

Development of allergen-specific T-cell memory in atopic and normal children

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Summary

Background In the past 20–30 years, there has been an increase in prevalence of allergic respiratory diseases, particularly amongst children. This study is a prospective analysis of the postnatal maturation of T-helper cell (Th) responses to aeroallergens in atopic and non-atopic infants.

Methods We measured mononuclear-cell proliferative and cytokine responses to specific allergens and tetanus toxoid in blood samples from atopic and non-atopic infants every 6 months from birth to 2 years of age. Cytokine analyses of responses to housedust-mite allergen used ELISA and reverse-transcriptase PCR. We also measured responses to *Fel d 1* (cat allergen) and tetanus toxoid.

Findings Samples from 18 atopic and 13 non-atopic infants showed low-level Th2-skewed allergen-specific responses at birth, with little accompanying specific interferon- γ production. Neonatal Th2 responses were lower in the atopic group than in the non-atopic group; the differences were significant for interleukin-4 (mRNA: β -actin ratio 0.48 [SE 0.15] vs 0.15 [0.06], $p=0.049$), interleukin-6 (4750 [48] vs 1352 [51] pg/mL culture fluid, $p=0.003$), interleukin-10 (1162 [228] vs 485 [89], $p=0.015$), and interleukin-13 (7.1 [0.9] vs 0.9 [0.3], $p=0.008$). There was rapid suppression of Th2 responses during the first year of life in non-atopic children, but there was consolidation of responses in atopic children, associated with defective neonatal interferon- γ production.

Interpretation The continuation of fetal allergen-specific Th2 responses during infancy is a defining feature of the inductive phase of atopic disease, and is associated with decreased capacity for production of the Th1 cytokine interferon γ by atopic neonates. These findings provide a plausible mechanism for persistence of the fetal Th2 responses during early childhood in atopic individuals and subsequent expression of disease.

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See Commentary page ????

Introduction

In the past 20–30 years, there has been a progressive increase in the prevalence of allergic respiratory diseases, in particular atopic asthma,¹ with an associated increase in management costs. Primary prevention aims to identify at-risk individuals at an early stage and to begin prophylactic therapy to prevent development of chronic disorders. Targets for primary prevention are the underlying immune

responses that potentially trigger allergic reactions to inhaled antigens.^{2,3} Although only some atopic people develop chronic asthma, the substantial overlap between current skin-test reactivity to one or more inhalant allergens and active asthma in schoolchildren and in adults⁴ suggests that atopy is an important risk factor for expression of asthma. Interruption of the process of primary allergic sensitisation to inhalants might delay or prevent the onset of allergic diseases such as atopic asthma.

A detailed understanding of the T-cell immunology underlying the sensitisation process is required. Atopy in adults is associated with the long-term expression of allergen-specific immunity, characterised by production of T-helper 2 (Th2) cytokines such as interleukin-4 and interleukin-5, which promote IgE production and eosinophilia.^{5,6} By contrast, non-atopic people show mainly T-helper 1 (Th1) immunity characterised by production of interferon γ , which inhibits the growth of Th2 cells.

Research into the pathogenesis of atopic diseases is focused on how these patterns of allergen-specific T-cell memory develop, particularly in early childhood, when the immature immune system first comes into direct contact with inhalant allergens. Compartmentalisation of allergen-specific T-cell immunity into adult-equivalent Th1 patterns rather than Th2 patterns occurs in most children before the age of 5 years,⁷ which suggests that research should focus on the immediate postnatal period. In addition, the DNA genotyping of T-cell clones has proved the existence of fetally derived allergen-reactive T cells in cord blood, indicative of intrauterine T-cell priming.⁸ Moreover, these primed cells typically display a Th2-polarised pattern of cytokine production.⁸

We tested the hypothesis that individuals who develop atopic disorders in infancy and those who do not develop such disorders show opposing patterns of regulation of allergen-specific, in-utero-primed, Th2 responses. We undertook a prospective study of peripheral-blood mononuclear cells (PBMC) taken from children regularly from birth to 2 years. We analysed T-cell cytokine responses to a panel of allergens and sought clinical and laboratory markers of atopy.

