

TH₂-polarized immunological memory to inhalant allergens in atopics is established during infancy and early childhood

A. YABUHARA, C. MACAUBAS, S. L. PRESCOTT, T. J. VENAILLE, B. J. HOLT, W. HABRE, P. D. SLY and P. G. HOLT

TVW Telethon Institute for Child Health Research, West Perth, Western Australia

Summary

Background There is increasing evidence that the T-cell reactivity to environmental allergens underlying expression of allergic disease in adulthood, develops initially during childhood. However, there is little information available on the kinetics of these early responses, or on the patterns of cytokine production during this period.

Objective The purpose of this study was twofold: to obtain further information on the reported differences between responses to food versus inhalant allergens during early childhood, and to ascertain the age-range over which T-cell responses to inhalant allergens become polarized towards the TH₂ cytokine profile, in potentially atopic children.

Methods *In vitro* cytokine responses to house dust mite (HDM) and egg (OVA) were assessed by semiquantitative RT-PCR in panels of 2- and 5-year-old children and adults; lymphoproliferative responses to OVA were subjected to epitope analysis.

Results At age 2 years IL-4/IL-5 responses to HDM grouped with positive atopic family history, and by age 5 years cytokine responses correlated strongly with individual SPT reactivity to HDM. In contrast, OVA responses were restricted to weak and transient IL-5 signals in the 2-year-old family history positive group. Lymphoproliferation assays performed in parallel indicate a log-scale greater postnatal expansion of T-cell reactivity to the inhalant allergen; preliminary epitope analysis of OVA responses indicate that the number of OVA epitopes recognised decrease during early childhood.

Conclusions Inhalant allergen-specific *in vitro* cytokine production associated with positive skin-prick test (SPT) reactions, one of the hallmarks of adult atopy, manifests in children at or before 5 years of age; additionally, cytokine responses in SPT negative 5 year-olds are restricted to IFN γ , as per normal adults. In contrast, T-cell responses to a typical food allergen appear to be deleted during early childhood.

Keywords: Allergy, TH₁/TH₂, tolerance/suppression, immunotherapy, oral tolerance, immunotherapy

Clinical and Experimental Allergy, Vol. 27, pp. 1261–1269. Submitted 30 September 1996; revised 28 January 1997; accepted 20 February 1997.

Introduction

The repeated local expression of TH₂-polarized immunity to inhaled environmental antigens at the level of the airway mucosa is now recognized as being central to the pathogenesis of atopic respiratory disease [1–5]. For reasons which

are not yet clear, both the prevalence and severity of these diseases are progressively increasing in the developed countries, and they represent a major drain on contracting health budgets.

Current treatments for established atopic respiratory diseases are essentially symptomatic, and their effectiveness varies greatly between patients. Immunotherapeutic approaches aimed at desensitizing or deviating the TH₂-like responses which drive these disease processes theoretically offer more effective treatment alternatives [4];

Correspondence: Dr P.G. Holt, TVW Telethon Institute for Child Health Research, PO Box 855, West Perth, Western Australia 6872.

This work is supported by GlaxoWellcome and the NH&MRC of Australia. S.L. Prescott is supported by the W.A. Asthma Foundation.

however, they may be of limited value in chronic allergic disease which is often characterized by the development of hypersensitivity to a progressively expanding range of environmental agents.

There is therefore increasing interest in early therapeutic intervention in these diseases, at a stage prior to that at which allergen-specific immunity in potential atopics becomes locked permanently into a pathogenic TH₂-like pattern. It is accordingly important to establish the time frame over which environmental allergen-specific T-cell immunity most commonly develops in atopics, and to identify the major classes of regulatory mechanisms which shape these early responses.

Seroepidemiological studies in paediatric populations (review in [6]) hint strongly that allergen-specific antibody production can be initiated in infancy. Moreover, T-cells which proliferate *in vitro* in response to a variety of environmental allergens, have been identified in neonatal blood samples [7–10]. However, it has not been established whether these early T-cell responses are directed against native allergens or crossreacting antigens, and more importantly no information is yet available relating to their cytokine profiles relative to those in adults, and the latter represents the principal aim of the present study.

Methods and materials

Subjects

Infants: Twenty-seven subjects (aged 17.5 ± 2 months) were studied. The children were classified according to positive or negative history of atopy in first degree relatives employing a standard questionnaire [11]: 18 children had a familial history of atopy, and nine were negative.

Five-year-old children: 40 subjects (aged 5 years $\pm$ 3 months) were selected on the basis of skin-test reactivity (SPT) to house dust mite (HDM): 20 SPT positive and 20 SPT negative.

Adults: as internal controls for the studies below on allergen-specific cytokine production by T-cells from infants and children, a panel of 10 volunteer staff members were selected on the basis of presence (five individuals) or absence (five individuals) of HDM-specific IgE as detailed previously [12]; an additional group of 34 randomly selected adults was employed for lymphoproliferation studies.

Cord Blood (CB): a panel of 16 CB samples collected as part of an ongoing population study, was employed for some of these experiments.

