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Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE

A number of structurally diverse antigens preferentially stimulate the synthesis of IgE antibodies, but no unifying principle has been proposed that explains the nature of isotype selection. In the present study, we show that common allergens present in bee venom, house dust mite emanations and parasite proteins induce mast cell and basophil degranulation and stimulate interleukin-4 synthesis, and secretion in the absence of antigen-specific IgE. These data point to a linkage between the initial activation of cells of the innate immune system and subsequent adaptive immune responses. They suggest that IgE-independent mast cell and basophil degranulation is predictive of potential allergenicity and can be evaluated by means of a cellular assay. Our study indicates that non-immunological degranulation by prototypic allergens, such as bee venom phospholipase A₂ or proteases associated with house dust mite emanations, is critically dependent on enzymatic activity. These findings have potentially important implications for vaccine design in allergic and parasitic disease.

1 Introduction

Allergy occurs when the immune system responds to an initial encounter with antigen by producing an antibody of the IgE isotype. Cross-linking of high-affinity receptor (FcεRI)-bound IgE by antigen/allergen results in the release of pharmacologically active agents that cause the clinical symptoms associated with immediate and delayed-hypersensitivity reactions [1–3]. There is evidence that these mechanisms may have evolved to confer protective immunity against pathogens, particularly parasites [2, 4].

A number of environmental factors have been linked to the development of atopic diseases, which affect more than 20 % of the human population. It is not known why many diverse and often harmless substances act as allergens or adjuvants to stimulate the synthesis of antibodies of the IgE isotype [3]. In many viral infections, there is an increase in total and virus-specific IgE levels [5, 6]. Despite detailed information regarding the molecular structure of many allergens, a common allergenic principle has not been identified, and the pathogenesis of allergy and asthma still remains to be established.

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Abbreviations: **H:** Histamine **5-HT:** 5-Hydroxytryptamine **h:** Human **IgE-VNP:** NIP-specific IgE **FcεRI α:** Ligand binding domain of the high-affinity receptor for hIgE **Der p:** *Dermatophagoides pteronyssinus* **HDM:** House dust mite **SGM:** Spent growth medium **NIP-HSA:** 4-hydroxy-3-nitrophenyl-acetyl coupled to human serum albumin **LDH:** Lactate dehydrogenase **SE:** Staphylococcal enterotoxin **HLMC:** Human lung mast cells **HSMC:** Human skin mast cells **ASA:** Acetylsalicylic acid

Key words: Allergen / Mast cell activation / Interleukin-4

The molecular mechanisms controlling antibody isotype selection following encounter with a given antigen are unclear. The regulatory role of cytokines is established, and interleukin (IL)-4 has a central role in the induction of IgE antibody responses. Eosinophils, mast cells, and basophils have been shown to be a source of IL-4, in addition to T lymphocytes [7–12]. It has been proposed that the interaction of helminths or allergens with these cells induces IL-4 release. The only known physiological stimulus inducing IL-4 production by these cells is the cross-linking of FcεRI, but this could occur only after specific IgE antibody responses are established [13]. Following the engineering of the RBL-2/2C cell line (RBL-2H3.1 cells [14] transfected with the α subunit of the human FcεRI [15, 16]), we observed IgE-independent mediator release when cells were challenged with allergens, including house dust mite emanations and bee venom, prior to sensitization with antigen-specific IgE [3, 17]. Enzymatically active bee venom allergen phospholipase (PL)A₂, but not an inactive variant, is able to induce IgE-independent mediator release, including IL-4 from this cell line [17], and it is only when mice are immunized with active PLA₂ that high levels of PLA₂-specific IgE occur [17]. Since IL-4 directs T helper cell differentiation [13] and B cell class switching to both IgG and IgE, we suggested that cells of mast cell, basophil, or both lineages might be the initial source of IL-4 and that interaction of allergens or pathogens at mucosal surfaces will generate a stimulus capable of steering subsequent adaptive immune responses [17].

Evidence indicates that the action of antigens on components of the innate immune system determines subsequent adaptive responses [18]. Following infection with *Listeria monocytogenes* [19], macrophages become a source of IL-12 which induces Th1 development of naive CD4⁺ T cells. FcεRI⁺ cells are the major source of IL-4 following *Schistosoma mansoni* infestation [20]. The protective role of mast cells in bacterial infections has just become apparent [21–24]. Although the molecular basis for cell activation remains to be established, we have already proposed that a number of allergenic and parasitic proteins stimulate

secretion of mediators of the allergic response independently of IgE sensitization, and that this action precedes subsequent IgE responses [3, 17]. We have examined this hypothesis further by extending our studies to a number of potential allergens and assessed IgE-dependent and independent induction of mediator release from RBL-2/2/C cells [15], from human leukocyte preparations enriched in basophils, and from human lung and skin mast cells. We demonstrate the non-immunological release of mediators of the allergic response following challenge with aspirin-based drugs, lectins, lipopolysaccharides, endotoxins, viral particles, allergenic, and parasite antigens, such as bee venom, house dust mite emanations and parasite secretions which are rich in hydrolytic enzymes.