Methods

Study groups

We compared allergen-specific T-cell responses during infancy in two groups of children—one group at high genetic risk of atopy, with a positive family history, who developed definite atopic disease during the 2-year study period; and the other at low risk, with no family history, who remained symptom-free at 2 years of age.

Participants were selected from a cohort of children followed from birth to 2 years old. All babies entered the study at birth, before cord-blood collection, and were assessed every 6 months, when a detailed history was taken, and physical examination, allergen skin tests, and blood collection were done. Atopic eczema was diagnosed by a paediatric allergist. Children with recurrent wheezing, urticaria, or rhinitis were included in the atopic group only if they had other evidence of allergic sensitisation (eg, atopic eczema, positive skin-prick test, positive radioallergen sorbent test).

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Child	Recurrent wheeze	Eczema	Urticaria	Possible rhinitis	Environmental allergen	
					SPT	RAST
03	+	—	+	—	Nuts	..
09	+	+	—	+	Egg	..
14	—	+	—	—	..	..
16	+	+	—	—	Egg	FX5
20	+	+	—	—	..	FX5
24	+	+	+	—	Egg	..
34	—	+	+	+	..	..
36	+	+	+	+	..	..
43	+	+	—	+	HDM	HDM
44	+	+	+	—	..	..
46	+	+	—	—	..	..
51	+	+	—	—	..	..
54	+	+	—	—	..	..
55	+	+	+	—	HDM	HDM
56	+	—	—	—	..	..
57	+	—	+	—	HDM	HDM
58	+	+	+	—	..	..
59	+	+	+	+	..	..

SPT=skin-prick test. RAST=radioallergosorbent test. FX5=mixture of common food allergens (egg, milk, fish, wheat, peanut, soy). HDM=housedust-mite allergen.

Signs in children with atopy

Eczema was diagnosed by a doctor if there were chronic erythematous pruritic skin eruptions on the face (except nose and lips), trunk, or limbs. Mild cases in which the diagnosis was in doubt were excluded. Inspection of records confirmed that participating children had received the appropriate routine vaccinations for their age.

The study was approved by the ethics committees of the Princess Margaret Hospital and the King Edward Memorial Hospital (Perth, Western Australia). Informed consent was obtained from parents for cord-blood collection and for longitudinal follow-up.

Data collection

For collection of cord blood at birth, the placental vessels were wiped with alcohol three times and allowed to dry before venepuncture. Samples were placed in heparinised RPMI culture medium for transport at room temperature. Subsequent collection of peripheral blood was by venepuncture of the cubital fossa; the same transport medium was used.

Mononuclear cells, from cryopreserved samples collected at birth (CBMC) and from fresh PBMC samples collected at each subsequent visit, were cultured with allergen for lymphoproliferation and for production of Th1 and Th2 cytokines. For lymphoproliferation, CBMC were cultured in triplicate for 7 days in microwells at 10^5 cells per L in serum-free medium,^{8,9} either alone or in the presence of aeroallergens (housedust-mite-allergen extract 10 µg/mL, *Fel d 1* cat-allergen, 30 µg/mL, ovalbumin 100 µg/mL, and tetanus toxoid 0.5 Lf units/mL). Data were expressed as the difference in disintegrations per min (dpm) between the stimulated culture and background (unstimulated) control cultures. Responses that were greater than twice the background counts and more than 1000 dpm above the background counts were classed as positive.⁹ PBMC collected during subsequent visits were cultured in the same way for 6 days.

The response to ovalbumin protein was subjected to epitope analysis by use of cell samples collected at birth, at 6 months, and at 12 months. The analysis used a series of 28 sequential overlapping peptides spanning the ovalbumin molecule, consisting of 19 aminoacid residues per peptide, overlapping each other by five residues.⁸ The peptides were pooled in sequential sets of three, and introduced into replicate microcultures at final individual concentrations of 10 µg/mL. Incorporation of tritium into DNA was measured after 7 days for CBMC, and after 6 days for PBMC collected at 6 and 12 months of age. Individual responses were scored as positive or negative as for the lymphoproliferation assays.

