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Allergen-induced interleukin-9 production *in vitro*: correlation with atopy in human adults and comparison with interleukin-5 and interleukin-13

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Clinical and Experimental Allergy

Summary

Background The contribution of IL-9 to human atopy is supported by genetic studies. However, IL-9 production in response to allergen *in vitro* has been reported only in children. **Objective** Study IL-9 induction by allergen in adults, compare it with IL-5 and IL-13 and evaluate its association with atopy.

Methods Peripheral blood mononuclear cell (PBMC) from control adults and from atopic patients were cultured with various allergens or phytohaemagglutinin (PHA) and secreted IL-5, IL-9 and IL-13 were measured by ELISA.

Results IL-9 was produced in response to *Dermatophagoides pteronyssinus* (Der p) by PBMC from Der p-hypersensitive adults at levels equivalent to those induced by PHA but with slower kinetics. The induction of IL-9 was allergen specific, reflecting donor RAST profile. In Der p-triggered reactions of non-atopic and atopic subjects, IL-9 showed the highest selectivity for atopics, IL-5 and IL-13 being produced more frequently in non-atopic donors. Significant correlations with specific IgE titres were found for IL-9 with all allergens tested (Der p and two peptides of Bet v 1 birch allergen). For IL-5 and IL-13, they were in the same range for Der p but more variable for birch allergens. Patterns of cytokine production by individual patients in response to allergen reflected these differences: for Der p, IL-5, IL-9 and IL-13 productions were strongly correlated but for birch IL-5 differed from the latter two. The *in vitro* production of IL-9 reflected clinical hypersensitivity profiles and was higher in individuals with asthma than in those with disease limited to rhinitis and/or conjunctivitis.

Conclusions Allergen-triggered IL-9 production *in vitro* is an excellent marker for atopy in adults given its virtual absence in allergen-stimulated PBMC from non-atopic individuals and its correlation with allergen-specific IgE and asthma.

Keywords allergen, atopy, interleukin

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Introduction

IL-9 has recently attracted much attention by the discovery of its potential role in asthma [1]. The notion that this T-helper type 2 (Th2) cytokine, initially discovered as a mouse T cell [2] and mast cell [3] growth factor, could play an important role in human asthma emerged from genetic studies. In man, a cluster of candidate genes possibly involved in the control of susceptibility to the disease, including IL-4, IL-5, IL-9 and IL-13, was identified on chromosome 5q31–q33 [4–6]. In mice, the gene segment conferring asthma susceptibility in BALB/c-C57Bl/6

crosses was localized on chromosome 13 [7], which comprises the sequence encoding IL-9 [8] but not that encoding the other Th2 cytokines. These observations suggested that IL-9 could be an essential element in asthma susceptibility.

Multiple functional *in vitro* observations corroborate this idea, including the action of IL-9 on mast cells and eosinophils [9–11], as well as its ability to stimulate IL-4-triggered IgE production *in vitro* [12]. Moreover, IL-9 activity also extends to non-haematopoietic cells implicated in asthmatic reactions, such as lung epithelial and smooth muscle cells. Indeed, IL-9 was reported to stimulate the release of mucins [13] and chemotactic factors

[14] by these cells. The significance of these *in vitro* observations has been strongly reinforced by the enhanced asthma susceptibility observed in mice overexpressing the IL-9 gene [13, 15] and by its inhibition in animals treated with anti-IL-9 antibodies [16, 17]. While these observations provide strong evidence in favour of the implication of IL-9 in asthma, they do not exclude the existence of an IL-9-independent pathway to bronchial hypersensitivity in view of undiminished pulmonary inflammation reported in IL-9-deficient mice [18].

Evidence that human IL-9 is produced in asthmatic airways was obtained by detection of IL-9 mRNA via *in situ* hybridization in bronchial mucosa [19], by immunocytochemistry performed on asthmatic lungs [20] and by the up-regulation of IL-9 in these tissues after allergen challenge [21]. Moreover, *in vitro* stimulation of peripheral blood mononuclear cell (PBMC) from preschool children with house dust mite *Dermatophagoides pteronyssinus* extract (Der p) and other allergens was found to induce IL-9 message [22, 23], data later confirmed by IL-9 protein determinations [24].

To further evaluate the association of IL-9 with atopy, we investigated IL-9 as well as IL-5 and IL-13 production by PBMC from adult individuals in response to several allergens and examined correlations between the produced cytokines and allergy parameters.

Material and methods

Study subjects

Thirty adult volunteers (17 women and 13 men) were recruited for the study with Der p. Atopic individuals were identified on the basis of allergen-specific IgE determined by RAST performed using UniCAP specific IgE fluoroenzyme-immunoassays (Pharmacia Diagnostics, Uppsala, Sweden) according to manufacturer's guidelines. Values above 0.70 kU/L were considered positive. Mean age of subjects with a positive RAST for Der p ($n = 13$) was 27 ± 8 years and that of negative subjects ($n = 17$) was 35 ± 13 . Clinical and allergology data for these donors are given in Table 1. All subjects with a positive RAST for Der p have allergic complaints (asthma: $n = 4$ or rhinitis and/or conjunctivitis: $n = 9$). Among individuals negative for Der p, two, including one with asthma, have a positive RAST for other allergens. Blood was also obtained from a second series of 24 atopic subjects (17 women and seven men aged 36 ± 13 years) at the Pneumology Unit, Cliniques Universitaires Saint-Luc. These patients were allergic to birch and/or Der p. RAST was performed as part of the clinical follow-up (Table 1). Skin prick tests (SPT) (data not shown) with commercial standardized extracts of aeroallergens (Stallergènes®, Antony, France) were done on the forearm of the patient. A glycerol-saline solution and a 9% codeine phosphate solution were used as negative and positive controls, respectively. The test was read after 20 min.

