

Immune responses to birch in young children during their first 7 years of life

M. F. Böttcher, M. C. Jenmalm and B. Björkstén*

Department of Molecular and Clinical Medicine, Division of Pediatrics, and Clinical Research Centre, Faculty of Health Sciences, Linköping University, Linköping and *Centre for Allergy Research and Institute of Environmental Medicine, Karolinska Institute, Karolinska, Sweden

Summary

Background The character of immune responses to allergens during the first years of life may decide whether the individual will become tolerant or develop allergy later in life.

Objective To study the development of immune responses to the seasonal inhalant allergen birch over the first 7 years of life.

Methods Blood samples were obtained from 21 children who were followed prospectively from the second to the seventh pollen season of life. Birch-induced cytokine production and IgG subclass antibodies to rBet v 1 were analysed with ELISA, mRNA expression with real time PCR, IgE antibodies to birch with Magic Lite™ and birch-induced mononuclear cell proliferation with ³H-thymidine incorporation.

Results Birch-induced IFN- γ and IL-10 production increased with age, both in atopic and non-atopic children, while birch-induced IL-13 production decreased. The two children who were sensitized and developed clinical allergy to birch showed persistent IL-4 and IL-5 production and IL-9 mRNA expression, as well as Th2-associated IgG₄ responses. Transient Th2-like responses were observed among the other children. Proliferative responses and IgG₁ antibodies were seen in all children.

Conclusions Immune responses to birch can be demonstrated in all children, during the first 7 years of life, regardless of atopic status. A transient early Th2-like response is down-regulated after the fourth pollen season, except in children who develop clinical allergy to the particular allergen.

Keywords atopy, birch, childhood, IgG subclass antibodies, T cell responses

Submitted 19 November 2001; revised 4 March 2002; accepted 8 April 2002

Introduction

Allergic diseases are believed to be due to Th2-like immunity to allergens in affected tissue, and immune responses to allergens are characterized by a cross-regulation between Th1 and Th2 cells [1]. Th2 cells produce IgE synthesis inducing IL-4 [2], eosinophilia promoting IL-5 and IL-9 [3, 4], while IFN- γ from Th1 cells down-regulates IgE synthesis [2]. Interleukin-9 also enhances IgE production [5] and stimulates T cell and mast cell growth [6, 7]. Interleukin-10 is an anti-inflammatory cytokine, which inhibits both Th1 and Th2 cells and IgE production [8], as well as shortens eosinophil survival [9]. Less is known about Th1/Th2 development in atopic children.

Prospective studies have shown that IgE antibodies to inhalant allergens are commonly detected both in atopic and non-atopic infants during the first years of life [10]. In non-atopic children, the IgE responses to inhalant allergens are eventually down-regulated, whereas they reach higher levels and tend to persist in atopic children. Furthermore, IgG responses, primarily of the IgG₁ subclass to inhalant allergens, are also common in infancy, while high levels of IgG₄ subclass antibodies to

allergens are associated with sensitization and allergic symptoms [11]. The differences in IgG₄ antibody levels to birch in atopic and non-atopic children increase from 18 months to 8 years, largely due to a down-regulation of IgG₄ antibody responses in the non-atopic children [11].

It has been demonstrated that birch-induced IFN- γ is associated with IgG₁ antibody production, while IL-4 production relates to IgG₄ antibody subclass responses to Bet v1 [12]. The balance between Th1 and Th2 memory cells, which has been suggested to be set during early life by a process of allergen-driven Th1/Th2 selection, might be reflected by these see-sawing IgG subclass and IgE responses to different allergens. Prescott and colleagues, in a prospective 2-year follow-up study, demonstrated a persistent proliferative response to house dust mite [13]. The cytokine responses of cord blood mononuclear cells (CBMC) showed a Th2-skewed profile in all children, but the Th2 responses were lower in individuals who later developed atopic diseases than in those who did not. Non-atopic individuals showed a gradual decline in detectable IL-4 mRNA and IL-13 protein responses to house dust mite, while atopic individuals had persistent IL-4 mRNA and IL-13 protein responses. There was a similar increase in house dust mite-induced IL-6 and IL-10 production in the atopic and non-atopic groups. The IFN- γ mRNA responses were rarely detectable from CBMC but were easily detectable after 6 months of age in the non-atopic group but not in the atopic group.

Correspondence: Malin F. Böttcher, Department of Molecular and Clinical Medicine, Division of Pediatrics, Faculty of Health Sciences, Linköping University, Linköping, Sweden. E-mail: malfa@kfc.liu.se

So far, only allergen-specific T cell responses to house dust mite have been prospectively studied and then only up to 2 years of age [13]. The aim of this study was therefore to investigate the Th1/Th2 cell selection process to the seasonal inhalant allergen birch over several years. Birch is in some respect a model allergen, as the season of exposure is short, mostly less than 6 weeks.

Methods

Study group

Twenty-four families with their newborn babies were randomly invited at the maternity clinic at the University Hospital of Linköping, to participate in a prospective study regarding the development of immune responses to birch early in life. All children were born at term from July 1991 to March 1992. Twenty-one families agreed to participate and their children were followed annually during their first 7 years of life. Clinical examinations, venous blood sampling and skin prick tests were performed during or shortly after each birch pollen season, i.e. between the middle of May and the middle of June, 1993 to 1998.

Atopic dermatitis was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as four or more episodes of bronchial obstruction, at least once verified by a physician. Allergic rhinoconjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. Skin prick tests were done in duplicate on the volar aspects of the forearms with thawed egg white, fresh skimmed cow's milk (lipid concentration 0.5%) and birch, timothy, cat, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/mL) was used as a positive control, and albumin diluent was included as a negative control. The test was regarded as positive if the mean diameter was > 3 mm.

