

Regulation of T-helper cell responses to inhalant allergen during early childhood

C. MACAUBAS, P. D. SLY, P. BURTON, K. TILLER, A. YABUHARA, B. J. HOLT, T. B. SMALLACOMBE, G. KENDALL, M. C. JENMALM* and P. G. HOLT

TVW Telethon Institute for Child Health Research, Perth, WA, Australia and *Clinical Research Centre, University Hospital, Linköping, Sweden

Summary

Background Recent evidence suggests that preschool children manifest patterns of allergen-specific skin prick test (SPT) reactivity and *in vitro* T-cell cytokine production which are similar to that of either atopic or nonatopic adults. However, published studies on this age group involve small sample sizes and a restricted number of cytokines, usually in response to polyclonal stimuli.

Objective To elucidate the relationship between *in vivo* and *in vitro* immune responses to a major inhalant allergen house dust mite (HDM) in preschoolers.

Methods Peripheral blood mononuclear cells (PBMCs) from matched groups of HDM-SPT⁺ and SPT[−] 6-year-olds ($n = 30$ and 29 , respectively) tested for PBMC responses to HDM, and cytokine production measured at both the protein and mRNA levels. Immunoglobulin (Ig) E and IgG subclass antibody titres were determined in serum. Interrelationships between *in vitro* and *in vivo* HDM responses were examined via multivariate analyses.

Results SPT reactivity to HDM was associated with *in vitro* production by putative T cells of interleukin (IL) -4, IL-5, IL-9, IL-10, IL-13 and low level IFN γ , and with production *in vivo* of IgE and (all) IgG subclass antibodies; HDM responses in the SPT[−] group were restricted mainly to IL-10 and IFN γ and very low levels of IL-4; IL-6 production from non-T-cell sources was common. The cytokine most associated with positive SPT responses was IL-9; SPT wheal diameter correlated positively with IL-4, IL-5 and IL-13 and negatively with IL-10.

Conclusion Detailed analysis of cytokine responses in this very young age group have the potential to uncover subtle relationships between *in vivo* and *in vitro* allergen reactivity which may be less clear in adults, in whom T-cell response patterns are modified via chronic stimulation. The present findings which suggest potentially important roles for IL-9 and IL-10 in the early phase of allergic disease, may be one such example.

Keywords: atopy, childhood, cytokines, T-helper cells

Clinical and Experimental Allergy, Vol. 29, pp. 1223–1231. Submitted 26 October 1998; re-revised 12 February 1999; accepted 18 March 1999.

Introduction

The underlying immunological basis for allergic diseases in humans is currently viewed from the perspective of the murine TH1/TH2 paradigm [1,2], viz. responses to inhalant allergens in adult atopics appear polarized towards the

TH2 [3,4] or TH0 [5,6] cytokine phenotype, in contrast with clinically unresponsive normals who manifest low-level allergen-specific T-cell reactivity which is dominated by IFN γ /IL-2 production. This dichotomy was originally described at the clonal level, and has been supported by results from bulk culture experiments with peripheral blood mononuclear cells (PBMCs) which have demonstrated specific lymphoproliferation [7] and accompanying TH1 or TH2-polarized cytokine responses

Correspondence: P. G. Holt, Division of Cell Biology, TVW Telethon Institute for Child Health Research, PO Box 855, West Perth WA 6872, Australia.

[8] to common airborne allergens, in essentially all adults.

A number of recent studies (reviewed in [9]) suggest that with chronic exposure to environmental allergens, the nature of these T-helper cell reactions in atopics may become more complex, and in particular that the later stages of the response to allergen challenge may involve a significant TH1 component [10]. Nevertheless, the contribution of the key TH2 cytokines interleukin (IL) -4, IL-5 and IL-13 remains as the unique defining feature of the atopic T-cell response to allergen. The questions of when/how apparently contrasting patterns of allergen-specific memory are developed within atopics and nonatopics is thus key to our understanding of the aetiology of allergic disease.

With respect to the issue of when allergic sensitization is most commonly initiated, accumulating evidence from a range of epidemiological and laboratory-based studies (reviewed in [11]) have focused attention on early childhood, particularly in view of the demonstration by several groups of T cells in cord blood (CB) which proliferate in response to allergens. Our laboratory has recently demonstrated the foetal origin of these allergen-responsive cells in CB via cloning and subsequent DNA genotyping, and has additionally shown that their cytokine production profiles are markedly TH2-polarized in virtually all subjects, regardless of respective atopy genotypes [12]. This infers that initial T-cell priming may occur by transplacental leakage of allergen fragments from the maternal circulation. The TH2 polarity of these early responses may be an indirect result of the generalized damping of TH1-associated responses within the foetal microenvironment via the immune deviating functions of the placenta [13,14].

We have presented preliminary evidence that during the first few years of life, these weakly primed foetal T-cell responses are reshaped by direct environmental allergen exposure, and furthermore that this process varies with different classes of allergens. Thus, lymphoproliferative responses to dietary allergens typified by ovalbumin (OVA) display an age-related decline post-natally, in contrast with inhalant allergen-specific responses which expand progressively [15]. These declining lymphoproliferative responses to OVA are paralleled by shrinkage in the number of OVA epitopes recognized [16]. In contrast, parallel lymphoproliferative responses to the archetypal inhalant allergen house dust mite (HDM) progressively increase during early childhood [15], accompanied by a generalized increase in allergen-specific IFN γ reactivity and variable patterns of IL-4/IL-5 production as measured at the mRNA level [16]. The present study focuses upon responses to HDM in PBMCs from preschool children, in particular on patterns of cytokine production in SPT⁺ vs SPT⁻ subjects, as measured at both the protein and mRNA levels.

