

Allergen-induced Th1 and Th2 cytokine secretion in relation to specific allergen sensitization and atopic symptoms in children

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Summary

Background Allergic diseases are believed to be due to T helper (Th)2-like immunity to allergens in affected tissues, and immune responses to allergens are characterized by a cross-regulation between Th1 and Th2 cells. Atopic individuals may develop IgE antibodies to only one or more allergens. However, the mechanisms behind sensitization to a specific allergen, e.g. why an individual develops IgE to cat but not birch, are not known. Our aim was to study birch- and cat-induced Th1 and Th2 cytokine secretion in children who were sensitized to birch but not to cat, and vice versa.

Materials and methods The subjects in the study were 60 12-year-old children. Seventeen of the children were sensitized (skin prick test and circulating IgE positive) to birch but not cat, 13 were sensitized to cat but not birch, 11 were sensitized both to birch and cat, and 19 children were skin prick test and circulating IgE negative. Forty-six children had a history of atopic symptoms, and 42 of them had current symptoms. Peripheral blood mononuclear cells were separated from venous blood and stimulated with cat or birch allergen. The levels of IL-4, IL-5, IL-9, IL-10, IL-13 and IFN- γ in the cell supernatants were analysed by ELISA.

Results Sensitized children produced more of the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 than non-sensitized atopic and non-atopic children in response to stimulation with the allergen they were sensitized to. High levels of the Th2 cytokines IL-4 and IL-5 and low levels of the anti-inflammatory cytokine IL-10 were associated with atopic symptoms, and high cat-induced IL-9 levels with asthma.

Conclusions The Th2 cytokines IL-4, IL-5, IL-9 and IL-13 were all commonly detected in sensitized children after stimulation with the specific, in contrast to an unrelated, allergen. Atopic symptoms were associated with increased levels of IL-4 and IL-5 and tended to be associated with low levels of IL-10, and asthma with high cat-induced IL-9 levels.

Keywords: allergen, cytokine, Th1, Th2, childhood

Clinical and Experimental Allergy, Vol. 31, pp. 1528–1535. Submitted 27 October 2000; revised 12 March 2001; accepted 14 April 2001.

Introduction

Atopic diseases are believed to be due to excessive T helper (Th)2-like immunity to allergens in affected tissues [1,2].

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The Th1/Th2 concept was originally described in murine models (reviewed in [3]), where immune responses to allergens delivered to the respiratory mucosa are characterized by a cross-regulation between Th1 and Th2 cell populations [4]. Th2 cells produce immunoglobulin E (IgE) synthesis, inducing interleukin-(IL)-4 [5] and IL-13 [6], eosinophilia promoting IL-5 and IL-9 [7,8], while interferon

(IFN)- γ from Th1 cells down-regulates IgE synthesis [5]. IL-9 also enhances IgE production [9] and stimulates T cell and mast cell growth [10,11]. Interleukin-10 is an anti-inflammatory cytokine [12] that inhibits both Th1 and Th2 cells and IgE synthesis [13], as well as shortening eosinophil survival [12]. The Th1/Th2 dichotomy was later also described in humans [14].

Allergen-specific T cell clones from atopic adults appear to be polarized towards a Th2 [14] or Th0 [15] phenotype. More recently, atopy was shown to be associated with increased levels of IL-4 [16–19], IL-5 [20–24] and IL-13 [19,21,23,24], accompanied by decreased [17], similar [16,21,22,24] or increased [20] levels of IFN- γ in allergen-stimulated bulk cultures with peripheral blood mononuclear cells (PBMC). Allergen-induced IL-9 secretion has not been investigated, due to previous lack of appropriate antibodies, although we could recently show that increased IL-9 mRNA expression after allergen stimulation was associated with atopy [24].

However, the mechanisms regulating sensitization to a specific allergen, e.g. why an atopic individual develops IgE antibodies to cat but not birch, are not known. Previous studies of allergen-induced IL-5 and IL-13 secretion in PBMC were performed in atopic individuals who were both symptomatic and sensitized to the studied allergens, while the controls were neither [21,22]. Therefore, it was not possible to determine whether high levels of those cytokines were related to atopic symptoms or to sensitization to the specific allergen. In another study [18], PBMC from birch-sensitized patients secreted IL-4 after stimulation with birch, but not cat, extract. However, the only other studied cytokine was IFN- γ . Distinct Th2 cytokines, e.g. IL-4 and IL-5, are differentially regulated [25–27].