Anti-histaminic drugs were stopped 10 days before sampling and none of the patients studied received systemic corticosteroids in the last 3 months. Experimental protocols were approved by the Ethical Committees of the Academic Hospital of the VUB and the Cliniques Universitaires St. Luc (Brussels, Belgium).

Peripheral blood mononuclear cell stimulation with allergen or phytohaemagglutinin

PBMC prepared by Lymphoprep (Axis-Shield Poc AS, Oslo, Norway) density gradient centrifugation were incubated (4×10^5 cells in 200 μ L) in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C, 30 min) FCS (Gibco Cat No: 011-6290, Merelbeke, Belgium). Phytohaemagglutinin (PHA, reagent grade HA 15, Murex, Dartford, Kent, UK) was added at 2 μ g/mL. Der p extract was from HAL Allergen Lab (Haarlem, the Netherlands). Recombinant allergens were from Biomay (Vienna, Austria). They included Bet v 1 N-terminal (N-ter) and C-terminal (C-ter) fragments [25] and timothy *Phleum pratense* Phl p 5 and p 6 [26]. All allergens were used at 10 μ g/mL. Polymyxin B (5 μ g/mL) was included in cultures to prevent any influence of putative contamination with lipopolysaccharide, according to Prescott [27]. PHA stimulation was included for all donors as internal viability control.

Determination of cytokine concentrations

Cytokine production was measured by ELISA using commercial reagents for IL-5 (PeproTech, London, UK or Pharmingen, Erembodegem, Belgium) and IL-13 (Santquin, Amsterdam, the Netherlands). IL-9 was determined as described in [24]. The sensitivities of these assays were in the range of 1–10 pg/mL. In experiments where responses of individual donors to allergen are compared and correlated with RAST values, cytokine levels produced in the absence of stimulus (IL-5; 66 ± 19 , IL-9; 18 ± 6 pg/mL and IL-13; 286 ± 186 pg/mL) were subtracted from those measured after stimulation in order to restrict the comparison to allergen-induced cytokine production.

Statistics

Statistical analysis was performed with InStat 3 software for Macintosh (version 3 GraphPad Software). Correlation analyses were performed with Spearman's rank order correlation test, cytokine concentrations between groups were compared by Mann-Whitney and group comparisons were analysed by Fisher's exact test.

Results

Allergen-specific interleukin-9 induction in adult atopics

Preliminary experiments were carried out to determine the kinetics of IL-9 production. PBMC from two donors with

Table 1. Subject classification

VUB donors					UCL St Luc patients						
Subject*	Gender	Age (years)	Symptom	Der p RAST	Patient†	Gender	Age (years)	Symptom	Der p RAST	Birch RAST	Bet V1 RAST
N1	F	27	N	<	P1	F	27	RC	<	58	48.7
N2	F	24	N	<	P2	M	48	RC A1	<	85.2	88.5
N3	F	24	R	<	P3	F	38	RC	23.2	n d	3.54
N4	F	24	N	<	P4	M	28	RC A1	<	52.5	56.6
N5	M	30	N	<	P5	F	33	RC A1	18.3	<	n d
N6	F	41	N	<	P6	M	61	RC	1.15	17.6	20.6
N7	M	27	A	<	P7	F	20	R	3.33	<	n d
N8	F	29	N	<	P8	F	28	R	1.29	<	n d
N9	M	44	N	<	P9	F	57	RC A1	1.3	101	n d
N10	M	24	N	<	P10	F	51	RC A1	<	6.47	6.24
N11	M	23	N	<	P11	F	35	RC A1	4.5	78.5	86.7
N12	F	53	N	<	P12	F	32	R A1	2.5	<	n d
N13	F	30	N	<	P13	F	27	A2	6.3	<	n d
N14	F	60	N	<	P14	F	21	RC	1.8	19.8	n d
N15	F	51	N	<	P15	M	43	R A1	<	91.8	n d
N16	M	45	N	<	P16	M	34	RC A3	16.5	100	100
N17	M	59	N	<	P17	F	48	RC A1	<	8	n d
D1	F	25	R	0.86	P18	M	29	RC A1	<	14.3	9.69
D2	M	41	RC	23.3	P19	M	62	R	17.5	nd	n d
D3	F	26	RC	1.31	P20	F	45	RC A1	<	9.5	9.25
D4	F	26	RC	58.8	P21	F	25	R	2.9	<	<
D5	M	16	A	43.2	P22	F	34	RC A1	13.2	<	n d
D6	M	20	RC	46	P23	F	19	R	9.2	<	n d
D7	M	23	A	20.5	P24	F	24	R	6.3	53.5	n d
D8	F	27	RC	6.94							
D9	F	26	RC	25.2							
D10	M	46	RC	37.4							
D11	F	27	A	40.7							
D12	M	27	A	16.2							
D13	F	25	RC	68.1							

*Subjects from VUB are classified as N1–N17 or D1–D13, based on negative or positive RAST for Der p, respectively. RAST in kU/L. Subject N3 has a positive RAST for grasses and trees, N7 for trees.

†Patients from UCL St Luc hospital are all atopic and numbered P1–24.