The atopic status of the parents was established by a typical clinical history (i.e. allergic rhinoconjunctivitis, allergic asthma or flexural itchy dermatitis).

Separation of PBMC

Blood samples were collected in tubes with preservative-free heparin (Beckton Dickinson, Stockholm, Sweden). Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll Paque density gradient (Pharmacia Biotech, Sollentuna, Sweden), and washed three times in RPMI-1640 (Life Technologies AB, Täby, Sweden) containing 2% fetal calf serum (Life Technologies AB). They were then cryopreserved by standard methodology in 10% DMSO (Sigma-Aldrich, Stockholm, Sweden), 50% fetal calf serum and 40% RPMI-1640.

Proliferation

After thawing and washing, triplicate cultures of 2×10^5 PBMC in 200 µL of AIM-V serum-free medium (Life Technologies) supplemented with 20 µM mercaptoethanol (Sigma-Aldrich) were cultured at 37 °C with 5% CO₂ with medium alone or 10 000 Standard Quality Units (SQU)/mL Aquagen birch extract (ALK). Phytohemagglutinin (PHA) (Sigma-Aldrich),

5 µg/mL, served as positive control. After 5 days, the cells were pulsed with ³H-thymidine (Amersham, Stockholm, Sweden) and harvested 24 h later for detection of ³H-DNA synthesis by liquid scintillation counting. Proliferative responses were expressed as a proliferative index, i.e. counts per minute (cpm), divided by background responses (cultures with medium only). A proliferative index > 2 was regarded as positive.

Cytokine production

After thawing, the cells were resuspended at 1×10^6 viable cells/mL AIM-V serum-free medium (Life Technologies) supplemented with 20 µM mercaptoethanol (Sigma-Aldrich). One mL aliquots (1×10^6 cells) were cultured at 37 °C with 5% CO₂ with medium alone, 10 000 SQU/mL Aquagen birch extract (ALK) or 2 µg/mL PHA (Sigma-Aldrich). To enable measurement of IL-4, separate cultures were performed with monoclonal antibodies to human IL-4 receptor, 2 µg/mL (clone 25463.111, R & D Systems, Abingdon, UK). After 96 h, the samples were centrifuged at 2000 g for 5 min, and the supernatants were aspirated and stored at -20 °C. The cells were lysed with 300 µL RLT Lysis Buffer (Qiagen, Hilden, Germany) and stored at -70 °C.

ELISA for detection of IL-4, IL-5, IL-10, IL-13 and IFN-γ

The levels of IL-4, IL-10, IL-13 and IFN-γ were determined by commercially available ELISA kits (CLB Pelikine Compact™, Research Diagnostics Inc., Flanders, NJ, USA), as described by the manufacturer. The levels of IL-5 were determined using an in-house ELISA as described elsewhere [14]. Briefly, Costar 3690 plates (Life Technologies AB) were coated with 50 µL/well of 0.25 µg/mL monoclonal rat anti-human IL-5 (clone TRFK5, Pharmingen, Becton-Dickinson). Free plastic spaces were blocked with 100 µL/well of a blocking solution (CLB). Recombinant human IL-5 (Pharmingen, Becton-Dickinson) diluted twofold (range 3.1 to 200 pg/mL) in AIM-V medium (Life Technologies) was used as a standard. Standards, samples, or for controls, AIM-V medium (Life Technologies) only, 50 µL/well, were added to the plates. For detection, 50 µL/well of a 1.0-µg/mL monoclonal biotinylated rat anti-human IL-5 antibody was used followed by 50 µL/well of streptavidin conjugated polyhorseradish peroxidase (CLB) diluted 1/10 000 in dilution buffer (CLB). Tetramethylbenzidine (Sigma-Aldrich), 50 µL/well, was used as substrate and addition of 50 µL/well of 1.8 M sulphuric acid stopped the reaction. The sensitivity limits for quantitative determinations were 6.25 pg/mL for IL-4 and IL-5, 4.7 pg/mL for IL-10, 62.5 pg/mL for IL-13, and 25 pg/mL for IFN-γ.

RNA extraction and reverse transcription PCR of mRNA

Total ribonucleic acid (RNA) was isolated according to the RNeasy® 96 Protocol (Qiagen). In brief, the lysed cells were mixed with ethanol and applied to a RNeasy® 96-well plate. When the membrane had dried, the RNA was eluted in RNase-free water.

RNA was converted to complementary DNA using an oligo dT-primer, random decamer primer and M-MLV reverse transcriptase for 1 h at 42 °C followed by 10 min at 75 °C according to the Reverse-it protocol of the supplier (Abgene Advanced Biotechnologies Ltd, Epsom, Surrey, UK). The samples were run in a GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Quantification of IL-4 and IL-9 mRNA with quantitative real time PCR

To a 96-well reaction plate (MicroAmp Optical, Perkin Elmer Applied Biosystems), 24 µL of Reaction Mix (TaqMan Universal PCR Master Mix, forward and reverse primers and probes, for concentrations see Table 1) was added to each well followed by 1 µL samples, standards (cDNA from PHA-stimulated mononuclear cells) or water as negative control. Oligonucleotide primers (MedProbe, Oslo, Norway) were designed for human IL-4 and IL-9 sequence using Primer Express (Perkin Elmer) (for sequences see Table 1). The TaqMan probe with the fluorophore FAM (6-carboxy-fluorescein) at the 5'-end and the quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3'-end were bought from MedProbe. Both primers and probe were purified with HPLC.

The wells were covered with optical caps (Perkin Elmer Applied Biosystems), and the plate was centrifuged for some seconds at high speed and thereafter placed into the thermocycler (ABI PrismTM 7700 Sequence Detector, Perkin Elmer Applied Biosystems). The thermal cycle conditions were 50 °C for 2 min followed by 95 °C for 10 min and then 40 cycles were run for 15 s at 95 °C and for 1 min at 60 °C, as recommended by Perkin Elmer for the SDS 7700 system.