For cytokine analysis, CBMC or PBMC were cultured for 24 h in medium alone, or supplemented with optimum stimulating concentrations of housedust-mite allergen 10 µg/mL, *Fel d 1* 30 µg/mL, tetanus toxoid 0.5 Lf, or phytohaemagglutinin 1 µg/mL. Preliminary studies used commercially available low-level ELISA on allergen-stimulated culture supernatants from children within this age range,⁸ but the only cytokines within the

readable range were interleukin-6, 10, and 13. Responses involving interleukins 4, 5, and 9 (no ELISA available), and interferon γ were detectable only in mRNA concentrations which showed the low precursor frequency of specific T cells in these immature immune responses. Accordingly, allergen-stimulated supernatants in this study were assayed for the presence of interleukin-6, 10, and 13 with commercial ELISA kits (CLB Laboratories, Amsterdam). By contrast, interleukins 4, 5, and 9, and interferon γ and β -actin were detected as specific mRNA measured in cell pellets (or the same 24 h cultures) by standardised semiquantitative reverse-transcriptase PCR.⁸ The intensity of each test sample was expressed as a ratio relative to that of its respective β -actin control. Interferon- γ responses triggered by polyclonal stimuli were within the readable ELISA range and are given here in terms of cytokine protein. Other results are given as either the amount of cytokine mRNA in cell pellets, or as the amount of protein in the antigen-stimulated culture supernatants, after subtraction of the amounts observed in unstimulated control cultures.

In a separate series of experiments, a subset of CBMC from 11 atopic children and 12 normal children, selected randomly on the basis of cell availability, were tested with stimuli independent of antigen-presenting cells (APC), to find out whether previously observed impaired interferon- γ responses of atopic children are due to intrinsic T-cell defects or to immature APC signalling. CBMC and purified T cells were stimulated with combinations of antibody to human CD2 (100 ng/mL T11, Coulter Immunology, Hialeah, FL, USA), antibodies to human CD3 (1 in 100 culture supernatant from OKT3 cell line, ATCC, USA), antibodies to human CD28 (50 ng/mL anti-Leu 28, Becton Dickinson, San Jose, CA, USA), and recombinant interleukin-2 (20 U/mL). A combination of phorbol myristic acetate (20 ng/mL) and phytohaemagglutinin (1 µg/mL) was also used in parallel cultures, as an alternative method of APC-independent stimulation. T cells were purified from resting CBMC by means of CD4-Dynabeads (Dyna M-450; Dynal AS, Oslo, Norway).⁸ The cell content was assessed by flow cytometry, and all of the samples achieved more than 97% purity for CD4 cells, with less than 3% contamination with cells positive for HLA DR.

Statistical analysis

The non-parametric Mann-Whitney *U* test was used for comparisons of all variables (cytokine protein, mRNA expression,

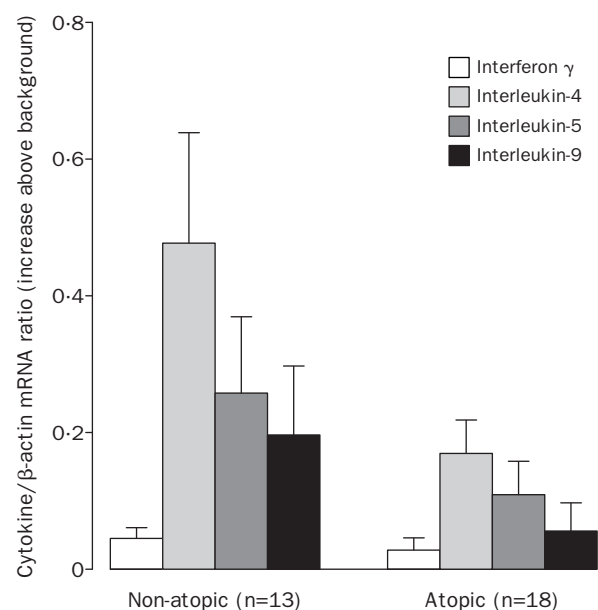


Figure 1: Allergen-specific cytokine mRNA production by CBMC from individuals who were atopic or non-atopic at age 2 years

Bars show SE. $p=0.049$ for difference in interleukin-4 values between non-atopic and atopic groups.

antibody concentrations), since no variable was normally distributed. For comparisons within groups at different ages, the paired Mann-Whitney *U* test was used, and the unpaired test was used for comparisons between groups. SPSS (version 6.1.1 for Macintosh) and Instat (version 2.0 for Macintosh) were used for data analysis.