The aim of this study was to relate birch- and cat-induced IL-4, IL-5, IL-9, IL-10, IL-13 and IFN- γ secretion to sensitization to birch or cat and atopic symptoms.

Materials and methods

Subjects

The study included 60 12-year-old children who had been identified from the Linköping part of the phase 2 International Study of Asthma and Allergies in Childhood (ISAAC). The blood was drawn outside the birch pollen season, i.e. between October 1999 and March 2000. Atopic symptoms were determined by the ISAAC phase 2 questionnaire. Asthma was defined as four or more yearly episodes of bronchial obstruction, atopic rhinoconjunctivitis (ARC) as sneezing or a runny or blocked nose in the absence of a cold or the flu, accompanied by itchy-watery eyes, and atopic dermatitis as an itchy rash, which was coming and going for at least 6 months. Symptoms were

Table 1. Atopic symptoms of the children

Symptoms*	Current	Cumulative
Any	42	46
Airway symptoms†	37	45
Atopic rhinoconjunctivitis	36	41
Asthma	8	15
Atopic dermatitis	18	31
Atopic dermatitis only	5	4
None	18	14

Atopic symptoms were determined by the ISAAC phase 2 questionnaire, and defined as current if occurring within the last 12 months. *Several children had more than one atopic symptom.

†Atopic rhinoconjunctivitis or asthma.

defined as current if occurring within the last 12 months. The symptoms of the children are described in Table 1.

Sensitization to an allergen was defined as the presence of both a positive skin prick test and circulating IgE antibodies. Seventeen of the children were sensitized to birch but not cat, 13 were sensitized to cat but not birch, 11 were sensitized both to birch and cat, and 19 children were skin prick test and circulating IgE negative. The non-atopic children had no skin test reactivity to a panel of allergens. All the children who were sensitized and had no current symptoms ($n = 4$) had had symptoms previously. Three of the children who had symptoms (one with eczema, one with asthma and one with both ARC and eczema) were non-sensitized.

Skin prick tests were performed in duplicate on the volar aspects of the forearms with standardized birch, timothy, cat, dog and two house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*) extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/mL) was used as a positive control and albumin diluent as negative control. The test was regarded as positive when the mean diameter was ≥ 3 mm.

The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping.

IgE antibody analysis

The levels of IgE antibodies to birch and cat were analysed with Magic Lite™, a commercial chemoluminometric method, according to the recommendations of the manufacturer (ALK). Samples above 1.43 SU/mL were regarded as positive.

Cell culture

Venous blood was drawn into heparin-treated tubes

(Vacuette, Greiner Labour technik, Kremsmünster, Austria). PBMC were isolated on a Ficoll Paque density gradient (Pharmacia Biotech, Sollentuna, Sweden) as described in detail elsewhere [28].

Aliquots of 1 mL, containing 1×10^6 cells, were cultured in AIM-V serum-free medium (Life Technologies AB, Täby, Sweden) with 20 μM mercaptoethanol (Sigma-Aldrich, Stockholm, Sweden). Optimal allergen doses and time points for cytokine secretion were determined in a pilot study including cells from six adults. Doses tested were 1, 10 and 50 kSU/mL and time points tested were 24, 48, 72, 96, 144 and 192 h. The cells were stimulated with birch (10 kSU/mL) and cat allergen (10 kSU/mL) extracts from ALK. Separate cultures were performed with 2 $\mu\text{g}/\text{mL}$ monoclonal antibodies to the IL-4 receptor (R & D Systems, Abingdon, UK), enabling measurement of IL-4 [29]. Two $\mu\text{g}/\text{mL}$ of phytohaemagglutinin A (PHA) was used as a positive control. The cells were cultured at 37 °C with 5% CO_2 (Forma CO_2 -incubator model 3862, Forma Scientific Inc., Marietta, OH, USA) for 24 h or 144 h. The supernatants were collected after centrifugation and stored at -70 °C.