Samples were run with primers and probes for IL-4 or IL-9 and for rRNA in two different wells. The results from the rRNA reactions were used as internal controls, i.e. the amount of IL-4 and IL-9 mRNA was calculated relative to the amount of rRNA present in each sample.

Antibody analyses

Birch-specific IgE antibodies were analysed with the high sensitivity protocol of Magic LiteTM according to the recommendations of the manufacturer (ALK).

The determination of IgG subclass antibodies to the recombinant major birch allergen Bet v 1 was performed as described in detail elsewhere [15]. Briefly, microtitre plates were coated with 1 µg/mL rBet v 1 (kindly provided by Professor D. Kraft, Institute of General and Experimental Pathology, AKH, University of Vienna, Vienna, Austria) in PBS. Human serum or buffer only was added to duplicate wells, followed by monoclonal murine antibodies to human IgG₁ (clone HP6069) and IgG₄ (clone HP6011) (Bio Zac AB, Järfälla, Sweden). Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma-Aldrich, Stockholm, Sweden) was then added, and p-nitrophenyl

phosphate (Sigma-Aldrich) was used as substrate. The reference serum, which was calibrated according to a semiquantitative method as described earlier [15], was from a patient undergoing birch immunotherapy. Values were expressed as arbitrary units (AU) deduced from the ODs of the reference serum curve, after subtracting the blanks.

Samples with undetectable levels of IgG₄ antibodies with the conventional ELISA (described above), were analysed with a more sensitive ELISA [15]. For the substrate reaction an amplified enzyme system, AMPAK (Dakopatts AB, Älvsjö, Sweden), was used.

Pollen counts

Total levels of birch pollen for each year were obtained from the Palynological Laboratory, Swedish Museum of Natural History. Birch pollen was counted in Norrköping, a city 40 km north of Linköping.

Statistics

As the concentrations of cytokines and antibodies were not normally distributed, comparisons between paired groups were analysed using Wilcoxon Signed rank test and trends over time were analysed using ANOVA for repeated measurements. A probability level of < 5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (Abacus Concepts Inc., Berkely, CA, USA).

Ethics

The Regional Ethics Committee for Human Research at Linköping University approved the study.

Results

Sixteen of the children had at least one parent with a history of atopic symptoms (Table 2). Eight children developed allergic symptoms during the 6-year follow-up period and are referred to as atopic. Seven of them were SPT positive (Table 2). Two children developed symptoms of allergic rhinoconjunctivitis during the birch pollen season and persistent positive skin prick tests to birch pollen extract from the age of 4 years (Table 2). They were the only children who had measurable IgE to birch (Fig. 1). These two children are referred to as birch allergic.

Table 1. Sequences of primers and probes

Target	Primer/probe	Sequence (5' to 3')	Conc (nM)	Position	Amplicon length
rRNA	FP	CGGCTACCACATCCAAGGAA	200	*1	*1
	Probe	TGCTGGCACCAGACTTGCCCTC	50	*1	*1
	RP	GCTGGAATTACCGCGGCT	100	*1	*1
IL-4	FP	ACAGCCTCACAGAGCAGAA G ACT*2	300	178–201	72
	Probe	TGTGCACCGAGTTGACCGTAACAGACA	200	203–229	
	RP	TG TCTT GGAGGCAGCAAAGA	300	250–229	
IL-9	FP	GATCCAGCTTCCAAGTGCCA	300	128–148	76
	Probe	AAACAGAGACA AACTGGTCA CTTAGCACTGCA	100/175		
	RP	TGCAGTTGT C AGAGGGAATGC	300	204–181	

FP = forward primer. RP = reverse primer. *1 = recommended by PE Biosystems. *2 = Letters in bold indicate an exon-exon junction.

Proliferative responses to birch were detected in most atopic and non-atopic children, already during their second pollen season in 1993, and the magnitude of the responses was similar over the following years (Fig. 2).

Similarly, birch-induced IFN- γ production was detected in most of the children during the second pollen season (Fig. 3a). The levels were largely similar up to the age of 5 years and then increased. The birch-induced IFN- γ levels increased statistically significantly from 1993 to 1997 and 1998 ($P = 0.01$ and 0.03 , respectively, Wilcoxon signed rank test). Birch-induced IL-10 production increased during the first years of life (Fig. 3b). The IL-5 and IL-13 production (Figs 3c and d), on the other hand,

tended to decrease with age. This was statistically significant from 1995 to 1998 ($P = 0.02$) and from 1996 to 1998 ($P = 0.03$) for IL-5 and from 1993 to 1995, 1996 and 1998 ($P = 0.02$, 0.04 and 0.048 , respectively, Wilcoxon signed rank test) for IL-13. Two children, however, showed persistent birch-induced IL-5 responses (Fig. 3c). Both of them developed allergic symptoms and had positive SPT to birch from the age of 4 years. One of the two birch-allergic children also showed persistent birch-induced IL-13 production (Fig. 3d). Birch-induced IL-4 protein production and mRNA expression, as well as IL-9 mRNA expression, was only demonstrated in some children. The age-related decrease of IL-9 mRNA expression reached statistical significance from 1994 to 1997 and 1998 ($P = 0.049$ and 0.046 , respectively), from 1995 to 1997 and 1998 ($P = 0.004$ and 0.01 , respectively) and from 1997 to 1998 ($P = 0.02$, Wilcoxon signed rank test). Only the two birch-allergic children showed persistent IL-4 protein production (Fig. 3e), IL-4 mRNA (data not shown) and IL-9 mRNA (Fig. 3f) expression.