Results

After 2 years of prospective follow-up, there were 13 low-risk non-atopic children without symptoms and 18 atopic children with diagnosed allergic disease in the study (table). Age-related changes in allergen-specific lymphoproliferation (data not shown) confirmed contrasting patterns in response to food and aeroallergens.^{7,9} Neonates with an immediate family history of atopy tended to show higher allergen-specific CBMC proliferative responses than neonates at low risk of allergy (data not shown), but these differences were significant only for *Fel d 1* ($p=0.002$). With age, proliferative responses to ovalbumin showed a progressive decline and were virtually absent by 12 months. By contrast, responses to the aeroallergens housedust-mite and *Fel d* were more persistent. There were few detectable lymphoproliferative responses to tetanus toxoid until after the first diphtheria-pertussis-tetanus vaccination. Age-related patterns of lymphoproliferative response were consistent throughout the study groups, with no significant differences in response frequency or magnitude when infants were compared on the basis of atopic family history or expression of atopic signs.

The age-dependent decline in responsiveness to whole ovalbumin protein was confirmed by epitope analysis, which showed reaction to two or more epitopes by 65% of CBMC samples—this degree of responsiveness was observed in only 10% of samples taken at 6 months (data not shown). This observation is consistent with the suggestion that high-zone tolerance to food allergens may occur very early in infancy.⁷

Cord-blood cytokine responses specific to housedust-mite allergen in all samples showed a Th2-skewed profile similar to that in atopic adults (figure 1). Similar results have been shown for ovalbumin responses.⁸ There was very little detectable interferon γ either as protein (data not shown) or as mRNA, after reverse-transcriptase PCR amplification up to 47 cycles, irrespective of atopic risk or later atopic disease. The neonatal allergen-specific Th2 responses (figure 1) tended to be lower in individuals who later developed atopic disease than in non-atopic individuals, and this difference was significant for interleukin-4 mRNA ($p=0.049$). This pattern was even more obvious for allergen-stimulated Th2 cytokines interleukin-6 ($p=0.003$), interleukin-10 ($p=0.015$), and interleukin-13 ($p=0.008$) detectable as protein in culture supernatants (figure 2). Identical differences in the interleukin-6 responses between the atopic and non-atopic groups were observed with both *Fel d 1* allergen and tetanus antigen (data not shown).

Although atopic and non-atopic individuals showed similar proliferative responses after birth, there were distinct differences in patterns of allergen-specific cytokine response with age (figure 3). Non-atopic individuals showed a gradual fall in detectable interleukin-4 mRNA responses to housedust-mite allergen. This process started within the first 6 months of life, and by 18 months interleukin-4 production in response to housedust-mite allergen barely differed from that in unstimulated control

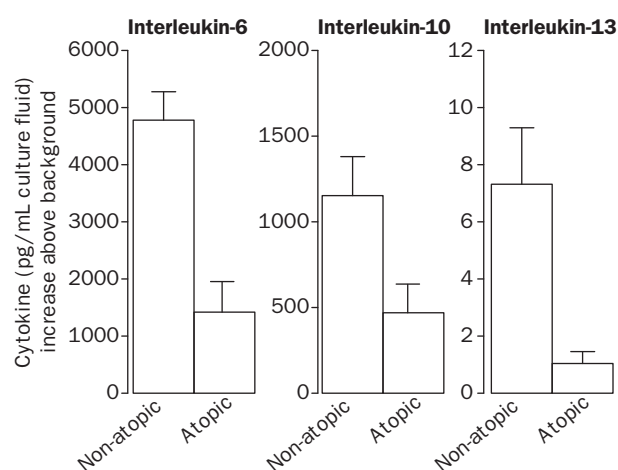


Figure 2: **Production of interleukin-6, interleukin-10, and interleukin-13 cytokine protein specific to housedust-mite allergen by CBMC**

Note vertical scales differ.

cultures ($p=0.04$ for difference birth to 18 months). By contrast, atopic individuals showed persistent interleukin-4 mRNA responses to housedust-mite allergen up to age 18 months. For interferon γ , mRNA responses were easily detectable after 6 months of age in the non-atopic group with a significant increase in response soon after birth ($p=0.04$ for difference from birth to 6 months). No significant increase occurred in the atopic group.

