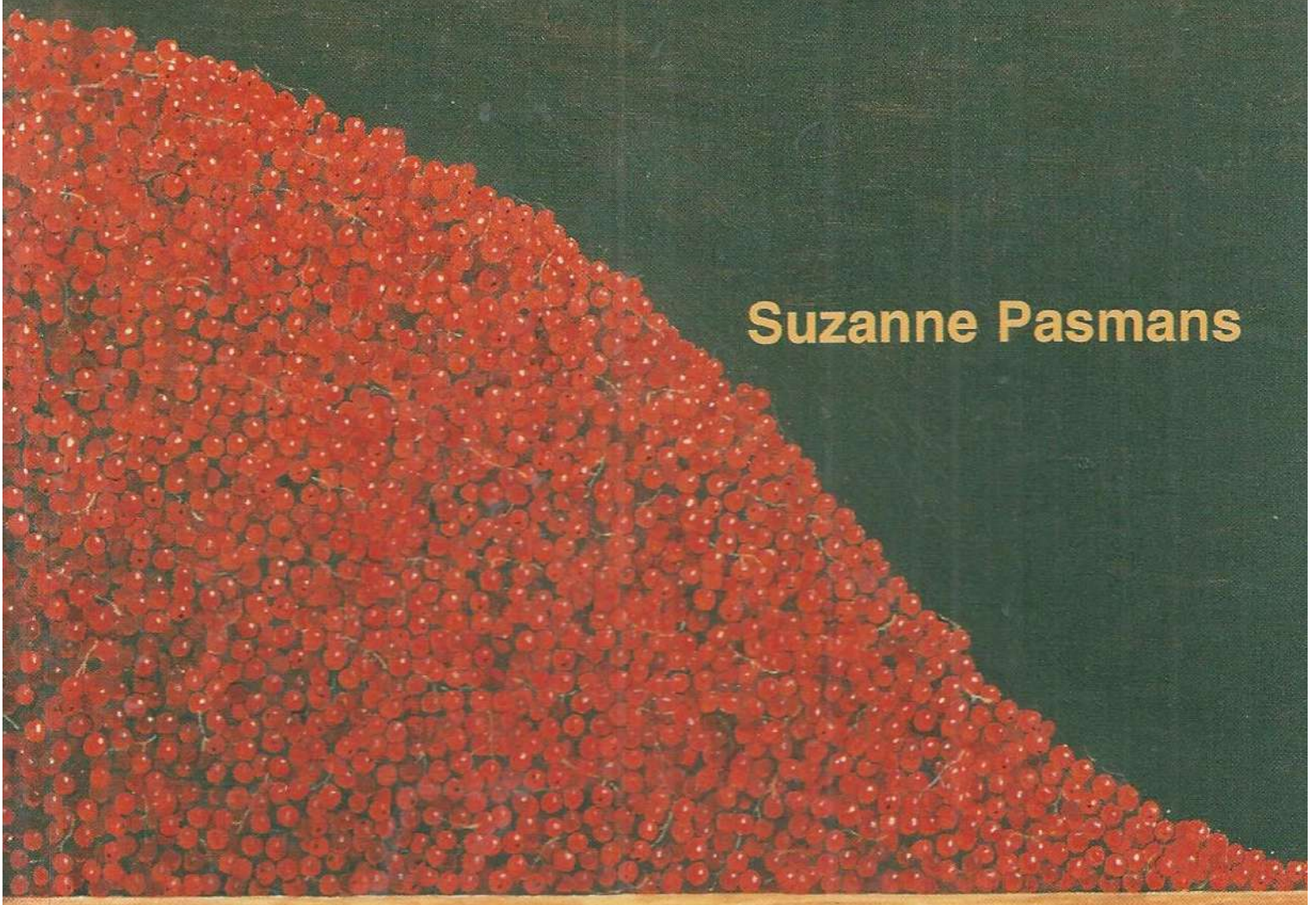
IgE-DEPENDENT HISTAMINE-RELEASING ACTIVITY IN ATOPY



Suzanne Pasmans

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IgE-DEPENDENT HISTAMINE-RELEASING ACTIVITY IN ATOPY

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Entia non sunt multiplicanda praeter necessitatem.

(No more things should be presumed to exist than are absolutely necessary.)

William of Ockham (circa 1284-1349)

In memory of F. Pot, dermatologist

List of abbreviations

BSA	bovine serum albumin
ΔHRF	increase in histamine release between 1 and 60 minutes to HRF-MN-hep, expressed as percentage of the reference IgE^+ serum
DNP	dinitrophenyl
FcεRI/FcεRIα	high affinity IgE-receptor / α-chain of FcεRI
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FEV_1	forced expiratory volume in 1 second
FMLP	formyl-methionyl-leucyl-phenylalanine
FVC	forced vital capacity
HRA/HRF	histamine-releasing activity / factor
HRF-MN	HRA/HRF in the supernatant of SK/SD-stimulated mononuclear cells
HRF-MN-hep	chemokine-depleted HRF-MN supernatant
IL	interleukin
IU	international units (1 IU IgE = 2.4 ng)
MAR	mouse anti-rat
MCP	monocyte chemotactic protein
moAb	monoclonal antibody
NP	nitrophenacetyl
PBS	phosphate-buffered saline
PC_{20}	provocative concentration of histamine eliciting a 20 % decrease of FEV_1
PMA	phorbol-myristate acetate
RAST	radio-allergosorbent test
RBL	rat basophilic leukemia cell
RBL-T	RBL transfected with human FcεRIα

Contents

Chapter 1

INTRODUCTION

Aim of this thesis

Do endogenous IgE-binding factors exist that are capable of triggering mast cells and / or basophils in the late phase and the chronic-allergic reaction ?

These topics will be discussed separately in the following paragraphs. The sequences of events in the allergic reaction to an allergen will be described, including the acute, the late and the chronic-allergic reaction. Subsequently, the role of IgE in the allergic reaction will be outlined as well as the role of the cells that have a high affinity receptor for IgE: the mast cells and the basophilic leucocytes. Next, stimuli that might activate the mast cell and the basophil in the allergic reaction are discussed: IgE-independent stimuli (interleukins and chemokines) and IgE-dependent stimuli (autoanti-IgE, CD23, lectins and autoallergens). Another group of stimuli that activate basophils in an IgE-independent and IgE-dependent way and play a role in the allergic reaction are called "histamine-releasing factors" (HRFs). HRFs are defined in this thesis as a heterogeneous group of proteins produced by several human cells, that cause histamine release from human basophils. Both the cells that produce HRFs (such as lymphocytes and monocytes) and the cells that are activated by HRFs (basophils particularly) are present in the late-phase reaction of an allergic inflammation and in chronic-allergic inflammation. This suggests that HRF might be involved in the late-phase response and in chronic-allergic inflammation.

Allergic reaction: acute, late and chronic response

When an allergen enters the body of patients with allergic asthma, allergic rhinitis or allergic eczema, the allergen can crosslink the IgE present on the surface of mast cells. These mast cells degranulate and release their proinflammatory products: preformed mediators, newly synthesized mediators and cytokines. In the target organs (lung, nose and the skin) these mediators cause the allergic symptoms of the acute response (e.g. in the airways of the lung in patients with asthma: bronchoconstriction, mucosal oedema and hyper-secretion), occurring within 5-10 min after contact with the allergen [1].

Subsequently, some individuals develop a recrudescence of symptoms within 3-11 hr after allergen challenge [1-4]. This late response is characterized by oedema and erythema in the skin [5] and by increased resistance to airflow in the upper and lower airways [6].

The pathogenesis of this late response is unknown. The following factors in the acute allergic response might contribute to this late response: IgE, the mast cell and its mediators and cytokines. IgE involvement was demonstrated not only for the immediate reaction but also for the late-phase reaction in the skin [5] and the lower airways [7]. In this thesis we questioned what endogenous IgE-binding factors might play a role in triggering the cells that have a high affinity receptor for IgE (mast cells and / or basophils) in the late phase and the chronic-allergic reaction.

Microscopic evaluation of the late response in the skin, nose and lungs shows cellular infiltrates that resemble chronic-allergic inflammation: eosinophils, neutrophils, monocytes and lymphocytes [8-13]. Also basophils are present in the late reaction: in the bronchioles of the airways of the lung [14-20], in the skin [8, 21] and in the nose [22-24]. These inflammatory cells are attracted into the affected site by the mediators and cytokines released by the mast cell [2, 25]. Inflammatory changes are seen even in the mildest forms of allergic diseases. At the site of inflammation the inflammatory cells are in turn activated or primed. This process causes the late response and is responsible for the maintance of the process of chronic-allergic inflammation in the target organs. Topical steroids inhibit the late allergic reaction and the influx of inflammatory cells [23, 26, 27] that are due to active inflammation [28]. The chronic-allergic inflammatory response together with the structural changes due to the chronic inflammation in the target organs are probably responsible for the hyperreactivity in the target organs (such as an increased bronchial histamine sensitivity in asthma [29-32]). Allergic patients are usually exposed over a long period of time to very low concentrations of allergens. This changes allergic diseases as asthma, rhinitis and eczema into diseases of chronic-allergic inflammation.

In the chronic-allergic inflammation IgE is bound to the cells with a high affinity receptor for IgE: mast cells and basophils. In the immediate allergic reaction the mast cell is the predominant source of mediators of inflammation (such as histamine and prostaglandin D_2); the pattern of mediator release in the late phase more resembles the response of the basophil (histamine, but no prostaglandin D_2) [15, 33-36]. These data suggest an important role in the late-phase reaction and the chronic-allergic inflammation for the basophils besides the mast cell. But the stimulus for their IgE-dependent activation in the late phase of the allergic reaction is unknown.

Stimuli that might activate basophils in the late-phase reaction

Basophils and mast cells have IgE on their surface bound to high affinity IgE-receptors that after crosslinking can activate these cell types. Moreover, these cells have other receptors by which they interact with IgE-independent stimuli. Basophils and mast cells differ in their response to different stimuli; basophils are considered more excitable cells than mast cells. Different mast cell types have been described, each with a different response pattern [37]. As in the work presented in this thesis responses of basophils are described, the responses on mast cell will not be discussed.

IgE-independent stimuli: interleukins and chemokines

Cytokines are produced by inflammatory cells present in the chronic-allergic reaction. In vitro some cytokines induce histamine release from human basophils: IL-1 [38] and IL-3 [39, 40]. The time course of histamine release is slower compared to anti-IgE and crossdesentization with anti-IgE is not possible. Moreover, the relevance of these results is debatable. These phenomena need high concentrations of cytokines, high concentrations of Ca^{2+} or the presence of D_2O (a microtubuli-stabilizing agent) [41]. More relevant is the priming of basophils by cytokines to respond to other stimuli [42-52].

Chemokines are structurally related cytokines with a molecular weight of approximately 8 kDa. There are two subgroups of chemokines both containing four conserved cysteine residues [53]. The α chemokines have a single amino acid between one pair of the cysteines (CXC) and are encoded on chromosome 4, in contrast to the β group (CC) that are encoded on chromosome 17. All the chemokines bind to heparin, although their relative affinities vary. Chemokines are produced also by the inflammatory cells present in the chronic-allergic reaction. They possess chemotactic activities that attract inflammatory cells to the tissue and subsequently activate these cells [53].

Several chemokines are known to induce rapid degranulation of basophils, particularly RANTES, MCP-1 and MCP-3 [54-61]. MCP-1 and MCP-3 have comparable histamine-releasing potency to which all donors respond. RANTES is less potent in inducing histamine release and not all basophil donors respond to RANTES. IL-8 induces or inhibits histamine release at different concentrations [40, 62].

IgE-dependent stimuli

IgE-dependent stimuli require the presence of IgE on the surface of the basophil. Their likely mode of action is therefore: to bind to the cell-bound IgE, inducing cell activation by crosslinking the IgE-receptors. Several endogenous IgE-binding factors have been described that might potentially be involved in IgE-dependent histamine-releasing activity. Factors that bind to human IgE can be divided in nondiscriminating and discriminating IgE-binding factors depending on the extend of recognition of different IgE molecules: discriminating IgE-binding factors react selectively with some subpopulation of IgE.

Nondiscriminating IgE-binding factors

Autoanti-IgE

IgG anti-IgE antibodies have been found using different testsystems, like RIAs and immunoblotting [63-71]. Allergic patients have IgG anti-IgE that migt be capable of crosslinking the surface IgE and inducing histamine release. When noncrosslinking autoanti-IgE antibodies are bound to cell-bound IgE, this complex might subsequent be crosslinked by addition of another stimulus, for example autoanti-IgG [72]. It is unlikely that IgG autoanti-IgE or autoanti-IgG antibodies are responsible for histamine-releasing activity (HRA) in culture supernatants (see below, because the molecular weight of HRA is reported to be < 45 kDa). Untill now we have not been able to detect convincingly IgG anti-IgE using the test systems described in the literature. An often used system is based on murine anti-human IgE catching IgE antibodies; one of the main difficulties in our hands is, that many sera contained IgG anti-mouse activity [73, 74] (unpublished results).

Soluble CD23 (sCD23)

Soluble CD23 (sCD23) might activate basophils and is produced by the cells present in the chronic-allergic reaction: e.g. lymphocytes and monocytes [75]. Soluble CD23 consists of cleavage fragments (37 kDa, 33 kDa, 25-27 kDa and 12 kDa) of the low affinity receptors for IgE (CD23) and generated by autoproteolysis. The 37 kDa, 33 kDa and 25-27 kDa fragments of sCD23 are IgE-binding proteins. sCD23 has been described to play a role in the regulation of the IgE-synthesis [75]. However, sCD23 did not have histamine-releasing activity [76].

Discriminating IgE-binding factors

To differentiate allergic from nonallergic individuals an IgE-binding factor relevant in the chronic-allergic reaction has to discriminate between some subpopulations of IgE.

Lectins such as human ε binding protein (εBP)

The involvement of IgE-dependent basophil activation might also be due to binding by a lectin-like molecule of differentially glycosylated IgE, due to post-translation modification. The human IgE molecule is coded for by one gene [77], but isoforms have been described based on differential splicing. IgE is heavily glycosylated, e.g. with N-linked oligosaccharides. Differential glycosylation of myeloma IgE is reported: one myeloma protein (ND) contains 30 % more carbohydrates than another IgE myeloma (PS) [78]. HRFs (see below), culture supernatants of cells present in the allergic inflammation, were unable to induce IgE-mediated histamine release in basophils with these different IgE myelomas [79].

A β-galactoside-specific lectin with S-type carbohydrate-recognition capacity that binds human IgE is εBP (also named L-34, RL-29/HL-29, Mac-2) [80-82]. This protein of 31 kDa is present in many tissues. Both εBP and CD23, the low affinity receptor for IgE, have a lectin domain and bind IgE. In contrast to εBP the binding of CD23 to IgE is not carbohydrate dependent. εBP distinguishes between different glycoforms of IgE. However, addition of εBP to basophils did not induce histamine release [83].

Autoallergens

An explanation for IgE-dependent activation in the chronic-allergic reaction might be that an autoallergen is released by the inflammatory cells that can activate the basophils and mast cells.

One candidate autoallergen might be the allergen involved in human dander skin reactivity. Storm Van Leeuwen et al. [84] were the first to report positive immediate type skin reactions to human dander in atopic dermatitis patients. Atopic dermatitis patients also reacted to extracts from their own dander [85]. Berrens and Guickers demonstrated IgE-binding in a RAST to human dander [86]. Probably much of this reactivity is due to mites and yeasts, such as Pityrosporum orbiculare [87, 88] (unpublished results).

An autoallergen that has been described to bind human IgE and to induce histamine release in basophils, is human profilin. Profilin is a protein found in all eukaryotic cells. Profilin in pollen from grasses, trees and weeds was found to be a major crossreactive component, largely responsible also for crossallergenicity between pollen and vegetable foods [89, 90]. Crossreactivity was shown for human IgE antibodies between profilin from birch pollen and profilin from human platelets [91]. Both human and birch profilin were found to release histamine from basophils [91]. No information is available on release of profilin in vivo. In this thesis (in chapter 6) is shown that human profilin is not an IgE-dependent HRF (see below) for basophils.

Histamine-releasing factors

Dvorak et al. [92] reported about "cutaneous basophil hypersensitivity" in guinea pigs and speculated that antigen stimulation could induce lymphocytes to synthesize and release factors to attract basophils and trigger mediator release. Thueson et al. [93, 94] were the first to report such a factor in the supernatant of cultured human mononuclear cells that caused histamine release from human basophils. Initially, this factor was called histamine-releasing activity (HRA) and subsequently termed histamine-releasing factor (HRF). These initial studies [93, 94] have been confirmed by other investigators [95-99]. The mechanism of histamine release has been reported to be IgE-dependent [100, 101] or IgE-independent [94]. Variability between individuals has been described for the production of HRF and for the response to HRF.

In the allergic reaction, discrepancies exist between the level of allergen specific IgE and the severity of clinical symptoms of allergic individuals such as the late-phase reaction (6 to 11 hours after allergen exposure) and the chronic-allergic response [102]. Lichtenstein et al. [102] speculated that IgE-dependent histamine-releasing factor (HRF) might be involved. These differences might be explained by variability in production of HRF and / or reactivity to HRF. This thesis will be focused on the response to HRF and on differences between individuals in their response to HRF.

HRFs cause non-cytotoxic, calcium and temperature-dependent mediator release in vitro from human basophils [103]. Both histamine and leukotriene D are released from basophils stimulated with HRF [104].

Also lung mast cells [95, 105] and mast cells from synovial tissue [106] degranulate on exposure to HRF. However, IgE-dependency is unknown. The sensitivity of mast cells for HRF is lower than that of basophils [36, 93-95, 107]. Lung mast cells usually respond poorly to known stimuli compared to cutaneous and mucosal mast cells of the gastrointestinal tract [55].

In animals the IgE-independent HRFs: MIP-1α, MCP-1, MCP-2 and MCP-3 induce mast cell degranulation and monocyte infiltration on intradermal injection [108-110]. The degranulation by MCP-1 was only modestly compared to MIP-1α.

Criteria for IgE-dependency of histamine release [79]

The IgE-dependency of the activity of some HRFs has been demonstrated in several ways:

1) The kinetics of IgE-dependent HRF-induced histamine release resemble those of IgE-mediated stimuli [79] in that they are slower than MCP-1 and FMLP, but faster than PMA [111] (figure 1). The maximal histamine release response has been reached for FMLP after 1 minute, for anti-IgE after 15 minutes and for PMA after 60 minutes (figure 1). A measure of IgE-mediated release is the increase in the histamine release between 1 and 60 minutes (chapter 3).

2) After crossdesensitization basophils by incubating them at 37 °C with optimal concentrations of a histamine-releasing anti-IgE antibody in the absence of calcium, the subsequent release with IgE-dependent HRF in the presence of calcium was ablated [79].

3) Inhibition of the IgE-dependent HRF release by wortmannin (chapter 2), a potent inhibitor of IgE-mediated release [111] by inhibiting PI3 kinase [112, 113].

4) Removal of IgE from the basophil surface with lactic-acid buffer decreased the response to IgE-dependent HRF [79, 114]. This procedure leads to removal of 80-90 % of the IgE from the surface of the cells.

5) Passive sensitization of lactic-acid treated cells with IgE^+ serum renders these cells responsive to IgE-dependent HRF in contrast to IgE^- serum (see below: IgE heterogeneity) [79].

Preincubation of lactic-acid treated cells with IgE^- serum inhibits the ability of IgE^+ serum to bind to the basophil and to interact with IgE-dependent HRF [79].

Affinity purified IgE derived from an IgE^+ individual sensitizes lactic-acid treated cells for the release induced by IgE-dependent HRF [79].

IgE heterogeneity; definition of IgE^+/IgE^-

The IgE-dependent HRF interacts with IgE of some but not all donors, indicating a functional heterogeneity of IgE. IgE^+ is IgE which after sensitization of lactic-acid treated basophils renders them responsive to HRF; the remaining IgE molecules are IgE^- [79].