A, asthma (from stage 1 to 4 of GINA asthma classification);

C, conjunctivitis; R, rhinitis; VUB, Vrije Universiteit Brussel; UCL, Université Catholique de Louvain; Der p, *Dermatophagoides pteronyssinus*; RAST in kU/L.

positive RAST for Der p and bronchial hyper-responsiveness (BHR) were incubated with allergen or PHA and culture supernatants were collected at different time-points. IL-9, which was undetectable in the absence of stimuli (data not shown), reached near-maximal levels 24 h after stimulation with PHA (Fig. 1a) but, in response to Der p, it became detectable only after 48 h and concentrations continued to rise with maximal levels reached after 4–5 days. The amounts of IL-9 produced in response to Der p exceeded those induced by PHA. For subsequent experiments, a 6-day incubation period was selected for medium collection. This incubation time was also optimal for IL-5 and IL-13 (data not shown).

To ascertain that the Der p-induced IL-9 response was allergen specific, IL-9 production was tested in a subject with positive RAST and BHR for birch and grass pollen but negative for Der p, and in one with opposite reactivity.

IL-9 levels were measured after stimulation with Der p extract, a recombinant Bet v 1 C-ter fragment or grass pollen proteins *P. pratense* Phl p 5 or 6. Figure 1b shows that IL-9 was selectively induced in accordance with donor hypersensitivity.

Interleukin-9 production in response to Dermatophagoides pteronyssinus: comparison with interleukin-5 and interleukin-13

The significance of *in vitro* cytokine production induced by allergen is highly dependent on the reproducibility of this response. To test this parameter, we measured IL-9 induced by Der p in PBMC of four individuals hypersensitive to this allergen and repeated the experiments over a period of several months. Experimental variability was

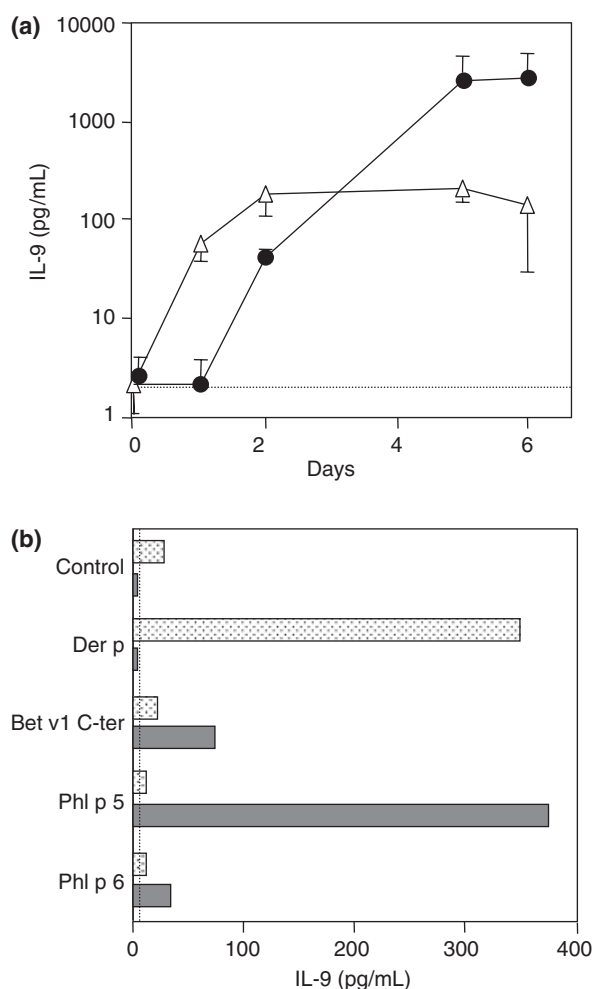


Fig. 1. (a) Kinetics of IL-9 production in response to *Dermatophagoides pteronyssinus* (Der p) or phytohaemagglutinin (PHA). Peripheral blood mononuclear cell from two donors with positive RAST for Der p were incubated with this allergen (black circles) or with PHA (white triangles). Culture supernatants were collected at different time-points and IL-9 concentration was determined by ELISA (means $\pm$ SEM for duplicate cultures from both donors). In this and subsequent figures, dotted lines indicate the limit of detection. (b) Allergen-specific IL-9 induction in atopic patients. PBMC from two donors, one allergic to birch and grass pollen but not to house dust mite (dark bars) and one with opposite reactivity (dotted bars) were incubated with Der p extract, a recombinant C-terminal fragment of Bet v 1, recombinant *Phleum pratense* Phl p 5 and 6 or control medium for 6 days before IL-9 determination in culture supernatants (means from duplicate cultures).

assessed by performing three separate stimulations, each in triplicate cultures, from a PBMC sample of two donors (Table 2). Variation coefficients (SD/mean) were 0.85 and 0.10, respectively. The stability of IL-9 production level with time in individual donors was also evaluated. Donor D11 and D2 remained the highest and lowest producers over a 1-year interval. For the two donors, where five and four different samples could be collected, variation coefficients between different bleedings were 0.80 and 0.73,

respectively, with maximal differences of 4–5-fold. Together, these data indicate that IL-9 production profiles are sufficiently stable to categorize donors although significant differences can be seen with time, possibly because of recent allergen exposure.