Cell culture

Peripheral blood mononuclear cells which had been cryopreserved at collection were thawed and resuspended at

1×10^6 viable cells/mL in mercaptoethanol-supplemented AIM V medium (Gibco; [12]); the cryopreservation procedure does not significantly affect *in vitro* T-cell response patterns [12]. For cytokine analyses, aliquots of 1 ml from each sample were cultured for 24 h at 37°C with 5% CO₂ as follows: with medium alone, medium containing unfractionated House Dust Mite allergen (HDM; aqueous extract of lyophilized *Dermatophagoides pteronyssinus*, supplied by CSL Limited, Australia) at a final concentration of 10 µg/mL, or medium with Ovalbumin (Grade V OVA, Sigma Chemicals) at a final concentration of 100 µg/mL. For lymphoproliferative studies analysing responses to HDM, OVA or OVA peptides, a standard microplate assay was used as previously [12].

Total RNA extraction and cDNA synthesis

Cell pellets were lysed with 250 µL of RNeasy Lysis solution (Qiagen, Australia) and 25 µL of chloroform. The RNA pellets were resuspended in 5.5 µL DEPC-treated water, and used immediately. First strand synthesis of cDNA was performed in a total volume of 25 µL at 42°C using 250 ng of oligo [(dT)₁₅] (Biotech International, Australia) and 2.5 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in the presence of 12.5 U of ribonuclease inhibitor (Biotech).

Polymerase chain reaction (PCR)

cDNA was amplified for IL-4, IL-5, Interferon-γ and β-actin. Reactions were performed in 12.5 µL total volume overlaid with mineral oil, using respective primers and *TthPlus* DNA polymerase (Biotech). The PCR was run as follows: an initial denaturation step of 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. All reactions were performed in a programmable thermocycler (Perkin-Elmer). The number of cycles for β-actin and the cytokines was determined for each set of samples to ensure we reached detection levels and that the reaction remained in the linear phase.

Primers

The sequence for the primers were as follows: β-actin: 5' CgT gAC ATT AAg gAg AAg CTg TgC and 5' CCT Agg Agg AgC AAT gAT CTT gAT (expected size product: 375bp). IFNγ: 5' TTT ggg TTC TCT Tgg CTg TT × 5' TgT CTT CCT TgA Tgg TCT CC (expected product size: 230bp). IL-5: 5' CAT gAg gAT gCT TCT gCA TT × 5' TTC ggC CgT CAA TgT ATT TC (expected product size: 305bp). IL-4: 5' CAA gTg CgA TAT CAC CTT ACA gg 3' × 5' CCT TCA CAg gAC Agg AAT TCA AgC 3' (expected product size: 306bp).

Dot-blotting, hybridization and detection

Following denaturation with sodium hydroxide, PCR products were manually blotted on gridded nylon membranes (Hybond N+, Amersham), one for each cytokine tested. The membranes were pre-hybridized for 4–6 h, followed by hybridization overnight at 42°C with the respective probes diluted at 100 ng/mL. Double-stranded probes were produced by PCR reactions using biotin-16-dUTP (Boehringer Mannheim, Germany) at a ratio of 7:1 dTTP:dUTP. The template for probe synthesis were: chicken cDNA β -actin plasmid (gift from Dr Bruce Robinson, UWA, Australia); human IL-4 cDNA plasmid (DNAX, USA); human IL-5 cDNA plasmid (clone 118), and human γ IFN cDNA plasmid (clone C12), both donated by Dr Colin Sanderson (TVW Telethon ICHR, Australia). Following washing under increasing stringency conditions, the binding of the probes to the DNA was visualized by chemiluminescence using a commercial kit (ECL, Amersham) following manufacturer's instructions. The membranes were exposed to Hyperfilm (ECL, Amersham) and developed. The intensity of each dot was then determined using a densitometer (Scan

Analysis 2.02, Apple Macintosh) and the results expressed as a ratio of cytokine to β -actin density. In this way, cytokine levels could be compared between samples though different cytokines cannot be compared to each other.

Reproducibility of PCR and dot blot

cDNA from PHA-stimulated adult mononuclear cells was amplified in parallel for β -actin and cytokines (all in triplicates) for a cycle analysis. In the linear range of the amplification, the mean intra-subject variation of the densitometry values (minus matched background) was 15%. Also, cDNA samples from six children were tested for β -actin and cytokines in two completely separate experiments. The mean variation for the densitometry values was 28%.

Statistical analysis

Non-parametric analysis (Mann–Whitney two-tailed) were performed, using the program Instat 2.0 (GraphPad Software, San Diego, CA, USA).