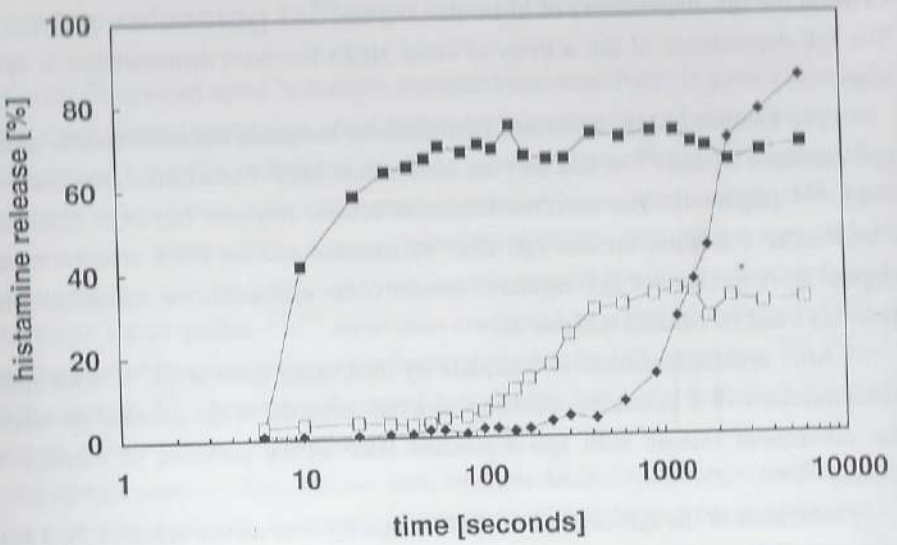

Figure 1. *Time course of histamine release. Purified basophils were incubated at 37 °C with PMA (100 ng/ml, closed diamonds), FMLP (1 μM, closed squares) or anti-IgE (100 ng/ml, open squares). After incubation, histamine release was stopped by diluting the cells in ice-cold 0.9 % NaCl. Printed in slightly modified form with permission from dr. E.F. Knol et al. 1991 Eur. J. Immunol. 21: 881-885.*

Characterization

Most of the activity of the IgE-independent HRA has been characterized. Supernatants were prepared by activation of mononuclear cells with streptokinase/streptodornase (SK/SD, a recall-antigen, reflecting the anti-streptococcal immune response) [115, 116]. After fractionation on a QMA anion exchange column with a salt gradient, HRF activity was recovered in three different fractions; the effluent, at 0.5 M NaCl and a peak preceding the 1.0 M NaCl wash. These three different peaks were subjected to SDS gel electrophoresis and revealed heterogeneous areas of HRF activity with predominant activity in three molecular mass ranges: 11-12 kDa, 15-17 KDa and 41 kDa. The first peak contained CTAP-III/NAP-2. The second peak contained: IL8, MCAF/MCP-1 and CTAP-III/NAP-2. The third peak contained: RANTES, MIP-1α and β. The presence of MCP-2 or MCP-3 was not tested. Peak 1 and 3 were active on basophils from both atopic and nonatopic individuals, whereas peak 2 was

preferentially active on cells from atopic donors. Il-8 has the size and specificity of the histamine-releasing inhibitory factor (HRIF) that has been described [117].

Recently one of the IgE-dependent HRFs has been characterized by MacDonald et al. [118]. Supernatant from the U937 cell line (a monocyte-macrophage-like cell line) was fractionated by chromatography on Sephadex G75, MONO Q and Superdex 75 columns. Four bands were obtained: a 60 kDa (later found to be albumin), a 39 kDa band that was N-terminally blocked and a doublet at 23 kDa. The upper band of the doublet was nucleolin, a nucleophosphoprotein involved in the synthesis and maturation of ribosomes. An antibody to nucleolin did not inhibit IgE-dependent HRA. The lower band in the doublet was p21, which function is unknown. The cDNA of p21 was inserted into the pGEX-2T plasmid and induced with IPTG to produce a protein fused with gluthionine-S-transferase. The histamine release induced by this protein was found to be dependent on IgE^+ on the surface of the basophil. The recombinant material is active only at high concentrations (10-100 μg/ml); this concentration is in orders of magnitude higher than expected for natural IgE-dependent HRF. No information on the IgE-binding activities of the recombinant material is available.

The role of interleukins in HRA was investigated by inhibition of the activity with antibodies. A combinantion of anti-IL1, anti-IL3, anti-TNFα, anti-GM-CSF and anti-IL8 abolished a major portion of the HR activity [50]. This might indicate that these cytokines possess a priming effect, and that the detectable HRF activity reflects the net result of interaction of the various cytokines present in the culture supernatant.

Production

IgE-independent HRFs have been obtained *in vitro* from a variety of cell sources including vascular endothelium cells, B and T lymphocytes [119], mixed mononuclear cell cultures [119], thoracic duct lymphocytes [120], embryonic cells [121]. Mononuclear cells produce IgE-independent HRF either spontaneously or upon stimulation with mitogens, bacterial antigens, and allergens [94, 122, 123]. IgE-independent HRF appeared in the supernatant as early as four hours after the start of the cell culture and its production level remained high even at 48 hours [50, 124]. In *body fluids* IgE-independent HRFs have also been found. IgE-independent HRFs have been recovered from blister fluid obtained from chronic idiopathic urticaria [125]. MCP-1 and IL-8 were detected in nasal lavage fluids [126]. RANTES and MIP-1α were measured in bronchoalveolar lavage fluid (BAL) fluids [127]. In bronchial tissue of asthmatic patients MCP-1 expression was reported [128].

IgE-dependent HRFs have been obtained *in vitro* from a variety of cell sources including alveolar macrophages [100, 102, 105], platelets [129], mononuclear cells [114],

T cells [114], B cells [114], U937 monocyte/macrophage-like cell line [102, 130] and RPMI 8866 B cell line [102]. In *body fluids* the IgE-dependent HRF has been found in nasal lavages [131], late-phase blister fluids [132] and bronchial alveolar lavages [114, 133]. All these fluids contain both IgE-dependent and IgE-independent HRF.

Clinical relevance

Association between the responsiveness to HRF and disease status

Most HRF-preparations were ill-defined mixtures of IgE-dependent and IgE-independent HRF activity.

Some investigators have shown that HRFs are also active *in vivo*: Alam and Rozniecki [134] reported that lymphocyte supernatant induced bronchoconstriction and a wheal and flare reaction in asthmatic patients which was not due to histamine in the supernatants. Weiss et al. [135] showed that 9 out of 12 patients with extrinsic asthma had immediate wheal and flare reactions to intradermal administration of autologous platelet-derived HRF, while non of the 5 healthy controls individuals, the 5 patients with intrinsic asthma or the 5 patients with allergic rhinitis responded.

The basophils of allergic donors, especially donors with asthma, released a higher percentage of cellular histamine in response to HRF derived from mononuclear supernatants than basophils from nonallergic individuals [114, 136]. The response of basophils from allergic patients with asthma was poorly associated with the severity of disease [114].

In the clinical studies reported in the following paragraphs IgE-dependency of at least part of the HRF activity was demonstrated.

HRF obtained from nasal and bronchial lavage and lung macrophages did activate selectively the cells of atopic donors and not from nonatopic donors [100, 102, 114, 137].

In 55 patients with ragweed hayfever, a significant correlation between the intensity of the symptoms in the late-phase reaction and the basophil histamine release to IgE-dependent HRF was found [138]. A decrease in responsiveness was seen after the ragweed season [139].

Basophils from allergic asthmatics responded to a platelet-derived HRF, whereas basophils from nonallergic asthmatics did not. In the allergic asthmatics, the responsiveness to HRF was associated with metacholine sensitivity and asthma symptoms [140].

Association between the production of HRF and disease status.

The stimulation of HRF synthesis by delayed hypersensitivity antigens was correlated with the delayed cutaneous response of the cell donor to these antigens [93, 99]. Alam et al.[141] studied 20 patients with mild to severe asthma. They cultured peripheral mononuclear cells for 24 hr and correlated the spontaneous production of HRF by these cells with the patients' state of bronchial hyperreactivity as assessed by inhaled histamine. The same was observed for subjects with extrinsic and intrinsic asthma [141]. An inverse correlation was noted between the magnitude of the spontaneous HRF production by mononuclear cells and the sensitivity of the patient to bronchial histamine [141].

The quantity of HRF released by mononuclear cells from pollen sensitive patients is increased when these cells are cultured with allergen (pollen extract), especially during the pollen season [124]. Kuna et al. [142] investigated a group of grass-pollen allergic patients before and after 2 years of preseasonal immunotherapy. These investigators found a good correlation between the change in spontaneous HRF production by mononuclear cells and the change in PC_{20} for histamine upon allergen provocation. This observation was confirmed by others [123, 143].

MCP-1 and IL-8 were detected in nasal lavage fluids of rhinitis patients with symptoms during the pollen season [126]. Higher levels of MCP-1, RANTES and MIP-1α were measured in BAL fluids from asthmatics compared to a healthy control group [127]. However, the level of IL-8 was higher in the healthy control group [127]. In bronchial tissue of asthmatic patients MCP-1 expression as determined by immunohistochemical techniques, was increased [127, 128].

In the clinical studies reported in the following paragraphs IgE-dependency of part of the HRF activity was demonstrated.

Sampson et al. [144] reported that mononuclear cells of food allergic children with atopic dermatitis in culture spontaneously secrete an IgE-dependent HRF. After a diet that eliminated the offending food allergen for a long time the spontaneous production of IgE-dependent HRF decreased [144].

The production of p21 was investigated in the mononuclear cells derived from allergic and nonallergic donors. Although cells from atopic donors secreted higher levels of histamine-releasing activity, both groups had comparable expression of p21 mRNA in their mononuclear cells [145].

Summary of the clinical relevance of HRF in allergic diseases

Measurement of the parameters of IgE-(in)dependent HRF production and IgE-dependent HRF response, might be good clinical parameters of

1) the efficacy of immunotherapy

In patients treated with immunotherapy a good correlation was reported between the change in spontaneous HRF production by mononuclear cells and the change in PC_{20} for histamine upon allergen provocation [123, 142].

2) the efficacy of diet in individuals with food allergy

In children with food allergy the spontaneous secretion of IgE-dependent HRF by mononuclear cells decreased after a diet that eliminated the offending food allergen [144].

3) the efficacy of medication: corticosteroid treatment

Topical corticosteroids prevent the development of the late-phase reaction [146]. Prolonged treatment with topical corticosteroids has been shown to inhibit mediator release in nasal allergic responses [146]. The underlying mechanism is probably the inhibition of the influx of inflammatory cells to the nasal mucosa [23]. In patients with allergic rhinitis that were treated with topical steroids a low, but significant correlation was found between the net changes in symptom scores and the net differences in HRF activity, in contrast to the placebo group [147].

4) disease severity.

IgE-(in)dependent HRF production and IgE-dependent HRF response are associated with disease severity (see before). In asthma the IgE-independent HRF production is associated with the bronchial histamine sensitivity [141] and IgE-dependent HRF response with bronchial metacholine sensitivity and asthma symptoms [140]. In eczema IgE-dependent HRF production is associated with increased hyperirritability [148] and in allergic rhinitis IgE-dependent HRF response is associated with allergic symptoms [138].

Scope of this thesis

In this thesis we questioned whether endogenous IgE-binding factors exist that might play a role in the allergic reaction.

To investigate a possible role for IgE-dependent HRA in supernatants of mononuclear cells, mononuclear supernatants derived from single donors were tested for their IgE-independent and IgE-dependent histamine-releasing activity (chapter 2). As a measure for IgE-independent HRA the quantitative differences in MCP-1 content were measured in mononuclear cell supernatants (chapter 3). The presence of

IgE-dependent HRA in mononuclear supernatants was analyzed after depletion of chemokine activity and by investigating the time course of the histamine response. In chapter 4 the clinical relevance of the IgE-dependent HRF in chemokine depleted supernatants of mononuclear cells was evaluated.

In chapter 5 we reinvestigated IgE-binding to human profilin and its relevance as an IgE-dependent HRF.

Moreover an attempt was made to develop a more convenient system for testing IgE reactivity with the IgE-dependent HRF. The results with RBL-2H3 cells transfected with human FcεRIα are described in chapter 6.

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Chapter 2

VARIABILITY OF IgE-DEPENDENT HISTAMINE-RELEASING ACTIVITY IN SUPERNATANTS OF HUMAN MONONUCLEAR CELLS

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ABSTRACT

Histamine-releasing factors (HRF) that release mediators from human basophils by interacting with IgE have been identified from different cell sources, including lymphocytes, monocytes, thrombocytes and endothelial cells.

These factors are studied in view of their potential importance as a stimulus in chronic inflammation. In this report we investigated the qualitative variability of the histamine-releasing activity in the supernatants of activated mononuclear cells.

Purified human mononuclear cells of 8 donors were activated with streptokinase/streptodornase (SK/SD) and the supernatants (HRF-MN) were tested for histamine-releasing activity (HRA) in both allergic (RAST positive for inhalant allergens) and nonallergic individuals. Four of the eight HRF-MN supernatants were discriminating, i.e. showing no histamine-release response with nonallergic individuals, whereas four supernatants were not. Two of the HRF-MN supernatants that exhibited discriminating properties were studied in more detail.

The response to HRF-MN was tested (1) in a direct bioassay on basophils of allergic (RAST positive for inhalant allergens) and nonallergic individuals and (2) in an indirect bioassay with 70% pure basophils of RAST negative donors after passive sensitization with sera of allergic donors. An association was found between the response to HRF-MN and the RAST for inhalant allergens: none (0/12) of the RAST-negative but 15/23 of the RAST-positive individuals were HRF-MN responders.

The IgE-dependency of HRF-MN was shown e.g. by inhibition of passive sensitization by preincubating a responder serum with monoclonal anitbody (moAb) anti-IgE MH25-1.

Our results are in contrast with findings of other investigators who use pooled supernatants and demonstrated HRF-MN responsiveness with both allergic and nonallergic donors. We conclude that mononuclear cell supernatants derived from different mononuclear cell donors vary not only quantitatively but also qualitatively with respect to IgE-dependent histamine-releasing activity.

Introduction

Thueson et al. [1,2] were the first to report that supernatants of streptokinase/ streptodornase (SK/SD) activated human mononuclear cells (HRF-MN) induce histamine release from human basophils. Histamine-releasing factors (HRFs) have now been identified from different sources [3]. These HRFs represent a heterogeneous group of proteins. It seems likely that there are two major mechanisms of histamine release: (1) in an IgE-dependent way , or (2) in an IgE-independent way [3-6]. MacDonald et al. [7] showed that at least one form of HRF is IgE-dependent. Basophils of approximately 50% of the allergic donors, called IgE^+ donors, respond to this IgE-dependent HRF. IgE^- donors are individuals who do not respond to the IgE-dependent HRF, although their basophils have IgE on their membranes. MacDonald et al. hypothesize that this heterogeneity of IgE (IgE^+/IgE^-) is due to a differential glycosylation of IgE [3].

Lichtenstein et al. [8] also showed that it is not the production of HRF but the ability to respond to HRF that differentiates allergic from nonallergic individuals. Several cell types produce IgE-dependent HRA, including the U937 monocyte/macrophage-like cell line [9], thrombocytes [10], alveolar macrophages [11] and mononuclear cells [12, 13]. In vivo IgE-dependent HRA has been found in several biological fluids e.g. nasal lavages fluid [7], in bronchoalveolar lavage fluid [14] and in skin blister fluids obtained during the late phase of an allergic reaction [15].

The mechanism of histamine release of HRF is rather debated. In order to obtain more information on mechanisms of HRF-induced histamine release, wortmannin was included in our experiments. Wortmannin is an inhibitor of myosin light-chain kinase in basophils [16]. Used at a concentration of 10 nM it is a potent inhibitor of the anti-IgE-mediated response of basophilic granulocytes without affecting the response to phorbo-myristate (PMA) or formyl-methionyl-leucyl-phenylalanine (FMLP) [17]. At higher wortmannin concentration, the PMA response is also blocked.

In this report we established a qualitative variability of the histamine-release response induced by supernatants of SK/SD-activated mononuclear cells of different donors. Some supernatants were discriminating (i.e. showed no histamine release in individuals

RAST negative for inhalant allergens), whereas other supernatants induced a histamine release in both allergic and nonallergic individuals (i.e. nondiscriminating). Two discriminating HRF-MN supernatants were selected to investigate the histamine release in allergic patients.

Materials and methods

Reagents

The following reagents we used: Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), HEPES (SIGMA Chemicals Co, St. Louis, Mo, USA), human serum albumin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands), Earle's balanced salt solution (Flow Laboratories, Ayrshire, UK), bovine serum albumin (Organon,Teknica B.V., Oss, The Netherlands), fetal calf serum (Sera-Lab, Sussex, UK), streptokinase-streptodornase (SK/SD) (Lederle Laboratories, Pearl River, NY, USA), Iscove's (Gibco/BRL, Paisley, UK).

Phorbol-myristate acetate (PMA) (Sigma) and wortmannin (kindly supplied by Dr T.G.Payne, Preclinical Research, Sandoz Ltd., Basel, Switzerland) were dissolved in dimethylsulfoside (DMSO) and were stored at -20 °C. These agents were diluted at least 100 fold in the cell incubations. The final concentration of DMSO (<0.3 %v/v) had no effect on cell viability or histamine release. The concentrations used were: PMA (100 ng/ml) and wortmannin (10 nM).

Three anti-IgE preparations were used: a monoclonal antibody (moAb) for immunofluorescense studies to detect IgE (Sigma, clone GE-1), a moAb to inhibit the passive sensitization of IgE (CLB, nr MH25-1) and a stimulating polyclonal sheep antiserum to detect anti-IgE-mediated histamine release (CLB, nr SH25P01). In the sheep serum the concentration of anti-IgE antibodies was calculated by comparing affinity-purified antibodies from the same antiserum for histamine release from human basophils. The concentration polyclonal anti-IgE used was 100 ng/ml.

Amicon YM2 (1000 MW) membranes (Amivan Danvers, MA, Ireland), FITC labeled goat anti-mouse antibodies (CLB, nr GM17-01-F07), moAb Y2 against thrombocyte GP IIa (Dakopatts, Copenhagen, Denmark).

Allergens for skin tests were obtained from ALK (Copenhagen, Denmark).

Buffers

Incubation medium for the cells contained 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human albumin, pH 7.4. Elutriation medium contained phosphate-buffered saline (PBS; 0.01 M phosphate, 0.14 M NaCl), 10 % human serum albumin, 13 mM trisodium citrate and 1mM EDTA, pH 7.4.

Culture medium (Iscove's modified Dulbecco's medium) containing Iscove's, fetal calf serum (5 % v/v, heat inactivated), supplemented with 50 µM 2-mercaptoethanol, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Skin test

Skin tests were performed with inhalant allergens as described by Van Der Zee et al. [18].

RAST and the total IgE assay.

The sera were all RAST tested on inhalant allergens and the total amount of IgE was measured as described previously [18].

Histamine release bioassay for HRF activity

Purified or unpurified basophils (see below) were resuspended in HEPES buffer supplemented with 1 mM $CaCl_2$. Incubations were performed in 300 µl (250 µl celsuspension and 50 µl stimulus), containing about $5x10^4$ basophilic granulocytes, as described by Knol et al. [19]. The incubations with HRF-MN were performed in 350 µl (250 µl celsuspension and 100 µl HRF-MN supernatant).

Histamine release

Histamine was measured by fluorometric analysis as described by Siraganian [20]. Histamine release was calculated as percentage of the total amount of histamine in the cells. The results were corrected for the spontaneous release. The spontaneous histamine release was below 10 % with the exception of 5 patients (11, 11, 12, 17, 26 %) (table 1).

An individual was scored as HRF-MN responder when the histamine release was ≥10 % after correction for spontaneous release [21].

Immunofluorescence

The effect of stripping the IgE from the basophils with lactic-acid buffer and resensitization of the basophils was analyzed in a FACScan flowcytometer (Becton and Dickinson, San José, CA, USA) [22]. Purified basophils were incubated with a moAb anti-IgE (Sigma, clone GE-1) and a control moAb against the cat allergen *Fel d* I. The binding of a FITC-labeled goat anti-mouse antibody was determined by flow cytometry.

HRF-MN

Cell donors

Donors were selected, after informed consent, from the Department of Plasmapheresis of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. The mononuclear cell donors were tested in a RAST to common inhalant allergens. HRF-MN 2, 12, 14, 16 were derived from RAST positive donors and HRF-MN 8, 9, 10, 11 were derived from RAST negative donors.

Purification of human mononuclear cels

Buffy coat were derived from 1000 ml of human blood (Haemonetics Plasma Collection System, Haemonetics Corporation, Braintree, Massachussetts). The mononuclear cells were purified as described by Roos et al. [23]. Supernatant MN2 was prepared from the buffy coat derived from 500 ml blood. To prevent thrombocyte adherence and activation, 1mM EDTA was added to the media. The thrombocyte content in the mononuclear cells was checked by flowcytometry in a FACScan flowcytometer (Becton and Dickinson). Using the moAb Y2 (against thrombocyte GP IIIa) < 1400 thrombocytes/10^6 mononuclear cells were detected [22]. Counting the mononuclear cells electronically on a Coulter counter (model ZF; Coulter Electronics, Dunstable, UK) and counting the thrombocytes on a Whole Blood Platelet Counter (Cell-DYN 100, Sequoia-Turner, Mountain View, CA) 400.000 thrombocytes/10^6 mononuclear cells were detected.

Preparation of mononuclear cell supernatants

The mononuclear cells were washed twice with Earle's medium supplemented with heat inactivated fetal calf serum (5 % v/v) and resuspended at a concentration of 5×10^6 cells/ml in tissue culture medium. SK/SD, dialyzed against PBS, was added to

the cell culture at 13 U/10^6 cells and the cells were cultured for 17 hr at 37 °C with 5 % CO_2 [24]. Eight supernatants of different mononuclear cell donors were concentrated by ultrafiltration using a YM2 membrane. The final concentrations were as followed: MN2: 3-fold, MN8: 7-fold, MN9: 13-fold, MN10: 7-fold, MN11: 10-fold, MN12: 7-fold, MN14: 8-fold, MN16: 9-fold. The experiments were performed with MN2, unless indicated otherwise.