The correlation of Der p-induced IL-9 production with atopy was first tested in adult volunteers positive or negative for anti-Der p IgE as determined by RAST assays and was compared with IL-5 and IL-13. Production of IL-5 and IL-9 reached at least twice the background in 11/13 (85%) and those of IL-13 in 12/13 (92%) RAST-positive individuals. In these subjects, production of IL-9 correlated significantly with that of IL-5 ($r=0.623$ with $P=0.022$) and IL-13 ($r=0.589$ with $P=0.034$). A similar level of correlation was calculated between IL-5 and IL-13 ($r=0.714$ with $P=0.006$). In the 17 donors negative for anti-Der p IgE, IL-9 was found in only 1 (6%), while IL-5 and IL-13 were up-regulated in four (24%) and nine (53%), respectively (Fig. 2). As the sensitivity of IL-9 determination was at least equivalent to that of IL-5 and IL-13, these results suggest that IL-9 production *in vitro* is a very specific marker of Der p hypersensitivity, closely followed by IL-5, but more selective than IL-13 (P -values between RAST-positive and -negative donors were <0.0001 , 0.0025 and 0.042 for IL-9, IL-5 and IL-13, respectively). Der p non-allergic donors (N8, N15 and N17), whose IL-13 production was in the same range as that of most Der p-hypersensitive individuals, illustrate the more permissive regulation of *in vitro* IL-13 production as compared with that of IL-9. This difference in 'false' positives between IL-9 and IL-13 was very significant ($P=0.007$ by Fisher's test). The greater selectivity of IL-9 determination was not restricted to non-atopic individuals as donors N7 and N3, which are hypersensitive to trees (and grasses in the case of donor N3) but not to Der p, produced no IL-9 (nor IL-5) in response to Der p but were clearly positive for IL-13.

Correlation of allergen-specific immunoglobulin E with allergen-induced interleukin-5, interleukin-9 and interleukin-13

The clinical significance of cytokines induced by allergen was evaluated by testing the correlation with Der p-specific IgE serum concentrations in the RAST-positive donors (>0.7 kU/L). Significant and similar correlations were observed for IL-5 and IL-9 ($r=0.561$ with $P=0.029$ for IL-5 and $r=0.588$ with $P=0.024$ for IL-9). For IL-13, the correlation coefficient was not significant ($r=0.470$ with $P=0.101$) (Fig. 3a). The two donors that failed to produce IL-9 (D3 and D8) also had the lowest RAST scores.

The analysis was extended to PBMC from a group of adult atopic patients with different hypersensitivity profiles. Plots of Der p-induced cytokines as a function of the corresponding IgE titres are shown in Fig. 3b for patients

Table 2. Stability of IL-9 production patterns in response to Der p with time

Donor	Symptomatology	Reactivity	Date bleeding	IL-9 (pg/mL) in response to Der p
D2	R-C	Der p, birch	19 November, 2003	154
			3 March, 2004	158
D5	Asthma	Der p, trees	24 April, 2003	926
			27 May, 2003	523
			5 February, 2004	335
			21 April, 2004	178 $\pm$ 51 ($n = 9$)
D11	Asthma	Der p, trees, grasses	20 March, 2003	6336
			18 February, 2004	2248 $\pm$ 393 ($n = 3$)
			10 March, 2004	6060 $\pm$ 283 ($n = 3$)
			28 April, 2004	1517 $\pm$ 51 ($n = 9$)
			9 August, 2004	2879
D12	Asthma	Der p	18 February, 2003	588
			18 June, 2003	211

PBMC from four donors collected on the indicated dates were incubated with Der p for 6 days. Depending on the available sample size, IL-9 levels were measured in single or multiple wells as indicated (mean $\pm$ SEM). The first line of each donor is incorporated in Fig. 2. PBMC, peripheral blood mononuclear cell; Der p, *Dermatophagoides pteronyssinus*.