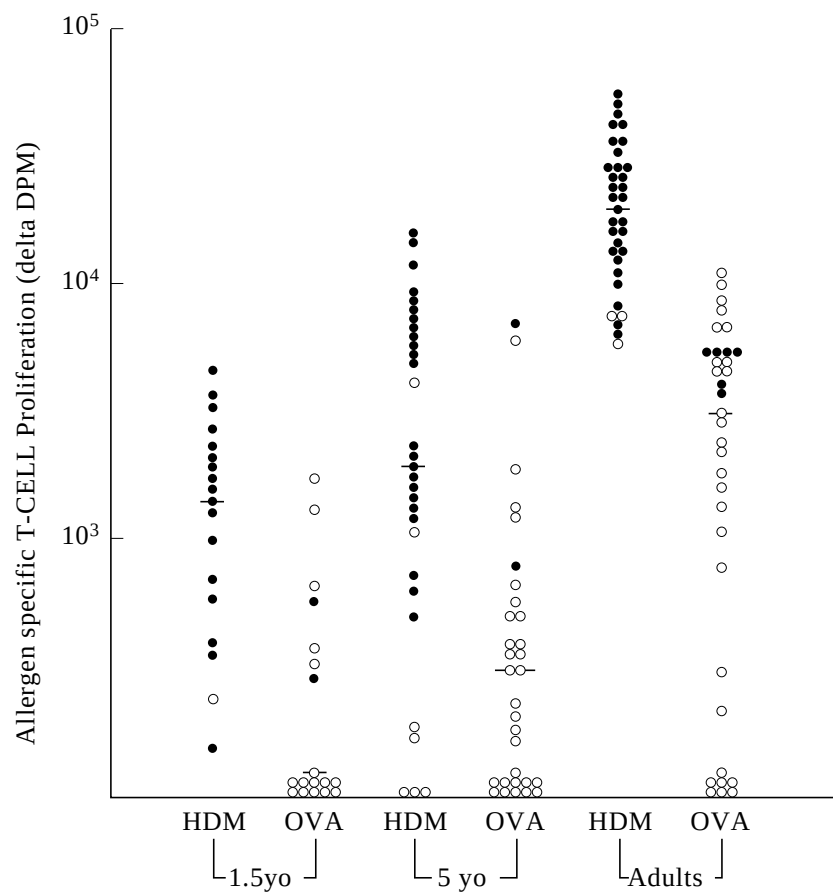


Fig. 1. Lymphoproliferative responses to food vs inhalant allergen at different ages. Data shown are ^3H -DNA synthesis as median Δ DPM from replicate cultures. ●, positive responses defined here as Stimulation Indices (test/background control) above 3 independent of DPM, ○, below 3. Population medians are designated by horizontal bars.

Results

Lymphoproliferative responses to dietary vs inhalant allergen

We focused upon the archetypal airborne (inhalant) allergen House Dust Mite (HDM) and the archetypal food allergen ovalbumin (OVA). In the initial phase of these experiments we sought to extend earlier findings on the qualitative and quantitative differences in postnatal development of T-cell immunity to these two classes of allergens, as detectable by allergen-induced lymphoproliferation. It has recently been demonstrated [7–10] that a significant proportion of cord blood T-cell samples proliferate in response to OVA and HDM, and further that these responses diverge postnatally [10]. This phenomenon was further investigated in the experiments illustrated in Figs 1 and 2.

As shown in Fig. 1 and in our earlier studies [10], $\geq 90\%$ of adult PBL samples demonstrate moderate-large lymphoproliferative responses to HDM (median approximately $20 \times 10^3 \Delta$ DPM per culture), compared with the low frequency and low intensity (median approximately $2 \times 10^3 \Delta$ DPM) of responses to the food antigen OVA, and these differences are already evident by age 1.5 years. However, we and others [7–10] have previously demonstrated that OVA and HDM responses occur with comparable (and relatively high) frequency in cord blood (CB), which suggests selective censoring of responses to the food antigen may have occurred postnatally. This possibility was further explored in the experiments of Fig. 2, involving crude epitope mapping of responses to OVA in CB vs 5-year-old samples. The simultaneous expression of reactivity against several regions of the OVA molecule by CB T cells make it unlikely that these early responses are against cross-reacting antigens; the findings are instead more consistent with primary immune responses in which immunodominance is yet to be established. These experiments further demonstrate that at the population level, the frequency of lymphoproliferative responses to OVA, and the number of epitopes recognized by each positive responder, was higher in the CB relative to the 5-year-old samples; these population findings require confirmation in individual subjects via prospective cohort studies spanning this age range, and these are in progress in several centres, including our own.

However, on the basis of the present findings we hypothesize that, by analogy with murine high dose Oral Tolerance [13], the regulatory process which eventually dominates these responses to OVA is high zone tolerance (T-cell anergy and/or deletion) induced by postnatal exposure to this ubiquitous allergen in the normal diet, leading to effective censorship of the peripheral OVA-reactive T-cell population. The nadir of OVA-specific T-cell reactivity appears to be during infancy, after which a small degree