Basophils

Allergic individuals

(1) Individuals to select HRF-MN supernatants: volunteers with an allergic history and RAST positive for inhalant allergens were selected, after informed consent, from the Department of Plasmapheresis of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

(2) Individuals to investigate the histamine-release response to HRF-MN2 and HRF-MN8: in the Pulmonology and Ear, Nose and Throat Departments of the Academic Medical Center in Amsterdam, The Netherlands, 22 allergic individuals were selected on the basis of a positive skin test and a positive radioallergosorbent test (RAST) to at least one inhalant allergen. The study, performed after informed consent of the patients, was approved by the local Medical Ethics Committee.

Controls

Donors without an allergic history and RAST negative for inhalant allergens were selected, after informed consent, from the department of Plasmapheresis of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

IgE for in vitro sensitization derived from allergic individuals

(1) Two sera were obtained after informed consent from allergic individuals selected as described above in the Pulmonology and Ear, Nose and Throat Departments of the Academic Medical Center in Amsterdam, The Netherlands.

(2) Serum 152, an IgE $^+$ serum (see below), derived from an allergic individual RAST positive for inhalant allergens (2,200 IU/ml) was obtained, after informed consent, from one of the above-described volunteers from the Department of Plasmapheresis of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

Table 1. *The histamine release responses in the direct bioassay, the RAST results for inhalant allergens and the total IgE level of the tested allergic and non-allergic individuals.*

P/C (1)	No	purity (2) [%]	PMA (3)	anti-IgE (3)	total IgE [IU/ml]	RAST (4)	HRF-MN (5) [%]
Patient							
P	1	1~2	63	28	390	+ +	68
P	2	1~2	79	49	8	+ +	67
P	3	1~2	90	16	190	+ +	55
*P	4	1~2	68	12	803	+ +	55
P	5	1~2	75	13	915	+ +	53
P	6	1~2	86	9	2200	+ +	49
P	7	1~2	ND	27	2300	+ +	46
P	8	1~2	85	27	82	+ +	42
P	9	1~2	70	29	583	+ +	32
P	10	1~2	54	49	66	+ +	29
*P	11	1~2	63	0	425	+ +	29
P	12	1~2	58	24	94	+ +	28
*P	13	1~2	40	7	1353	+ +	25
P	14	1~2	74	56	2000	+ +	22
*P	15	1~2	76	38	270	+ +	15
P	16	1~2	81	15	370	+ +	6
P	17	1~2	74	24	492	+ +	3
P	18	1~2	77	5	1700	+ +	2
P	19	1~2	83	2	48	+ +	2
P	20	1~2	31	39	339	+ +	-4
*P	21	1~2	34	6	26	+	4
P	22	1~2	86	60	34	+	1
Control							
#C	1	1~2	41	13	13	-	9
#C	2	50	38	45	53	-	8
#C	3	55	61	26	1	-	7
#C	4	80	45	20	3	-	6
#C	5	1~2	43	3	241	-	6
#C	6	1~2	27	30	4	-	5
C	7	68	45	2	19	-	3
C	8	1~2	82	32	11	-	3
C	9	25	76	26	159	-	2
C	10	87	38	50	6	-	1
C	11	1~2	15	-1	452	-	1
C	12	1~2	ND	30	14	-	-5

*(1) patient (P), control (C); (2) in most individuals the histamine release response to HRF-MN was tested using unpurified basophils (1~2), some control individuals were tested using purified basophils; (3) anti-IgE (100 ng/ml) and PMA (100 ng/ml); (4) RAST for inhalant allergens; (5) the results are presented as the percentage histamine release substracted with the spontaneous histamine release; #eight control individuals, RAST negative for inhalant allergens, were tested with the discriminating supernatant HRF-MN8. The other individuals were tested with the discriminating supernatant HRF-MN2; *the spontaneous release of the patients 4,11,13,15, 21 were 26,11,17,11%, respectively. The other patients and controls had a spontaneous histamine release <10%; (ND) not determined.*

Direct ***histamine release bioassay to test unpurified basophils***

Fifty ml of blood was collected in 50 ml PBS-13 mM trisodium citrate 1mM EDTA. Erythrocytes, eosinophils and neutrophils were removed with Percoll of 1.078 g/cm^3. The Percoll-plasma interlayer was collected, washed with PBS supplemented with 13 mM trisodium citrate and 1mM EDTA, and resuspended in HEPES buffer.

Purification of human basophils for the indirect histamine release assay

The basophils were purified from buffy coats (500 ml of human blood) by successive isopycnic centrifugation, elutriator centrifugation and isopycnic centrifugation as previously described [25], with minor modifications. During the elutriation procedure the fraction was collected between 2,550 and 1,000 rpm. This fraction was further purified over a discontinuous Percoll gradient of 1.068 g/cm^3 and 1.074 g/cm^3. The number of cells in this preparation was about 10x10^6, the purity of the basophils was about 50-70%.

Removing the IgE with lactic-acid buffer from the (un)purified basophils.

To dissociate IgE from (un)purified basophils, the cell suspension (1-20x10^6 cells), was washed twice in unbuffered saline, resuspended in 1 ml of elution buffer, pH 3.9 (containing 10 mM lactic acid, 140 mM NaCl, 5 mM KCl) and incubated at 23 °C for 3.5 min [26]. The cells were diluted with 2 ml HEPES buffer and washed once with HEPES buffer.

Indirect ***histamine release bioassay with purified basophils.***

Passive sensitization of lactic-acid treated basophils: lactic-acid treated purified basophils were incubated (37 °C, 90 min) [27] with 150 µl serum (>100 IU IgE) in HEPES buffer containing 4 mM EDTA and 10 µg/ml heparin to a final volume of 1 ml. After sensitization, the cell suspension was diluted with 2 ml HEPES buffer and washed twice with HEPES buffer. Subsequently, the cells were resuspended for 30 min. at 37 °C in HEPES buffer supplemented with 1 mM $CaCl_2$.

Inhibition of sensitization with moAb anti-IgE antibody [28].

Purified moAb anti-IgE (CLB, nr MH25-1) was dialyzed against PBS (24 h, 4 °C). The effect of passive sensitization was tested by preincubating a HRF-MN responder serum (150 µl, total IgE 2200 IU/ml) with 250 µl anti-IgE (0.25 mg/ml) for 4 hr at 23 °C. As a control, a moAb against a protein from birch pollen was used. The responsiveness to HRF-MN8 was tested.

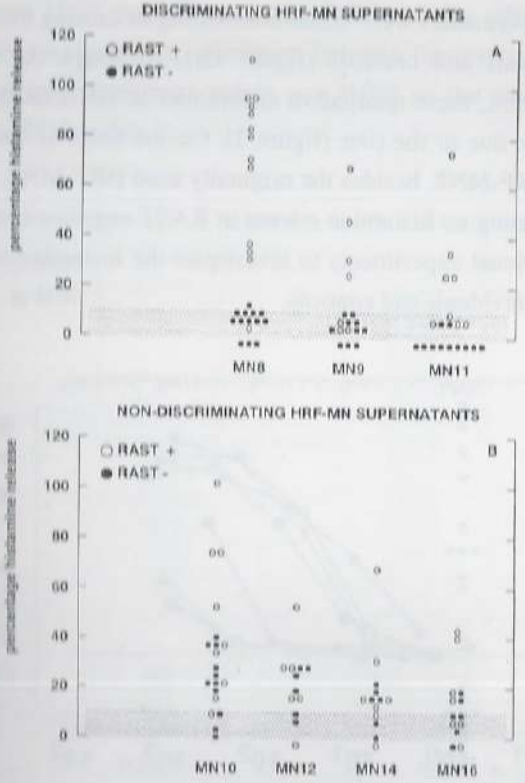

Figure 1. Histamine release from the basophils of allergic individuals and nonallergic individuals induced by SK/SD-activated mononuclear cell supernatants from 7 different donors. Individuals RAST negative for inhalant allergens (closed circles), individuals RAST positive for inhalant allergens (open circles). (A) Histamine response of the discriminating supernatants, (B) Histamine response of the nondiscriminating mononuclear supernatants.

Results

Selection of the HRF-MN supernatants

The mononuclear cells of 8 different donors were activated with SK/SD. Four out of eight mononuclear cell supernatants discriminated between allergic (RAST positive for inhalant allergens) and nonallergic (RAST negative) individuals, i.e. showing no histamine release response in control individuals (figure 1A and figure 3 for HRF-MN2). Four of the eight supernatants were nondiscriminating in causing histamine release both in allergic individuals and controls (figure 1B). Although the supernatants were quantitatively different, these qualitative differences in HRA on basophils of control individuals were not due to the titer (figure 2). On the basis of these experiments we have also chosen HRF-MN8, besides the originally used HRF-MN2, as a discriminating supernatant (i.e. causing no histamine release in RAST negative control individuals) to perform some additional experiments to investigate the histamine response in selected groups of allergic individuals and controls.

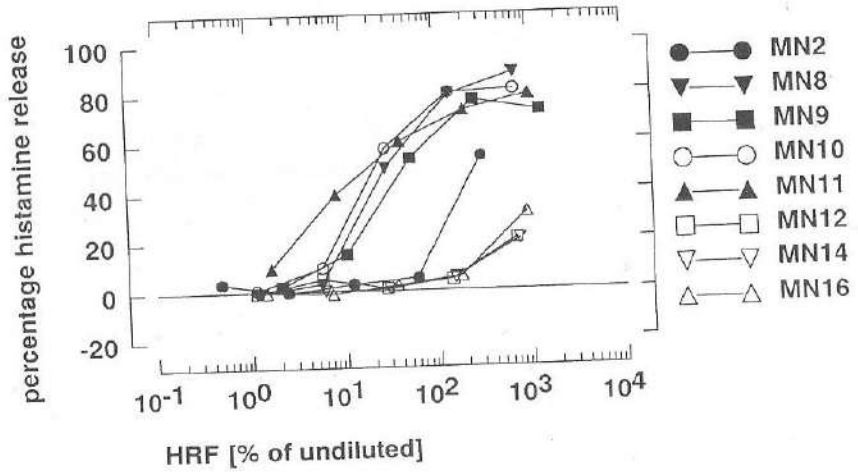

Figure 2. Histamine release from the basophils of an allergic individual induced by SK/SD-activated mononuclear cell supernatants derived from 8 different donors. Discriminating supernatants (closed symbols), nondiscriminating mononuclear cell supernatants (open symbols).

Histamine release response to HRF-MN in allergic individuals and controls by the *direct* bioassay (unpurified basophils)

The basophilic granulocytes of 22 allergic individuals and 6 normal donors were challenged with HRF-MN2, anti-IgE and PMA (table 1). Six additional controls were tested with HRF-MN8 (the histamine release of one responder to HRF-MN2 and HRF-MN8 were respectively 47 % and 72 %). None of the controls, RAST negative for inhalant allergens, showed histamine release with HRF-MN2 or HRF-MN8 (figure 3). Of the allergic patients 15/22 showed more than 10% histamine release with HRF-MN2 (figure 3). There was no significant correlation between the response to HRF-MN and the response to anti-IgE (Spearman r=0.0, $p > 0.05$) or the total serum IgE level (Spearman r=0.3, $p > 0.05$) (table 1).

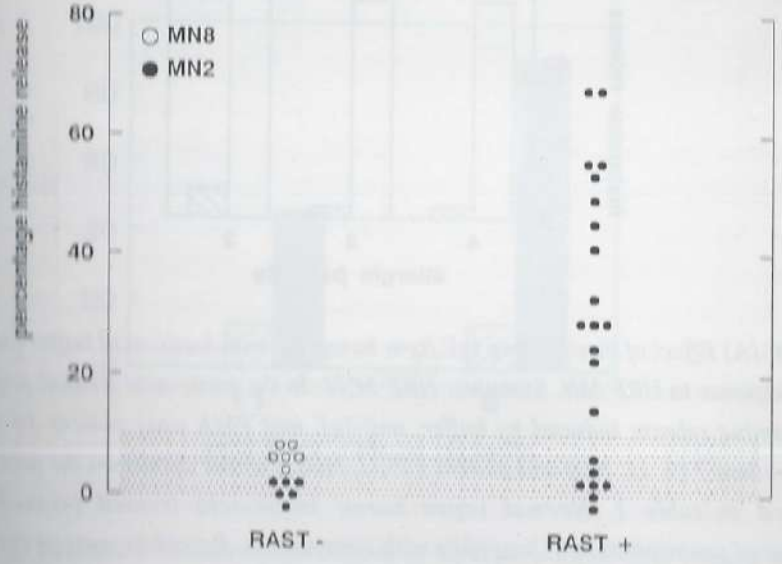

Figure 3. HRF-MN-induced histamine release in relation to the RAST to inhalant allergens. HRF-MN2 (closed symbols), HRF-MN8 (open symbols). Further details on the patients are presented in table 1.

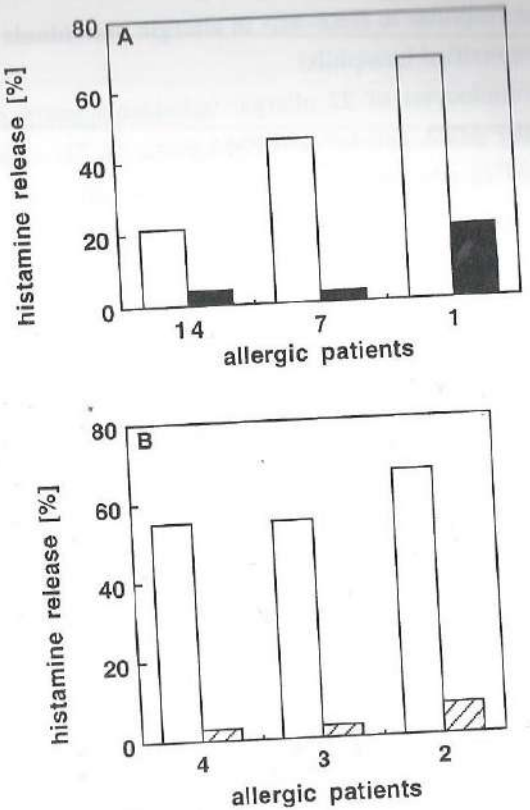

Figure 4. *(A) Effect of dissociating IgE from basophils with lactic-acid buffer (stripped) on the response to HRF-MN. Stimulus: HRF-MN2. In the lactic-acid treated population the histamine release induced by buffer, anti-IgE and PMA was: patient 14 (13, 24, 66 %), patient 7 (9, 11, ND) and patient 1 (5,-1, ND). Further details on the patients are presented in table 1. Normal (open bars); lactic-acid treated (closed bars). (B) Effect of preincubation of basophils with wortmannin. Basophils were preincubated at 37 °C for 5 min without or with the inhibitor (10nM). After this preincubation, HRF-MN2 was added (100 µl). In the cell population preincubated with wortmannin, the histamine release induced by buffer, anti-IgE and PMA was: patient 4 (16, -2, 51 %), patient 3 (10, 2, 70 %) and patient 2 (7, 5, 75 %). Further details on the patients are presented in table 1. Normal (open bars); wortmannin (hatched bars).*

IgE-dependency of HRF-MN

Direct bioassay (unpurified basophils)

By the direct bioassay with unpurified basophils support for IgE-dependency of HRF-MN was obtained in two ways. First, removing most of the surface IgE from the basophils with lactic-acid buffer (stripping) resulted in a decrease of the HRF-MN2 induced histamine release (figure 4A). Second after preincubating the basophils with wortmannin, an inhibitor of the IgE-mediated pathway in human basophils [17], inhibition was demonstrated (figure 4B). As expected wortmannin decreased the anti-IgE response, without affecting the histamine release by PMA (data not shown).

The effects observed with HRF-MN were dependent on divalent cations and on temperature (data not shown).

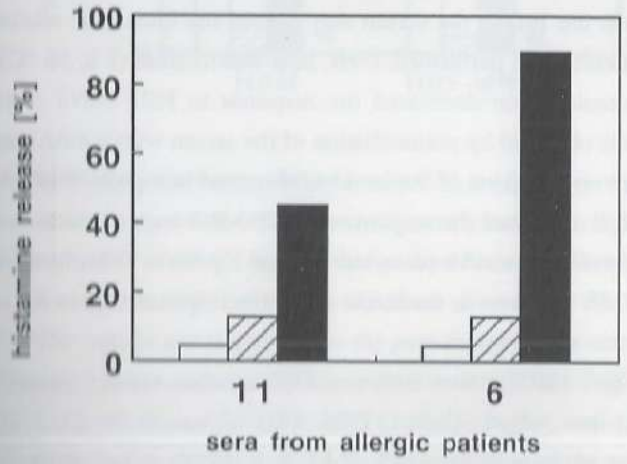

Figure 5. *Histamine release to HRF-MN8 of 50% pure lactic-acid treated basophils (stripped) of a nonallergic donor and after passive sensitization with the sera of the allergic patients 11 and 6. Both patients are IgE+. In the sensitized populations, the histamine release induced by buffer, anti-IgE and PMA were: patient 11 (5, 17, 57 %) and patient 6 (5, 16, 39 %). Further details on the patients are presented in table 1. Normal (open bars); lactic-acid treated (hatched bars); sensitized (closed bars).*

IgE-dependency of HRF-MN

Indirect bioassay (purified basophils)

By the indirect bioassay with purified basophils the IgE-dependence of HRF-MN is investigated by sensitizing lactic-acid treated basophils of a nonallergic and HRF-MN nonresponder donor with serum. Lactic-acid treated purified basophils were sensitized with sera of allergic donors, RAST positive for inhalant allergens, and incubated with HRF-MN2. Binding of IgE to the lactic-acid treated basophils was verified by FACS analysis (data not shown). In this indirect bioassay HRF-MN2 induced more than 10 % histamine release in 3/7 of the sensitizing sera (control sera positive for inhalant allergens) (14 %, 15 %, and 32 %, data not shown). Sera from 2 allergic individuals (patient 6 and 11) who were positive in the direct bioassay for HRF-MN2 (49 % and 29 %) responded to HRF-MN8 in the indirect bioassay after passive sensitization of lactic-acid treated HRF-MN nonresponder basophils (figure 5).

To investigate that it was the IgE in the serum that caused the histamine release with HRF-MN8, two experiments were performed. First, heat-inactivation (2 h, 56 °C) of the serum before passive sensitization decreased the response to HRF-MN8 (figure 6). A more definite proof was obtained by preincubation of the serum with a moAb anti-IgE (CLB nr MH25-1) before sensitization of the lactic-acid treated basophils. Preincubation with the moAb against IgE abolished the response to HRF-MN8 and to house-dust mite. Preincubation with an irrelevant moAb (directed against a protein from birch pollen) did not inhibit the HRF-MN response or the house-dust mite response (figure 6).

Discussion

HRA in the supernatants of cells has to be differentiated in IgE-dependent and IgE-independent HRF [3-6]. We investigated the IgE-dependency of two discriminating HRF-MN supernatants. This was shown in several ways. (A) In the direct bioassay with unpurified basophils: (1) removing cell-bound IgE with lactic acid decreased the HRF-MN response, (2) preincubation with wortmannin (inhibitor of the IgE-mediated activation pathway in human basophils [17]) decreased only the HRF-MN and anti-IgE response and not the response to PMA. (B) In the indirect bioassay with purified basophils of healthy donors: (3) basophils from nonresponder donors became responsive

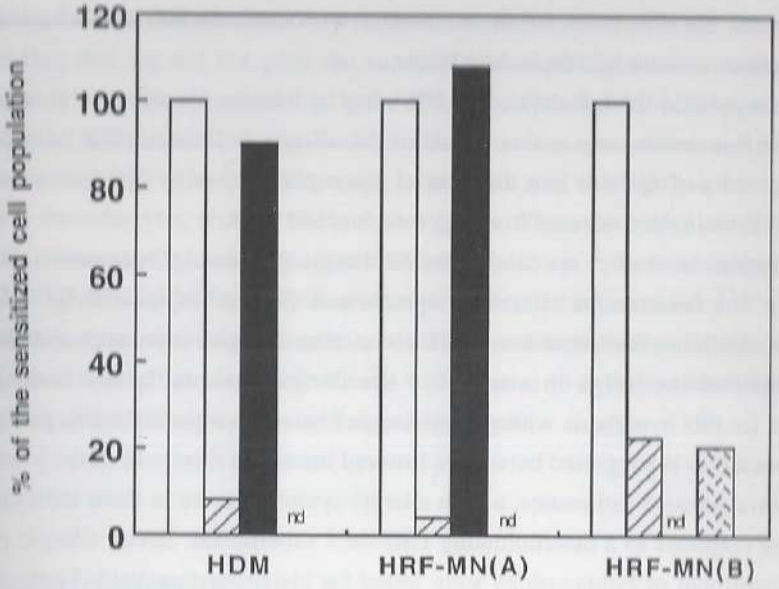

Figure 6. Inhibition of sensitization of serum 152, an IgE$^+$ serum: (1) by incubation of the serum before passive sensitization with moAb anti-IgE and (2) by incubating the IgE$^+$ serum for 2hr at 56 °C before passive sensitization. These are the results of two different experiments with cells from the same donor: (A) 50 % and (B) 75 % pure basophils. The results are presented as the percentage of the sensitized cell population. The histamine release induced by house-dust mite (HDM), buffer, anti-IgE and PMA were: 26, 5, 12, 49 % and for HRF-MN8 (100µl), buffer, anti-IgE and PMA were:(A) 33, 3, 12, 49 % and in (B) 16, 4, 8, 14 %. Preincubation of the serum with an irrelevant moAb did not affect the response to HRF-MN8 nor the house-dust mite response. nd: not determined; sensitized (open bars); anti-IgE (hatched bars); anti-birch pollen (closed bars); 56 °C (double hatched bars).

after passive sensitization with a responder serum, (4) treatment of the sensitizing serum at 56 °C destroyed this activity (5) likewise, preincubation of the sensitizing serum with a nonreleasing moAb anti-IgE (moAb MH25-1) abolished the response to HRF-MN.