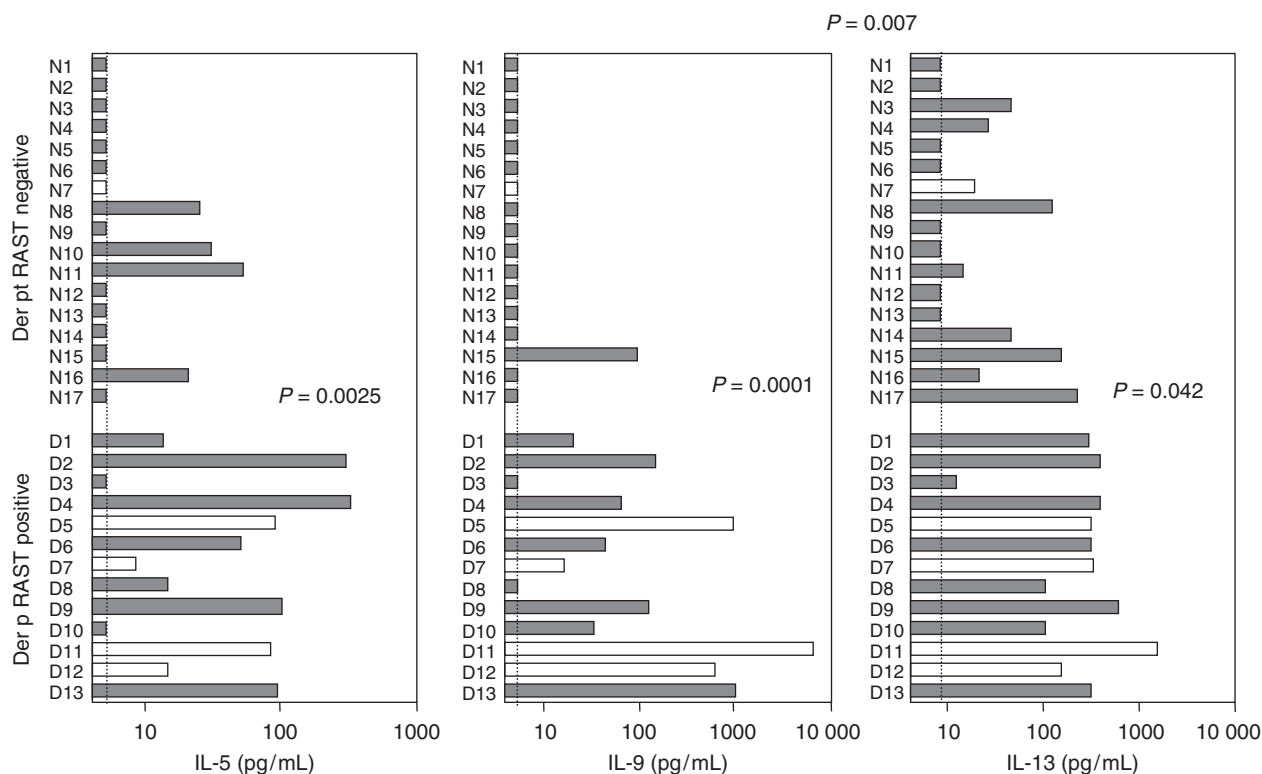


Fig. 2. IL-5, IL-9 and IL-13 production by peripheral blood mononuclear cell in response to *Dermatophagoides pteronyssinus* (Der p) PBMC from Der p RAST-negative (N, $n = 17$) and -positive (D, $n = 13$) adults were stimulated with Der p. Cytokine concentrations were determined on day 6. Open bars correspond to asthmatics. Donor N7 has a positive RAST for trees and donor N3 is positive for grasses and trees but both are negative for Der p. P -values between RAST-positive and -negative donors were analysed by Fisher's exact test.

with RAST ≥ 0.7 kU/L for Der p ($n = 16$). In this patient group, the best correlation with RAST measurements was found for IL-9 ($r = 0.660$ with $P = 0.005$). For IL-5, it was somewhat weaker ($r = 0.583$ with $P = 0.019$) and for IL-13 it was again not significant ($r = 0.353$ and $P = 0.200$).

PBMC from 15 patients with positive RAST either for total birch allergen extract ($n = 14$) and/or Bet v 1 ($n = 10$) protein were also tested for their response to N-ter and C-ter fragments of Bet v 1. These two peptides were selected because N-ter and C-ter have been reported to

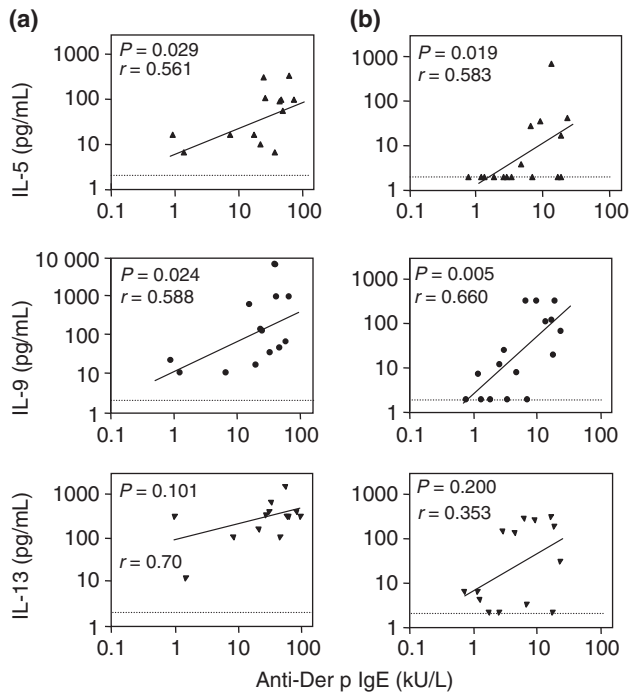


Fig. 3. Correlations between cytokine production and RAST values in responses to *Dermatophagoides pteronyssinus* (Der p). Cytokines induced by Der p in the group with positive RAST for Der p shown in Fig. 2a and in a second cohort of atopic patients also reactive with Der p (b, $n = 16$ for IL-5 and IL-9 and $n = 14$ for IL-13).

induce Th2- and Th1-biased responses, respectively [28]. Strong correlations with total anti-birch and anti-Bet v 1 IgE were seen for IL-9 induced by both N-ter and C-ter peptides (Fig. 4). The correlation coefficient was particularly elevated ($r = 0.939$) for C-ter-induced IL-9 and anti-Bet v 1 IgE. For IL-13, it was at the limit of significance for the N-ter and significant with the C-ter fragment, while for IL-5 there was a positive trend for the N-ter polypeptide only. In fact, the C-ter fragment induced significantly less ($P = 0.015$) IL-5 (57.8 ± 25.6 pg/mL) than the N-ter equivalent (115 ± 27.7 pg/mL), in agreement with previously published data [28]. The opposite was seen for IL-9 (58 ± 13 and 126 ± 39 pg/mL for N-ter and C-ter, respectively $P = 0.038$) and also, to some extent, for IL-13 (71 ± 15 for N-ter and 91 ± 21 for C-ter). This difference between IL-5 and IL-9 was also apparent from their lack of correlation in individual patients for both forms of Bet v 1 ($r = 0.154$ with $P = 0.62$ for N-ter and $r = 0.32$ with $P = 0.28$ for C-ter). By contrast, correlations between IL-9 and IL-13 were very significant for the responses to C-ter ($r = 0.769$ with $P = 0.002$).