2 Materials and methods

2.1 Source, isolation and characterization of antigens

Recombinant mite protease *Dermatophagoides pteronyssinus* (*Der p*) I [25] was provided by Dr. T. Merrett, Allergy Analysis Centre, Euro/DPC, Ltd., Glyn Rhonwy, Llanberis, GB. House dust mite (HDM) extracts from mites of the genus *Dermatophagoides* (D1912, 4001, 4002, 4020) were purchased from SmithKline Beecham Pharmaceuticals, GB. Despite a low proteolytic activity, these preparations retain allergenic epitopes. HDM colonies (gift of Dr. M. Colloff, University of Glasgow, GB) were maintained in 9-cm petri dishes at 75% relative humidity at room temperature, fed with baker's yeast and subcultured weekly by replacement of two-thirds of the dish contents with fresh yeast. For large-scale preparation of crude extracts containing mite emanations, spent growth medium (SGM) from several mite cultures was pooled and separated from growth medium (baker's yeast) by passage through a sieve. SGM, mite debris and feces were resuspended in 2 ml PBS following homogenization, and extracts were kept at -20°C until further use. Proteolytic activity associated with HDM emanations was isolated from 200 mg freeze-dried SGM which was taken up in 4 ml PBS and loaded onto a Sephadex G200 column equilibrated with the same buffer for size exclusion chromatography (Pharmacia LKB Biotechnology). Fractions in the regions corresponding to 25 and 30 kDa were pooled and concentrated by ultrafiltration using a YM-10 membrane (Amicon). Concentrated pooled samples were reloaded onto a Superdex-75 column for separation of the 30 and 25 kDa components. In each instance SDS-PAGE analysis [26] of the peak fractions showed the presence of one major (>95%) and several minor bands. The major bands were recognized in immunoblots probed with serum (1:10 dilution) from a skin test-positive HDM-allergic individual (T.A.). Blots were developed with a horseradish peroxidase-labeled rabbit anti-human IgE antibody (P0295, Dako, GB) [27].

For the isolation of cercarial proteases, a Puerto Rican isolate of *Schistosoma mansoni* was maintained by routine passage through LACA mice and *Biomphalaria glabrata*. Snails harboring a patent infection were induced to shed cercariae, which were concentrated by sedimentation at 4°C [28]. Resulting schistosomula were cultured for 3 h in 50-ml flasks at 37°C in medium 169 in 6% CO_2 in air. The parasite suspension was then centrifuged at $150 \times g$ for

90 s and the culture supernatant removed for concentration by ultrafiltration with a 3-kDa-cutoff Diaflo membrane (Amicon) under N_2 pressure (40 psi). SDS-PAGE [26] analysis revealed the presence of several bands. Serine protease activity was shown to be associated with bands in the 20–25-kDa region.

The catalytic activity of proteolytic enzymes was assayed using Azocoll (A9409), tosyl sulfonyl L-arginine methyl ester (TAME, T4626) and benzoyl-L-arginine ester hydrochloride (BAPNA, B1007) as substrates [29], all purchased from Sigma. Cysteine proteases were activated in buffer containing 0.01 M EDTA, 0.05 mM 2-mercaptoethanol, and 5 mM cysteine, and passed through a Sephadex G-20 spun column immediately before use. Overnight storage of enzyme preparations at room temperature resulted in the almost complete loss of proteolytic activity (>99%) due to autoproteolysis, as evidenced by SDS-PAGE analysis (data not shown).

All other proteases, lectins and staphylococcal enterotoxins were purchased from Sigma (see Table 1). Dispersible aspirin (Mandaprin) was purchased from M&A Pharmachem, Bolton, GB.

Influenza virus strains Hong Kong (H_3N_2) containing 270 μg hemagglutinin/ml, Texas-like strain containing 520 μg hemagglutinin/ml, X1AC, F and ALUSSR (H_1N_1), herpes virus and equinus flu virus were provided by Prof. R. Jennings (Department of Experimental and Clinical Microbiology, University of Sheffield, GB), and purified influenza virus hemagglutinin was from Prof. J. J. Skehel (MRC, Mill Hill, London, GB).

2.2 Cell culture and measurement of mediator secretion with or without sensitization with antigen-specific IgE

Mediator release from RBL-2/2/C has been described in earlier studies [15–17, 30] and human (h)Fc ϵ Ia chain expression is not required to assess IgE-independent mediator release. Cell culture procedures and measurement of 5-hydroxytryptamine (5-HT) and β -hexosaminidase secretion, and IL-4 and MHC class II up-regulation, have been described [16, 17]. The percentage of histamine released by RBL-2/2/C was evaluated by measurement of histamine in 50 μl cell supernatant using an ELISA kit (Miles Inc., CT).

2.3 Lactate dehydrogenase (LDH) assay

Antigen cytotoxicity was determined using a nonradioactive cytotoxicity assay (CytoTox 96, G1780, Promega, Southampton, GB) to measure the activity LDH in 50 μl cell supernatant containing antigen-induced mediators.

2.4 Preparation of human lung and skin mast cell suspensions

Cell suspensions containing human lung mast cells (HLMC) and human skin (foreskin) mast cells (HSMC) were prepared by physical and enzymatic dispersion [31].