This inhibition of passive sensitization by the moAb anti-IgE is probably due to steric hindrance. On the basis of these results, we conclude that these two HRF-MN supernatants contain IgE-dependent HRA.

In our opinion the IgE-dependent HRF may be a lectin-like agent or an autoallergen. That HRF interacts only with a subset of the allergic individuals can be explained by heterogeneity of IgE due to a differential glycosylation [3] or by IgE antibodies reactive with an autoallergen released from e.g. mononuclear cells.

Lichtenstein et al. [8] speculate that in allergic patients IgE-dependent HRF is the missing link between the clinical symptoms and the level of specific IgE. HRF might explain the late-phase reaction (6-11 hours after antigen exposure) and the chronic inflammation that occur in a subset of the allergic patients. In this study we found support for this hypothesis with preliminary in vivo data on the asthmatic patients tested. An association is suggested between a lowered histamine threshold of the lower airways in hyperresponsive asthmatics, a high allergic symptom score in these individuals and a positive response to a discriminating HRF-MN supernatant. Seven allergic patients of the Department of Pulmonology were tested for histamine threshold. Three of the four patients that were responsive to HRF-MN had a low histamine threshold ($PC_{20} \leq 8$ mg/ml), whereas the 3 patients that were HRF-MN negative had a normal histamine threshold ($PC_{20} > 8$ mg/ml). At the moment we are investigating this association between disease severity and a positive response to a discriminating HRF-MN supernatant in individuals with asthma.

Alam et al. [13], Baeza et al. [12] and Sim et al. [29] reported HRA in the supernatants of mononuclear cells. According to some of these investigators monocyte chemotactic and activating factor (MCAF), an intercrine, represents part of the activity in their HRF-MN supernatant [30, 31]. They found histamine release in a subset of both allergic and nonallergic individuals (i.e. nondiscriminating HRF-MN). Their results seem to be in contrast with the findings of MacDonald et al. [7], who reported a discriminating HRF response in a subset of the allergic individuals and in none of the nonallergic individuals. A possible explanation for these contradictory results might be the differences in cell types used as source of HRF. But in contrast with other investigators we found a qualitative variability in histamine-releasing activity of different mononuclear cell supernatants. We report that mononuclear cell supernatants derived from different donors are discriminating or nondiscriminating with respect to HRA. The reasons for

these differences in HRA are yet not fully understood. Moreover the ratio in discriminating HRA is different for every supernatant. These differences are likely to be due to the fact that we did not pool the supernatants of different mononuclear cell donors [32]. So this qualitative variability in the HRA in different HRF-MN supernatants may explain the differences in results between other investigators and us, and between them. Other potentially relevant differences in protocol are, that: (1) we use at least 20 times less thrombocytes, so most likely the histamine release caused by our HRF-MN is not due to products of thrombocytes like CTAP-III [33], (2) we culture the mononuclear cells at a lower cell density [32] and (3) we use less concentrated supernatants [32].

Our results indicate that the supernatants of different mononuclear cell donors differ not only quantitatively but also qualitatively with respect to IgE-dependent HRA on basophils derived from allergic and nonallergic individuals.

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Chapter 3

HISTAMINE-RELEASING ACTIVITY IN SUPERNATANTS OF MONONUCLEAR CELLS: CONTRIBUTION OF MCP-1 ACTIVITY COMPARED TO IgE-DEPENDENT ACTIVITY

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Submitted

Abstract

We found a high correlation between the MCP-1 content and the histamine-releasing activity (HRA/HRF) to basophils for 280 supernatants of mononuclear cells (Spearman rho 0.80; 95 % confidence interval: 0.76-0.84). After replacing the IgE^- on the basophils (IgE that does not respond to the IgE-dependent HRF) with IgE^+ serum (IgE that does respond to the IgE-dependent HRF) the correlation between the MCP-1 content and histamine release was much lower (rho=0.12, n=18 versus rho=0.80, n=280; p for difference between rho's: <0.05).

After depletion of chemokines in 3 HRF-MN supernatants with heparin-Sepharose, a decrease in MCP-1 content and in IgE-independent HRA was found whereas IgE-dependent HRA was still present, as indicated by: 1) a more marked increase of histamine release between 1 and 60 minutes; 2) a more marked effect of stripping basophils and 3) a more marked effect of loading the basophils with IgE^+.

We conclude that in HRF-MN supernatants the IgE-independent HRA masks the IgE-dependent HRA. The latter can be more clearly detected after depletion of chemokine activity with heparin-Sepharose.

Introduction

The immediate allergic reaction is frequently followed by a late response after 6-11 hours, resembling chronic inflammation [1-3]. As both basophils and histamine-releasing factors (HRFs) are present in this late reaction, their interaction is likely to be relevant for the late response [1, 4-8]. HRFs cause histamine release from human basophils. They are a heterogeneous group of proteins between 5 and 50 kDa produced in supernatants of different human cells [4-8]. Recently [9] we have reported that supernatants of mononuclear cells contain both IgE-independent and IgE-dependent histamine-releasing activity (HRA) in a highly variable ratio in individual mononuclear supernatants.

Only a subpopulation of the allergic individuals respond to the IgE-dependent HRF, defined as IgE^+ donors [4, 10]. Nonallergic donors and some of the allergic donors do not

respond to the IgE-dependent HRF and are defined as IgE^- donors. We reported earlier that the IgE in the IgE^+ serum is responsible for the interaction with HRF by inhibition of passive sensitization with a monoclonal anti-IgE antibody [9]. MacDonald et al. [4] assume that the IgE-dependent HRF is a lectin-like agent, which interacts only with a subset of the allergic individuals because of differences in glycosylation of IgE [4]. An alternative explanation for the IgE-dependent HRA would be one or several autoallergens released from human cells. IgE^+ would then be an IgE autoantibody. One possible candidate autoallergen, human profilin, was excluded [11].

Chemokines are chemotactic cytokines that attract neutrophils (CXC chemokines) or mononuclear cells (CC chemokines) and are thought to be involved in different types of inflammation [12]. Chemokines bind to heparin-Sepharose. Some CC chemokines activate both eosinophils and basophils, suggesting a role for these chemokines in the allergic reaction [13]. Monocyte chemotactic protein 1 (MCP-1) has a potent histamine-releasing effect on human basophils which is maximal after 1 minute [14-18]. Another CC chemokine with 71% sequence similarity [13, 19] with MCP-1 and with high histamine-releasing effect is MCP-3 [18]. Reactivity to MCP-1 [14-18] and MCP-3 [18] is not IgE-dependent.

The histamine-releasing activity of the IgE-independent HRF supernatant is not correlated with the allergic status of the donor [20, 21]. Of the IgE-independent HRA in the supernatants derived from mononuclear cells, 50-60% is due to MCP-1 [14, 22].

In the present paper, we further analyzed the contribution of MCP-1 to the histamine-releasing activity in supernatants of mononuclear cells. Moreover, we investigated the effect of chemokine-depletion on both IgE-independent and on IgE-dependent HRA.

Materials and methods

Iodination

Ten µg of recombinant (r) MCP-1 (Pepro-Tech Inc., Rocky Hill, NJ) was radiolabeled by the chloramine-T method with 1 mCi carrier free ^{125}I (Amersham International Ltd., Buckinghamshire, U.K.). Unbound ^{125}I was separated from the labeled MCP-1 on a Sephadex G-50 column equilibrated in PBS with BSA (3 mg/ml).

Competitive RIA for MCP-1

New Zealand white rabbits were immunized with 500 µg of bovine thyroglobulin (BTG) in complete Freund's adjuvant on day 0, followed by one injection of 100 µg of rMCP-1 (Pepro-Tech Inc., Rocky Hill, NJ) coupled to BTG in Freund's complete adjuvant after one week. At weekly intervals four injections of 50 µg rMCP-1 coupled to BTG in complete Freund's adjuvant followed.

Fifty µl rabbit anti-MCP-1 (diluted 1/1500) was incubated with 250 µl Protein A Sepharose (2 mg/ml), 50 µl sample and 50 µl ^{125}I-MCP-1 to a final volume of 0.65 ml. After an overnight incubation, the Sepharose was washed, and the radioactivity bound to the Sepharose was measured. The MCP-1 content was calculated relative to rMCP-1 (sensitivity ≥ 1 ng/ml). rMCP-3 (1 µg/ml, Pepro-Tech Inc., Rocky Hill, NJ) did not inhibit the binding of ^{125}I-MCP-1 in the competitive RIA for MCP-1.

RAST and the total IgE assay

Donors were selected, after informed consent, from the department of Plasmapheresis of the CLB. The sera from all individuals were RAST tested on inhalant allergens and the total amount of IgE was measured as described previously [23]. All individuals were interviewed for an allergic history. The study, performed after informed consent of the individuals was approved by the local Medical Ethics Committee.

IgE for in vitro sensitization

Three sera were obtained of donors from the department of Plasmapheresis of the CLB. Two IgE^+ sera, # 152 and # 163 (total IgE respectively 2200 and 2600 IU/ml) derived from one donor at different times, contained IgE to inhalant allergens and one IgE^- serum # 27 (total IgE 513 IU/ml) contained IgE positive for penicillin. IgE^+ is IgE derived from some allergic individuals that binds HRF; in contrast to IgE^- that does not bind HRF [4].

Purified basophils were isolated, lactic-acid treated and sensitized as described previously [9].

The effect of stripping the IgE from the purified basophils with lactic-acid buffer and resensitization of the basophils was analyzed in a FACScan flowcytometer (Becton and Dickinson, San José, CA, USA) [24]. Purified basophils were incubated with polyclonal, FITC-labeled goat anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

Histamine release in the bioassay was performed as described previously [9].

The results in the bioassay for histamine release were not corrected for the spontaneous release. The spontaneous histamine release was below 10%.

HRF-MN

Preparation of mononuclear supernatants

Three buffy coats were derived from three donors (two negative and one positive in a RAST for inhalant allergens). The mononuclear cells were isolated as described previously [9]. Mononuclear cells derived from these three donors were resuspended at concentrations of 0.19, 0.56, 1.67 and $5x10^6$ cells/ml in tissue culture medium and activated with: 0, 65, 195, 585 U/ml streptokinase/streptodornase (SK/SD, Lederle Laboratories, Pearl River, NY), dialyzed against PBS, anti-CD2 (CLB-T II.1/1 and CLB T II.2/1; each 1 µg/ml) and/or anti-CD28 (CLB 28/1; 5 µg/ml) and/or PMA (1 ng/ml). After the cells were cultured for 24 hr or 96 hr at 37°C with 5 % CO_2 the supernatant was harvested and tested for HRA unconcentrated.

Under standardized conditions the mononuclear cells derived from 18 buffy coats (from 14 different donors: 5 RAST positive and 9 RAST negative to inhalant allergens) were resuspended at a concentration of 5×10^6 cells/ml and after SK/SD was added to the cell culture at 65 U/ml, the cells were cultured for 17 hr at 37 °C with 5 % CO_2 [25]. A supernatant was concentrated three times by ultrafiltration using Amicon YM1 (1000 MW) or YM3 (3000 MW) membranes (Amivan Danvers, MA, Ireland).

Immunoabsorption of HRF-MN supernatant with rabbit anti-MCP-1

After washing 33 mg Protein A Sepharose with incubation medium (without albumin and glucose) 27.5 µl rabbit serum anti-MCP-1 (or rabbit serum anti-*Lol p* II [26] as

a negative control), was added (room temperature, head-over-end, 1 hr). After washing with incubation medium (without albumin and glucose), 500 µl HRF-MN supernatant was added overnight head-over-end at 4 °C. The supernatant was tested for MCP-1 content and histamine-releasing activity.

Depletion of HRF-MN supernatants with heparin-Sepharose

Three HRF-MN supernatants (derived from 2 donors RAST negative and from 1 donor RAST positive to inhalant allergens), that were already three times concentrated (YM3), were pooled. Seventy-five ml of this pool was incubated with 3 gram heparin-Sepharose CL-6B (washed with PBS, Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After stirring overnight at 4 °C, the supernatant was separated from the heparin-Sepharose on a filter. The depleted pool was further concentrated three times (YM3).

Results

Histamine release induced by rMCP-1 and rMCP-3 is IgE-independent

To investigate the mechanisms of mediator release, the histamine release by rMCP-1 and rMCP-3 compared to house-dust mite were tested using the purified basophils derived from a blood donor with IgE antibodies to house-dust mite. Both rMCP-1 and rMCP-3 have been described as IgE-independent HRF [14-18]. We investigated the effect on the histamine release by (i) treating the basophils with 30 nM wortmannin [27], a potent inhibitor of the IgE-mediated response by inhibiting PI3 kinase [28, 29]; (ii) incubating the basophils with lactic acid, to remove the IgE from the surface of the basophils and (iii) investigating the kinetics of histamine release at 1 minute (figure 1). The histamine release by house-dust mite decreased after treating the basophils with wortmannin or lactic acid at 60 minutes. Moreover the histamine release was lower at 1 minute than at 60 minutes. The histamine release response to rMCP-1 and rMCP-3 showed no significant difference under these different conditions, indicating that both rMCP-1 and rMCP-3 demonstrated IgE-independent histamine release, in contrast to house-dust mite (figure 1).

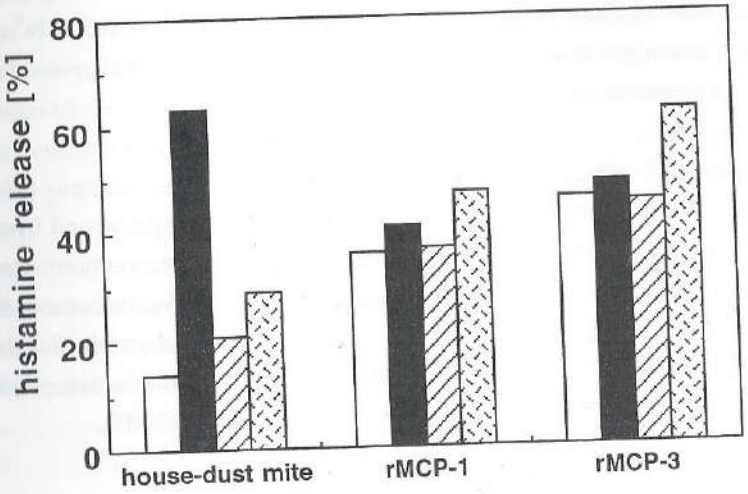

Figure 1. The mechanism of histamine release by rMCP-1, rMCP-3 and house-dust mite allergen (HDM). Basophils, derived from an individual with IgE antibodies to HDM, were incubated with 1667 SQ /ml HDM and 10^{-7} M rMCP-1 or rMCP-3. The percentage of histamine release was measured. Histamine release was stopped by adding ice-cold 0.9 % NaCl after 1 minute (open bars) or after 60 minutes (closed bars). Basophils were preincubated with wortmannin (hatched bars) or treated with lactic-acid (double hatched bars). This experiment is representative for 3 experiments.

We also investigated the effect of sensitization of lactic-acid treated basophils with an IgE^+ serum containing IgE antibodies to house-dust mite (# 163) on the histamine release response to rMCP-1 and rMCP-3. The basophils were obtained from a blood donor RAST negative to inhalant allergens. After sensitization of the lactic-acid treated basophils the histamine release to house-dust mite increased between 1 minute and 60 minutes. No significant increase was demonstrated for the histamine release response to rMCP-1 and rMCP3 between 1 minute and 60 minutes (lactic-acid treated basophils, rMCP-1: 3 %, rMCP-3: 3 %; after sensitization with # 163, rMCP-1: 3 %, rMCP-3: 1 %).

HRF-MN production

Effect of differences in culture conditions of mononuclear cells on the histamine release by these supernatants

With the mononuclear cells derived from three donors (two RAST positive and one RAST negative in a RAST to inhalant allergens) we investigated the influence of the culture conditions, cell density and activation, on the HRF-MN production. All supernatants were each tested on three basophil donors: two RAST positive and one RAST negative in a RAST to inhalant allergens. Besides the spontaneous HRF production, the mononuclear cells were activated with different SK/SD concentrations (0, 65, 195, 585 U/ml) and different combinations of anti-CD2, anti-CD28 (1 µg/ml and 5 µg/ml, respectively) and PMA (1 ng/ml) for 24 hr or 96 hr. Compared to the spontaneous HRF production, activation of the mononuclear cells increased the HRA levels of the supernatants. No significant differences in the HRA levels were found when cultured at cell densities of 1.67×10^6 cells/ml and 5×10^6 cells/ml were compared.

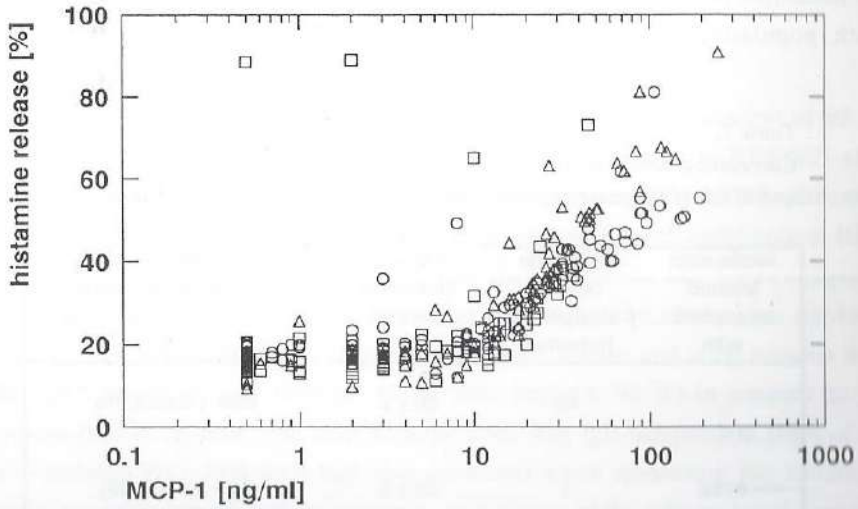

Figure 2. Correlation between MCP-1 content in small cultures and histamine release. The mononuclear cells were derived from: two RAST negative donors (MN-NA: open circles and open squares) and one RAST positive donor (MN-A: open triangles).

Correlation between MCP-1 content and the histamine release by these HRF-MN supernatants

Next we investigated the relation between the MCP-1 concentration in the supernatants and the histamine release by these supernatants (figure 2). The MCP-1 content of the supernatants highly correlated with the histamine release induced by the supernatants (Spearman's rho 0.80; 95 % confidence interval: 0.76-0.84).

IgE-independent and IgE-dependent HRA by HRF-MN: differences in correlation between MCP-1 content and the histamine release by the HRF-MN supernatants at 1 minute and at 60 minutes

We next investigated the relation between the MCP-1 content and an IgE-independent and/or IgE-dependent HRA in 18 mononuclear supernatants (the mononuclear cells were cultured under standardized conditions). Basophils derived from a blood donor, IgE^- and RAST negative to inhalant allergens, were divided in three populations: (1) lactic-acid treated, (2) lactic-acid treated and subsequently sensitized with IgE^+ serum # 152 and the histamine release stopped at 1 minute and (3) as (2) but stopped at 60 minutes. Each population was incubated with 18 mononuclear supernatants (table 1).

Table 1.
Correlation between MCP-1 content of 18 HRF-MN supernatants and HRA of the same supernatants on different basophil populations.

lactic-acid treated basophils with	histamine release stopped at [minutes]	mean % histamine release ± SEM	Spearman's rho (95 % confidence interval)
-	60	30 ± 2	0.44 (-0.04/0.75)
#152	1	33 ± 2	0.59 (0.17/0.83)
#152	60	58 ± 4	0.12 (-0.37/0.56)

Lactic-acid treated basophils obtained from a non-allergic donor were sensitized with IgE+ serum #152 and incubated with 18 HRF-MN supernatants.

The correlation between MCP-1 content in the HRF-MN supernatants and the difference in histamine release in the sensitized population at 1 minute and 60 minutes was: rho -0.20; 95 % confidence interval: -0.61-0.30.