To further evaluate the clinical significance of the *in vitro* produced cytokines, patients with hypersensitivity symptoms and RAST tests positive for either Der p ($n = 7$) or birch ($n = 12$) were selected from the previous group and their *in vitro* responses to both allergens were compared. IL-9 production perfectly matched that expected on

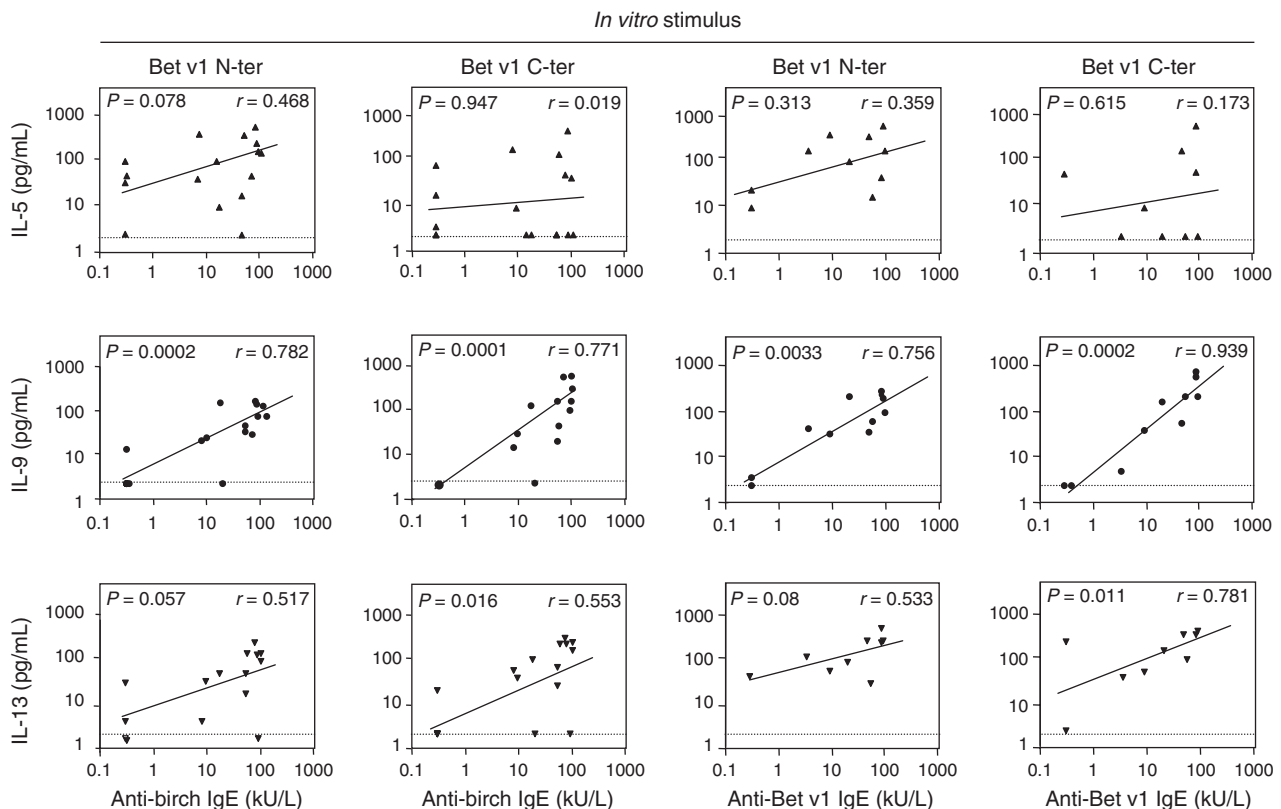


Fig. 4. Correlations between cytokine production and RAST values in response to birch allergens. Peripheral blood mononuclear cell were stimulated with N-ter and C-ter fragments of Bet v 1 for 6 days. Cytokine levels were plotted vs. anti-birch or anti-Bet v 1 IgE levels.