HLMC and HSMC purity ranged from 10 % to 20 % and from 2 % to 5 %, respectively, as determined by Alcian blue staining [32]. HLMC were purified (enriched to 50–81 %) by countercurrent elutriation [33, 34] or by incubating the cells for 1 h at 4 °C with anti-c-kit or anti-IgE antibody, followed by incubation with magnetic beads coated with anti-rabbit antibody for 1 h at 4 °C. Cells coated with magnetic beads were isolated using a magnetic particle concentrator and washed three times with PBS prior to use.

2.5 Preparation of white blood cells enriched with basophils

Mixed leukocyte preparations were obtained from venous blood of healthy donors. Basophil enrichment was achieved by centrifugation at $1200 \times g$ for 15 min over a discontinuous Percoll gradient (50–60 %) prepared on a LeucoSep tube (Greiner Labortechnik, Frischenhauser, Germany). The basophil-enriched fraction was collected and washed three times with 30 ml 25 mM Pipes, 110 mM NaCl, 5 mM KCl, 0.1 % glucose, and 0.003 % HSA, pH 7.2 by centrifugation at $1500 \times g$ for 10 min. The cell pellet was resuspended with PBS containing 1 mM CaCl_2 , 1 mM MgCl_2 and 5.5 mM glucose. Basophil purity (3–10 %) was determined by Alcian blue staining.

2.6 Measurement of histamine and IL-4 released by HLMC, HSMC, and basophils

Cell suspensions were centrifuged at $1000 \times g$ and resuspended in PBS containing 1 mM CaCl_2 , 1 mM MgCl_2 and 5.5 mM glucose to a cell density of 2×10^6 mast cells/ml. The cell suspension (100 μl) was challenged with antigens dissolved in PBS in a total reaction volume of 300 μl . HLMC and basophils were incubated at 37 °C and HSMC at 32 °C. After a 2-h incubation, samples were centrifuged at $1000 \times g$ and 50 μl supernatant was assayed for hista-

mine release using an automated spectrofluorimeter. The remaining supernatant was stored at -20°C for IL-4 measurement. Spontaneous release of histamine was determined by incubating the cells with PBS. The total cellular histamine content was obtained after lysing the cells with 1.6 % perchloric acid.

Human IL-4 was determined using commercial kits purchased from Endogen (Human IL-4 ELISA kit, EH-IL-4) or from R&D Systems (Quantikine Human IL-4, D4000). The former test kit proved to be more sensitive than the latter by an order of magnitude.

3 Results and discussion

3.1 Stimulation of mediator release from RBL-2/2/C cells

3.1.1 Effect of acetylsalicylic acid (ASA)-based drugs, endotoxins, lipopolysaccharides, lectins, and viral particles

Table 1 summarizes the IgE-independent induction of mediator release from RBL-2/2/C cells in response to agents reported to cause allergic reactions. Aspirin and other ASA-based drugs, including food dyes, are known to cause severe anaphylactic reactions in some individuals [35, 36]. RBL-2/2/C cells respond to this drug with mediator release (Table 1). Although some investigators suggest an IgE-dependent mechanism in ASA sensitivity [35], our data show IgE-independent mediator release and this cell line appears to be useful to study the molecular basis of mast cell activation by ASA.

Histamine release in response to lectins, bacteria, viruses, and LPS is well documented [5, 37–40]. No apparent variation in the reactivity of IgE from different individuals to specific lectins has been observed, although it has been suggested that lectin-induced histamine release is an IgE-dependent reaction [39]. However, results shown in Table 1