These results indicate that besides MCP-1 another HRA is present in the mononuclear supernatants that has the characteristics of an IgE-dependent HRA.

Removing the IgE-independent HRA from the HRF-MN supernatants:

As we were interested in the IgE-dependent HRA in the mononuclear supernatants we investigated the effect of removal of IgE-independent HRA from supernatants.

By preincubation with rabbit serum anti-MCP-1

Preincubation of a HRF-MN supernatant with rabbit serum coupled to protein A Sepharose caused a decrease in (i) MCP-1 content of > 99 %, from 144 ng/ml to ≤ 1 ng/ml and in (ii) histamine release of 70 % (data not shown); preincubation with a control rabbit serum anti-*Lol p* II, resulted in no decrease in MCP-1 concentration and < 5 % decrease in histamine release.

By treatment with heparin-Sepharose

The treatment with heparin-Sepharose caused a decrease in MCP-1 content of 83 %, from 149 to 25 ng/ml. The effect on the histamine release response to HRF-MN after heparin depletion was investigated after sensitization of lactic-acid treated basophils with an IgE^+ serum (#163, figure 3). The histamine release of the IgE-independent HRA decreased with 47 % after chemokine depletion. An IgE^- serum (#27) was compared with an IgE^+ serum (#152) in the histamine release by the chemokine depleted supernatant. The histamine release increased between 1 minute and at 60 minutes only for the IgE^+ serum (before heparin: 19 %; after heparin: 44 %) in contrast to the IgE^- serum (before heparin: 3 %; after heparin: 2 %). The IgE-independent HRA of the heparin-depleted HRF-MN pool had also decreased when measuring the histamine release of basophils treated with wortmannin, an inhibitor of the IgE-mediated response (data not shown). Another pool of supernatants was depleted with 15 or 3 gram heparin-Sepharose. This resulted in a higher decrease in MCP-1 content (≤ 1 ng/ml) using 15 gram heparin-Sepharose, but also the IgE-dependent HRA was not clearly detectable anymore (data not shown). These results indicate that the presence of an

IgE-dependent histamine-releasing activity is more convincingly demonstrated after heparin depletion and is dependent on the presence of IgE^+ on the basophils (figure 3).

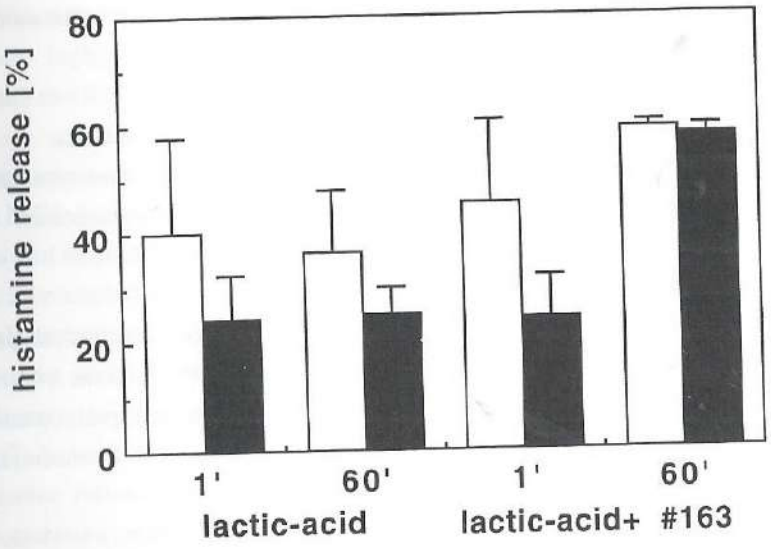

Figure 3. Effect on the histamine release by HRF-MN after heparin depletion. A pool of three HRF-MN supernatants (open bars) was depleted with heparin-Sepharose (closed bars). The histamine-releasing activity was measured after sensitization of lactic-acid treated basophils with IgE^+ serum #163. The histamine release was stopped at 1 minute and at 60 minutes by adding ice-cold 0.9 % NaCl. Results are presented as the mean ± SEM of two experiments.

Discussion

Our results confirm that both rMCP-1 [14-18] and rMCP-3 [18] activate the basophil via an IgE-independent mechanism [14-18]. In this report the IgE-independent histamine-releasing effect of MCP-1 and MCP-3 was shown by lactic-acid treatment of

the basophils, by wortmannin preincubation of the basophils, by the time course of histamine release and by sensitization of lactic-acid treated basophils with serum.

Alam et al. [14] reported a significant reduction of histamine release to rMCP-1 after lactic-acid treatment of the basophils, whereas we found no decrease and perhaps even an increase.

Despite the high correlation between MCP-1 levels and the histamine-releasing activity in the HRF-MN supernatants, a quantitative discrepancy was found between the immunochemical reactivity for MCP-1 and the histamine-releasing activity in the HRF-MN supernatants. The histamine release by rMCP-1 was about 1000 times less active compared to the histamine-releasing activity of the HRF-MN supernatants on the basis of their MCP-1 content. Several explanations are possible for this phenomenon: 1) MCP-3 or other factors are more relevant than MCP-1 for the histamine-releasing activity in the HRF-MN supernatants, 2) the HRF-MN supernatants might contain (an)other factor(s), e.g. IL-3 [8, 15, 17, 22], that primes the basophils (within 1 minute) for histamine release or induces histamine release from the basophils, 3) the recombinant material has less biological activity but its immunological activity is comparable to the native protein (the polyclonal anti-MCP-1 serum was obtained by immunisation with the same recombinant material) or 4) the natural material has less immunological reactivity.

MCP-1 in pooled mononuclear supernatants has been reported to be responsible for 50-60 % of the HRA [14, 22]. Kaplan et al. [30] have found a reduction of 75-80 % of the HRA after immunoabsorption in pooled HRF-MN supernatants of MCP-1, RANTES and IL-3 [30].

In this report we showed that in supernatants containing a high level of MCP-1 it is difficult to differentiate between IgE-dependent and IgE-independent HRA because the histamine release is already near maximal at 1 minute.

After immunoabsorption of the MCP-1 activity in the HRF-MN supernatants with polyclonal serum anti-MCP-1 a decrease of > 99 % in MCP-1 content and of 70 % in the histamine release by the IgE-independent HRA was found. Using a suboptimal amount of heparin-Sepharose, that binds many if not all chemokines, a decrease of 83 % in MCP-1 content and of 47 % in the histamine release in the IgE-independent HRA was shown. The lower decrease in MCP-1 depletion with heparin-Sepharose and the comparable decrease in IgE-independent HRA indicates that besides MCP-1 another IgE-independent HRA is present in HRF-MN.

The IgE-dependent HRA was not a PMA-like response as the histamine release response to PMA at 60 minutes did not increase by sensitization of basophils (data not shown).

We conclude that removal of IgE-independent HRA in HRF-MN with heparin-Sepharose and measuring the increase of histamine release after 1 minute is a more reliable way to investigate IgE-dependent HRA in supernatants of cells and/or the patients who respond to it.

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Chapter 4

REACTIVITY TO IgE-DEPENDENT HISTAMINE-RELEASING ACTIVITY IN SUPERNATANTS OF MONONUCLEAR CELLS IN PATIENTS WITH ASTHMA OR RHINITIS

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Submitted

Abstract

We investigated the relation between the occurence of IgE reactive with histamine-releasing factor (HRF) and the clinical status of the patients. We included 18 patients with allergic asthma, 19 patients with nonallergic asthma, 17 patients with allergic rhinitis and 19 healthy control individuals. Sera were used to passively sensitize purified, lactic-acid treated basophils. IgE-independent histamine-releasing activity due to chemokines was removed from mononuclear cell supernatants with heparin-Sepharose. IgE-dependent histamine-releasing activity was determined by measuring the increase in histamine release between 1 minute and 60 minutes: ΔHRF.

In the patient groups and the controls, the number of sera with more than 12 % ΔHRF histamine release were: 8/18 patients with allergic asthma, 2/19 patients with nonallergic asthma, 5/17 patients with allergic rhinitis and 0/19 of the controls. The capacity of a serum to sensitize basophils for reactivity to HRF was associate: 1) with IgE to inhalant allergens: 40 % of the RAST positive individuals and 8 % of the RAST negative individuals showed more than 12 % ΔHRF histamine release (Odds ratio 7.77, 2.00-30.29, p=0.003); 2) with bronchial sensitivity to histamine in all asthmatic patients (geometric mean PC_{20}: 1.50 versus 0.51 mg/ml, p for difference 0.004) and 3) with bronchial sensitivity to histamine in allergic asthmatic patients (geometric mean PC_{20}: 1.27 versus 0.37 mg/ml, p for difference 0.017).

These findings support the hypothesis that IgE-dependent HRF might contribute to the chronic-allergic reaction.

Introduction

The response of an allergic patient to an allergen is characterized by an immediate response, occuring within 5-10 minutes and in some individuals followed by a late-phase response after 6-11 hours [1-3]. The late-phase of the allergic reaction correlates better with the disease severity of the patient [4]. The pathogenesis of this late response is complex and not completely understood. After binding of the allergen to the IgE on the surface of mast cells and/or basophils, these cells degranulate and release their

mediators. These mediators cause the allergic symptoms of the acute response and attract inflammatory cells (neutrophils, eosinophils, lymphocytes, basophils, monocytes) into the affected site which in turn are primed or activated at the site of inflammation (late-phase response and chronic-allergic inflammation) [2, 3].

Of the FcεRI expressing cells, in the immediate allergic reaction the mast cell is the predominant source of mediators, whereas basophils rather than mast cells release their mediators in the late phase [5-7]. These data suggest that the basophil plays a role in the late phase of the allergic reaction [8]. However, the stimulus for the basophil activation in the allergic late phase reaction and chronic-allergic reaction is still unknown.

HRFs [9-13] are a heterogeneous group of proteins between 5 and 50 kDa found in supernatants of different human cells. They induce histamine release from human basophils. As both basophils and histamine-releasing factors (HRFs) are present in this late reaction , HRFs are thought to be involved in the late response [1].

Part of the histamine release induced by HRF has been found to be due to chemokines. Especially monocyte chemotactic protein-1 (MCP-1) is thought to be important for the IgE-independent HRF histamine release in the cell supernatants described above [14-16]. A reduction of 75-80% of the histamine-releasing activity has been found after immunoabsorption of MCP-1, RANTES and IL-3 [17] in pooled HRF-MN supernatants.

Recently [18], we reported that supernatants of mononuclear cells contain a mixture of both IgE-independent and IgE-dependent HRF; the composition is qualitatively and quantitively different in mononuclear supernatants of individual donors. The histamine release induced by the IgE-independent chemokines is already maximal after 1 minute [14, 16, 19-22]. However, the histamine release induced by IgE-dependent activators (like anti-IgE and allergen) is low at 1 minute compared to the maximal response after 15 minutes [23]. In supernatants derived from mononuclear cells (HRF-MN) that contain a high level of IgE-independent HRF, the IgE-dependent HRF is difficult to detect [16] and its clinical relevance difficult to evaluate.

We therefore modified the method to measure the IgE-dependent HRF activity [16]. We discriminated the IgE-dependent HRF in HRF-MN supernatants from the IgE-independent HRF by: 1) depletion of most of the IgE-independent chemokine activity from the HRF-MN supernatants by its affinity to heparin-Sepharose,

2) substraction of the histamine release at 1 minute from the histamine release response at 60 minutes.

In this report we investigated the correlation between the response to the IgE-dependent histamine release in HRF-MN and the disease status in asthma and allergic rhinitis. By sensitization of basophils derived from nonallergic donors with serum IgE of the patients, we were able to avoid variation due to (hypothetical) in vivo priming of the basophils. We investigated the presence of IgE reactive with HRF in four groups of subjects: patients with allergic asthma, nonallergic asthma, allergic rhinitis and healthy controls.

Patients and methods

Human subjects

IgE for in vitro sensitization

This study was performed after informed consent of the patients and was approved by the local ethical committee.

Allergic asthma patients (table 1)

Twenty-one patients with allergic asthma were selected from the outpatient Department of Pulmonology. Asthma was diagnosed according to the criteria of the American Thoracic Society [24]. Patients with asthma had a history of paroxysms of dyspnoe, wheezing and coughing. Patient selection was based on a low histamine threshold ($PC_{20} \leq 8$ mg/ml), positive skin-prick test and a RAST positive to at least one inhalant allergen. All patients were in a stable phase of their disease. None of the patients was treated with immunotherapy. Three sera were excluded because they demonstrated in the bioassay >10 % histamine release after addition of concentrated culture medium with streptokinase/streptodornase (SK/SD was used to activate the mononuclear cells [18]).

Table 1. *Characteristics of patients with allergic asthma and non-allergic asthma.*

Pt No	b^	AGE	SEX	S	FEV1 [% pred]	rev FEV1 [%]	FEV1 /FVC [%]	PC20 [mg /ml]	dHRF [%]	TIgE [IU /ml]	G	R B	R M	R C	R D	*
A-asthma																
A6	A	34	M	1	69	ND	60	0.07	143	627	4	0	3	4	3	4
A10	A	43	M	0	73	11	69	3.97	5	12	0	0	2	0	0	2
A11	A	50	F	0	98	19	65	1.04	10	420	0	0	0	4	0	4
A12	A	45	F	1	62	18	57	1.15	5	49	0	0	0	1	0	1
A13	A	24	F	0	96	8	84	0.66	8	246	0	0	5	0	0	5
A14	A	38	F	1	68	4	76	0.25	25	112	0	0	3	4	2	4
A15	A	22	F	0	98	4	94	0.30	178	1400	4	2	2	1	4	4
A16	A	41	F	1	106	5	82	4.90	3	14	0	0	1	0	0	1
A17	A	27	F	1	97	8	86	3.53	3	396	0	0	0	1	1	1
A18	A	45	F	0	91	13	67	0.20	0	8	0	0	2	0	0	2
A19	A	23	F	0	108	8	81	1.60	-3	117	0	0	0	1	0	1
A20	A	36	M	1	67	0	74	0.20	13	927	0	0	5	0	0	5
A21	B	29	F	0	117	5	77	1.20	69	466	2	0	0	4	4	4
A22	B	27	M	0	82	4	78	0.40	346	257	5	0	4	1	0	5
A24	B	41	M	1	72	3	70	0.50	-15	297	0	0	4	2	0	4
A25	B	24	M	0	68	5	83	0.90	15	911	0	0	4	2	0	4
A26	B	28	F	1	83	21	65	0.90	238	205	0	0	0	4	4	4
A27	B	43	M	1	88	33	51	0.32	38	362	0	0	0	5	5	5
NA-asthma																
B9	A	38	F	1	80	14	69	2.90	5	45	0	0	0	0	0	0
B10	A	24	F	0	130	0	92	0.17	10	19	0	0	0	0	0	0
B11	A	33	F	0	113	3	76	1.31	23	7	0	0	0	0	0	0
B12	A	51	M	1	88	1	69	1.57	13	5	0	0	0	0	0	0
B13	A	33	F	1	105	ND	77	2.50	8	225	0	0	0	0	0	0
B14	A	21	F	0	118	6	96	0.86	8	63	0	0	0	0	0	0
B15	A	53	F	0	106	12	75	0.79	5	67	0	0	0	0	0	0
B16	A	30	F	0	66	9	68	3.60	5	209	0	0	0	0	0	0
B17	A	53	M	1	62	40	45	1.20	50	414	0	0	0	0	0	0
B18	A	33	F	1	100	11	80	3.74	5	6	0	0	0	0	0	0
B19	A	24	F	0	100	7	86	3.72	5	24	0	0	0	0	0	0
B20	A	56	F	1	61	-11	58	1.40	5	309	0	0	0	0	0	0
B21	B	48	M	1	57	16	42	1.10	0	51	0	0	0	0	0	0
B22	B	55	M	1	65	8	64	3.30	0	15	0	0	0	0	0	0
B23	B	42	F	0	85	1	81	1.90	8	104	0	0	0	0	0	0
B31	A	44	M	0	116	5	69	0.10	4	233	0	0	0	0	0	0
B32	A	33	F	1	86	1	68	3.10	4	34	0	0	0	0	0	0
B34	A	35	M	1	73	2	80	3.80	-2	137	0	0	0	0	0	0
B35	A	36	F	1	111	11	79	6.45	4	84	0	0	0	0	0	0

*Legend of table, definition of abbreviations: A-asthma= allergic asthma; NA-asthma = non-allergic asthma; b^= basophil donor; M= male; F= female; S= smoking; 0= no; 1= yes; rev FEV1= reversibility of FEV1 with salbutamol; dHRF= delta HRF; TIgE= total IgE; G= grass; B= birch pollen; M= house-dust mite; C= cat dander; D= dog dander; *= the highest RAST score to a panel of inhalant allergens*

Table 2. *Characteristics of patients with allergic rhinitis and of the healthy controls.*

Pt No	B^	AGE	SEX	dHRF [%]	TIgE [IU /ml]	G	R B	R M	R C	R D	*
All. rhinitis											
D9	A	43	M	10	69	0	0	4	0	0	4
D10	A	36	M	5	105	0	0	2	0	0	2
D11	A	23	M	10	62	0	0	4	0	0	4
D12	A	32	F	5	115	1	0	4	0	0	4
D13	A	28	F	8	78	0	0	2	0	0	2
D14	A	47	F	85	67	3	2	0	0	0	3
D15	A	35	M	5	826	2	0	2	0	0	2
D16	A	28	F	105	387	4	1	1	2	0	4
D17	A	27	M	23	209	0	0	3	0	0	3
D18	A	43	F	8	125	0	0	2	0	0	2
D20	A	16	M	5	86	0	0	3	0	0	3
D21	B	33	M	46	911	0	0	0	0	0	5
D22	B	29	M	23	71	0	0	0	4	0	4
D31	A	73	F	0	58	0	0	3	2	0	3
D32	A	33	M	-2	163	0	4	0	0	0	4
D34	A	50	F	2	98	0	4	0	0	0	4
D35	A	39	M	2	111	0	3	0	0	0	3
Controls											
C1	A	21	F	0	12	0	0	0	0	0	0
C2	A	29	F	0	29	0	0	0	0	0	0
C3	A	28	M	0	1	0	0	0	0	0	0
C4	A	28	M	4	16	0	0	0	0	0	0
C5	A	29	F	4	61	0	0	0	0	0	0
C6	A	33	F	2	45	0	0	0	0	0	0
C7	A	32	F	9	88	0	0	0	0	0	0
C8	A	28	F	2	42	0	0	0	0	0	0
C9	A	26	F	9	69	0	0	0	0	0	0
C10	A	40	M	6	26	0	0	0	0	0	0
C11	A	33	F	6	25	0	0	0	0	0	0
C12	A	29	M	4	23	0	0	0	0	0	0
C13	A	31	F	2	2	0	0	0	0	0	0
C14	A	38	F	0	48	0	0	0	0	0	0
C17	A	59	F	-2	0	0	0	0	0	0	0
C18	A	23	F	0	19	0	0	0	0	0	0
C19	A	45	M	4	241	0	0	0	0	0	0
C20	A	51	F	4	48	0	0	0	0	0	0
C21	A	30	F	2	18	0	0	0	0	0	0

*Legend of table, definition of abbreviations: b^= basophil donor; M= male; F= female; S= smoking; 0= no; 1= yes; dHRF= delta HRF; TIgE= total IgE; G= grass pollen; B= birch pollen; M= house-dust mite; C= cat dander; D= dog dander; ND= not determined; *= the highest RAST score to a panel of inhalant allergens*

Nonallergic asthma patients (table 1)

Twenty patients with nonallergic asthma were also selected from the outpatient Department of Pulmonology. Asthma was diagnosed according to the criteria of the American Thoracic Society [24]. Patients with asthma had a history of paroxysms of dyspnoe, wheezing and coughing. Patient selection was based on a nonallergic history, low histamine threshold ($PC_{20} \leq 8$ mg/ml), negative skin-prick test and RAST negative to a panel of common inhalant allergens. All patients were in a stable phase of their disease. None of the patients was treated with immunotherapy. One serum was excluded because it demonstrated in the bioassay >10 % histamine release after addition of concentrated culture medium with SK/SD (described below).

Allergic rhinitis (table 2)

Twenty patients with allergic rhinitis were selected from the outpatient Department of Otorhinolaryngology. Patients with allergic rhinitis had a history of sneezing, itching in the nose, a running nose or a blocked nose, but no history of current or past episodes of paroxysms of dyspnoe, wheezing and coughing. Patient selection was based on an allergic history, positive skin-prick test and RAST positive to at least one inhalant allergen. All patients were in a stable phase of their disease. None of the patients patients was treated with immunotherapy. Three sera were excluded because they demonstrated in the bioassay >10 % histamine release after addition of concentrated culture medium with SK/SD (described below).

Control group (table 2)

Nineteen employees without an history of allergy or upper and lower airways diseases and RAST negative for a panel of common inhalant allergens were selected from the Departments of Allergy and Pulmonology. Of the 19 controls individuals, 13 were tested for bronchial histamine reactivity, which was normal ($PC_{20} > 8$ mg/ml) in all tested control individuals.