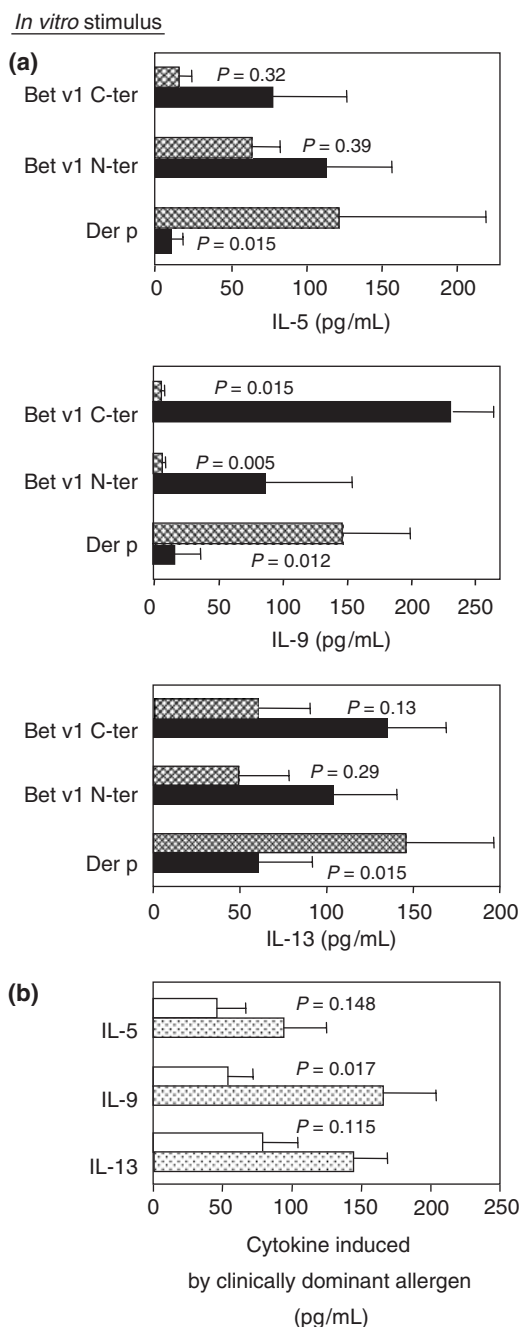


Fig. 5. Clinical relevance of *in vitro* cytokine production in response to allergens. Cytokine production (mean $\pm$ SEM) by peripheral blood mononuclear cell (a) from patients with hypersensitivity limited to *Dermatophagoides pteronyssinus* (Der p) ($n = 7$, grey bars) or birch ($n = 12$, black bars) in response to either allergen and (b) from patients with symptoms limited to rhinitis and conjunctivitis ($n = 13$, open bars) or including asthma ($n = 9$, dotted bars) stimulated by the clinically relevant allergens.

the basis of RAST levels (Fig. 5a). IL-5 induction by Der p was also more elevated in Der p allergics than in birch allergics but the difference was not statistically significant. Moreover, and unlike what was seen for IL-9, Bet v 1 N-ter stimulated the production of IL-5 in Der p allergics

with a negative RAST for birch. As to IL-13, its induction was less selective for all allergens.

Finally, cytokine production in asthmatics was compared with that of patients whose symptoms were restricted to rhinoconjunctivitis. In donors responsive to either Der p or birch, cytokines induced by the clinically relevant allergen, identified by anamnesis, RAST and SPT, were compared in the two patient groups. They were higher in asthmatics for all cytokines but the difference was statistically significant only for IL-9 (Fig. 5b).

Discussion

The present results show that IL-9 production is specifically induced in PBMC from hypersensitive adults by stimulation with allergen and correlates with allergen-specific IgE titres. Two-thirds of Der p reactive and 90% of birch-sensitive individuals (i.e. with a positive RAST for these allergens) produced IL-9 after allergen challenge. This confirms results reported for atopic children [24]. The failure by some allergic individuals to produce IL-9 in response to allergen, particularly in one patient group in response to Der p was not because of poor cell viability as these cells produced IL-9 after stimulation with PHA (data not shown). It was rather associated with low anti-Der p IgE (< 2 kU/L). As the same individuals were also low producers of IL-5 and IL-13, it is likely that the lack of IL-9 production observed in some atopics reflects a low level of responsiveness and not a selective down-regulation of IL-9.

A recent report describing *in vitro* cytokine production by PBMC in response to *Der farinae* has shown a correlation between IL-5 and IL-13 production in asthma patients [29]. Such correlation was also found in the response to Der p reported here and was extended to IL-9. Combining the Der p-sensitive individuals in our two donor groups ($n = 29$), highly significant ($P < 0.001$) correlation coefficients were calculated for IL-9-IL-5 ($r = 0.579$), IL-9-IL-13 ($r = 0.682$) and IL-5-IL-13 ($r = 0.759$). For birch allergens, a different picture emerged, as N-ter Bet v 1 induced more IL-5 than C-ter, while the opposite was seen for IL-9. The ratio of IL-9/IL-5 in individual patients was 1.8 ± 0.9 for N-ter, and 16.1 ± 6.2 for C-ter ($P = 0.005$). The same difference was seen for IL-13/IL-5 ratios (2.7 ± 1.9 for N-ter and 12.6 ± 6.1 for C-ter, $P = 0.005$). IL-9/IL-13 ratios remained identical for both fragments (3.2 ± 2.2 for N-ter and 3.5 ± 2.2 for C-ter). These observations reinforce the notion that the molecular nature of the birch allergen has a major influence on the pattern of cytokine production [30] and that, in response to Bet v 1, IL-9 and IL-13 show significant similarities.

Differences between IL-9 and IL-13 were however observed with respect to their expression by non-atopic individuals in response to Der p. Induction of IL-9 appeared more selective than that of IL-13, as more than half (14/25) of the subjects with a negative RAST for Der p up-regulated

IL-13 in response to this allergen, as compared with 4/25 for IL-9. Obviously, subtle differences are operating in the control of *in vitro* expression of the three Th2 factors in response to allergen that will need further clarification.

The clinical significance of cytokine production in response to allergen was evaluated by testing its correlation with allergen-specific IgE. For IL-9, this was excellent for all allergens tested. For IL-5 and IL-13, it was more variable: for Der p it was fairly similar to IL-9 but, for birch, major differences were seen, particularly with IL-5. Moreover, when cytokines produced in response to clinically relevant or clinically irrelevant allergens were compared, only IL-9 production significantly reflected clinical symptomatology in a group of patients hypersensitive to either Der p or birch. To our knowledge, such clear correlation between IL-9 protein biosynthesis and allergen-specific IgE levels and atopy has not been reported previously in adults and confirms data obtained at mRNA [22] and protein [24] level in children. IL-9 production also tended to be higher in asthmatic individuals than in atopics without asthma. This difference was not significant for IL-5 nor for IL-13. The preferential association of IL-9 with asthma is also in line with observations reported in children [31] and with genetic studies in the mouse [7].

In conclusion, our results strengthen the link between IL-9 expression and atopy and asthma. The fact that IL-9 production was more strictly linked to the latter condition than IL-13 does not challenge the notion that IL-13 is an essential effector in asthma [32, 33]. However, our data suggest that the *in vitro* production of IL-9, which in mice is a predominant inducer of IL-13 *in vivo* [34], is a more accurate marker for atopy-promoting T cell responses.