Background responses (cultures with medium only) were subtracted from the allergen- and PHA-induced responses. IL-4, IL-5, IL-9, IL-13 and IFN- γ were analysed in the 144-h samples, while IL-10 was analysed in the 24-h samples. The differing kinetics of secretion of IL-10, as compared with the other cytokines, is probably due to different cellular sources. It is likely that monocytes are activated to secrete IL-10 early in the cultures [24].

Cytokine analysis

The production of IFN- γ , IL-4, IL-5, IL-9, IL-10 and IL-13 was detected with Enzyme-Linked Immunosorbent Assay (ELISA). Anti-human IL-9 monoclonal antibodies MH9A4 (IgG2b) and MH9A3 (IgG1) were derived from C57Bl/6 mice immunized with human IL-9 coupled to ovalbumin, as described previously for mouse IL-9 [11]. MH9A3 was biotinylated using an EZ-Link™ Sulfo-NHS-LC-biotinylation kit (Pierce Chemical Co., Rockford, IL, USA), according to the protocol of the manufacturer. Costar 3690 plates (Corning Inc., Corning, NY, USA) were coated overnight at room temperature (22°C). The following mouse anti-human coating antibodies were used in carbonate buffer, pH 9.6: IL-4, IL-13 and IFN- γ (clone no. 3010.211, 32116.11 and 2571811, respectively, R & D Systems); IL-5 (clone no. TRFK 5, PharMingen, San Diego, CA, USA); IL-9 (clone no. MH9A4); and IL-10 (coating antibody, PeliPair™ reagent set, CLB, Amsterdam, the Netherlands). The concentrations used were 2 $\mu\text{g}/\text{mL}$ for IFN- γ , 0.25 $\mu\text{g}/\text{mL}$ for IL-5, 5 $\mu\text{g}/\text{mL}$ for IL-9 and 4 $\mu\text{g}/\text{mL}$ for IL-4, IL-10 and IL-13. Excess free antibodies

were washed off (Wellwash Ascent, Labsystems, Stockholm, Sweden) with $4 \times 250 \mu\text{L}$ PBS-Tween (0.05% Tween 20 in PBS, pH 7.4), and the wells were incubated for 1 h at room temperature on a plate shaker (Thermostar, Labvision, Stockholm, Sweden) with 100 μL per well of low-fat milk to block non-specific protein binding sites.

The plates were washed as before and 50 μL of samples or standards, recombinant human IFN- γ , IL-4, IL-9 and IL-13 (R & D Systems), IL-5 (PharMingen) and IL-10 (CLB) were added to duplicate wells. A control using AIM-V serum-free medium with 20 μM mercaptoethanol was also included. The plates were incubated and washed as before.

Biotinylated detection antibodies, goat anti-human IFN- γ , IL-4 and IL-13 (R & D Systems), rat anti-human IL-5 (clone no. JES1-5A10, PharMingen), mouse anti-human IL-9 (clone no. MH9A4) and IL-10 (CLB), were then added (50 μL per well). All were diluted in high performance ELISA-dilution buffer (CLB), to the final concentrations of 0.20 $\mu\text{g}/\text{mL}$ for IFN- γ , 1 $\mu\text{g}/\text{mL}$ for IL-5, 90 ng/mL for IL-9, and 0.50 $\mu\text{g}/\text{mL}$ for IL-4 and IL-13. The antibodies to IL-10 were diluted 1 : 100. This was followed by another incubation and wash.

Streptavidin-poly horse radish peroxidase (50 μL per well, 0.1 μM , CLB) was added and the plates were incubated as before, but for 30 min, and then washed. Thereafter 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) was added (50 μL per well), and incubated as before, but for 30 min in the dark; 1.8 M H_2SO_4 (50 μL per well) was finally added to stop the reaction. The amount of substrate converted to product was thereafter detected as optical densities (OD) at 450 nm in an Anthos ht II ELISA reader (Anthos ht II, Labdesign, Täby, Sweden). Values were expressed as pg/mL deduced from the OD of the standard curve after subtracting the blanks. The sensitivity limits for quantitative determinations were 2 pg/mL for IL-10, 3 pg/mL for IL-4 and IL-5, 10 pg/mL for IL-9, 12 pg/mL for IFN- γ and 16 pg/mL for IL-13.