Reference plasma: IgE+

MacDonald et al. have defined IgE derived from some allergic individuals that interacts with HRF as IgE^+ ; in contrast to IgE^-, that does not interact HRF [9]. One IgE^+ serum # 163 (total IgE 2600 IU/ml) derived from an individual with allergic asthma and a histamine threshold $PC_{20} \leq 8$ mg/ml was obtained from a donor of the Department of Plasmapheresis.

Cell donors

Mononuclear cell donors

Three donors were selected from the Department of Plasmapheresis. All individuals were interviewed for an allergic history. In a RAST for inhalant allergens, 2 donors were negative and 1 was positive.

Basophil cell donors

Two donors were selected from the Department of Plasmapheresis. Both donors were RAST negative for inhalant allergens and had a negative allergic history.

RAST and the total IgE assay [18]

The sera from all individuals were RAST tested in a panel of common inhalant allergens and the total amount of IgE was measured as described previously [25]. The RAST was scored as follows: percentage binding of radioactivity between 0-2 %: 0; 2-5 %: 1; 5-10 %: 2; 10-20 %: 3; 20-40 %: 4; > 40 %: 5. The highest score in the RAST for one of the inhalant allergens tested is shown in table 1 and 2.

Skin-prick test [18]

Allergens for skin-prick tests were obtained from ALK (Copenhagen, Denmark). Skin-prick tests were performed with a panel of common inhalant allergens as described by Van der Zee et al. [25].

Lung function tests

FEV_1 and FVC were measured with a calibrated, water-sealed spirometer, according to standardized guidelines [26]. Bronchial hyperreactivity to histamine was determined by a 2-minutes tidal breathing method [27]. The histamine threshold was defined as the interpolated concentration of histamine that caused a decrease in FEV_1 of 20% of the baseline value.

Histamine release

Histamine release was performed as described previously [18]. A stimulating polyclonal sheep anti-human IgE antiserum (100 ng/ml [18], CLB, nr SH25P01) and house-dust mite extract (HDM, 1667 SQ /ml, ALK, Copenhagen, Denmark) were used to detect IgE-mediated histamine release. rMCP-1 (Pepro-Tech Inc., Rocky Hill, NJ) was used at concentrations of 10^{-7} M. The incubations with HRF-MN were performed in 350 µl (250 µl cell suspension and 100 µl HRF-MN supernatant). Concentrated culture medium with SK/SD was tested as a negative control. The histamine release was stopped by diluting the cells in ice-cold PBS. The results were not corrected for the spontaneous release. The spontaneous histamine release was below 10%.

Chemokine-depleted HRF-MN supernatants

HRF-MN supernatants were prepared and concentrated as described previously [18]. Three HRF-MN supernatants that contained IgE-dependent HRF were pooled. Seventy-five ml of this pool was incubated with 3 gram heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After stirring overnight at 4 °C, the supernatant was separated from the heparin-Sepharose on a filter. The depleted pool was concentrated three times (YM3): HRF-MN-hep.

Highly purified basophils were isolated and sensitized as described previously [18].

The effect of stripping the IgE from purified basophils with lactic-acid buffer and resensitization of the basophils was investigated. Binding of polyclonal, FITC-labeled goat anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was analyzed in a FACScan flowcytometer (Becton and Dickinson, San José, CA, USA) [28].

Detection of the IgE-dependent histamine release in HRF-MN

We discriminated the IgE-dependent HRF in HRF-MN from the IgE-independent HRF by [16]: 1) depletion of most of the IgE-independent chemokine activity from the HRF-MN supernatant with heparin-Sepharose, 2) subtraction of the histamine release response at 60 minutes with the histamine release response at 1 minute (the increase in histamine release between 1 minute and 60 minutes in the lactic-acid treated cells that were not sensitized was < 10%) and 3) sensitization of lactic-acid treated basophils derived from a nonallergic donor with serum derived from the patients.

HRF activity expressed to reference: ΔHRF

As a reference in each of the three experiments the same IgE^+ serum was tested. In experiment 1, 2 and 3 with basophil donor A, B and A, respectively. The increase in histamine release between 1 and 60 minutes to HRF-MN-hep after sensitization with IgE^+ were: 40, 13 and 47 %, respectively. Of each patient serum the increase in histamine release between 1 and 60 minutes to HRF-MN-hep was expressed as percentage of the reference IgE^+ serum: ΔHRF. The number of patient sera tested in the three experiments were: 35, 11 and 27, respectively.

Statistics

The cut off value for ΔHRF was determined as the mean +3SD of the ΔHRF release found with sera derived from control individuals. Associations were determined by means of Chi-square test; the difference of the geometric mean ± SEM by Student's t-test.

Results

Comparing the four patient groups

The histamine release response to chemokine-depleted mononuclear supernatant in the 4 groups is shown in figure 1. The results represent the increase in histamine release from 1 minute to 60 minutes. ΔHRF is expressed as percentage of the reference serum. Using the control group (n=19) we defined the cut off value for being IgE^+ (mean +3SD of ΔHRF) of this study at 12 % of the reference serum. All the controls were below this value (figure 1). In the patient groups, the number of sera with more than 12 % ΔHRF histamine release were: 8/18 in the allergic asthma group, 2/19 in the nonallergic asthma group and 5/17 in the allergic rhinitis group.

The percentage of patients in the allergic asthma group with more than 12 % ΔHRF histamine release differed significantly from the healthy controls (0%) (Odds ratio 38.00, 1.98-730.54, p=0.003). In the atopic patient groups (allergic asthma and allergic rhinitis) 40 % of the individuals showed more than 12 % ΔHRF histamine release. This differed significantly from the nonallergic patient groups (nonallergic asthma and

healthy controls) in which 8 % of the individuals showed more than 12 % ΔHRF histamine release (Odds ratio 7.77, 2.00-30.29, p=0.003). No significant differences were found comparing the other patient groups with each other.

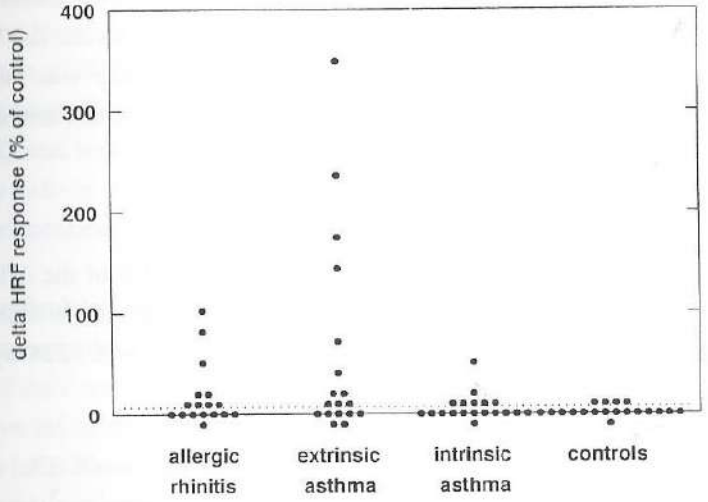

Figure 1. The histamine release response to chemokine-depleted mononuclear supernatant in 4 patient groups is shown. The results represent the increase in histamine release from 1 to 60 minutes. ΔHRF is expressed as percentage of the reference serum.

Association between ΔHRF histamine release response and the different parameters of atopy

The ΔHRF histamine release response (n=73) was associated significantly with: ① the total IgE level (cut off value 200 IU/ml): Odds ratio 11.04, 3.17-38.44, p<0.001 ; ② the highest score in the RAST for inhalant allergens (cut off value >2) : Odds ratio 19.10, 4.66-78.24, p<0.001; ③ and a positive RAST positive for grass pollen (cut off value >2): Odds ratio 49.72, 2.58-958.75, p<0.001; cat dander (cut off value >2): Odds ratio 30.00, 3.28-274.52, p<0.001 and dog dander (cut off value >2): Odds ratio 49.72, 2.58-958.75, p<0.001. The associaton between ΔHRF and IgE to house-dust mite (Odds ratio 3.82, 1.07-13.62, p=0.07) or birch pollen (Odds ratio 0.44, 0.02-8.88, p=0.96) was not significant.

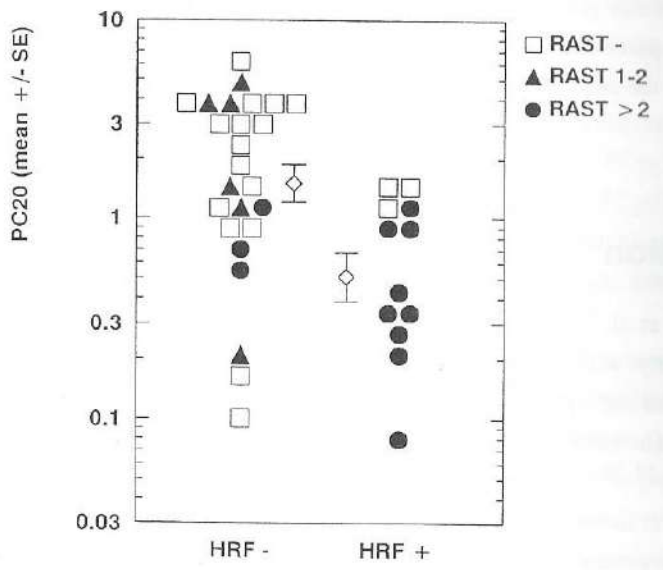

Figure 2. Comparison between the asthmatic patients (allergic and nonallergic) of the HRF positive sera (IgE^+) and HRF negative sera (IgE^-) with PC_{20} histamine (mg/ml). The asthma patients are subdivided in sera RAST negative for inhalant allergens, nonallergic asthma patient (open squares), sera RAST positive for inhalant allergens, allergic asthma patients (closed triangles ≤ 2 in the RAST) and sera RAST positive for inhalant allergens, allergic asthma patients (closed circles > 2 in the RAST).

Correlations in the asthma groups

In patients with asthma the association between the bronchial histamine threshold and reactivity to HRF differed significantly (IgE^+ , geometric mean PC_{20} 0.51 mg/ml, 0.39-0.68, versus IgE^-, geometric mean PC_{20} 1.50 mg/ml, 1.21-1.88; p for difference 0.004, figure 2). This association was also significantly different in patients with allergic asthma (IgE^+ , geometric mean PC_{20} 0.37 mg/ml, 0.27-0.50, versus IgE^-, geometric mean PC_{20} 1.27 mg/ml, 0.89-1.81; p for difference 0.017).

In the asthma groups IgE^+ was not significantly associated with age, sex, being a smoker, the percentage of reversibility of FEV_1 with salbutamol, FEV_1 as percentage of predicted or the ratio FEV_1/FVC.

Discussion

MacDonald et al. [9] postulated that the IgE-dependent HRF is a lectin-like agent, which interacts only with a subset of the allergic individuals because of differences in glycosylation of IgE. An alternative explanation for the histamine release by IgE-dependent HRF would be: IgE antibodies to (an) autoallergen(s) released from human cells [18, 29].

Looking at the responsiveness to HRF, the results reported by different investigators are contradictionary. Some authors reported that basophils derived from only a subset of the allergic individuals and not from nonallergic individuals respond to HRF. This response correlates with the atopic status of the basophil donor [30-33], the disease severity and is dependent on the IgE^+ [9, 34] on the surface of the basophils. Others, however, found that basophils derived from both allergic and nonallergic individuals respond to HRF, although allergic individuals showed a higher response [35, 36]; this response did not correlate with the disease severity [36]. Some of these investigators also described that this response to HRF is IgE^+ mediated [36].

The sensitization of lactic-acid treated basophils derived from two nonallergic donors with sera derived from the different patients excludes in vivo priming of the basophils and the variability caused by using basophils of every patient. The only variable in the method we used was the serum of the patient.

A disadvantage of this serum-based bioassay as a test system is, the possible involvement of serum factor(s) that modify the IgE-dependent histamine release. However, both the spontaneous histamine release and the anti-IgE mediated histamine release were comparable upon sensitization with the IgE^+ and IgE^- containing sera (data not shown).

The low prevalence of IgE^+ in the RAST negative asthmatic patients suggests that the IgE-dependent HRF is not an important factor in nonallergic asthma, confirming results obtained by others, who used platelets supernatants as HRF source [31-33].

In all asthma groups, the geometric mean PC_{20} histamine was significantly different between the IgE^+ patients and IgE^- patients. We did not measure the PC_{20} histamine in the allergic rhinitis group. The association between IgE^+ and a low PC_{20} histamine in asthma is not necessarily contradictory to the finding of IgE^+ in patients with allergic rhinitis. We [37] like others [38, 39] found in some patients with allergic rhinitis without lower airway symptoms a low PC_{20} histamine level.

As both the cells that respond to IgE-dependent HRF and the inflammatory cells that produce HRF are present in the late-phase response and the chronic-allergic reaction, our results support the hypothesis that the IgE-dependent HRF contributes to the late-phase response and the chronic-allergic reaction in a subpopulation of the atopic patients.

We conclude that in allergic rhinitis and in allergic asthma the response to the IgE-dependent HRF is highly correlated with the atopic status of the patient. In all asthmatic patients and in allergic asthmatic patients, the presence of IgE^+ is associated with a low histamine threshold (PC_{20} histamine < 2 mg/ml).

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Chapter 5

IgE-BINDING TO A HUMAN PROFILIN CONTAINING FRACTION DUE TO A CONTAMINATION WITH YEAST-DERIVED MATERIAL

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J Allergy Clin Immunol (in press)

Brief communication

Profilin is a protein found in all eukaryotic cells. Profilin in pollen from grasses, trees and weeds was found to be, largely responsible for crossallergenicity between pollen and vegetable foods [1, 2]. Valenta et al. [3] described crossreactivity for human IgE antibodies between profilin from birch pollen and profilin from human platelets, using immunoblots as assay system. We identified 11 sera with IgE to a human profilin containing fraction in a preliminary investigation [4].

In the present study we re-investigated the occurrence of IgE against human profilin in view of the possibility that human profilin, acting as autoallergen, might be implicated in IgE-dependent histamine-releasing activity [5].

Human profilin was isolated from human platelets by its affinity to poly-l-proline [3]. IgE to profilin described by others [2, 3] as well as by us [1] reacts with profilin from pollen and vegetable foods. It was therefore unexpected to find IgE-binding to the fraction containing human profilin in sera that were negative for IgE to pollen allergens [4]. Since all these sera were positive in the RAST for *Aspergillus Fumigatus*, other moulds and yeast, we assumed that this might indicate crossreactivity between profilin from moulds and human profilin. Alternatively, we considered the possibility that tissue damage by the mould was responsible for the release of autoallergenic human profilin.

However, none of these sera were positive on an immunoblot of an extract of human platelets. A rabbit serum obtained by immunization with the human profilin containing fraction showed IgG-binding with both a 14 kDa band (presumably human-platelet profilin) and a 41 kDa band (presumably human-platelet actin, data not shown).

The profilin preparation used in the RAST was prepared using an ATP-containing buffer, and was subsequently dialyzed. The results of control experiments suggested the presence of a nondialyzable contaminant in one of the buffer components. Indeed, a high-molecular-weight contaminant in the "ATP" proved to be responsible, as was shown by RAST with Sepharose-coupled "ATP" and by RAST-inhibition. The commercially obtained "ATP" (Boehringer, Mannheim, FRG, catalogue nr 127531) was found to be isolated from yeast. The IgE-binding to "ATP" was inhibited by yeast extracts (figure 1). We have no indication that the reaction with yeast is due to yeast profilin. The protein content of the dialyzed "ATP" used for the profilin isolation was

0.45 mg protein / g ATP. For 50% inhibition of the "ATP" RAST, 6 µl of 1 mM "ATP" was required (figure 1). On a protein basis, this corresponds to 1.6 ng. We consider it therefore unlikely that the inhibitory activity is due to a protein. Purified yeast mannan had little inhibitory effect (figure 1).

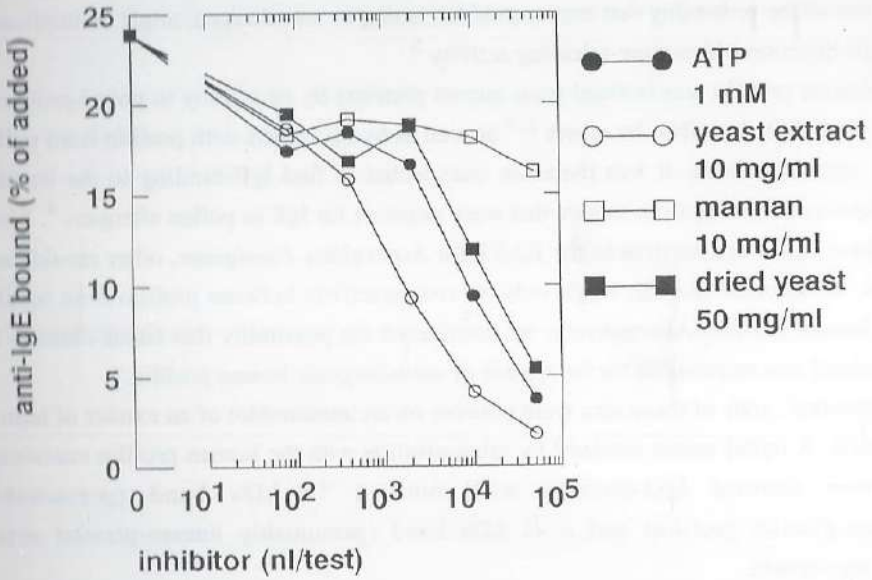

Figure 1. RAST-inhibition assay of the IgE-binding of one serum to "ATP"-Sepharose by "ATP", yeast extract and mannan. Inhibitors: ATP (1 mM, closed circles), yeast extract (10 mg/ml, open circles), yeast mannan (10 mg/ml, open squares) and dried yeast (50 mg/ml, closed squares).

Spectroscopically, no difference was found between the "ATP" used for the purification of profilin and a more purified ATP (Boehringer, catalogue nr 519987), either at 260 or at 280 nm. After purification of human profilin with the more purified ATP no IgE-binding to human profilin was demonstrated in: 1) the 11 sera positive in the RAST for *Aspergillus Fumigatus*, 2) in 23 sera positive in a RAST to grass profilin and 3) in 16 sera that were positive in a bioassay for the IgE-dependent histamine-releasing activity [5] in supernatants derived from mononuclear cells. As a positive control we used the rabbit anti-serum to human profilin that demonstrated IgG-binding with human profilin but not with grass-pollen profilin.

In conclusion: results obtained by direct RAST and RAST inhibition indicate that the IgE-binding to "human profilin" we previously described [4], was in fact due to yeast-derived material in the "ATP" used during the isolation of profilin. We did not find IgE-binding to human profilin in 23 sera with IgE to grass-pollen profilin.

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Addendum

IS HUMAN PROFILIN AN IgE-DEPENDENT HISTAMINE-RELEASING FACTOR ?

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J Allergy Clin Immunol 1992; 89: 276 (abstract)

Abstract

We tested the hypothesis that IgE antibodies to human profilin (Valenta et al., 1991) are involved in the IgE-dependent HRF-activity described by MacDonald et al..

Profilin (molecular weight 14 Kda) was isolated from human platelets. We identified 8 sera of allergic individuals with IgE-binding to this autoallergen and used one of these for quantitation of profilin. IgE-dependent HRF-activity was measured using purified stripped basophils sensitized with IgE^+ as defined by MacDonald. HRF was prepared from the supernatants of SK/SD-stimulated mononuclear cells. Experiments were also performed with human dander extract, which, on the basis of its histamine-releasing capacity on basophils, was considered to be a potential source of IgE-dependent HRF.

By a RAST-inhibition assay the profilin content of the HRF preparation was below the detection limit, but a surprisingly high inhibition of IgE-binding to profilin was found with the human dander extract.

In contrast to the HRF preparation and the human dander extract, the isolated profilin shows little or no histamine-releasing activity.

Two IgE^+ sera were tested in the profilin-RAST. One serum was negative, the other was weakly positive. Two sera with IgE antibodies to profilin were IgE-typed; both were IgE^-.

We conclude that: (1) allergic individuals show IgE-binding to human profilin, (2) the role of profilin as an IgE-dependent HRF is of minor importance and (3) human dander contains profilin.

Chapter 6

TRANSFECTION OF HUMAN FCεRIα INTO A RBL-2H3 CELL LINE WITH A HIGH IgE-MEDIATED RELEASING PHENOTYPE

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Abstract

Lack of a human basophilic or mast cell line with high IgE-mediated degranulation hampers research on human IgE and its receptor. To obtain such a cell line the cDNA of human FcεRIα was stably transfected into RBL-2H3 cells. Before transfection, RBL-2H3 cells with high IgE-mediated mediator release were selected by subcloning. The parental RBL-2H3 cells and two clones of RBL-2H3 were transfected with the plasmid NT zeo containing the cDNA of human FcεRIα. All transfectants expressed the human FcεRIα on the cell surface. Activation with the releasing moAbs anti-human FcεRIα (moAb 6F7 and 22E7) induced a serotonin release of 50 %. After sensitization with human IgE antibody the maximal release with allergen was 20 % and with anti-human IgE 50 %. Sensitization with human chimaeric IgE anti-NP and subsequent activation with antigen (NIP-BSA) resulted in a release of 40 %.