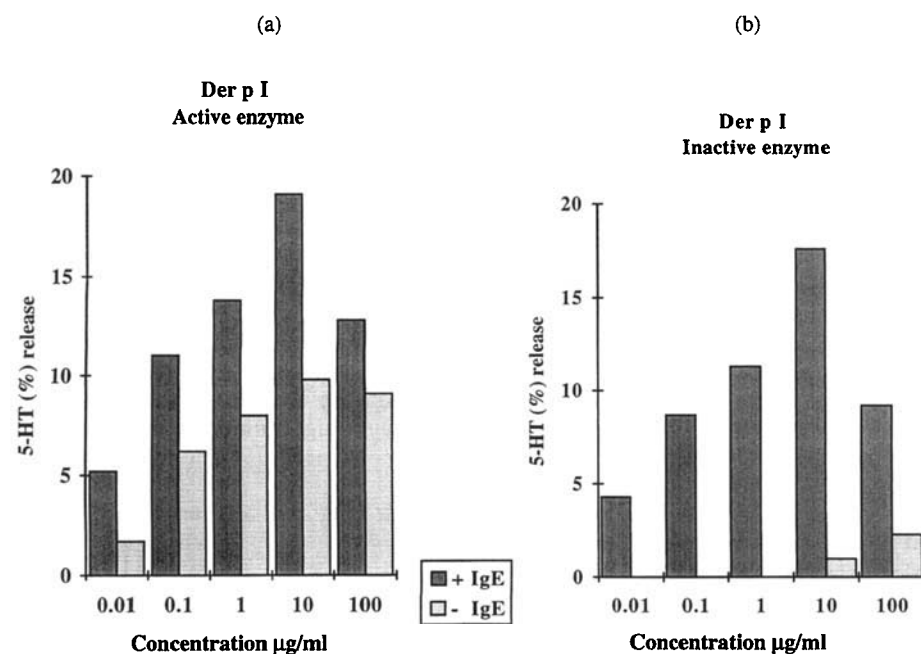


Figure 1. Percentage of 5-HT released by RBL-2/2/C cells sensitized with or without the IgE fraction from the serum of a HDM-sensitive (skin-test positive) individual. RBL-2/2/C cells were cultured for 24 h with or without HDM-specific IgE (1 $\mu\text{g}/\text{ml}$) in the presence of dexamethasone (10^{-6} M) and [^3H]5-HT [16]. Cells were challenged with enzymatically active *Der p I* (a) or with a preparation of *Der p I* which was kept for 24 h at 22 °C (b). After this time, less than 0.1 % of the original proteolytic activity remained. The concentration of active enzyme is based on substrate hydrolysis, that of inactive enzyme is based on protein concentration. 5-HT release was measured after a 15 min challenge with antigen at 37 °C. Release was corrected for background (<2 %) and expressed relative to the total amount of cellular 5-HT. Each bar represents the mean of two independent experiments carried out in duplicate.

Table 1. Effect of aspirin, lipopolysaccharide, superantigen, lectins, and viral suspensions on mediator release from RBL-2/2/C cells^{a)}

Antigen	% 5-HT released	
	+ IgE	– IgE
Aspirin (10 µg/ml)	72.1 ± 10.0	62.2 ± 19.2
LPS (<i>Salmonella typhosa</i>) (4 µg/ml)	n. t.	25.0 ± 1.5
SEB (10 µg/ml)	n. t.	16.8 ± 0.7
<i>Triticum vulgaris</i> (wheat germ) lectin (10 µg/ml)	16.6 ± 5.6	13.5 ± 3.7
<i>Lens culinaris</i> (lentil) lectin (10 µg/ml)	51.5 ± 1.3	62.9 ± 2.6
<i>Tetragonolabus purpurea</i> (asparagus pea) lectin (5 µg/ml)	2.5 ± 0.5	0
<i>Saphora japonica</i> (Japanese pagoda tree) lectin (250 µg/ml)	6.3 ± 0.3	0
Elder tree lectin (40 µg/ml)	17.3 ± 5.0	2.5 ± 0.6
Influenza virus (F) (1:5000)	4.4 ± 1.9	0
Influenza virus (H ₁ N ₁) (1:5000)	2.0 ± 0.5	1.1 ± 0.4
Influenza virus hemagglutinin (10 µg/ml)	8.6 ± 1.3	0

a) RBL-2/2/C cells were cultured for 24 h with or without 1 µg/ml NIP-specific IgE in presence of [³H]5-HT as described [15]. [³H]5-HT secretion from stimulated cells was determined after a 15-min exposure to antigens using established procedures [17]. During this period, less than 2% of 5-HT was released from LPS and SEB-stimulated cells. The data for LPS and SEB are the percentage of 5-HT secretion after 4 h. The release was corrected for background and expressed relative to the total [³H]5-HT incorporated. The values show the mean and SE of at least three tests performed in duplicate. n. t. = not tested.

demonstrate that some lectins stimulate mediator release from RBL-2/2/C in the absence of IgE or following sensitization with IgE, presumably by binding to carbohydrate residues present on the ligand. Pollen, fruits, and nuts are sources of lectins with different sugar specificities which can interact with carbohydrate structures on IgE, its receptor, or both. Since post-translational glycosylation is genetically determined, individual variations in responses to cell triggering by lectins are likely to occur.

In Table 1, we show that virus particles from different influenza virus strains induce degranulation. These results extend earlier observations that a variety of environmental agents enhance mediator release in the absence of antigen-specific IgE. The magnitude of this response can be as great as an IgE-mediated response. An exception is the IgE-dependent release observed with viral hemagglutinin. This depends on carbohydrate residues on the IgE molecule and is inhibited by the inclusion of sialic acid in the challenging buffer (data not shown). Viral enhancement of basophil degranulation is inhibited by carbohydrates [38], indicating a lectin-like action of viral products.

LPS are endotoxins released by invading bacteria and LPS receptors on macrophages have been reported [41]. LPS stimulates cytokine and leukotriene release from macrophages and co-stimulates IgE production by B cells [41]. Superantigens, including staphylococcal enterotoxins (SE), act via MHC class II antigens and there is some indication that these molecules are expressed on basophils [42]. We were only able to detect low levels of degranulation in response to LPS or SEB by 1 h, although after a 4-h incubation period, between 10% and 20% of 5-HT is released (Table 1).

Results presented in Table 1 confirm and extend earlier observations that a variety of environmental agents known to promote allergic responses in some individuals can activate cells of mast cell lineage independent of sensitization with antigen specific IgE.

3.1.2 Mediator release in response to enzymes associated with common allergenic agents

To examine the IgE-independent release mechanisms further, we investigated the action of enzymes associated with well-characterized allergens on RBL-2/2/C cells with or without sensitization with allergen-specific human IgE. We and others have suggested that enzymatic activity may be related to allergenicity [3, 17, 43]. Mast cell degranulation by phospholipases from a variety of species, trypsin, chymotrypsin, and thrombin is well established [44]. Phospholipases, proteases or both are associated with insect venoms, parasite secretions, HDM and cockroach emanations, fungal spores, grain dust, and plant pollens [45]. The major allergen from domestic cats, *Fel d* I, co-isolates with a kallikrein protease [46], and about 1% of patients treated by chemonucleolysis with papaya proteinases developed allergic responses [47]. The presence of hydrolytic enzymes in allergenic material suggested that these might initially stimulate allergic responses by an IgE-independent mechanism.