Statistics

As the cytokine levels were not normally distributed even after logarithmic transformation, non-parametric tests, corrected for ties, were used. Comparisons between unpaired groups were analysed with the Mann-Whitney *U*-test, and correlations were analysed with Spearman's rank order correlation coefficient test. The chi-square test was employed for categorical variables and Fisher's exact test was used when the expected frequency for any cell was less than five. A *P*-value of < 0.05 was considered as statistically significant. Calculations were performed with a statistical package, StatView 5.0 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA).

Table 2. Birch-induced cytokine secretion (% positive samples) in relation to sensitization

Sens	IL-4	IL-5	IL-9	IL-10	IL-13	IFN- γ
Birch†	44%***	71%**	32% ^P = 0.09	43%	50%*	43%
Birch & cat	36%*	82%**	27%	45%	45%	45%
Birch only	50%***	65%**	35% ^P = 0.11	41%	53%*	41%
Cat only	8%	31%	23%	46%	31%	23%
None	0%	26%	11%	47%	21%	32%

The proportion of children with detectable cytokine secretion in the birch- and/or cat-sensitized groups was compared with the proportion in the non-sensitized group using the chi-square test. Fisher's exact test was used when the expected frequency for any cell was less than five. Similar results were obtained when the cytokine levels in birch- and/or cat-sensitized children were compared with the levels in non-sensitized children with Mann-Whitney *U*-test (data not shown). †Children sensitized to birch and/or both birch and cat, *n* = 28. Eleven children were sensitized to both birch and cat, 17 to birch only, 13 to cat only and 19 were non-sensitized. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

Skin prick test reactivity in relation to cytokine secretion

Birch- and cat-induced cytokine secretion in sensitized and non-sensitized children is presented in Tables 2 and 3,

respectively. PHA-induced cytokine responses were not related to skin prick test reactivity (data not shown).

Birch-induced IL-4, IL-5 and IL-13 secretion was higher in children who were sensitized to birch than in those who were sensitized to cat or non-sensitized children, and the proportion of children with detectable levels of those cytokines was higher in the former, as compared with the latter, groups. A similar trend was observed for birch-induced IL-9 production. Furthermore, the size of the birch-induced skin prick test and the circulating IgE antibody levels to birch correlated with birch stimulated IL-4, IL-5 and IL-13 secretion in birch skin prick test and IgE positive children (Table 4). Birch-induced IL-10 and IFN- γ production was not related to sensitization and no significant correlations between those cytokines and the size of birch-induced skin prick test or the circulating IgE antibody levels were seen (Table 4).

Cat-stimulated IL-4, IL-5 and IL-9 production was enhanced in cat-sensitized children, as compared with birch or non-sensitized children, and the proportion of children with detectable levels of those cytokines was higher in the former, as compared to the latter, groups. However, cat-induced IL-10, IL-13 and IFN- γ production was not related to sensitization. Neither the skin prick test size nor the circulating IgE antibody levels correlated with cat-induced cytokine secretion in cat skin prick test and IgE positive children (data not shown).

Table 3. Cat-induced cytokine secretion (% positive samples or median (range) (pg/mL) for IL-5, IL-10 and IFN- γ † in relation to sensitization

Sens	IL-4	IL-5	IL-9	IL-10	IL-13	IFN- γ
Cat‡	42%*	66* (n.d. – 592)	54%*	140 (n.d. – 1442)	46%	61 ^P = 0.09 (n.d. – 600)
Cat & birch	36%	40* (n.d. – 376)	45%	104 (41 – 426)	27%	65 (n.d. – 431)
Cat only	46%*	69* (n.d. – 592)	62%*	141 (n.d. – 1442)	62%	nd ^P = 0.13 (n.d. – 600)
Birch only	12%	12 (n.d. – 108)	23%	145 (37 – 396)	29%	91 ^P = 0.12 (n.d. – 573)
None	11%	13 (n.d. – 188)	26%	196 (32 – 1175)	47%	176 (n.d. – 848)