These results confirm that signal transduction through the human FcεRIα chain with the endogenous γ (and β) chain is possible. However, after sensitization with patient serum and allergen, the number of human receptors is probably too low for a release comparable to that induced by endogenous receptors.

Introduction

For bioassays of reactions mediated by human IgE a suitable continuously growing cell line with an efficient IgE-receptor-dependent triggering pathway would be highly desirable. However, no human mast cell line or human basophil cell line exists that expresses the FcεRI and demonstrates high FcεRI-mediated release with human IgE. The FcεRI receptor is a tetramer (αβγ2), consisting of an α chain [1], a β chain [2], and two disulfide-linked γ chains [3]. The ectodomain of the α chain is responsible for ligand binding [4, 5]. The human receptor can be expressed in Cos 7 cells as a hybrid α human γ rat/mouse receptor or αγ human receptor [6, 7]. The human α chain can be expressed on the cell surface without the β chain. When the β chain is present part of the receptors consist of the tetrameric complex [6]. Moreover, substitution of the human FcεRI chain for

the rat FcεRI chain during signal transduction is possible [8-10]. However, the IgE-mediated release was < 10 % [9, 10].

In the present study the cDNA of the human FcεRIα was transfected into a rat basophilic leukemia cell line, RBL-2H3, with a high IgE-mediated releasing phenotype. This RBL-2H3 cell line is related to rat mucosal mast cells [11].

A cell line transfected with human FcεRIα is a useful tool to investigate the effect of new therapies in allergy on the level of IgE and its high affinity receptor.

Materials and methods

Immunoglobulins and reagents

Rat monoclonal IgE LO-DNP-30 was from IMEX (Université de Louvain, Bruxelles, Belgium). FITC-labeled polyclonal mouse anti-rat Ig (MAR) F(ab')2 fragments were from Jackson Immunoresearch laboratories (West Grove, PA).

Mouse IgE anti-DNP 2682-I mAb was a gift of dr. U. Blank (Institut Pasteur, Paris, France). MoAbs anti-human FcεRIα (moAb 6F7 and moAb 22E7) [12] were a gift of dr.J. Hakimi (Hoffman-La Roche, Nutley, NJ). Serum # 179 (total IgE 2600 IU/ml), obtained from a donor from the department of Plasmapheresis of the CLB, contained IgE to house-dust mite. Polyclonal sheep anti-human IgE antiserum was from CLB (nr SH25P01, Amsterdam, The Netherlands).

BSA was dinitrophenylated using 2-,4-dinitrobenzene sulfonic acic (Eastman-Kodak, Rochester, NY). After passage over Sephadex G25 (Pharmacia, Uppsala, Sweden), the average degree of substitution was 13 moles DNP/mole BSA. NIP_{20}-BSA was a gift from dr. T.A. Out (Academic Medical Center, Amsterdam, The Netherlands). Anti-NP has similar affinity to NIP as to NP.

Human chimaeric IgE anti-NP

The construct of the human chimaeric IgE anti-NP was kindly provided by Neuberger (MRC Centre, Cambridge, UK). Human chimaeric IgE anti-NP was prepared as described by dr. Neuberger et al. [13].

RBL cells and transfectants

The RBL-2H3 cells were cultured as a monolayer in DMEM supplemented with 10 % heat-inactivated FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin at 37 °C, in a humidified atmosphere of 95 % air/5 % CO_2. Only adherent RBL-2H3 cells were used. For secretion studies, the cells were grown to confluency. They were recovered with trypsin-EDTA. All culture reagents were from Gibco (Paisley, Scotland, UK). Colonies were harvested by clonal ring selection [14] and transferred to a 24-well cluster dish for expansion and analysis. The medium for the FcεRIα-transfected RBL in addition contained 0.5 mg/ml Zeocin (Cayla, Toulouse, France).

Plasmid constructions

Basic cloning procedures were performed as described [15]. The expression plasmid NT zeo was kindly provided by dr. C. Bonnerot (Institut Curie, Paris, France). The pCMV-4 neo plasmid containing human FcεRIα full-length cDNA was a gift from dr. U. Blank (Institut Pasteur, Paris, France). The human full-length cDNA was excised from pCMV-4 neo with Mlu I and Xba I (Boehringer, Mannheim, Germany), filled in with T7 DNA polymerase and cloned into the blunt-ended EcoR V site of NT zeo downstream of the promotor.

Transfection

Before transfection the RBL-2H3 cells were washed and resuspended in DMEM without FCS. A total of 50 µg of human FcεRIα DNA in the NT zeo vector, purified by centrifugation through cesium chloride gradient and linearized with Cla I, was incubated with 5 x 10^6 RBL-2H3 cells in gene pulse cuvettes (Biorad) and transfected at 260 V , 960 µF using a Biorad electroporation apparatus. The cells were then cultured in normal medium. Fourty-eight hours after transfection, the cells were placed in selective medium containing 0.5 mg/ml Zeocin.

FcεRIα expression

Endogenous receptor expression was screened after sensitization with rat monoclonal IgE LO-DNP-30 (1 hour at 0 ° C) in HBSS (Gibco) containing 5 % FCS and subsequently stained by FITC-labeled polyclonal mouse anti-rat Ig (MAR) F(ab')2

fragments. Fluorescense was analyzed by flow cytometry using a FACScan flow cytometer (Beckton Dickinson, Montain View, CA).

Nontransfected and transfected RBL-2H3 cells, expressing the human α subunit were identified by immunofluorescense in two ways. First by binding of human serum IgE (150 minutes, 37 °C), followed by incubation (60 minutes, 0 °C) with polyclonal FITC-conjugated anti-human IgE (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD), or second by incubating for 1 hour at 0 °C with 1 and 10 μg/ml moAbs anti-human FcεRIα (moAb 6F7 and moAb 22E7) in HBSS (Gibco) containing 5 % FCS and stained by FITC-labeled goat anti-mouse F(ab')2 (Jackson Immunoresearch, West Grove, PA).

Secretion studies [16]

Transfected or nontransfected RBL-2H3 cells were resuspended in RPMI medium supplemented with 10 % FCS at 1 x 10^6 cells/ml and incubated (60 minutes, 37 °C) with 3 μCi/ml [^{3}H] serotonin (Amersham, Les Ulis, France). After washing the cells were resuspended in the same medium and incubated for another hour at 37 °C. After washing again, the cells were resuspended in the same medium and added to 96-well microculture plates at 2 x 10^5 cells/well together with IgE to a final volome of 50 μl (150 minutes, 37 °C). Nonadherent cells were removed by washing 4 times with 200 μl HBSS, 25 μl medium was added to each well and cells were warmed at 37 °C for 15 minutes before challenge. Cells were challenged for 30 minutes at 37 °C with 25 μl of DNP-BSA or house-dust mite extract (ALK, Copenhagen, Denmark), previously warmed at 37 °C for 15 minutes. Reactions were stopped by adding 50 μl ice-cold medium and by placing plates on ice. The amount of [^{3}H] serotonin release in the supernatant was determined by mixing 50 μl of supernatant with 200 μl Aqualuma-Plus scintillation fluid (Lumac, The Netherlands) and counted in a β-plate counter (Pharmacia, Uppsala, Sweden). Using a 100 % cpm, the percentage of [^{3}H] serotonin release was calculated. The 100 % cpm was determined by taking 50 μl from wells containing the same number of cells lyzed in 50 μl 0.5 % SDS and 0.5 % NP40.

Results

Selection of RBL-2H3 clones with a high IgE-mediated releasing phenotype

To select a clone with a high IgE-mediated releasing phenotype before transfection, parent RBL-2H3 cells were subcloned. Colonies were harvested by clonal ring selection [14] and transferred to a 24-well culture dish for expansion and analysis. In the FACS analysis all 12 clones were comparable in rat receptor FcεRI expression, investigated by binding of rat monoclonal IgE LO-DNP-30 and subsequent FITC-labeled polyclonal mouse anti-rat Ig (MAR) F(ab')2 fragments (data not shown). Two subclones RBL-4 and RBL-7 showed a higher IgE-mediated release compared to the parent RBL-2H3 cells, 60 %, 50 % versus 40 %, respectively (data not shown).

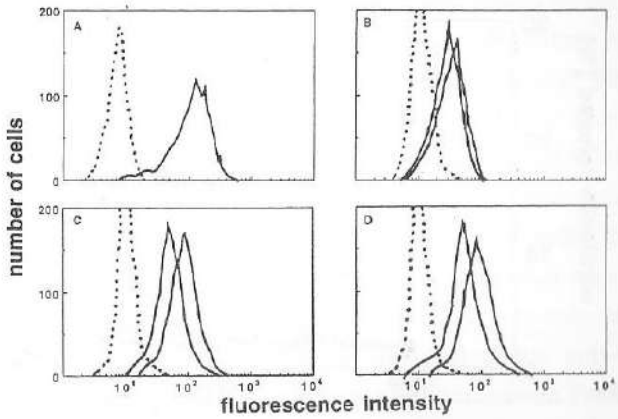

Figure 1. Surface expression of the rat FcεRIα chain on subclone RBL-7 and of the transfected human FcεRIα chain on RBL-7-T. A: surface expression of rat receptor FcεRIα expression on subclone RBL-7, investigated by binding of rat monoclonal IgE LO-DNP-30 and subsequent FITC-labeled polyclonal mouse anti-rat Ig (MAR) F(ab')2 fragments. Surface expression of human FcεRIα chain transfected RBL-7-T cells (B, C, D). Cells were analyzed after binding of B: human serum IgE or moAbs anti-human FcεRIα (C: moAb 6F7 and D: moAb 22E7) and by subsequent indirect immunofluorescence using respectively anti-human IgE- or MAR (Fab')2-FITC conjugated. The dotted graphs are the background fluorescence.

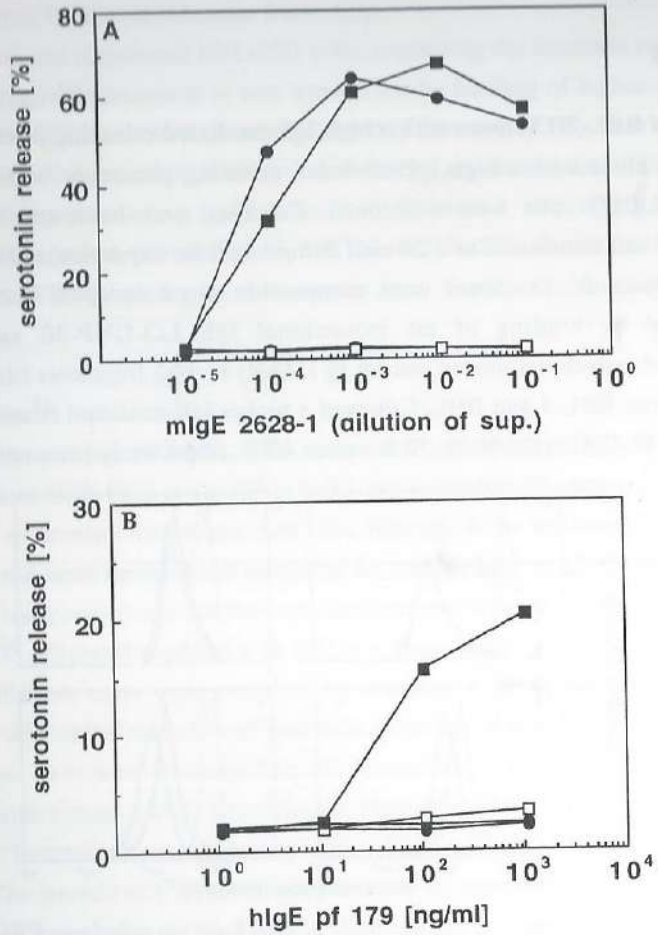

Figure 2. Release of [^{3}H] serotonin from nontransfected (circles) and transfected (squares) RBL-7 cells with human FcεRIα. Both cell lines were sensitized with A: mouse IgE anti-DNP and challenged with 1 µg/ml DNP-BSA (closed symbols) or control (open symbols) or B: sensitized with human serum IgE #179 and challenged with house-dust mite (HDM) 16667 SQ/ml (closed symbols) or control (open symbols). This experiment is representative for two experiments.

Transfection of human FcεRIα in RBL-2H3 cells

The cDNA coding for human FcεRIα chain was introduced into the expression vector NT zeo and transfected into three RBL-2H3 cell lines: parent-cloned RBL-2H3 and cloned RBL-4 and RBL-7. After selection in Zeocin containing medium and subsequent FACS analysis using human serum IgE or moAb anti-human FcεRIα chain, all transfectants, now designed as RBL-T, RBL-4-T and RBL-7-T, were found to express a functional α chain of the human IgE receptor. Figure 1 shows the analysis of expression of human receptors on transfected RBL-7-T. Comparable results were obtained with RBL-T and RBL-4-T. The nontransfected cells did neither bind human IgE nor moAbs anti-human FcεRIα chain (data not shown).

Serotonin release in human FcεRIα transfected RBL, RBL-4 and RBL-7

To examine whether the human FcεRIα chain transfected RBL-2H3 cells can be activated through this receptor, cells were sensitized with human serum containing IgE antibodies and challenged with house-dust mite extract and this release was compared to cells sensitized with mouse IgE anti-DNP and challenged with DNP-BSA. The RBL-T gave a maximal release of 40 % via the endogenous rat FcεRI receptor and a maximal release of 11 % induced by house-dust mite via human IgE bound to the human FcεRI receptor (data not shown). In the transfected and the nontransfected RBL-7 the endogenous rat FcεRI receptor gave a maximal release of more than 60 % with DNP-BSA (figure 2). The maximal release induced by house-dust mite via human IgE bound to the human FcεRIα in the transfected RBL-7-T was only 20 % (figure 2). However, when RBL-7-T were challenged with polyclonal anti-human IgE or unsensitized RBL-7-T cells with the releasing moAbs anti-human FcεRIα (moAb 6F7 and 22E7), the maximal human FcεRIα-mediated release was 50 % (figure 3). As complete antibodies were used to stimulate, interaction of the Fc portion of the antibodies with the IgG receptors on the RBL-2H3 cells might have influenced the response. Another explanation was that the transfected cells did not express enough human FcεRIα receptors on their surface to bind specific IgE to crosslink with house-dust mite. To test the latter hypothesis, the human FcεRIα transfected cells RBL-4-T were sensitized with mouse IgE anti-DNP and challenged with DNP-BSA or sensitized with human IgE and challenged with house-dust mite or sensitized with chimaeric IgE anti-NP and challenged with NIP-BSA (figure 4). The endogenous rat FcεRIα receptor

of RBL-4-T gave a maximal release of 45 %. The human FcεRIα of RBL-4-T gave a release of 40 % with the chimaeric IgE and 20 % with human serum IgE. For the RBL-7-T the release via the endogenous rat FcεRI receptor was more than 55 % and for the human FcεRIα receptor after sensitization with chimaeric IgE the maximal release was 40 % (data not shown). The RBL-T was not tested with the chimaeric IgE.

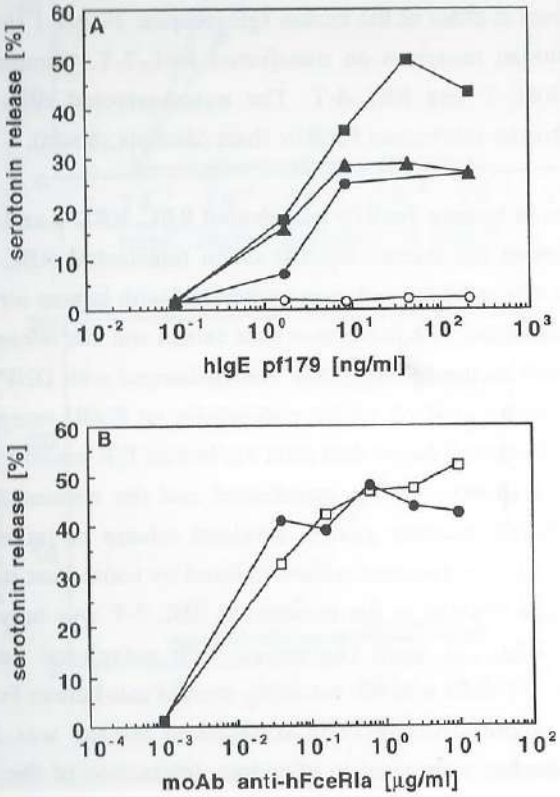

Figure 3. Release of [^{3}H] serotonin from transfected RBL-7-T cells with human FcεRIα. The cells were sensitized with human serum IgE #179 (A) and challenged with anti-human IgE 0 ng/ml (open circles), 10 ng/ml (closed circles), 100 ng/ml (closed squares) and 1000 ng/ml (closed triangles). Other cells (B) were not sensitized and activated with moAbs anti-human FcεRIα (moAb 6F7: closed squares and moAb 22E7: closed circles).

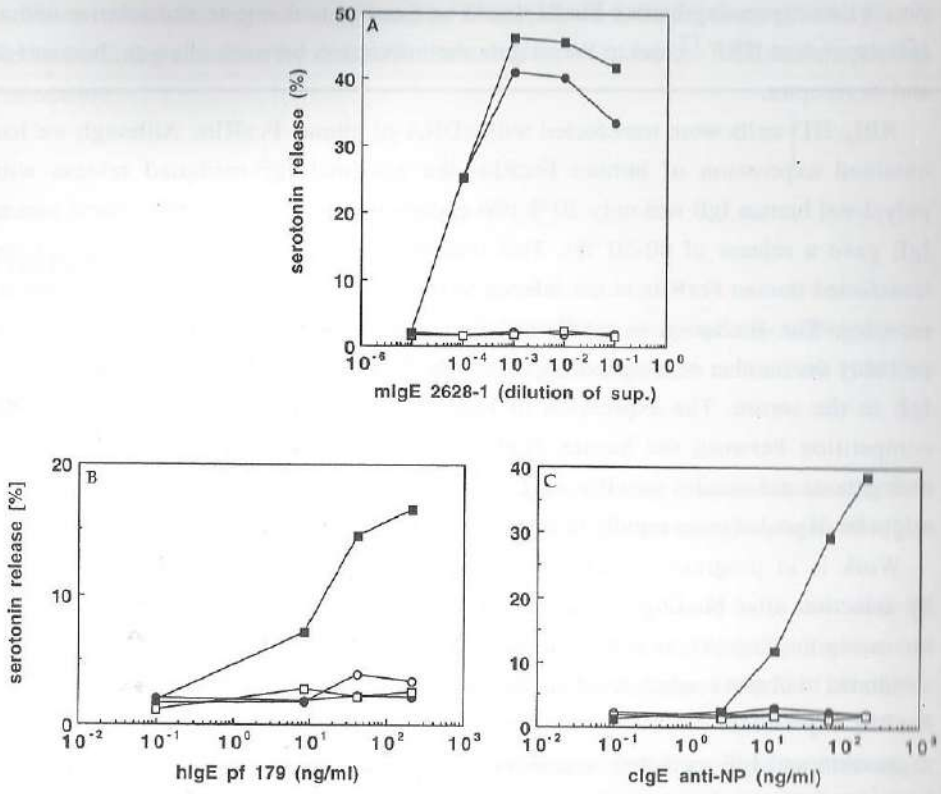

Figure 4. Release of [^{3}H] serotonin from nontransfected (circles) and transfected (squares) RBL-4 cells with human FcεRIα. Both cell lines were sensitized with A: mouse IgE anti-DNP and challenged with 1 µg/ml DNP-BSA (closed symbols) or control (open symbols) or B: sensitized with human serum IgE #179 and challenged with house-dust mite (HDM) 16667 SQ/ml (closed symbols) or control (open symbols) or C: sensitized with chimaeric IgE anti-NP and challenged with NIP-BSA 3 µg/ml (closed symbols) or control (open symbols). This experiment is representative for two experiments.

Discussion

A cell line expressing human FcεRI would be a useful tool: e.g. to characterize human IgE-dependent HRF [17] and to investigate the interaction between allergen, human IgE and its receptor.

RBL-2H3 cells were transfected with cDNA of human FcεRIα. Although we had obtained expression of human FcεRIα, the maximal IgE-mediated release with polyclonal human IgE was only 20 % (the endogenous receptor and monoclonal human IgE gave a release of 40-50 %). This indicates that the signal transduction of the transfected human FcεRIα is not inferior to the response mediated by the endogenous receptor. The limitation in mediator release with polyclonal human serum IgE is probably the number of receptors expressed on the cell surface compared to the specific IgE in the serum. The expression of human FcεRIα might be limited because of competition between the human FcεRIα and the endogenous rat FcεRI for the endogenous not-species specific rat FcεRIγ chains, alternatively the human α chains might be degraded more rapidly than the endogenous rat α chain [10].