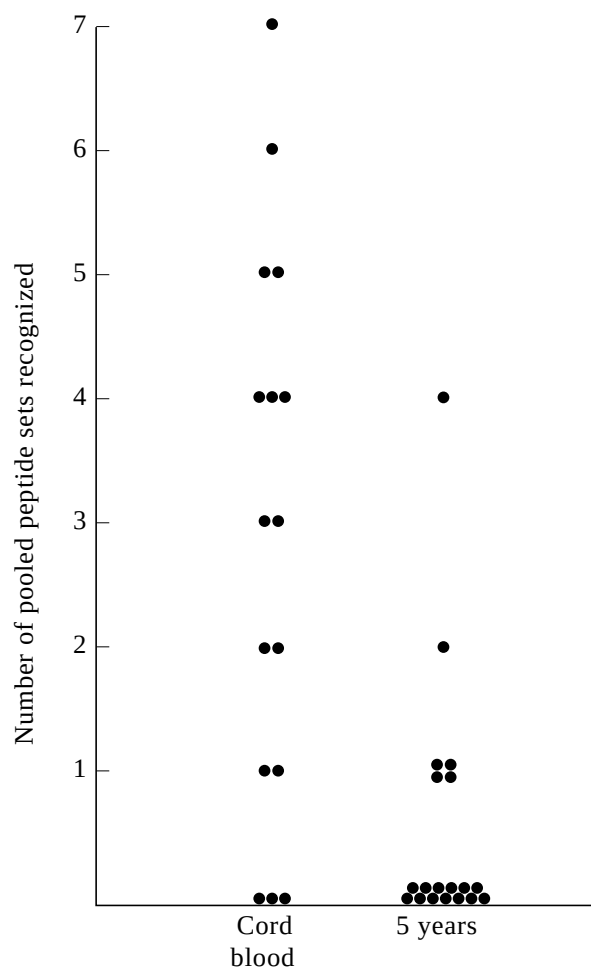


Fig. 2. Crude epitope analysis of T-cell responses to OVA. A peptide set spanning the OVA molecule comprising 19 mers with an overlap of 5 at each end was used; due to the restricted numbers of cells available, they were pooled in (consecutive) groups of 3; each peptide was used at a final concentration of $30 \mu\text{g/mL}$. Data shown indicate the number of peptide pools eliciting positive responses in individual PBL samples from cord blood and 5-year-olds.

of 'leakage' appears to occur between childhood and adulthood leading to the re-establishment of a small peripheral pool of reactive T cells; it is noteworthy, however, that the latter appears log-scale reduced relative to the anti-HDM T-cell pool.

The precise mechanisms underlying regulation of the response to the inhalant allergen remain to be defined in detail. However, given that positive HDM reactivity is essentially universal (Fig. 1 and reference [12]) and is polarised towards either TH_1 -like or TH_2 -like cytokine patterns [2–4], it may reasonably be postulated that the

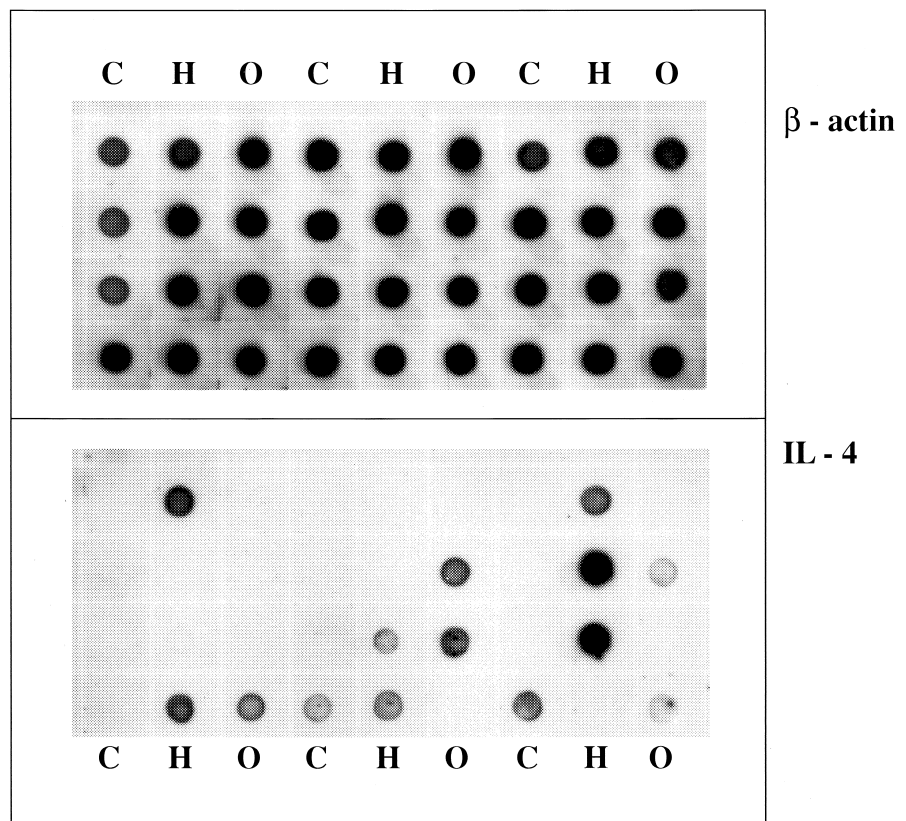


Fig. 3. Representative example of a Dot blotting analysis of β -actin (upper blot) and IL-4 (lower blot) specific amplified cDNA from 12 5-year-old children. Total RNA was extracted from mononuclear cells, reverse transcribed, amplified and blotted, followed by detection with double stranded biotinylated specific probes. The autoradiographs were scanned and the results expressed as a ratio of cytokine to β -actin density. C: control (only cell culture medium), H, House Dust Mite; O, Ovalbumin.