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References

- 1 Levitt R, McLane M, MacDonald D *et al.* IL-9 pathway in asthma: new therapeutic targets for allergic inflammatory disorders. *J Allergy Clin Immunol* 1999; 103:S485–91.

- 2 Uyttenhove C, Simpson R, Van Snick J. Functional and structural characterization of P40, a mouse glycoprotein with T cell growth factor activity. *Proc Natl Acad Sci USA* 1988; 85:6934–8.
- 3 Hültner L, Druez C, Moeller J *et al.* Mast cell growth enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin-9). *Eur J Immunol* 1990; 20:1413–6.
- 4 Marsh DG, Neely JD, Breazeale DR *et al.* Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum IgE concentrations. *Science* 1994; 264:1152–6.
- 5 Doull IJ, Lawrence S, Watson M *et al.* Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 1996; 153: 1280–4.
- 6 Levitt RC, Holroyd KJ. Fine-structure mapping of genes providing susceptibility to asthma on chromosome 5q31–q33. *Clin Exp Allergy* 1995; 25 (Suppl. 2):119–23.
- 7 Nicolaides N, Holroyd KJ, Ewart SL *et al.* Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci USA* 1997; 94: 13175–80.
- 8 Mock BA, Krall M, Kozak CA *et al.* IL-9 maps to mouse chromosome 13 and human chromosome 5. *Immunogenetics* 1990; 1:265–70.
- 9 Godfraind C, Louahed J, Faulkner H *et al.* Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. *J Immunol* 1998; 160:3989–96.
- 10 Louahed J, Zhou Y, Maloy WL *et al.* Interleukin 9 promotes influx and local maturation of eosinophils. *Blood* 2001; 97:1035–42.
- 11 Gounni AS, Gregory B, Nutku E *et al.* Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. *Blood* 2000; 96:2163–71.
- 12 Dugas B, Renauld J-C, Pène J *et al.* Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. *Eur J Immunol* 1993; 23:1687–92.
- 13 Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med* 1998; 188:1307–20.
- 14 Little FF, Cruikshank WW, Center DM. IL-9 stimulates release of chemotactic factors from human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2001; 25:347–52.
- 15 McLane MP, Haczku AH, van de Rijn M *et al.* Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. *Am J Respir Cell Mol* 1998; 19:713–20.
- 16 Kung TT, Luo B, Crawley Y *et al.* Effect of anti-mil-9 antibody on the development of pulmonary inflammation and airway hyperresponsiveness in allergic mice. *Am J Respir Cell Mol Biol* 2001; 25:600–5.
- 17 Cheng G, Arima M, Honda K *et al.* Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. *Am J Respir Crit Care Med* 2002; 166: 409–16.
- 18 Townsend JM, Fallon GP, Matthews JD, Smith P, Jolin EH, McKenzie NA. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity* 2000; 13:573–8.

- 19 Ying S, Meng Q, Kay AB, Robinson DS. Elevated expression of interleukin-9 mRNA in the bronchial mucosa of atopic asthmatics and allergen-induced cutaneous late-phase reaction: relationships to eosinophils, mast cells and T lymphocytes. *Clin Exp Allergy* 2002; 32:866–71.
- 20 Shimbara A, Christodoulopoulos P, Soussi-Gounni A *et al.* IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J Allergy Clin Immunol* 2000; 105:108–15.
- 21 Erpenbeck VJ, Hohlfeld JM, Volkmann B *et al.* Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol* 2003; 111:1319–27.
- 22 Macaubas C, Sly PD, Burton P *et al.* Regulation of T helper cell responses to inhalant allergen during early childhood. *Clin Exp Allergy* 1999; 29:1223–31.
- 23 Rudin A, Macaubas C, Wee C, Holt BJ, Sly PD, Holt PG. "Bystander" amplification of PBMC cytokine responses to seasonal allergen in polysensitized atopic children. *Allergy* 2001; 56:1042–8.
- 24 Jenmalm MC, Van Snick J, Cormont F, Salman B. Allergen-induced Th1 and Th2 cytokine secretion in relation to specific allergen sensitization and atopic symptoms in children. *Clin Exp Allergy* 2001; 31:1528–35.
- 25 Vrtala S, Hirtenlehner K, Vangelista L *et al.* Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997; 99:1673–81.
- 26 Vrtala S, Sperr WR, Reimitzer I *et al.* cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl pV allergen. *J Immunol* 1993; 151:4773–81.
- 27 Prescott SL, Macaubas C, Holt BJ *et al.* Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998; 160:4730–7.
- 28 Vrtala S, Akdis CA, Budak F *et al.* T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. *J Immunol* 2000; 165:6653–9.
- 29 Hashimoto T, Akiyama K, Kawaguchi H, Maeda Y, Taniguchi M, Kobayashi N. Correlation of allergen-induced IL-5 and IL-13 production by peripheral blood T cells of asthma patients. *Int Arch Allergy Immunol* 2004; 1341:7–1.
- 30 Niederberger V, Horak F, Vrtala S *et al.* Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004; 101 (Suppl. 2):14677–82.
- 31 Bottcher MF, Bjurström J, Mai XM, Nilsson L, Jenmalm MC. Allergen-induced cytokine secretion in atopic and non-atopic asthmatic children. *Pediatr Allergy Immunol* 2003; 14:345–50.
- 32 Wills-Karp M, Luyimbazi J, Xu X *et al.* Interleukine-13: central mediator of allergic asthma. *Science* 1998; 282:2258–61.
- 33 Grunig G, Warnock M, Wakil AE *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282:2261–3.
- 34 Temann UA, Ray P, Flavell A. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest* 2002; 109:29–39.