IgG₁ antibodies to rBet v1 were commonly detected both in atopic and non-atopic infants from the second pollen season

Table 2. Data regarding development of atopic dermatitis (AD), allergic rhinoconjunctivitis (ARC) and asthma (AB) and skin prick test (SPT) positivity in the individual children and the symptoms reported in the mother (M) and father (F)

Patient	Symptoms	SPT positivity	Atopic heredity
1			F (AD)
2		Egg –93, –94	M (ARC)
3	AD	Egg –93–97	M (ARC)
5			M (ARC)
6		Birch –94, –95	F (ARC)
7			
8	AD, ARC, AB	Egg –93–97, Birch, Cat, Timothy –95–98	M (ARC)
11		Timothy –96, –98	
12	AD	Milk, Cat –93, –95	M, F (ARC)
13			M (AD, ARC), F (ARC)
14	AD, AB		M (AD), F (ARC)
15	AD		M, F (ARC)
16	AD		M (AB)
17		Birch –98	M (ARC)
18			
19		Birch –94	M (ARC)
20			
21		Birch –94	
22	AD	Birch –95	F (ARC)
23	ARC	Birch, Cat –95–98, Timothy –97–98	M (ARC)
24			M, F (ARC)

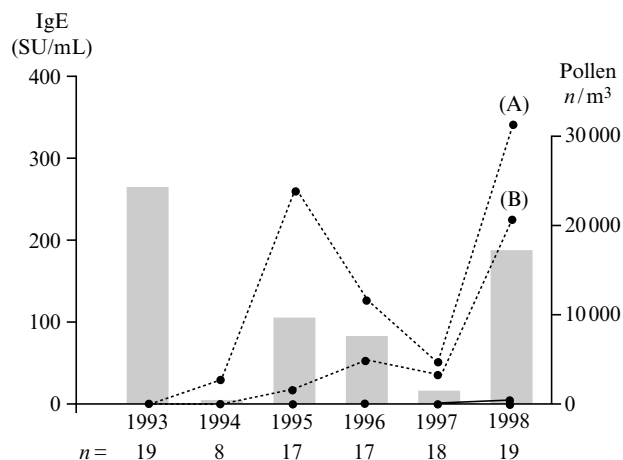
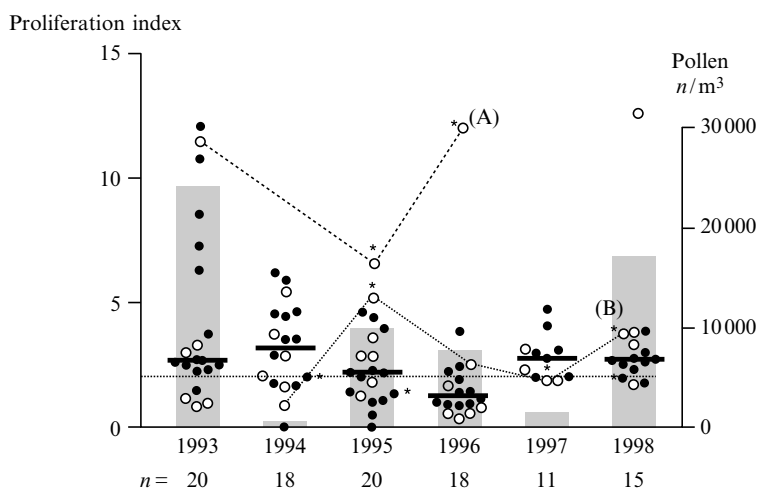


Fig. 1. IgE antibodies to birch in sera and birch pollen counts (grey bars) over the first 7 years of life. The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. Blood samples were collected during each pollen season of 1993 to 1998. The number of analysed samples is indicated (n).

Fig. 2. Birch-induced proliferation from peripheral blood mononuclear cells and birch pollen counts (grey bars) over the first 7 years of life in eight atopic (○) and 13 non-atopic children (●). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen season of 1993 to 1998. The number of analysed samples is indicated (n), as well as the median.