dominant regulatory process shaping these responses is classical Immune Deviation via mechanisms related to those in [14]. This suggestion is consistent with recent results from animal models on regulation of T-cell responses to aerosolised antigen [15]. Moreover, the disparity in regulatory mechanisms involved in OVA versus HDM responses may be explicable on the basis of the differences between respective levels of postnatal allergen exposure, viz. dietary antigens presented via the gastric mucosa in high concentrations (mg/g levels for food antigens) would appear to be in the dose range required to induce T-cell deletion/anergy [13], whereas the nanogram levels of inhalant allergens encountered at respiratory mucosal surfaces are sufficient only to drive low zone tolerance/immune deviation mechanisms [6,13].

Analysis of allergen-specific cytokine responses by semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (sqRT-PCR)

The remainder of this study focused on defining of the contribution of hallmark TH₁ and TH₂ cytokines to these immune responses, asking in particular whether the TH₁/TH₂ balance in adulthood is set during or subsequent to

infancy. The relatively low level of the infant T-cell responses (evident from Fig. 1) precluded the use of commercial assay systems for cytokine protein, and we were thus forced to rely upon sqPCR for determination of cytokine-specific mRNA. Rigorous standardization of this methodology was undertaken employing procedures developed elsewhere [16] (detailed below). In particular, cycle analyses were performed for each cytokine on samples from each age group, and cycle numbers were chosen that were in respective linear expansion ranges. PCR products in each sample were quantified on a relative basis by reference to endogenous β -actin after Dot blotting and probing with enzyme-conjugated homologous probes (representative example in Fig. 3), followed by densitometric scanning.

Given that the TH₂-skewed allergen-specific cytokine response profile characteristic of atopy in adults has been defined on the basis of cytokine protein production [2–4], we felt it was necessary to formally demonstrate that similar profiles would be revealed at the mRNA level by the sqPCR method used here. Therefore preliminary experiments were performed with PBL from HDM-IgE-positive and normal subjects, and as shown in Fig. 4 the adult allergic group exhibit the hallmark IL-4/IL-5 responses previously described [2–4].

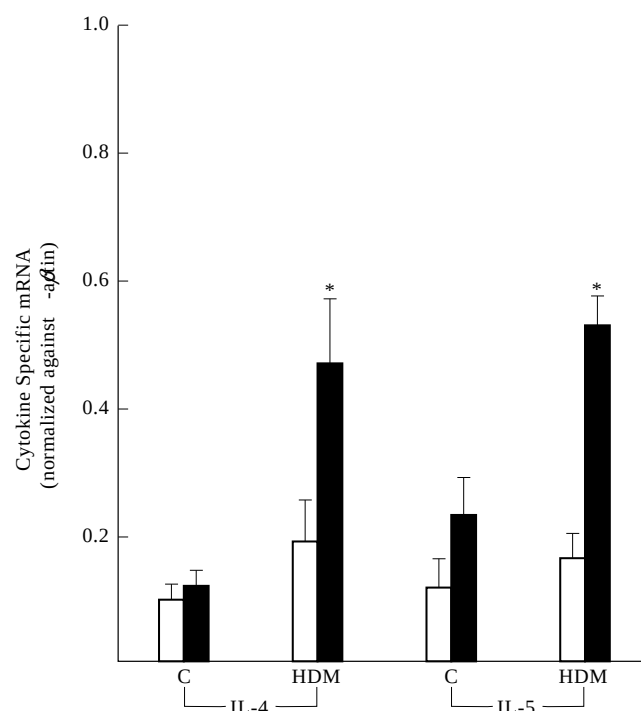


Fig. 4. Allergen-specific cytokine mRNA responses in adult atopics and normals. PBL from a panel of five atopic HDM-responders and five normals were stimulated *in vitro* with HDM for 24 h prior to RNA extraction and subsequent sqPCR analysis, as detailed in Methods. Data shown represent mean responses $\pm$ SE. *stimulated cultures > controls: $P < 0.05$. ■, HDM-IgE positive results, □, HDM-IgE negative results.

Allergen-specific cytokine responses to HDM and OVA in infancy and early childhood

Cytokine mRNA analyses on a group of 27 infants are shown in Fig. 5. Classification of infants as atopic/non-atopic is complicated by the fact that objective markers such as specific IgE antibody titres and skin-prick test (SPT) responses to inhalant allergens at this age are usually low, and symptom information provided by parents is often unreliable. It is accordingly difficult to accurately classify respiratory or gastric symptoms as atopic versus inflammatory/non-atopic. Indeed when cytokine responses within this population were analyzed by comparing 'symptom positive' to 'symptom negative' infants, no significant differences were found.