Table 2 summarizes IgE-independent mediator release (5-HT, histamine and IL-4) from RBL-2/2/C cells following exposure to bee venom, phospholipase, allergenic extracts containing proteases, and catalytically active and inactive proteolytic enzymes. LDH release was used as an indicator of cytotoxic damage. Cell degranulation induced by proteases was critically dependent on enzymatic activity. No mediator release is observed with catalytically inactive recombinant *Der p* I [25, 48]. Preparations of trypsin, thrombin, or papain lose mast cell secretagog activity following storage for more than 6 h at 4°C due to autoprolysis (data not shown). Activation of cell surface receptors by interaction with inactive protease fragments does not occur, although this mechanism has been described for the insulin-like activity of these enzymes [49].

We have already reported a link between allergenicity of recombinant bee venom PLA₂, catalytic activity, and IgE-independent non-cytotoxic mast cell mediator release, and suggested that PLA₂ induces mediator release and IL-4 synthesis and secretion in mast cells as a consequence of its enzymatic action [17]. Bee venom-induced degranulation of mast cells and the cytotoxic nature of the release, also demonstrated in the present study by LDH release, has

Table 2. Antigen-induced release of mediators of the allergic response from RBL-2/2/C cells without IgE sensitization^{a)}

Antigen	Mediators measured			
	5-HT	Histamine	IL-4 ^{b)}	LDH
Bee venom (1 µg/ml)	87.0 ± 8.4	85.0 ± 3.2	d	82.0
Bee venom PLA ₂ (10 µg/ml)	16.7 ± 1.0	7.3 ± 0.7	d	16.0
Phospholipase C (<i>Bacillus cereus</i> , 10 µg/ml)	46.4 ± 8.8	33.0 ± 9.6	n. t.	27.0
Thrombin (10 µg/ml)	6.4 ± 2.5	n. t.	n. t.	n. t.
Trypsin (10 µg/ml)	18.9 ± 2.7	n. t.	d	0
Papain (10 µg/ml)	17.0 ± 2.6	21.2 ± 3.2	n. t.	n. t.
HDM-SGM inactive (10 µg/ml)	0	0	n. d.	n. t.
HDM-SGM active (10 µg/ml)	26.5 ± 10.6	26.0 ± 5.5	d	n. t.
<i>Der p</i> I (cloned, 3 µg/ml)	0	n. d.	n. t.	n. t.
Schistosomal protease (3 µg/ml)	18.5 ± 1.7	19.7 ± 2.3	d.	0
<i>Aspergillus</i> protease (100 µg/ml)	24.5 ± 0.8	33.0 ± 7.1	d.	0

a) Enzymatic activity of all proteolytic enzymes was assayed immediately before use. The concentration of active enzyme is based on substrate hydrolysis and that of inactive enzyme is based on protein concentration. The percentage of mediators (5-HT, Histamine) and LDH secreted by RBL-2/2/C cells after 15 min of antigen stimulation was calculated as described [28]. The values show the mean and SE of at least three tests performed in duplicate. Similar observations were made with other proteases listed in Sect. 2.1 (data not shown).

b) In the absence of a quantitative assay for rat IL-4, the cytokine was detected by Western blotting: d = detected and n. d. = not detected, n. t. = not tested.

been attributed to the cytolytic peptide melittin [44]. Release of mast cell mediators *in vivo* following a bee sting therefore involves a cytolytic route, which is nevertheless of physiological importances since human mast cells and basophils contain preformed mediators which will be set free following cell lysis [8–12].

In contrast, SGM containing enzymatically active HDM proteases and purified *Der p* I stimulate mediator secretion by a noncytotoxic mechanism at low enzyme concentrations (0.5–50 µg/ml) with cytotoxic release at concentrations higher than 100 µg/ml. This dose-dependent effect has been observed for a number of serine proteases, including trypsin, which is commonly used to release RBL cells from plastic surfaces before studying the physiological consequences of IgE-mediated receptor activation in suspension cultures [50]. Our study indicates that the results of such experiments require re-evaluation.

The molecular mechanism by which proteases induce mediator release by a noncytotoxic mechanism remains to be determined. We have previously suggested that cleavage of the extracellular domain of the FcεRI α chain occurs at protease sensitive sites (residues 170–179) in close proximity to the cell membrane [43]. Receptor cleavage has also been reported for the extracellular portion of human FcεRII/CD23 by *Der p* I [51, 52]. Truncated receptors may aggregate due to removal of extracellular constraints, resulting in the generation of an activated receptor complex and ultimately degranulation.

Further evidence for a mechanism of activation by extracellular domain cleavage was obtained when we studied the dose-response relationship between *Der p* I protein concentration and 5-HT release from RBL-2/2/C cells. Fig. 1a shows the percentage of 5-HT released by RBL-2/2/C cells triggered with catalytically active *Der p* I with or without sensitization with IgE from an HDM-sensitive individual (T.A.). A bell-shaped dose-response pattern,

characteristic of receptor clustering, is observed in both cases. Comparable results (data not shown) were obtained when unsensitized cells were incubated with trypsin or papain. In contrast, as shown in Fig. 1b, inactive degraded *Der p* I possessing less than 1 % of its original activity causes degranulation only when cells are sensitized with IgE from an HDM-sensitive individual, indicating the persistence of allergenic epitopes in the degraded allergen. Similar observations were made when cells were incubated with catalytically inert recombinant *Der p* I protein (data not shown) [48].