For IL-4, IL-9 and IL-13, the proportion of children with detectable cytokine secretion in the birch- and/or cat-sensitized groups was compared with the proportion in the non-sensitized group using the chi-square test. Fisher's exact test was used when the expected frequency for any cell was less than five. Similar results were obtained when the cytokine levels in birch- and/or cat-sensitized children were compared with the levels in non-sensitized children using the Mann-Whitney *U*-test (data not shown). For IL-5, IL-10 and IFN- γ , cytokine levels in birch- and/or cat-sensitized children were compared with the levels in non-sensitized children using the Mann-Whitney *U*-test. †Median levels and range are given for IL-5, IL-10 and IFN- γ , as most of the samples were positive. ‡Children sensitized to cat and/or both birch and cat, *n* = 24. Eleven children were sensitized to both cat and birch, 13 to cat only, 17 to birch only, and 19 were non-sensitized. **P* < 0.05. n.d. = not detectable.

Table 4. Correlations between birch-stimulated cytokine secretion and size of birch-induced skin prick test and circulating IgE antibody levels to birch in birch-sensitized children

	IL-4	IL-5	IL-9	IL-10	IL-13	IFN- γ
SPT size	0.50**	0.54**	n.s.	n.s.	0.47*	n.s.
IgE level	0.73***	0.73***	n.s.	n.s.	0.61**	n.s.

Correlations were analysed with Spearman's rank correlation, and Rho values are given. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.s. = not significant.

Atopic symptoms in relation to cytokine secretion

As atopic symptoms were tightly associated with skin prick test reactivity, the differences between cytokine levels in symptomatic and non-symptomatic children were generally similar to the differences observed between skin prick test positive and negative children (data not shown). Thus, children with, compared with those without, atopic symptoms had higher levels of birch-induced IL-4 and birch- and cat-induced IL-5. For the cytokines that were less strongly associated with skin prick test reactivity, i.e. IL-9 and IL-13, the differences mostly disappeared, possibly due to the fact that children were included in the symptomatic group regardless of whether they were birch- or cat-sensitized. Two interesting exceptions were found, however. Asthmatic children produced higher IL-9 levels than healthy children after cat stimulation (Fig. 1). This was not observed for children with atopic rhinoconjunctivitis (data not shown). Children with atopic symptoms tended to have lower levels of birch- and cat-induced IL-10. This was particularly clear for children with airway symptoms (Fig. 2). Furthermore, high birch-induced (Mann-Whitney U -test, $P < 0.05$) and cat-induced (Mann-Whitney U -test, $P < 0.01$) IL-4 : IL-10 ratios were associated with airway symptoms. Similar findings were seen for a high birch-induced (Mann-Whitney U -test, $P < 0.01$) and cat-induced (Mann-Whitney U -test, $P < 0.01$) IL-5 : IL-10 ratio, as well as for a high cat-induced IL-9 : IL-10 ratio (Mann-Whitney U -test, $P < 0.01$).

Discussion

Th2 cytokine secretion from PBMC was commonly detected in sensitized children after stimulation with the specific, in contrast to an unrelated, allergen. Thus, sensitized children produced higher levels of the IgE-inducing cytokines IL-4 [5] and IL-13 [6]. IL-5, which is primarily related to atopic symptoms due to its eosinophil activating properties [7], was also strongly connected with specific sensitization. For IL-9, which enhances IgE