However, as shown in Fig. 5, significant differences were observed when the study population was subdivided on the basis of atopic family history. Thus, at mean age 1.5 years, the group with enhanced genetic risk for atopy defined by positive family history demonstrated a mixed cytokine response to both classes of allergens, characterized by IL-4/IL-5 and IFN γ mRNA production. In contrast, the family

history negative group displayed a low level TH₂ response involving IL-5-specific mRNA to OVA allergen only. The TH₂ cytokine component of the responses in the history positive group here (and in Fig. 6) may involve either TH₀ or TH₂ cells, and further studies involving generation of clones are in progress to address this issue.

Figure 6 extends these observations to the 5-year-old age group. Objective criteria for atopy in these children was obtained via a positive SPT to HDM; reactivity to OVA is rare in randomly selected children in this age range in our population (<5%), and is not tested unless dictated by distinct symptomology; the SPT group here were classified on the basis of reactivity to HDM. The findings in Fig. 6 reveal strong and consistent IL-4/IL-5 responses to HDM, again with concomitant IFN γ reactivity, within the atopic group; in contrast, the non-atopic group demonstrate IFN γ responses to HDM in the absence of TH₂ cytokines. Occasionally low level positive responses to OVA were observed, but these were not significant on a population basis in either group.

Discussion

The results of this study provide new insight into the aetiology of human allergic disease. One important issue which may now be approaching resolution, is the wide disparity in the prevalence of allergy to airborne allergens (high) vs food antigens (low). As noted above, recent studies on dose-response relationships in experimental oral tolerance in rodents [13] indicate that the particular control mechanism which is employed for immunoregulation of individual T-cell responses to protein antigens presented at the body's mucosal surfaces, is dictated by the antigen dose. Thus, high dose exposure appears to preferentially trigger T-cell deletion, while at lower doses T-cell anergy mechanisms dominate, and at the extreme (low) end of the range the only option available appears to be immune deviation [6,13].

It is likely that considerable overlap exists with respect to the ranges over which these mechanisms operate. However, the available published data suggest that the low exposure levels which constitute the norm for inhalant allergens are sufficient only for the triggering of immune deviation mechanisms. In contrast, it may be expected that immune responses to dietary antigens are mainly subject to regulation via deletion/anergy mechanisms triggered by the high exposure levels involved. Given the wide concentration range over which food antigens are commonly encountered (*viz.* as supplements vs primary dietary items), it is also likely that on many occasions exposure to the same antigens will occur within the range in which low-dose immune deviation operates, and hence this mechanism may remain available as a back-up for control of any food

Fig. 5. Allergen-specific cytokine responses in infants with or without atopic family history. PBL from 18 infants with positive family history and nine without history were cultured for 24 h at 10^6 cells/mL in medium alone (C) or with optimal stimulating levels of HDM or OVA allergen, prior to RNA extraction and subsequent sqPCR analysis for cytokine-specific mRNA, as detailed in Methods. Data shown represent mean responses $\pm$ SE. *stimulated cultures > controls: * $P < 0.005$. ** $P < 0.01$. ■, IL-4, ▨, IL-5, ■, IFN γ .

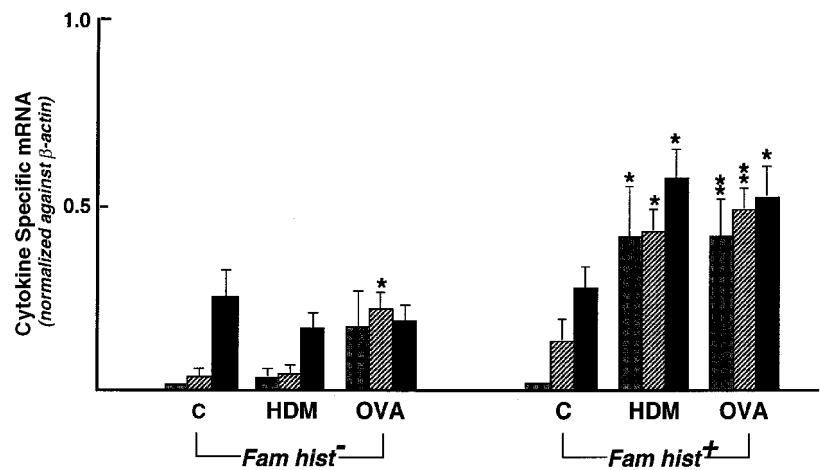
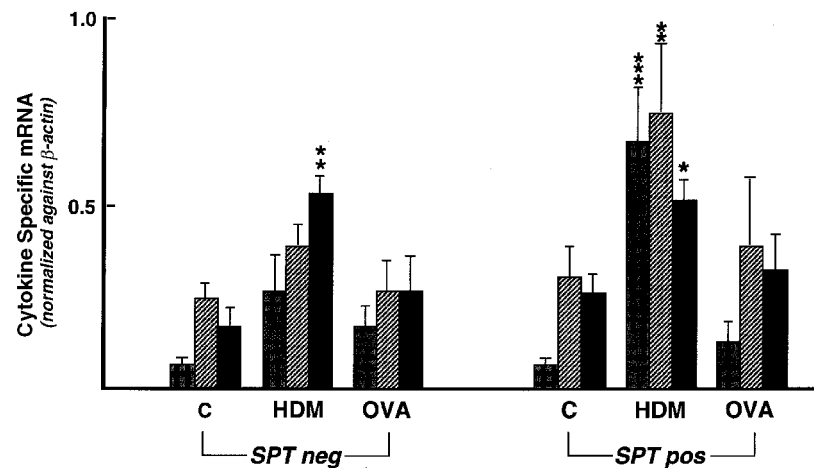