3.2 IL-4 metabolism in antigen-stimulated RBL-2/2/C cells

The release of IL-4 and increased synthesis in response to bee venom and *Der p* I can be demonstrated by Western blot analysis [17] as shown in Fig. 2A, panels a and b. It is observed within 15 min of challenge and peaks between 2–4 h after the initial stimulus. The kinetic pattern is similar to that observed following an IgE-mediated antigenic stimulus (Fig. 2B). Several anti-IL-4-reactive polypeptide bands are detected in Western blots of protein extracts from the cell pellets of stimulated RBL-2/2/C. We [17] and others [53] have made this observation previously. A FAST search of the SWISS PROT data base revealed no significant sequence homology between IL-4 and other known proteins, so we attributed these bands to the presence of different glycosylated forms of IL-4 and pro-IL-4 [17, 53], although the expression of alternatively spliced forms of IL-4 cannot be ruled out [54].

3.3 Up-regulation of class II/Ia major histocompatibility antigens

IL-4 induces the up-regulation of a number of membrane proteins, including FcεRII and MHC class II/Ia antigens.

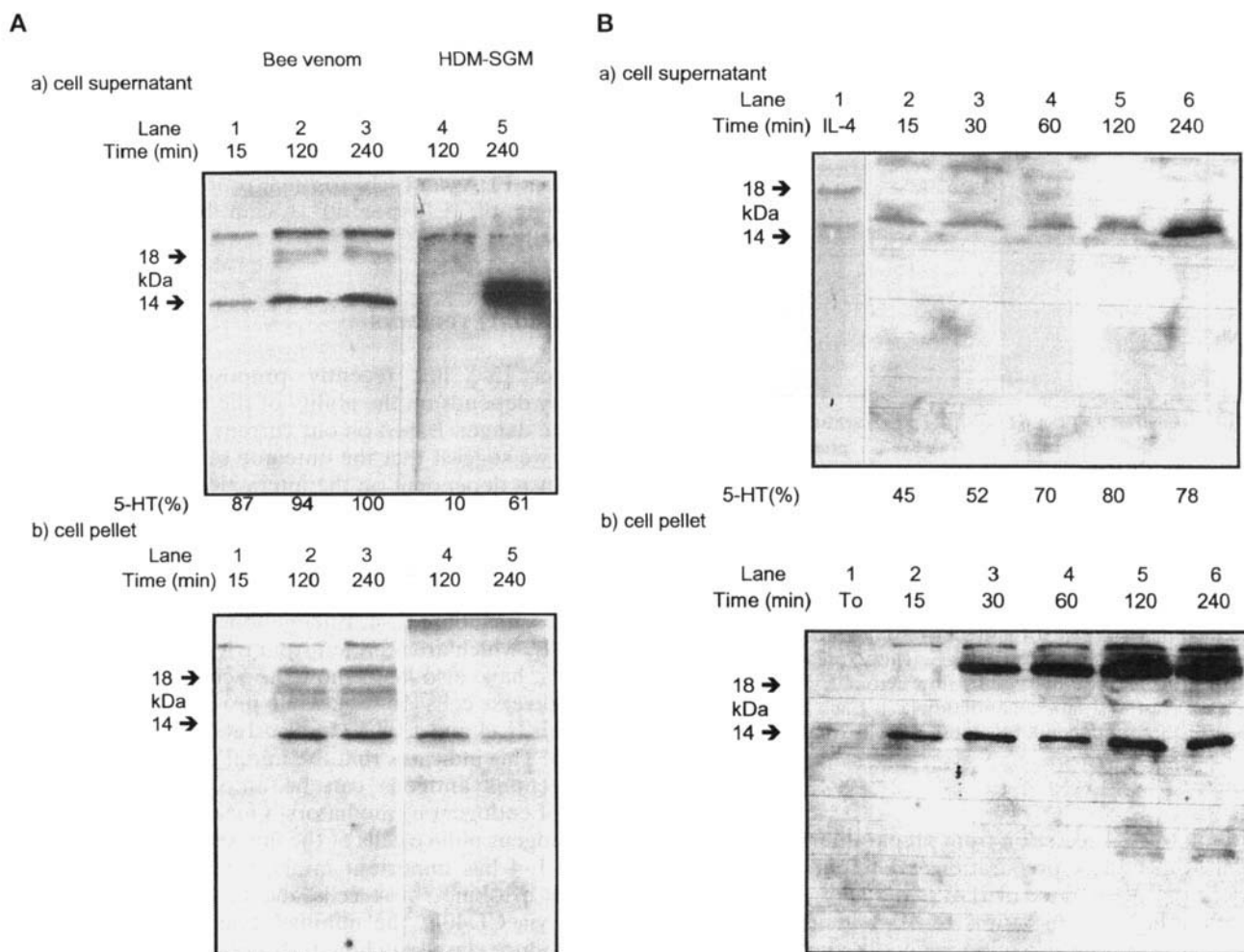


Figure 2. (A) The kinetics of 5-HT secretion and IL-4 synthesis and secretion in response to challenge with bee venom and spent growth medium from house dust mites is shown. RBL-2/2/C cells were cultured for 24 h in the presence [^{3} H]5-HT. Cells were washed twice before the addition of a bee venom suspension and HDM-SGM. Lanes 1–3 = bee venom suspension (1:1000), lanes 4–5 = house dust mite SGM (3 μ g/ml). Panel (a) shows the percentage of 5-HT secretion and immunoreactive IL-4 proteins, detected by Western blotting, secreted into the culture medium over a 4-h period; panel (b) IL-4 in cell pellets. (B) The kinetics of 5-HT secretion and IL-4 synthesis and secretion in response to an IgE-mediated antigenic challenge is shown. RBL-2/2/C cells were incubated for 24 h with NIP-specific IgE (1 μ g/ml) followed by challenge with 100 ng/ml NIP-HSA [16]. 5-HT and IL-4 secretion (panel a) and levels of IL-4 in cells pellets (panel b) were monitored over a period of 4 h. Panel (a): lane 1, positive control for anti-rat IL-4-reactive proteins secreted by CHO cells [53]; panel (b): lane 1, total cellular IL-4 content in RBL cells prior antigen stimulation; lanes 2–6, IL-4 in supernatants (a) and pellets (b) after antigen stimulation.

The increased expression of these molecules on rat B splenocytes in response to supernatants obtained from *Der p* I and schistosomal protease-triggered cells is shown in Fig. 3. It provides further support that the supernatant from RBL-2/2/C cells following non-immunological stimulation contain a factor which is capable of inducing a response similar to that caused by IL-4. The demonstration of increased expression of MHC class II/Ia antigens is important, since the quantity of antigen displayed by the processing cell is related to the density of these molecules.