Data on cytokine mRNA production are not available for the 2-year-olds because of transient difficulties with

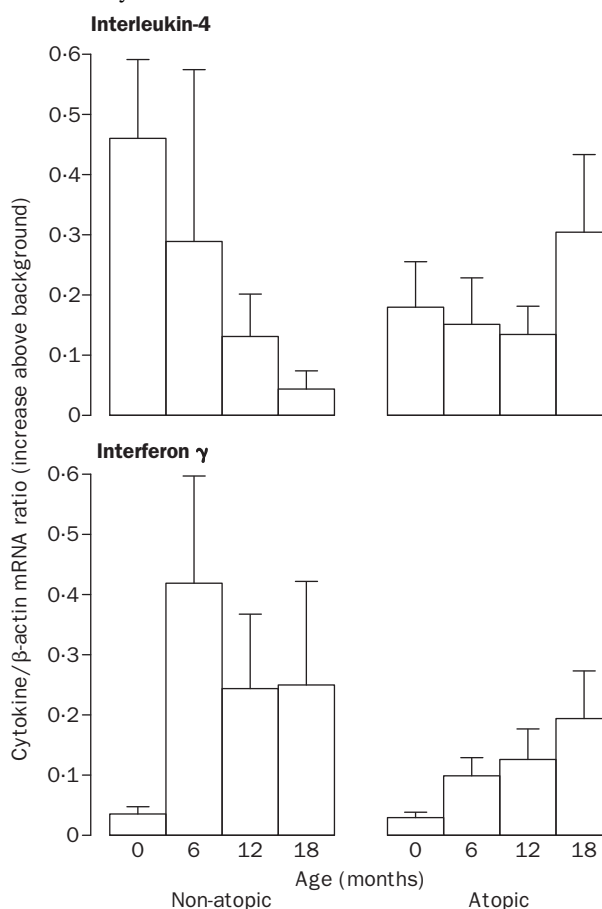


Figure 3: **Allergen-specific cytokine mRNA production by mononuclear cells from atopic and non-atopic individuals according to age**

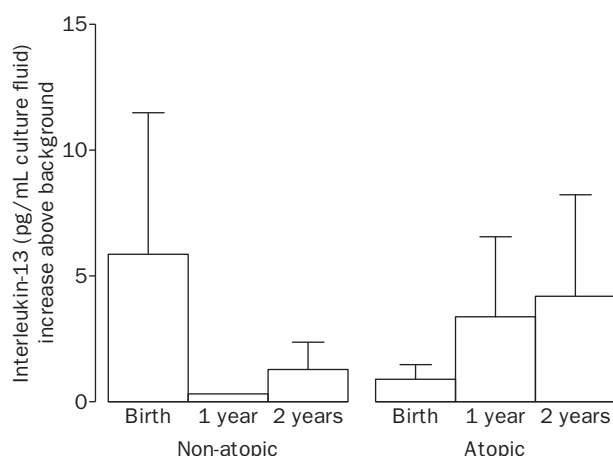


Figure 4: **Allergen-specific interleukin-13 production by mononuclear cells from atopic and non-atopic infants according to age**

PCR measurements. However, cytokine production concentrations were measured in these cultures, and results for interleukin-13 are available (figure 4). Interleukin-13 has similar biological activity to interleukin-4 but is more easily detectable as protein and showed similar patterns to interleukin-4 mRNA in response to house dust-mite allergen. Interleukin-6 and interleukin-10 production were also measured at the same times as interleukin-13, and the responses showed age-dependent increases of a similar magnitude in the atopic and non-atopic groups (data not shown). Low production of interferon γ was also detected in a subset of these samples but no significant differences were detected between groups (data not shown).