Fig. 6. Allergen-specific cytokine responses in 5 year olds with positive or negative SPT responses to HDM. PBL from 20 children with positive SPT responses to HDM and 20 who were SPT negative, were tested for allergen-specific cytokine responses to HDM and OVA, as per Fig. 5. *stimulated cultures > controls: * $P < 0.05$. ** $P < 0.01$; *** $P < 0.001$. ■, IL-4, ▨, IL-5, ■, IFN γ .



antigen-reactive T cells that escape censorship via anergy/deletion.

The key finding from this study relates to the time frame over which the immune system establishes control of these responses, i.e. during very early life. While formal prospective studies are required to ascertain the precise sequence of events in individual children, it is clear that on a population basis, the adult equivalent patterns of immunity characteristic of the two classes of allergens are imprinted within the first 5 years of life. The central question that can now be addressed in much more specific terms than was previously possible, is: how/why do these T-cell regulatory processes 'fail' in atopic individuals, particularly in the case of immune responses to inhaled antigens, leading to the development of potentially pathologic TH₂-skewed [2–5] or in some cases TH₀-like [1] TH-memory?

Recent developments in the experimental literature sug-

gest productive research avenues for pursuit of this question. In particular, a series of reports relating to murine neonatal tolerance have highlighted the importance of previously covert immune deviation phenomena in this process [17,18]. It is evident that at least in mice, the neonatal immune system is constitutively skewed towards TH₂ [19–22], such that early postnatal exposure to exogenous antigen can under certain conditions preferentially prime for TH₂-memory [17,18]. In addition, the nature of the antigen presenting cells (APC) involved in the priming process can further influence the subsequent direction of these immune responses [17].

These findings in experimental animals have important theoretical implications for the future study of TH-memory development against environmental allergens in humans. As noted earlier [23,24], human infants display reduced capacity to produce the principal TH₁ cytokine IFN γ , and this

deficiency is greatest in infants genetically at risk for development of atopy [9,11,25–28]. This suggests that biased TH-cell immunity and associated ‘tolerance’ phenomena are also likely to be operative in human neonates, and as a consequence may be expected to play a significant role in TH₁/TH₂ selection in the immune responses to environmental allergens encountered during this period. Accordingly, detailed analysis of the nature of T-cell immunity to environmental allergens in late fetal life, which in many cases appears to constitute the priming phase in these responses [7–10], merits a high priority in future research in this area.

As noted above, recent findings imply that TH₁/TH₂ switch regulation in murine neonatal tolerance is also subject to regulation by APC, and in particular that priming of neonates with mature Dendritic Cells (DC) can redress the effects of the underlying TH₂ skew in the neonatal T-cell compartment and thus facilitate the induction of TH₁-like immunity [17]. In this context, we have recently demonstrated that the principal APC population responsible for delivery of inhaled allergen to the adult immune system, comprising a network of DC that ramify throughout the epithelium lining the airways, is poorly developed at birth and does not fully mature until after weaning [29,30]. During this perinatal period the cells of this network express extremely low levels of MHC class II, respond poorly to inductive signals from cytokines such as GM-CSF, and exhibit low APC activity. Given that allergens delivered through the airway epithelium are likely to represent the principal stimuli for the TH₁/TH₂ selection process which shapes the responses denoted in Figs. 5 and 6, it is likely that factors which contribute to the functional maturation of this DC network during infancy may also play an important role in determination of allergen responder phenotype.