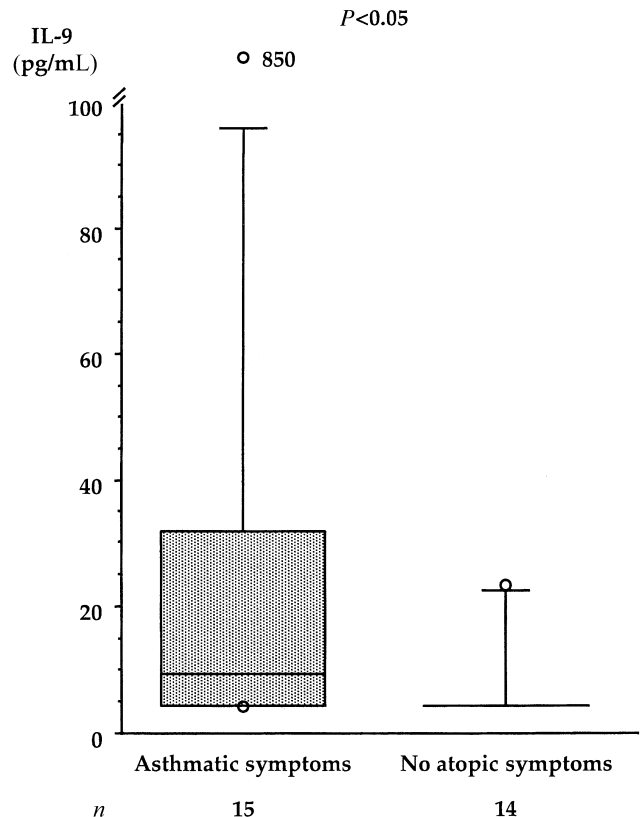
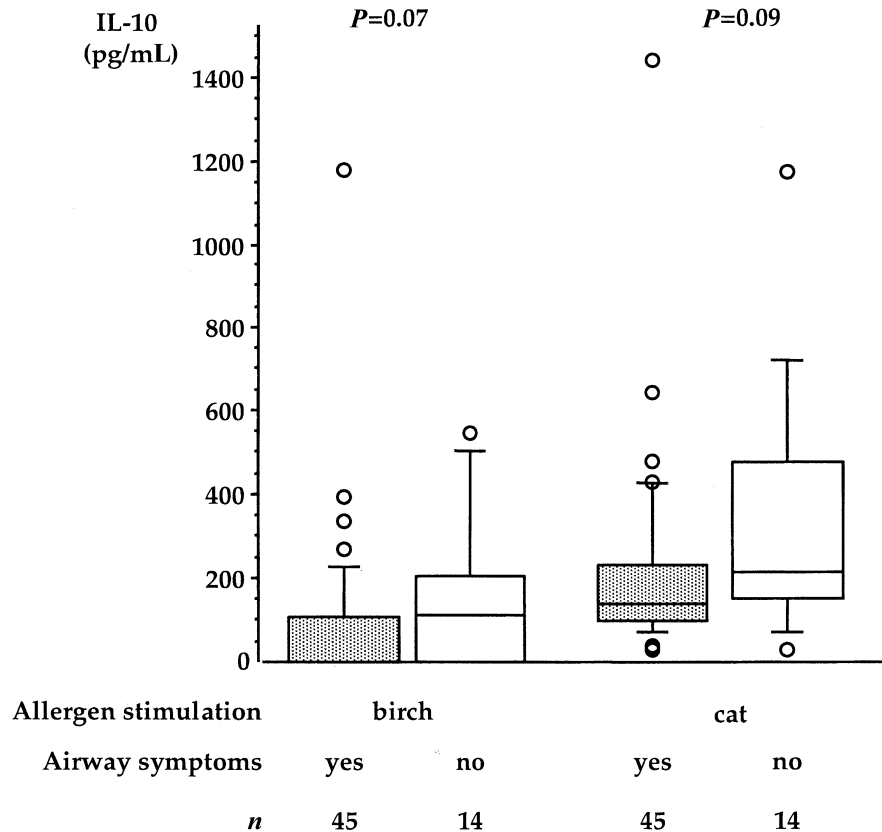


Fig. 1. Cat-induced IL-9 secretion in children with (shaded boxes) and without (open boxes) a history of asthmatic symptoms. 1×10^6 PBMC/mL were cultured for 144 h with cat allergen extract in AIM-V serum-free medium supplemented with $20 \mu\text{M}$ mercaptoethanol. IL-9 levels in the supernatants were measured with ELISA, and background responses (cultures with medium only) were subtracted. The 25th, 50th, 75th and 90th percentiles are indicated, as well as outliers.

production [9] and stimulates T-cell, mast cell and eosinophil proliferation [8,10,11], increased production was also observed in association with specific sensitization. Our results are in agreement with other studies on IL-4 [16–19], IL-5 [20–24] and IL-13 [19,21,23,24] secretion in allergen-stimulated PBMC of sensitized and non-sensitized individuals. Allergen-induced IL-9 secretion has not been investigated before, due to previous lack of appropriate antibodies, although we recently could show that increased IL-9 mRNA expression after allergen stimulation was associated with atopy [24]. However, the studies mentioned above did not investigate cytokine responses to allergens that the atopic individuals were not sensitized to. In another study [18], PBMC from birch-sensitized patients secreted IL-4 after stimulation with birch, but not cat, extract, supporting our findings. The only other studied cytokine was IFN- γ .