Lowered capacity for production of interferon γ in infancy has been identified as a marker of the atopic phenotype. Consistent with these findings, phytohaemagglutinin-induced production of interferon γ by CBMC from the children in this study who were atopic at age 2 years was much lower than that in non-atopic infants (atopic 224.2 pg/mL [SE 158.9–289.5] *vs* 66.5 [48.6–84.4] pg/mL culture fluid; $p < 0.03$). The production of interferon γ in these mixed-cell cultures is dependent on accessory cells, and hence the differences between the atopic and non-atopic groups may reflect variations in the functions of the accessory cells (APC) the T cells

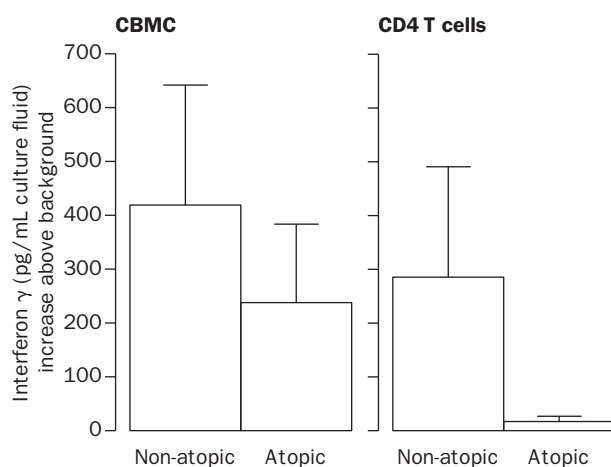


Figure 5: **Antigen-presenting-cell-independent stimulation of CBMC and CD4⁺ T-cell interferon- γ responses in atopic and non-atopic individuals at birth**

themselves, or both. In response to APC-independent stimuli (antibodies to CD2, CD3, and CD28 plus interleukin-2), interferon- γ production by CBMC was lower in the atopic group than in the non-atopic group (430 [230] *vs* 250 [150] pg/mL, figure 5). These differences were also apparent when purified CD4 T cells were stimulated in the absence of accessory cells (280 [220] *vs* 10 [5] pg/mL, $p = 0.01$). These findings were confirmed by use of phorbol myristate acetate plus phytohaemagglutinin as stimuli.

Discussion

Cross-sectional studies from our laboratory and elsewhere have given a broad outline of the events underlying primary allergic sensitisation. Initial T-cell priming commonly occurs across the placenta,¹⁰ particularly to allergens encountered by the mother in the last trimester of pregnancy.¹¹ The cytokine phenotype of these T cells is typically Th2-polarised.^{8,12}

During early childhood, these T-cell responses are subject to various regulatory mechanisms that are driven by direct exposure to environmental allergens. By the age of 5 years, almost all children have effectively downregulated T-cell reactivity to foods,⁷ with the parallel development of either Th1-polarised memory cells specific for inhalant allergens in non-atopic children or Th2-polarised memory cells in atopic children. Similar conclusions have been drawn from small prospective studies spanning the period from birth to early childhood.^{11,13}

Our prospective study showed an age-dependent decline in Th2 responses to inhalant allergens in non-atopic children, and a reciprocal pattern in those who developed atopy by the age of 2 years. These results support the conclusions of earlier cross-sectional studies^{7,9,12} that the defining feature of the atopic phenotype is the postnatal boosting of fetal allergen-specific Th2 immunity to inhalants, as opposed to redirection (immune deviation) of these responses towards the Th1 cytokine phenotype.

The failure of this immune-deviation process is associated with decreased capacity to produce the Th1 cytokine interferon γ . Previous studies have shown that the capacity to produce interferon γ is much lower in fetal T cells than in adults, and that production of interferon γ increases progressively from birth to age 5 years.¹⁴ This deficiency in normal infants is partly attributable to inefficient costimulation by neonatal accessory cells such as monocytes.¹⁵ However, the transient reduction in Th1 function is much greater in individuals genetically at risk of atopy, and can be shown in T-cell clones from children given APC-independent stimuli,¹⁶ which suggests that there is an additional defect in T cells in atopy. A significant part of the defect in interferon- γ production by CBMC is intrinsic to T cells (figure 5).