3.4 Mediator release from human mast cells and basophils

Although the RBL-2H3.1 cell line represents a model system for the study of mast cell function [14], responses to a number of stimuli may differ from those observed with HSMC, HLMC, and basophils.

Table 3 summarizes the effect of bee venom PLA_2 , HDM and schistosomal proteases on dissociated cell preparations of human lung and skin tissue, and partially purified HLMC and HSMC and basophils. Histamine release, which is a specific indicator of mast cell/basophil degranulation, occurs in response to catalytically active HDM, schistosomal proteases and PLA_2 , but not inactive antigens. Unlike mast cells, basophils do not respond to a PLA_2 -mediated stimulus. We have already demonstrated that the enzymatically inactive $\text{PLA}_2\text{H34Q}$ does not degranulate cells unless they are sensitized with IgE [17] and, as shown in Table 3, HLMC challenged with commercial HDM-SGM (4001), which retains only low levels of protease activity, release basal levels of histamine. It shows that preparations containing HLMC release histamine solely in response to enzymatically active *Der p* I and that degranulation cannot be attributed to cell triggering by allergen-specific IgE. We detect IL-4 secretion from mast cells after exposure to PLA_2 and proteases, and the latter

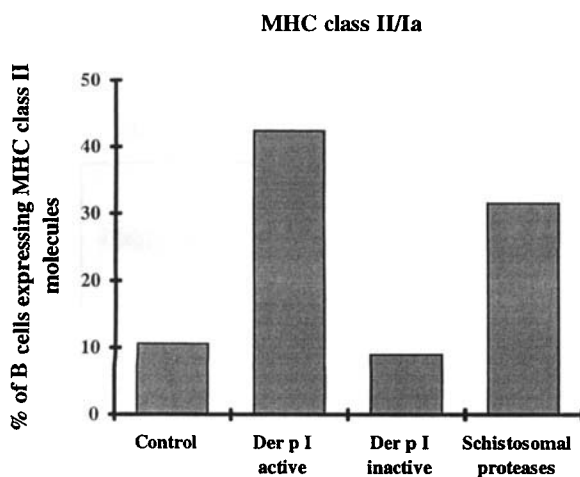


Figure 3. Up-regulation of MHC class II expression on rat B splenocytes by supernatants from RBL-2/2/C cells triggered with *Der p* I and schistosomal proteases. Rat splenic B cells were isolated by immunomagnetic depletion of splenic T cells as described [17], and incubated at 37°C for 24 h with culture medium (control) or supernatants from RBL-2/2/C cells, triggered with 3 µg/ml of active and inactive *Der p* I and schistosomal proteases without sensitization with antigen-specific IgE. The expression of MHC class II/Ia antigens was analyzed by flow cytometry using an anti-MHC class II/Ia FITC-labeled antibody [17]. Each bar represents the mean of three independent experiments carried out in duplicate.

also stimulate IL-4 secretion from preparations enriched in basophils. Since these preparations are impure, we cannot be certain that the source of IL-4 is the allergen-triggered mast cell. The demonstration of IL-4 generation from human tissues in response to an antigenic stimulus must in any case be considered significant in view of the pivotal role of this cytokine in steering adaptive immune respon-

ses. However, we found that the amount of IL-4 secretion based on mast cell content is similar for dissociated lung tissue and enriched preparations of mast cells and that this is not significantly influenced by purification procedures [33, 34]. Two HLMC preparations containing 50 and 81 % Alcian blue-stained mast cells, challenged with 3 µg/ml bee venom PLA₂ and schistosomal protease, released 300 and 120 pg of IL-4 per 10⁶ Alcian blue-stained cells, respectively.