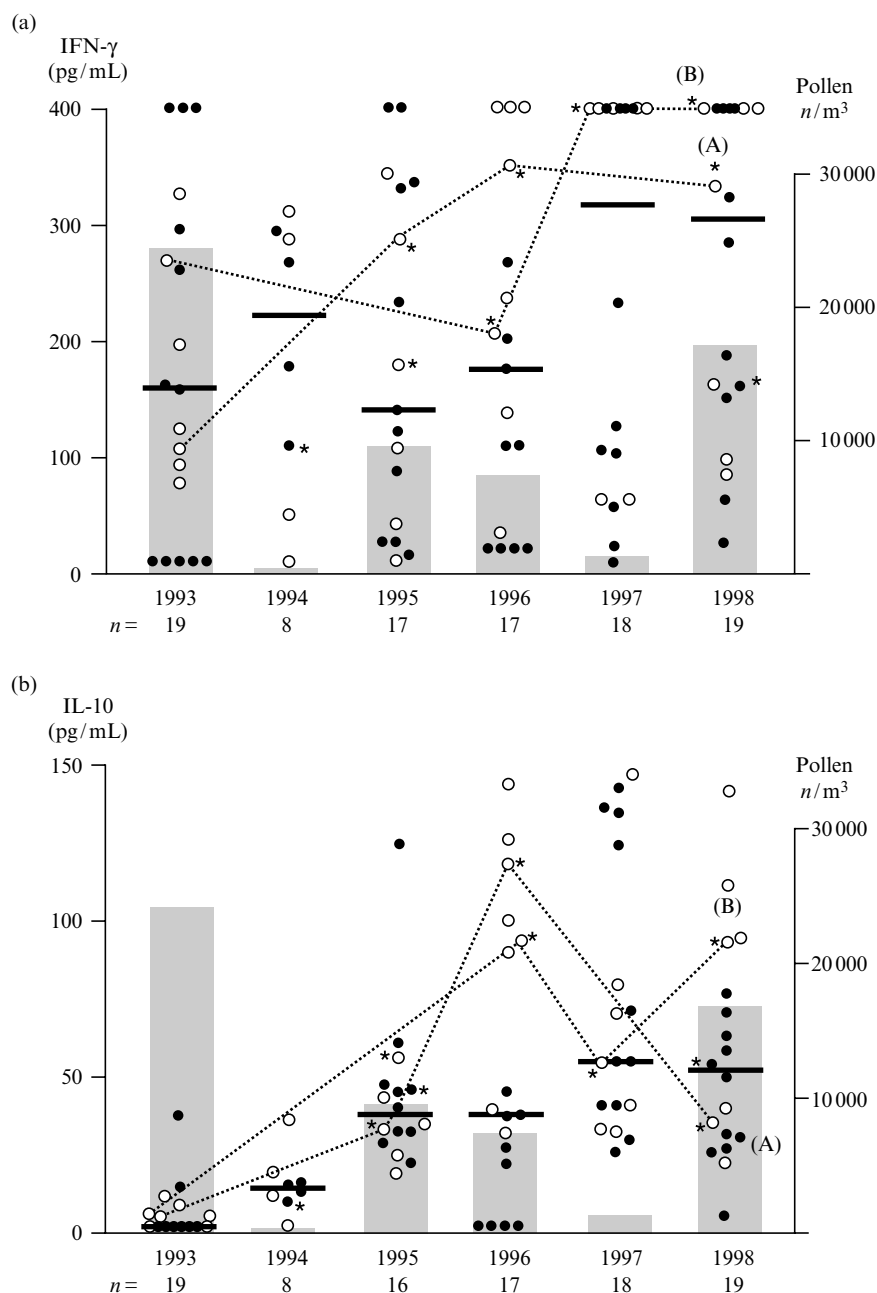


Fig. 3. (contd.)

and the levels increased with age. The four children with the highest levels of IgG₁ antibodies all had allergic symptoms and two of them were allergic to birch (Fig. 4a). In contrast, IgG₄ antibodies to rBet v1 were only sporadically demonstrated, and only the two birch-allergic children showed high and persistent IgG₄ levels (Fig. 4b).

The levels of birch-specific IgE antibodies in the two birch-allergic children seemed to be related to the counts of birch pollen from the third pollen season in 1994 (Fig. 1). There was, however, no association between pollen counts and birch-induced proliferation or cytokine production or the levels of IgG antibodies to birch (Figs 2–4).

Discussion

In this prospective study we show that immune responses to birch, during the first 7 years of life, are both Th1- and Th2-like. Thus, birch-induced IFN- γ (Th1) and IL-4, IL-5 and IL-13 protein and IL-4 and IL-9 mRNA (Th2), as well as Th2-associated IgG₄ production was documented, both in atopic and non-atopic children. The two children who developed allergy to birch were the only ones to have persistent IL-4, IL-5, IL-9, IgE and IgG₄ responses to birch, indicating a failure in down-regulating the neonatal Th2-like immunity. Thus, our study confirms and extends previous studies showing that