This interferon- γ production deficiency is reflected in the slower postnatal upregulation of allergen-specific Th1 responses, particularly during early infancy. Low capacity to produce interferon γ during initial extrauterine exposure to environmental allergens may compromise the immune-deviation process, given that the underlying process involves interferon- γ -mediated inhibition of Th2 cell growth.

The low production of interferon γ during infancy may be caused by the postnatal persistence of control mechanisms that limit capacity for intrauterine induction of Th1 responses, especially responses involving

interferon γ , which is highly toxic to the placenta.¹⁷ The lower ability of atopic T cells to produce this cytokine may reflect an exaggeration of this homeostatic mechanism.

The largest response to housedust-mite allergen was interleukin-6 production—we have shown that in CBMC this response is derived exclusively from the non-T-cell component of the mononuclear-cell population, probably monocytes activated via receptors primed with maternal antibody.⁸ This finding suggests a possible deficiency in monocyte cytokine production in atopic neonates or a deficiency in transplacental antibody transport.

Our results show that the differences in T-cell responses between atopic and non-atopic children are not restricted to production of the Th1 cytokine interferon γ . Allergen-specific production of interleukins 4, 6, 10, and 13 is also significantly lower in CBMC from the neonates who eventually develop atopy, and these findings are consistent with our study of T-cell clones from older infants.¹⁶ That study showed that the magnitude of the maturational defect in production of interferon γ in atopic children was greater than the corresponding defect in interleukin-4 production. Overall, the cytokine balance was tipped in favour of Th2 selection, and we speculate that this may also be the case in CBMC. In particular, the ability to increase rapidly the capacity to produce interferon γ during the early postnatal period, as shown in the non-atopic infants at age 6 months, may be a crucial factor. The absence of an effective Th2-inhibitory signal at this time may permit early expansion and maturation of Th2 memory cells, such that negative control via competing Th1 cells cannot be readily achieved.

These findings support the theory that early childhood is a unique period during which the immune responses that trigger disorders such as atopic asthma can potentially be manipulated therapeutically or prophylactically. In particular Th1 functions, either cytokine production capacity itself or responsiveness to exogenous inductive signals required for selective activation of Th1 cytokine genes, are logical targets for primary-prevention strategies.

The most pressing issue relating to allergic disease is the progressively increasing prevalence in more-developed countries, particularly among children. Our findings support the view^{18,19} that the environmental factors associated with this increased prevalence may exert their effects during early life, their most likely targets being mechanisms that regulate the maturation of adaptive immune functions.²⁰ Microbial exposure is the most potent of these environmental factors and is judged to be the archetypal selective stimulator of Th1 function.⁶ Sudo and colleagues' study of animals²¹ showed that if such stimuli are not available to the developing immune system during infancy, the maturation of Th1-dependent immune-deviation mechanisms is inhibited and lifelong dysregulation of Th2 responses may result. The main microbial stimulus in that study was from the gastrointestinal microflora, but epidemiological evidence shows that microbial pathogens, including viruses, may have similar Th1-stimulatory effects in human beings.^{6,18,22}

The nature of the relations between various types of microbial exposure during infancy and the subsequent expression of the atopic phenotype needs further detailed epidemiological study and investigation of the underlying molecular mechanisms. Capacity to respond to BCG vaccination during infancy is attenuated in individuals who subsequently develop atopy,²³ which suggests that delayed postnatal maturation of Th1 function in atopic children

may be related to a specific defect in recognition of and response to microbial signals.¹⁹

Contributors

S L Prescott, C Macaubas, P D Sly, and P G Holt shared responsibility for design, analysis, and interpretation of the study. Primer design and quality control of PCRs was done by C Macaubas and T Smallacombe. Laboratory work was done by S L Prescott, with assistance from T Smallacombe and B J Holt. All investigators contributed to writing the paper.

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