4 Concluding remarks

Matzinger [55] has recently proposed that acquired immunity depends on the ability of the immune system to recognize danger. Based on our current and previous studies [17], we suggest that the outcome of adaptive immune responses is dependent on the interaction of antigens with cells of the innate immune system. Our observations suggest that the reasons why diverse aero-allergens, insect venoms and invading parasites stimulate IgE synthesis is related to their ability to induce the release of cellular mediators, including IL-4. Endogenous mast cell proteases and PLA₂, which are secreted from triggered mast cells or basophils, have also been shown to activate cellular functions in diverse cells [56–61], while prostaglandin E₂ synergizes with IL-4 and LPS to induce IgG1 and IgE production [62]. This indicates that the initial stimulation of cells by exogenous antigens can be amplified through the release of endogenous mediators. Our demonstration that many antigens induce cells of the innate immune system to release IL-4 has important implications. In combination with this cytokine, mast cells and basophils are able to furnish, via CD40L, the minimal essential signals necessary to induce class-switching to IgE in B cells [63]. It suggests that antigen-activated cells can play a crucial role in the induction of Ig class switching. *In vivo*, where a cock-

Table 3. Release of histamine and IL-4 from preparations containing human lung (HLMC) and skin (HSMC) mast cells, and from basophil enriched leukocyte preparations stimulated with bee venom phospholipase A₂, house dust mite and schistosomal proteases^{a)}

Antigen	HLMC				HSMC				Basophils			
	Histamine (%)		IL-4 (pg/10 ⁶ cells)		Histamine (%)		IL-4 (pg/10 ⁶ cells)		Histamine (%)		IL-4 (pg/10 ⁶ cells)	
	n		n		n		n		n		n	
Bee venom PLA ₂ (3 µg/ml)	7	20.4 ± 5.7	4	168 ± 26.1	6	26.9 ± 18.3	1	6	3	0		n. t.
HDM-SGM (10 µg/ml)	4	15.3 ± 4.6	2	23 ± 8.4	7	29.1 ± 16.1	1	3	2	13.7 ± 3.6	2	52 ± 8.0
HDM-SGM (4001) (10 µg/ml)	2	3.2 ± 2.2		n. t.		n. t.		n. t.		n. t.		n. t.
<i>Der p</i> I (10 µg/ml)	4	13.6 ± 4.0	2	65.4 ± 0.8	5	28.7 ± 11.4	1	5	1	9.6		n. t.
<i>Der p</i> III (10 µg/ml)	4	12.4 ± 2.9	2	102 ± 3.3	7	13.1 ± 6.3	1	4	1	2.8		n. t.
Schistosomal protease (10 µg/ml)	3	23.2 ± 8.5	3	35 ± 18.7	6	18.4 ± 7.7	1	0	3	21.7 ± 9.8	2	39 ± 8.7

a) Experiments on preparations containing 1×10^6 – 2×10^6 cells were performed in duplicate. The proportion of Alcian blue-stained cells in cell preparations was: HLMC = 10–20 %, HSMC = 2–5 %, basophils = 3–10 %. Results for histamine and IL-4 are presented as the mean value ± SE; n = number of experiments performed in duplicate. The release was corrected for background and is expressed relative to total cellular histamine content. IL-4 values were corrected for spontaneous release (<3 pg/10⁶ cells). No histamine or IL-4 release was detected after cell challenge with enzymatically inactive proteases and PLA₂.

tail of antigens is usually encountered, substances which stimulate IL-4 secretion may act as IgE-specific adjuvants and induce an IgE response to bystander antigens [64]. Any substance which stimulates synthesis and secretion of IL-4 must therefore be considered as potentially allergenic. Non-immunological mediator release from RBL-2/2/C and human cells indicates that similar mechanisms operate in rodents and man, and suggests that RBL cells may be used to evaluate potential allergenicity.

The demonstration that enzymatically active allergens and parasite proteins activate cytokine secretion has important implications for the design of anti-parasite and anti-allergy vaccines. Immunization with active parasite protein or the use of IL-4 as an adjuvant may lead to a protective immune response. In contrast, for the treatment of allergies, immunization with biologically inactive antigens may stimulate an IgG response as demonstrated in our previous investigation [17].

The current study has concentrated on the role of antigens in activating cells of mast cell/basophil lineage. The action of phospholipases and proteases on the stimulation of metabolic pathways, which has been described for several different cell types [43, 44, 49, 56–60, 65], can be expected to modulate a variety of responses *in vivo*. The cleavage of FcεRII/CD23 by *Der p* I from cells of B cell lineage [51, 52] is a pertinent example which demonstrates that the encounter with a potential allergen can affect diverse cells in a physiologically significant way. Complement activation by protease antigens deserves attention in view of the fact that components of the complement cascade are activated by proteolytic cleavage [42] and complement receptors are found on many cells of the immune system, including mast cells and basophils.

The mode of activation of cellular responses and the route by which mediator secretion and induction of IL-4 and possibly that of other cytokines occurs, remains to be established. The identification of these pathways may provide important information for our understanding of the stimulation of basic immune mechanisms.

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