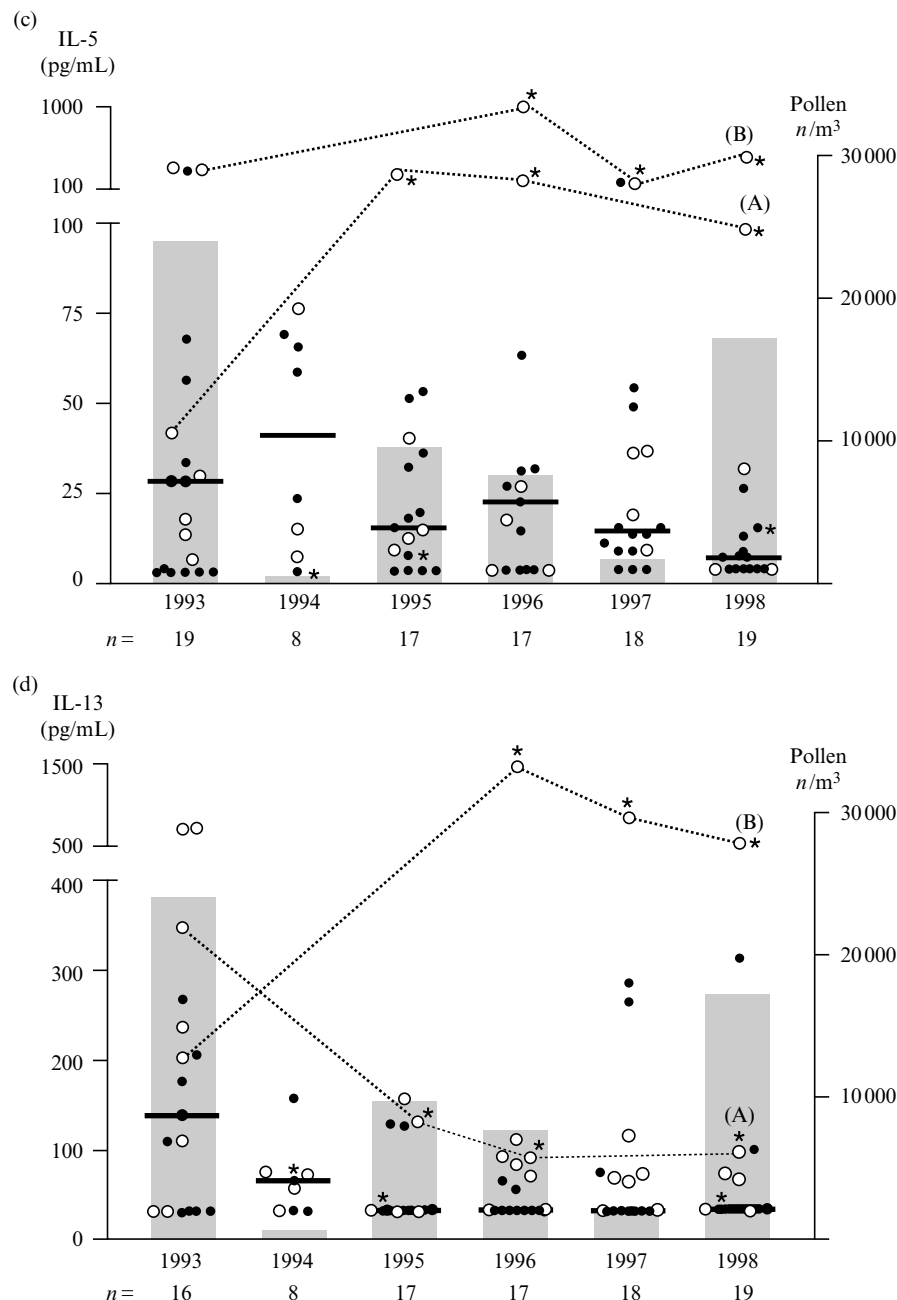


Fig. 3. (contd.)

postnatal Th2-like responses to allergens are common, independent of the atopic status later in life, and that these responses are later down-regulated, except in children who develop clinical symptoms to the allergen [10, 11, 16].

The failure of immune deviation to allergens among atopic individuals may be associated with poor neonatal capacity to produce IFN- γ [17, 18]. The IFN- γ producing capacity is much lower in neonatal T cells than in adults, and production increases with age [19]. The low IFN- γ production during infancy might be due to postnatal persistence of control mechanisms limiting intrauterine induction of potentially harmful Th1-like immunity [20]. In this prospective study, we show a similar birch-induced IFN- γ production in atopic and non-atopic

children. This is in agreement with a recent prospective study where house dust mite-induced IFN- γ production was low both in atopic and non-atopic children during their first 18 months of life [13]. In that study, however, house dust mite-induced IFN- γ mRNA expression increased with age only in the non-atopic individuals.

Although allergen-specific Th1-like immunity dominates in healthy individuals, IFN- γ -associated delayed-type hypersensitivity is not observed. Factors counteracting Th1 reactions are likely to be present and the anti-inflammatory cytokine IL-10 may be one candidate [9]. House dust mite-specific IL-10 production from CBMC is up-regulated in non-atopic, as compared with atopic, individuals [13]. The age-dependent

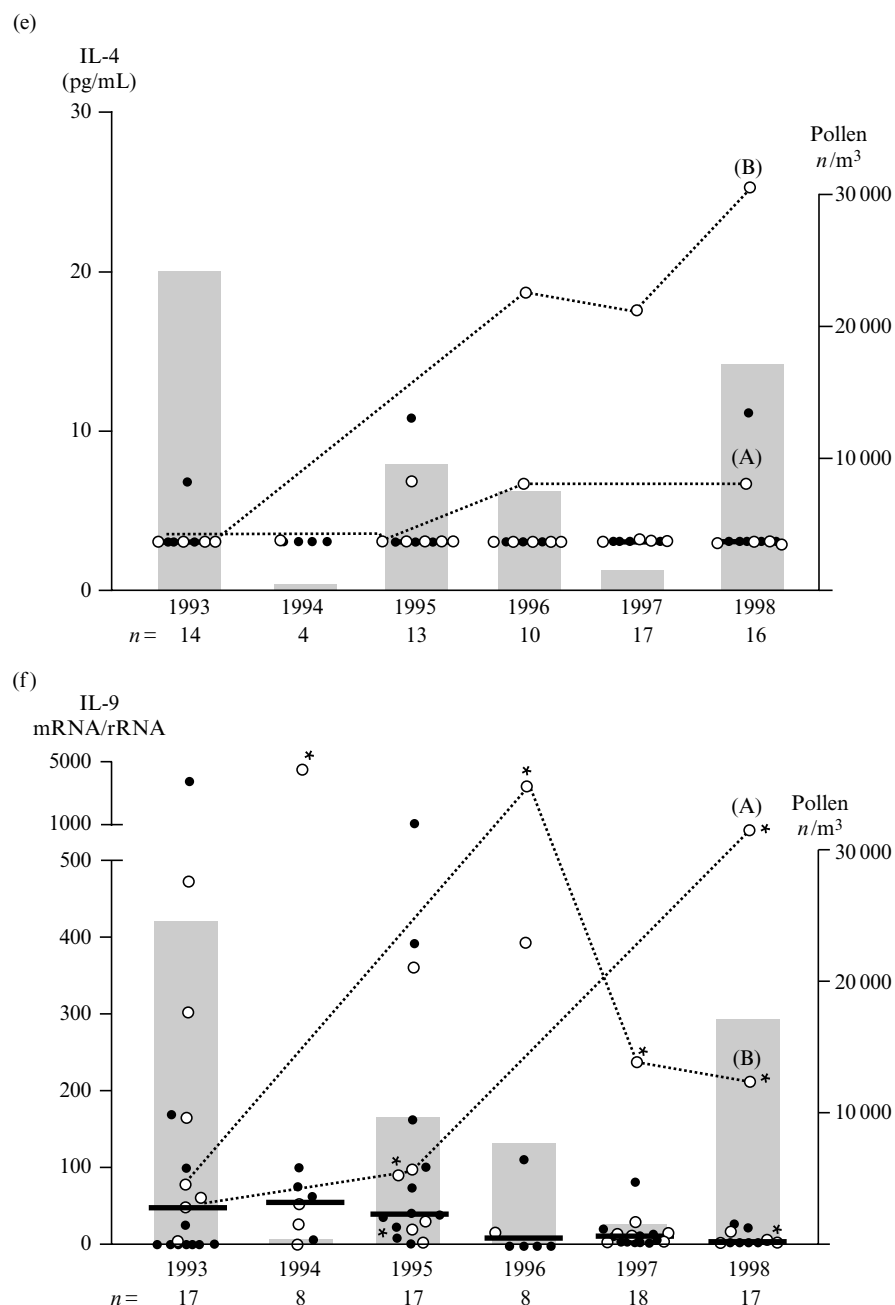


Fig. 3. Birch-induced IFN- γ (a), IL-10 (b), IL-5 (c), IL-13 (d), IL-4 (e) and IL-9 (f) responses from peripheral blood mononuclear cells and birch pollen counts (grey bars) over the first 7 years of life in eight atopic (○) and 13 non-atopic children (●). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen season of 1993 to 1998. The *P*-value for trends was < 0.05 for IL-10 (b). The number of analysed samples is indicated (*n*), as well as the median.