Work is in progress to increase the receptor expression of the human FcεRIα, by selection after binding to magnetic beads covered with house-dust mite and by increasing the Zeocin concentration of the selection medium. These cells are now being subcloned to obtain a subclone of the transfected RBL-7-T with higher and stable human FcεRIα expression. Another possibility to increase both the human FcεRIα receptor expression and IgE-mediated release with serum IgE and allergen, is to transfect the human γ chain into these transfectants.

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SUMMARY / SAMENVATTING

SUMMARY

In this thesis we tested the hypothesis that IgE-binding to an autoallergen is responsible for the discrepancy between the level of specific IgE and the severity of allergic symptoms.

A histamine-releasing factor (HRF/HRA) has been described that releases mediators from human basophils by interacting with surface IgE. The IgE that interacts with IgE-dependent HRA in cell supernatants was defined as IgE^+.

This HRA has been found in culture supernatants of different cell sources and in body fluids. The HRA of unpooled mononuclear supernatants derived from 8 donors was investigated. These supernatants were found to differ quantitatively and qualitatively in both IgE-independent and IgE-dependent HRA. The HRA in some supernatants discriminated between basophils derived from allergic and nonallergic donors (chapter 2). The IgE-dependency of the HRA was demonstrated by inhibition of passive sensitization after preincubating a responder serum with a nonreleasing monoclonal antibody to IgE.

The HRF production was investigated by activating the mononuclear cells with different stimuli (spontaneous, SK/SD, anti-CD2, anti-CD28, PMA) and at different cell densities. The contribution of the IgE-independent HRA was monitored by measuring MCP-1 in these 280 supernatants by an inhibition RIA for MCP-1. The additional presence of IgE-dependent HRA was shown by comparing in 18 mononuclear supernatants the histamine release from lactic-acid treated basophils sensitized with or without an IgE^+ serum. The difference in histamine release was measured between 1 and 60 minutes. Subsequently, IgE-independent chemokine activity was removed from the mononuclear supernatants by using the affinity of chemokines for heparin. The IgE-dependent HRA was unmasked after depleting the IgE-independent HRA with heparin-agarose (chapter 3).

The clinical relevance of the IgE-dependent HRA was investigated in three groups of patients and controls (chapter 4). 1) Eighteen patients with allergic asthma were selected with a positive skin test, a positive RAST to a panel of common inhalant allergens and an increased bronchial sensitivity to histamine ($PC_{20} \leq 8$ mg/ml). 2) Nineteen patients with nonallergic asthma were selected with a negative skin test, a negative RAST to a panel of common inhalant allergens, matched for age and an increased bronchial

sensitivity to histamine ($PC_{20} \leq 8$ mg/ml). 3) Furthermore 17 patients with allergic rhinitis were selected with a positive skin test and positive RAST to an identical panel of common inhalant allergens, with a history of sneezing, itching in the nose, a running nose or a blocked nose, but without a history of paroxysms of dyspnoe, wheezing and coughing. 4) Also 19 healthy controls were selected without a history of allergy or upper and lower airway diseases and a negative RAST to an identical panel of common inhalant allergens. Sera of these groups of patients and of the controls were used to sensitize lactic-acid treated purified basophils for IgE-dependent HRF using chemokine depleted mononuclear supernatants and measuring the increase in histamine release between 1 and 60 minutes. IgE^+ was found to be associated with atopy: 40 % of the RAST positive individuals and 8 % of the RAST negative individuals were IgE^+ (Odds ratio, 7.77, 2.00-30.29, p=0.003). Furthermore: IgE^+ was associated with bronchial sensitivity to histamine in all asthmatic patients (geometric mean PC_{20}: 1.50 versus 0.51 mg/ml, p for difference 0.004) and also in the allergic asthmatic patients (geometric mean PC_{20}: 1.27 versus 0.37 mg/ml, p for difference 0.017). In asthma patients disease severity and histamine sensitivity are reflected in the late allergic response. Since both the cells that respond to HRF and the inflammatory cells that produce the IgE-dependent HRF are present in the late allergic response and in the chronic-allergic response, our results suggest that the IgE-dependent HRF contributes to the late allergic response in some of the atopic asthma patients.

To characterize IgE-dependent HRF, the hypothesis was tested whether human profilin might be the IgE-dependent HRF. Human profilin is a protein present in all eukaryotic cells. Profilin is a major crossreactive component in pollen from grasses, trees and weeds. Crossreactivity for human IgE antibodies between profilin from birch pollen and profilin from human platelets was reported, using immunoblots as assay system. We demonstrated that the IgE-binding to human profilin we found, was due to a yeast contamination of the ATP by RAST and RAST inhibition. ATP was used in the buffers during the purification of profilin from human platelets. After purification of human profilin with more purified ATP, no reactivity with human profilin was demonstrated in sera containing IgE to birch-pollen profilin or in sera containing IgE^+. We concluded that human profilin was not an IgE-dependent HRF (chapter 5).

For further characterization of IgE-dependent HRF a more convenient and reliable, less variable assay was initiated. The α chain of the high affinity IgE receptor (FcεRI)

binds IgE. The cDNA of the human α chain of FcεRI was transfected in the rat basophilic leukaemia cell line (RBL-2H3) (chapter 6). Binding of human IgE and mediator release after crosslinking of human FcεRIα was demonstrated.

SAMENVATTING VOOR DE NIET-IMMUNOLOOG

Allergische patienten zijn gevoelig (gesensibiliseerd) voor allergenen (zoals bijvoorbeeld graspollen, katten of huisstofmijten). Tegen dit allergeen worden allergische afweerstoffen (immuunglobuline E, IgE antistoffen) gemaakt, die aangetoond kunnen worden: 1) in het bloed van de patient en 2) op het oppervlak van de allergische cellen in het bloed (basofielen) en in de weefsels (mestcellen). Wanneer een allergische patient in contact komt (bijvoorbeeld via de luchtwegen bij astma) met allergeen, worden de IgE antistoffen op het oppervlak van de mestcellen in het weefsel (in bijvoorbeeld de longen) van de patient met elkaar verbonden door het allergeen. De mestcellen worden hierdoor geactiveerd en stoten stoffen uit die binnen 5-10 minuten de allergische klachten (zoals benauwdheid) bij de patient veroorzaken (de vroege allergische reactie). Bovendien trekken de door de mestcellen uitgescheiden stoffen afweercellen (neutrofielen, eosinofielen, basofielen, lymfocyten en monocyten) uit de bloedbaan aan. Wanneer deze afweercellen uit het bloed in het weefsel (bijvoorbeeld de long bij astma) zijn aangekomen, worden ze geactiveerd en gaan op hun beurt vele stoffen uitscheiden, die bij sommige allergische patienten 6-11 uur na het eerste contact met het allergeen nogmaals klachten kunnen veroorzaken (de late allergische reactie). Deze late allergische reactie kan soms weken voortduren (de chronische allergische reactie). De wijze waarop afweercellen in de late en chronische allergische reactie in het weefsel worden gestimuleerd is niet geheel duidelijk. In de studies beschreven in dit proefschrift is gekeken naar het mogelijk bestaan van andere IgE-bindende factoren behalve allergenen, die een rol zouden kunnen spelen in de late en chronische allergische reactie. Dit zouden dan lichaamseigen eiwitten zijn waartegen IgE antistoffen bestaan, hetgeen op het bestaan van autoallergie zou kunnen wijzen.

De allergische cellen in het bloed (basofielen) hebben, net als de mestcellen in de weefsels, IgE antistoffen op hun oppervlak. In de literatuur is beschreven dat basofielen kunnen worden geactiveerd door lichaamseigen eiwitten uitgescheiden door afweercellen (lymfocyten en monocyten). Deze lichaamseigen eiwitten worden histamine vrijmakende factoren ("histamine-releasing factors", HRFs) genoemd. Wij hebben deze HRFs nader onderzocht en gekeken of de basofielen direct via het

celoppervlak werden geactiveerd door HRF (IgE-onafhankelijk) of via de IgE antistoffen gebonden op het oppervlak van de cel (IgE-afhankelijk). Gebleken is dat de basofielen op beide wijzen geactiveerd werden door HRFs (hoofdstuk 2). De activatie direct via het oppervlak van de cel is gekwantificeerd door de hoeveelheid van een van deze IgE-onafhankelijke HRFs, het zogenaamde MCP-1, te meten. Na verwijdering van de IgE-onafhankelijke HRFs met behulp van heparine-agarose werden de IgE-afhankelijke HRFs duidelijker aantoonbaar (hoofdstuk 3).

In hoeverre deze IgE-afhankelijke HRFs van belang zijn voor de allergische patienten werd onderzocht in een groep patienten met allergisch astma, een groep patienten met niet-allergisch astma, een groep patienten met allergische klachten in de neus en bij een groep gezonden als controles (hoofdstuk 4). Hieruit is gebleken dat een gedeelte van de allergische patienten reageerden op stimulatie met de IgE-afhankelijke HRFs in tegenstelling tot patienten met niet-allergische astma en de controles. Bovendien is bij patienten met astma een associatie aangetoond tussen een sterke overgevoeligheid van de longen en een reactie met de IgE-afhankelijke HRFs.

Het is nog niet duidelijk welk eiwit verantwoordelijk is voor de IgE-afhankelijke reactie met HRF. Een eiwit dat in alle lichaamscellen voorkomt is profiline. Een onderzoeksgroep heeft beschreven dat allergische patienten die IgE antistoffen hebben tegen profiline uit berkepollen ook IgE antistoffen tegen profiline uit menselijke cellen hebben. Wij hebben onderzocht of dit menselijk profiline misschien een IgE-afhankelijke HRF is. Aangezien wij het bestaan van deze IgE antistoffen tegen menselijk profiline niet hebben kunnen reproduceren, is profiline niet een IgE-afhankelijke HRF (hoofdstuk 5).

Voor verder onderzoek naar de identiteit van de IgE-afhankelijke HRF is geprobeerd een praktischer testsysteem te ontwikkelen. Hiervoor is met behulp van recombinant DNA technieken het DNA dat codeert voor het menselijk eiwit dat het IgE op het oppervlak van basofielen en mestcellen bindt, gebracht in een onsterfelijke cellijn (getransfecteerd) afkomstig van een ratte basofiel tumor (hoofdstuk 6). Aan deze getransfecteerde cellijn kunnen IgE antistoffen van patienten worden gebonden. Hiermee zal in de toekomst worden geprobeerd de IgE-afhankelijke HRF uitgescheiden door lymfocyten en monocyten te isoleren, te karakteriseren en de betekenis ervan verder te onderzoeken.

DANKWOORD

Scheepsjournaal

Na vier jaar van omzwermingen over nationale en internationale wateren is dan nu het moment aangebroken deze CLB-haven voor een ander te ruilen.

Het begon allemaal vier jaar geleden in een kleine 'Optimist' op een ogenschijnlijk stil watertje. Nadat een aantal eerste aanwijzigingen waren gegeven door Rob Aalberse van de zeilvereniging 'Allergie', werd ik meteen het water opgestuurd. Nog niet wetende hoe ik het vaste punt op de wal kon bereiken werd ik aanvankelijk geïnstrueerd door Rob vanaf de steiger en door professor Jansen vanaf zijn fiets op de wal vanaf de haven 'Longziekten van het AMC'. Regen, storm of zon, iedere dag weer zocht ik met veel plezier de uitdaging met de wind op in dit zeil-avontuur. Gelukkig hield de altijd enthousiaste havenmeester, Edward Knol, mij al die jaren nauwlettend in de gaten en heeft mij menigmaal in de branding bijgestaan: hartstikke bedankt. Bij het trimmen van mijn bootje voor de (inter)nationale wedstrijden en bij de optredende technische calamiteiten ben ik op voortreffelijke wijze geholpen door zeilvereniging 'Celchemie': Edward, Erik Mul, Anton Tool, Janine Schuurman, Dirk Roos en Arthur Verhoeven en nog vele anderen. Anton leerde mij met zijn geheel eigen didactische methoden het adrenaline peil onder controle te krijgen.

Na een half jaar werd de 'Optimist' verruild voor een Varuna 500 waarop ik een trouwe, supergezellige en karaktervolle (fokke)maat ontmoette, Marja Aalbers. Samen hebben wij genoten van mooie zomerdagen (en -nachten) en menige zware storm getrotseerd; bedankt voor alles! In deze tijd hebben Marja en ik dankbaar gebruik gemaakt van de uitstekende zeilen, die altijd met veel zorg werden gemaakt door Gisela Romijn en Ferrina Schotanus van de afdeling Plasmaferese. Een essentiele rol bij de produktie van de zeilen hebben onze fans, alle bloeddonoren, gespeeld, die altijd vrijwillig hun hulp aanboden en zelfs bereid waren hun tijd voor onze wedstrijden in te

delen. Verder konden wij altijd rekenen op alle medewerking van het RAST lab en van afdeling Inkoop.

Naast een goede teamgeest is coaching esssentieel voor een goede zeil prestatie. Met Rob als hoofdschipper heb ik de afgelopen jaren vanuit zijn zeilvereniging 'Allergie' de beginselen van wetenschappelijk onderzoek geleerd. Voor het te leveren bewijs stonden zelfstandigheid, vertrouwen en doorzettingsvermogen altijd hoog bij hem in het vaandel. Soms waren de stromingen zeer complex en was het moeilijk de beste koers te berekenen hetgeen resulteerde in vaak boeiende discussies. Bedankt dat ik op je afdeling mocht werken.

Meerdere malen is geprobeerd het aantal knopen op te voeren door extra zeilen bij te zetten: Michael Fijnenberg, Joke Boonstra, Karen Wijker, Aran Labrijn, Martina Kozel en Marcel Schutte, ofschoon het resultaat vaak anders was dan voorspeld, mede dankzij jullie geweldige inzet werd dit ogenschijnlijk ondoorzichtige parcours langzaam opgehelderd.

Vaak is ook gebruik gemaakt van de zeilvereniging 'Autoimmuunziekten', waar o.a. Lucien Aarden, Ed Nieuwenhuys, Els de Groot, Irma Rensink en Margreet Hart ons altijd met open armen ontvingen en met goede raad en materialen bijstonden. Voor het verkrijgen van nieuwe inspiratie kon ik altijd terecht in de Bibliotheek bij Liesbeth Loots en Anita Frankena. Zo ook met alle technische problemen bij Marja, Gerrard Perdok, Marcel Mulder en de Technische Dienst; met alle paperassen bij Heleen van Maanen, Wendy Merison en Lilian Hoekstra; en voor de leerzame 'appel-praatjes' bij tij en ontij bij Jos Barendregt.

Voor, tijdens en na het zeilen waren er de vele leuke, gezellige en leerzame momenten met de eigen leden van zeilvereniging 'Allergie': bedankt Steven, Ronald, Jaap, Agnes, Jolanda, Marjan, Peter, Gert-Jan en Maurits, ik heb ervan genoten. Zo ook van de dortslessende momenten in Bouke's corner. Verder wil ik bij deze collega's, familie en vrienden nogmaals danken voor jullie hartelijkheid toen de romp van mijn bootje beschadigd was.

Toen ontdekten we tijdens een wedstrijd in Zweden het schilderij 'miemelen'. Mede hierdoor kregen we na drie jaar stug volhouden de vaart goed in het schip. We waren aan een ander schip toe, een mooie houten Valk. Hiermee bezochten we geregeld de haven van professor Jansen, die samen met zijn goede en behulpzame team bestaande uit havenmeester Jaring van der Zee en de leden Agnes Witteman en Maurits van der Veen

ons van uitstekende adviezen en materialen van patienten voorzag, mede afkomstig van de afdeling KNO.

Voor het uitwerken van een nieuwe taktiek werd een reis buitengaats gepland 'in de moleculaire biologie' naar Parijs. Par la présente je voudrais encore remercier dr. W.H. Fridman, Marc Daëron et le laboratoire Immunologie Cellulaire et Clinique de Institut Curie pour le merveilieux temps que j'ai passé, toute la hospitalité que j'ai rencontrée at l'assistance excellente, notamment de Sylvain Latour et Odile Malbec.

Vooral tijdens de laatste weken waarin in korte tijd dit journaal over de zoektocht naar 'de zich onderscheidende miemel' werd gedocumenteerd ben ik op voortreffelijke wijze begeleid en ondersteund door Rob, professor Jansen, Edward en Jaring en op de achtergrond door Paul en mijn paranimfen Marja en Janine.

Paul wil ik danken voor zijn steun bij mijn eerste aarzelende en onzekere schreden richting de haven 'CLB' en tijdens mijn vaak ook voor hem turbulente verblijf aldaar. Mijn ouders dank ik voor de (studie) mogelijkheden die zij mij hebben geboden. Verder wil ik mijn (schoon)familie en vrienden danken voor het begrip, dat zij de afgelopen jaren tijdens de momenten dat ik weer eens op het water was, hebben weten op te brengen.

Zeilen kost veel geld, zonder alle steun van onze hoofdsponsor het Nederlands Astma Fonds en verder van het CLB, UCB en NWO was dit avontuur nooit mogelijk geweest. Verder wil ik degenen die ik niet genoemd heb maar ook aan de tot standkoming van dit scheepsjournaal hebben bijgedragen bij deze hartelijk danken.

Ik wens jullie allemaal een behouden vaart toe!

CURRICULUM VITAE

Suzanne Pasmans werd geboren in Heerlen op 30 juli 1963. Haar jeugd bracht zij door in Heerlen, alwaar zij haar eindexamen Gymnasium β behaalde aan het Bernardinuscollege. In 1982 startte zij met de studie Geneeskunde aan de Universiteit van Leiden. Stages in de immunologie werden hierbij gelopen op de afdelingen Nierziekten van het Academisch Ziekenhuis te Leiden en Ophthalmo-Immunologie in het Interuniversitair Oogheelkundig Instituut te Amsterdam. Na in 1990 het arts-examen cum laude te hebben behaald was zij korte tijd als arts-assistent werkzaam op de afdeling Oogheelkunde in het Ziekenhuis Leyenburg te 's Gravenhage. Vanaf september 1990 verrichtte zij een promotie onderzoek getiteld "IgE bindende factoren bij astma" onder begeleiding van Prof. Dr. R.C. Aalberse, Prof. Dr. H.M. Jansen en Dr. E.F. Knol op de afdeling Allergie van het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis in samenwerking met de afdeling Longziekten van het Academisch Ziekenhuis te Amsterdam. Vanaf 1 november 1994 was zij gedurende 3 maanden werkzaam bij Dr. M. Daëron op de afdeling Klinische Immunologie van het Instituut Curie te Parijs. Met ingang van 1 juli 1995 zal zij als assistent in opleiding tot dermatoloog werkzaam zijn in het Academisch Ziekenhuis te Utrecht (hoofd: Prof. Dr. W.A. van Vloten).

STELLINGEN

behorende bij het proefschrift

"IgE-dependent histamine-releasing activity in atopy"

1. Het principe van Ockham hanterend is IgE-afhankelijke 'histamine-releasende activiteit' een autoallergeen. IgE^+ is een autoantistof gericht tegen dit autoallergeen.
2. In supernatanten van humane cellen dient rekening te worden gehouden met de aanwezigheid van zowel IgE-afhankelijke als IgE-onafhankelijke 'histamine-releasende activiteit'. (Dit proefschrift.)
3. De associatie tussen de respons op IgE-ahankelijke 'histamine-releasende activiteit' en een gestoorde histamine drempel bij allergisch asthma suggereert een rol voor de IgE-afhankelijke 'histamine-releasende activiteit' bij allergisch astma. (Dit proefschrift.)
4. Het belang van IgE met betrekking tot de chronische allergische reactie (astma en eczeem) beperkt zich niet tot het initiëren van de acute allergische reactie.
5. Gezien het groot verschil in activiteit tussen recombinant en natuurlijk MCP-1 (mogelijk veroorzaakt door incorrecte vouwing van het recombinant eiwit) is niet uit te sluiten dat dit fenomeen ook de lage reactiviteit van recombinant 'histamine-releasende factor' verklaart.
6. IgE binding aan humaan profiline is klinisch niet relevant. (Dit proefschrift.)
7. Het bestaan van niet-IgE-gemedieerde anaphylaxie in IgE-'knock-out' muizen ontkracht niet de rol van de IgE-gemedieerde anaphylaxie in de mens, wel de kennis met betreking tot de historische literatuur van de referenten van Nature.
   (H.C. Oettgen, Nature 1994; 370: 367-370 en
   R.S. Nussensweig, J Exp Med 1964; 120: 315-328)
8. De associatie tussen de naam 'Susan' en onderzoek naar 'IgE-afhankelijke histamine-releasende activiteit' is niet causaal.
   (Susan MacDonald/Suzanne Pasmans)
9. Een promotieonderzoek is als fietsen op vals plat (gaat het omhoog of omlaag?) afgewisseld door de euforie van de cols.
10. Zonder toenemende emancipatie van de man is verdere emancipatie van de vrouw zinloos.
11. In deze tijd zijn nationaliteit en cultuur tegelijk vervagende en opruiende begrippen.
12. Onderzoek naar autoallergie is als zoeken naar de zich onderscheidende 'miemel' in een berg 'miemelen'. (Cover.)

Suzanne Pasmans, juni 1995