References

- 1 Byron KA, O'Brien RM, Varigos GA, Wootton AM. Dermatophagoides pteronyssinus II-induced interleukin-4 and interferon- γ expression by freshly isolated lymphocytes of atopic individuals. *Clin Exp Allergy* 1994; 24:878–83.
- 2 Wierenga EA, Snoek M, de Groot C, Chrétien I, Bos JD, Jansen HM, Kapsenberg ML. Evidence for compartmentalization of functional subsets of CD²⁺ T lymphocytes in atopic patients. *J Immunol* 1990; 144:4651–6.
- 3 Romagnani S. Induction of TH₁ and TH₂ responses: A key role for the ‘natural’ immune response? *Immunol Today* 1992; 13:379–81.
- 4 O'Hehir RE, Howne GF, Thomas WR, Lamb JR. House dust mite allergy: from T cell epitopes to immunotherapy. *Eur J Clin Invest* 1993; 23:763–72.
- 5 Corrigan CJ, Kay AB. T-cells and eosinophils in the pathogenesis of asthma. *Immunol Today* 1992; 13:501–7.
- 6 Holt PG. Immunoprophylaxis of atopy: light at the end of the tunnel? *Immunol Today* 1994; 15:484–9.
- 7 Kondo N, Kobayashi Y, Shinoda S, Kasahara K, Kameyama T, Iwasa S, Orii T. Cord blood lymphocyte responses to food antigens for the prediction of allergic disorders. *Arch Dis Child* 1992; 67:1003–7.
- 8 Piastra M, Stabile A, Fioravanti G, Castagnola M, Pani G, Ria F. Cord blood mononuclear cell responsiveness to beta-lactoglobulin: T-cell activity in ‘atopy-prone’ and ‘non-atopy-prone’ newborns. *Int Arch Allergy Immunol* 1994; 104:358–65.
- 9 Warner JA, Miles EA, Jones AC, Quint DJ, Colwell BM, Warner JO. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy* 1994; 24:423–30.
- 10 Holt PG, O'Keeffe PO, Holt BJ et al. T-cell ‘priming’ against environmental allergens in human neonates: sequential deletion of food antigen specificities during infancy with concomitant expansion of responses to ubiquitous inhalant allergens. *Ped Allergy Immunol* 1995; 6:85–90.
- 11 Holt PG, Clough JB, Holt BJ et al. Genetic ‘risk’ for atopy is associated with delayed postnatal maturation of T-cell competence. *Clin Exp Allergy* 1992; 22:1093–9.
- 12 Upham JW, Holt BJ, Baron-Hay MJ et al. Inhalant allergen-specific T-cell reactivity is detectable in close to 100% of atopic and normal individuals: covert responses are unmasked by serum-free medium. *Clin Exp Allergy* 1995; 25:634–42.
- 13 Friedman A, Weiner HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Immunology* 1994; 91:6688–92.
- 14 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–73.
- 15 McMenamin C, Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8⁺ T cell-mediated but MHC class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective suppression of IgE production. *J Exp Med* 1993; 178:889–99.
- 16 Doi S, Gemou-Engesaeth V, Kay AB, Corrigan CJ. Polymerase chain reaction quantification of cytokine messenger RNA expression in peripheral blood mononuclear cells of patients with acute exacerbations of asthma: effect of glucocorticoid therapy. *Clin Exp Allergy* 1994; 24:854–867.
- 17 Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 1996; 271:1723–1726.
- 18 Singh RR, Hahn BH, Sercarz EE. Neonatal peptide exposure can prime T cells, and upon subsequent immunization induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J Exp Med* 1996; 183:1613–1622.
- 19 Lin H, Mossmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal–fetal interface. *J Immunol* 1993; 151:4562–73.
- 20 Krishnan L, Guilbert LJ, Russell AS, Wegmann TG, Mossmann TR, Belosevic M. Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes

- decreased antigen-specific IFN- γ responses and increased production of T helper 2 cytokines. *J Immunol* 1996; 156:644–52.
- 21 Chen N, Gao Q, Field EH. Expansion of memory Th2 cells over Th1 cells in neonatal primed mice. *Transplantation* 1995; 60:1187–93.
- 22 Chen N, Field EH. Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance. *Transplantation* 1995; 59:933–41.
- 23 Holt PG. Postnatal maturation of immune competence during infancy and early childhood. *Pediatr Allergy Immunol* 1995; 6:59–70.
- 24 Holt PG. Primary allergic sensitisation to environmental antigens: perinatal T-cell priming as a determinant of responder phenotype in adulthood. *J Exp Med* 1996; 183:1297–1301.
- 25 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. *Pediatr Allergy Immunol* 1993; 4:60–4.
- 26 Tang MLK, Kemp AS, Thorburn J, Hill DJ. Reduced interferon- γ secretion in neonates and subsequent atopy. *Lancet* 1994; 344:983–86.
- 27 Martinez FD, Stern DA, Wright AL et al. Association of interleukin-2 and interferon- γ production by blood mononuclear cells in infancy with parental allergy skin tests and with subsequent development of atopy. *J Allergy Clin Immunol* 1995; 96:652–60.
- 28 Liao SY, Liao TN, Chiang BL et al. Decreased production of IFN γ and decreased production of IL-6 by cord blood mononuclear cells of newborns with a high risk of allergy. *Clin Exp Allergy* 1996; 26:397–405.
- 29 Nelson DJ, McMenamin C, McWilliam AS, Brenan M, Holt PG. Development of the airway intraepithelial Dendritic Cell network in the rat from class II MHC (Ia) negative precursors: differential regulation of Ia expression at different levels of the respiratory tract. *J Exp Med* 1994; 179:203–12.
- 30 Nelson DJ, Holt PG. Defective regional immunity in the respiratory tract of neonates is attributable to hyporesponsiveness of local Dendritic Cells to activation signals. *J Immunol* 1995; 155:3517–3524.