increase of IL-10 production was similar, however, in the two groups. Our results are consistent with that finding, as IL-10 production was detected in all children from the fourth pollen season.

The IL-4, IL-5 and IL-9 responses to birch declined with age, except in the two birch-allergic children. The allergen-induced production of those cytokines has not previously been prospectively studied at the protein level. Similarly, IL-13 responses declined with age except in one of the two birch-allergic children. Although IL-13 induces IgE switching independently of

IL-4 [21], it is not a typical Th2 cytokine, as both Th1 and Th2 clones produce it and it is not capable of inducing Th2 differentiation. No clear difference in IL-13 production was observed between atopic and non-atopic children in this study. In contrast, house dust mite-induced IL-13 production is down-regulated in non-atopic children, whereas the opposite was observed for atopic children, independently of what allergen the child was sensitized against [13]. There are, however, also differences in the methodology. In the study by Prescott et al. the cells were incubated with allergen for 24 h, in contrast to 96 h

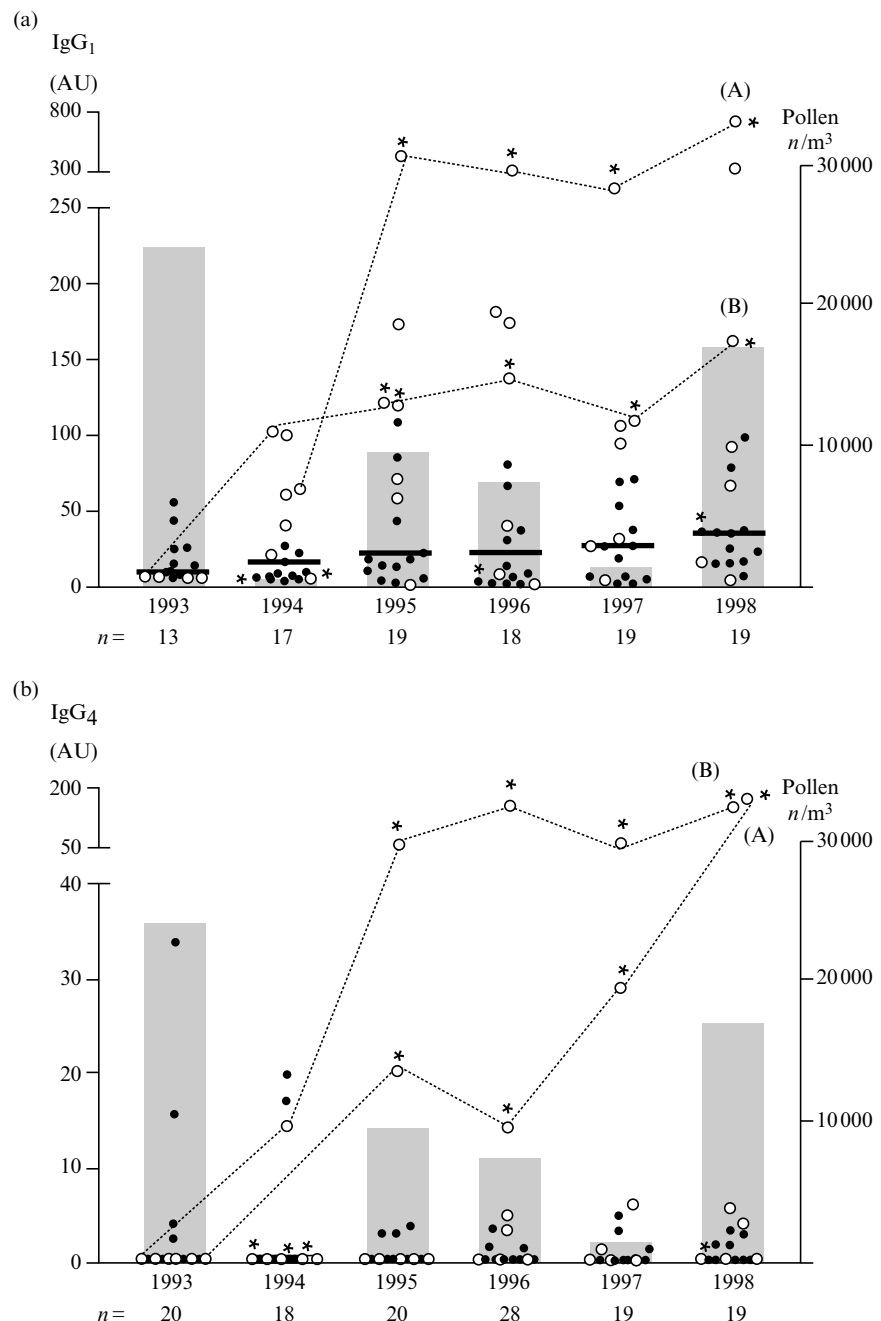


Fig. 4. Titres of IgG₁ (a) and IgG₄ (b) antibodies to rBet v 1 in sera and birch pollen counts (grey bars) over the first 7 years of life in eight atopic (○) and 13 non-atopic children (●). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen season of 1993 to 98. The *P*-value for trends was < 0.05 for IgG₁ (a). The number of analysed samples is indicated (*n*), as well as the median.

in our study. It has earlier been shown that allergen-induced IL-13, as well as IL-5 and IFN- γ production, increased over time and that the levels and number of individuals with detectable levels were significantly higher after 96 h, as compared with 24 h, of incubation [12]. Furthermore, the atopic status of the children in the study by Prescott et al. was based on a 2-year follow-up, whereas the children in our study were followed during their first 7 years of life.