Cat-induced production of IFN- γ , which inhibits IgE

Fig. 2. Birch- and cat-induced IL-10 secretion in children with (shaded boxes) and without (open boxes) a history of airway symptoms. 1×10^6 PBMC/mL were cultured for 24 h with birch or cat allergen extract in AIM-V serum-free medium supplemented with 20 μ M mercaptoethanol. IL-10 levels in the supernatants were measured with ELISA, and background responses (cultures with medium only) were subtracted. The 10th, 25th, 50th, 75th and 90th percentiles are indicated, as well as outliers.



synthesis [5], tended to be low in sensitized children regardless of skin prick test reactivity, e.g. both in birch- and cat-sensitized children. A low IFN- γ production in atopic individuals has been observed also after stimulation with antigens to which no skin prick test reactivity is observed, e.g. purified protein derivative of *Mycobacterium tuberculosis* [30]. In our study, the cat-induced, as compared with birch-induced, cytokine secretion was less distinctly associated with cat sensitization, and with the skin prick test size and the circulating IgE antibody levels. This may be due to the more varying exposure to cats, as compared with birch. All the blood samples were drawn out of the birch pollen season.

Current and cumulative atopic symptoms were associated with high allergen-induced IL-4 and IL-5 and tended to be associated with low IL-10 levels. In addition, asthmatic children, but not children with atopic rhinoconjunctivitis, produced higher IL-9 levels than healthy children after cat stimulation. Interestingly, transgenic mice overexpressing IL-9 show bronchial hyper-responsiveness [31], and the IL9 [32] and IL9R [33] genes are linked to human asthma. Furthermore, increased expression of IL-9 was recently found in bronchial biopsies from asthmatic patients, as compared with atopic or healthy control subjects [34]. A high birch- and cat-induced

IL-4 : IL-10, IL-5 : IL-10 and IL-9 : IL-10 ratio was associated with airway symptoms. IL-10 is an anti-inflammatory cytokine [12] that inhibits both Th1 and Th2 cells and IgE synthesis [13], as well as eosinophil survival [12]. In agreement with our results, low levels of lipopolysaccharide [35] and viral-induced [36] IL-10 production have been shown in allergic, as compared with non-allergic, individuals.

In conclusion, the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 were all commonly detected in PBMC from sensitized children after stimulation with the specific, in contrast to an unrelated, allergen. Atopic symptoms were associated with increased levels of IL-4 and IL-5 and tended to be associated with low levels of IL-10, and asthma was associated with high cat-induced IL-9 levels.

Acknowledgements

We thank Prof Bengt Björkstén, Department of Health and Environment, Division of Paediatrics, Linköping University Hospital, Sweden, and Dr Claudia Macaubas, TVW Telethon Institute for Child Health Research, Perth, Western Australia, for helpful discussion, and Ulrika Bengtsson, Jenny Fredriksson, Anne-Marie Fornander, Kerstin Hagersten, Lena Lindell and Ing-Marie Sandberg,

Department of Health and Environment, Division of Paediatrics, Linköping University Hospital, Sweden, for excellent technical assistance. Dr Lennart Bråbäck, Mid Sweden Research and Development Centre, Sundsvall Hospital, Prof Bengt Björkstén and Prof Max Kjellman, Division of Paediatrics, Linköping University Hospital, are acknowledged for organizing the Linköping part of the ISAAC phase II study. The Swedish Medical Research Council (grant #7510), The Swedish Foundation for Health and Care Sciences and Allergy Research, The National Heart and Lung Association and Glaxo-Wellcome (to Prof. Bengt Björkstén), The National Swedish Association against Allergic Diseases, The Samariten Foundation, Linköping University, The County Council of Östergötland and the First of May Flower Annual Campaign for Children's Health are acknowledged for financial support.

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