The IgG responses to Bet v 1 were largely restricted to the IgG₁ subclass, confirming the findings in a previous cohort [15]. The IgG₁ responses were commonly seen, regardless of atopic symptoms and sensitization, but, as reported previously, they were higher in atopic children [15, 22]. Similar to findings in

another cohort [11], there was an even stronger relationship between Th2-associated IgG₄ antibodies and atopy. In that study, the differences in IgG₄ antibody levels between atopic and non-atopic children were greater at 8 years than at 18 months, which was largely due to down-regulation of IgG₄ antibody responses in non-atopic children. This is supported by the findings in the present study.

The period when allergen-driven Th1/Th2 selection occurs, the so-called 'window of sensitization', has been suggested to occur during a narrow period after birth [23]. We have with this study extended this hypothesis to suggest that this may last for around 3 to 4 years after birth, at least for a seasonal allergen with a short period of exposure every year.

In conclusion, immune responses to birch can be demonstrated in children regardless of atopic status. A transient early Th2-like response is down-regulated after the fourth pollen season, except in children who develop clinical allergy to the particular allergen.

Acknowledgements

We thank Ulrika Bengtsson, Anne-Marie Fornander, Kerstin Hagersten, Lena Lindell and Kristina Warstedt for excellent technical assistance.

The Swedish Medical Research Council (grant no. 7510), the Swedish Foundation for Health Care Sciences and Allergy Research, the National Swedish Association against Allergic Diseases, the National Heart and Lung Association and the First May Flower Annual Campaign for Children's Health and Glaxo-Wellcome are acknowledged for financial support.

References

- Holt PG, McMenamin C. Defence against allergic sensitization in the healthy lung: the role of inhalation tolerance. *Clin Exp Allergy* 1989; 19:255–62.
- Pène JF, Rousset F, Brière F et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E2. *Proc Natl Acad Sci USA* 1988; 85:6880.
- Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000; 105:651–63.
- Levitt RC, McLane MP, MacDonald D et al. IL-9 pathway in asthma: new therapeutic targets for allergic inflammatory disorders. *J Allergy Clin Immunol* 1999; 103:S485–91.
- Dugas B, Renauld JC, 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.
- Renauld JC, Louahed J, Vink A, van Snick J, Uyttenhove C. Interleukin-9. *Adv Immunol* 1993; 54:79–97.
- Richard M, Grecis RK, Humphreys NE, Renauld JC, van Snick J. Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in *Trichuris muris*-infected mice. *Proc Natl Acad Sci USA* 2000; 97:767–72.
- Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat J-F, de Vries JE. IL-10 and viral IL-10 prevent induced IgE synthesis by inhibiting the accessory cell function of monocytes. *J Immunol* 1993; 151:1280–9.
- Koulis A, Robinson DS. The anti-inflammatory effects of interleukin-10 in allergic disease. *Clin Exp Allergy* 2000; 30:747–50.
- Hattevig G, Kjellman B, Björkstén B. Appearance of IgE antibodies to ingested and inhaled allergens during the first 12 years of life in atopic and non-atopic children. *Ped Allergy Immunol* 1993; 4:182–6.
- Jenmalm MC, Björkstén B. Development of immunoglobulin G subclass antibodies to ovalbumin, birch and cat during the first eight years of life in atopic and non-atopic children. *Ped Allergy Immunol* 1999; 10:112–21.
- Jenmalm MC, Björkstén B, Macaubas C, Holt BJ, Smallacombe TB, Holt PG. Allergen induced cytokine secretion in relation to atopic symptoms and IgE and IgG subclass antibody responses. *Ped Allergy Immunol* 1999; 10:168–77.
- Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; 353:196–200.
- Böttcher MF, Jenmalm MC, Garofalo RP, Björkstén B. Cytokines in breast milk from allergic and non-allergic mothers. *Ped Res* 2000; 47:157–62.
- Jenmalm MC, Holt PG, Björkstén B. Maternal influence on IgG subclass antibodies to Bet v 1 during the first 18 months of life as detected with a sensitive ELISA. *International Arch Allergy Immunol* 1997; 114:175–84.
- Prescott SL, Macaubas C, Holt BJ et al. Environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998; 160:4730–7.
- Rinas U, Horneff G, Wahn V. Interferon- γ production by cord-blood mononuclear cells is reduced in newborns with a family history of atopic disease and is independent from cord blood IgE levels. *Ped Allergy Immunol* 1993; 4:60–4.
- Tang MLK, Kemp AS, Thorburn J, Hill DJ. Reduced interferon- γ secretion in neonates and subsequent atopy. *Lancet* 1994; 344:983–5.
- Holt PG. Postnatal maturation of immune competence during infancy and childhood. *Ped Allergy Immunol* 1995; 6:59–70.
- Wegmann TG, Lin H, Gulbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; 14:353–6.
- Punnonen J, Aversa G, Cocks BG et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993; 90:3730–4.
- Ruiz RGG, Kemeny DM, Mariani F, Price JF. Early immune response to *Dermatophagoides pteronyssinus* and atopic predisposition. *Arch Dis Childhood* 1992; 67:1023–6.
- Holt PG. Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. *Ped Allergy Immunol* 1995; 6:1–10.