Materials and methods

Subjects

Two groups of age-matched children (1–5 months from their 6th birthday), respectively, atopic and nonatopic, were selected as a nested case control sub-sample from a large ongoing cohort study. Skin test reactivity (SPT) was assessed to a panel of allergens comprising house dust mite, rye grass, mould and cat; skin weal size was recorded as the mean of two diameters (one being the maximum diameter) measured at right angles. Reactivity to HDM as weal diameter ≥ 2 mm was used as the criterion to select the SPT⁺ group ($n = 30$, 20 male and 10 female); 19 members of this group responded to ≥ 3 of the allergens. The frequency of SPT responses to HDM within a sample from the overall cohort was 29.6%. SPT-negative children had no reactivity to any of the allergens tested ($n = 29$, 14 male and 15 female). The presence of IgE antibody in serum to a panel of environmental allergens was measured by the Phadiatop assay (Pharmacia Upjohn, Bridgewater, NJ, USA), and the overlap between Phadiatop^{+/+} and SPT^{+/+} groups was complete except for a single subject (who was Phadiatop⁺/SPT⁻).

Study design

The study was carried out with the approval of the Princess Margaret Hospital Ethics Committee, and its aims were twofold. Firstly, we sought to further elucidate differences in T-cell cytokine responses to a major inhalant allergen (HDM) between SPT⁺ atopic and SPT⁻ nonatopic children. Secondly, we sought to define relationships between *in vitro* cytokine responses and *in vivo* response to the test allergen, employing as an objective measure of the latter the diameter of the SPT weal. Accordingly, we cultured PMBCs from all subjects with optimal stimulatory levels of HDM (details below), and measured cytokine production at the earliest feasible time point i.e. 24 h – this time was chosen to minimize distortion of allergen-specific T-cell cytokine profiles from *in vivo* patterns, via *in vitro* differentiation of the stimulated T cells (we have previously shown that this sampling time successfully distinguishes between adult atopics and nonatopics with respect to HDM responses [16]).

We have elected, where possible, to employ two different techniques (detailed below) to measure cytokine responses, for the following reasons. Firstly, cytokine protein sampled in culture supernatants potentially underestimates overall production, due to *in vitro* consumption/degradation (e.g. [17]). Additionally, only a restricted range of the available cytokine ELISAs are sufficiently sensitive for studies on *in vitro* allergen-specific responses, particularly in young

children in whom frequency of responding T cells is relatively low. Accordingly, we have also employed RT-PCR to measure cytokine-specific mRNA in cell pellets from the cultures. However, this procedure provides information relevant to a single time point only, given that the half-life of most cytokine mRNA species are relatively short [18]. Therefore, we have elected where feasible to measure production of cytokine-specific mRNA and protein in parallel.

Cell preparation and culture

PBMCs were cryopreserved after collection and subsequently batch analysed. We have demonstrated in preliminary experiments that cryopreservation does not significantly affect *in vitro* T-cell responses against these allergens. After thawing, the cells were resuspended at a concentration of 1×10^6 viable cells/mL in AIM-V medium (Gibco) and cultured as detailed in [7]. Aliquots of 1-mL cells from each individual were cultured for 24 h at 37 °C with 5% CO₂ with medium alone or medium with HDM allergen (aqueous extract of lyophilized *Dermatophagoides pteronyssinus*, supplied by CSL Limited, Parkville, Australia) at a final concentration of 10 µg/mL. After culture the cells were collected by centrifugation; the supernatants were frozen at -20 °C for ELISA tests and the cell pellets were used immediately for RNA extraction. Replicate microplate cultures were established in parallel in 200-µL volumes of AIM-V, with or without 10 µg/mL HDM allergen, and lymphoproliferation measured at day 6 as detailed previously [7]. We have previously demonstrated via the use of PolymyxinB [12] that T-cell responses to HDM are not artefactually influenced by covert LPS contamination of the allergen preparation.

Semiquantitative RT-PCR for detection of cytokine-specific mRNA

Total RNA from the cell pellets was obtained using RNeasy B as previously described [16]. cDNA was transcribed using 250 ng oligo (dT)₁₅ (Biotech International, Bentley, Australia) and 2.5 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) in the presence of 12.5 U of ribonuclease inhibitor (Biotech) in a final volume of 25 µL. For the PCR reactions, the reaction mixture contained 1 µL of cDNA, 50 ng of the specific primers, 1 × PCR reaction buffer (Biotech), 0.2 mmol/L each dNTPs (Biotech), 1.5–2 mmol/L MgCl₂, and 0.5 unit of *Tth Plus* DNA polymerase enzyme (Biotech), in a total volume of 12.5 µL overlaid with mineral oil. 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 respective temperatures for 1 min and extension at 72 °C for 1 min.

All reactions were performed in a programmable thermocycler (Perkin-Elmer, Melbourne, Australia). Samples were amplified in groups comprising equal numbers of SPT⁺ and SPT⁻ children, and stimulated and unstimulated cultures from individual children were always tested together. A positive cDNA control was always amplified in parallel in all PCRs performed.

Primers

The following primers were used: annealing temperature 58 °C: β -actin (30 cycles), IL-4 and IFN γ (40 cycles), IL-5 (35 cycles) as described previously [16]; IL-13 (42 cycles) expected product size: 481 bp (5' CCA CGG TCA TTG CTC TCA CTT 3' and 5' CCC GCC TAC CCA AGA CAT TTT3') and IL-10 (30 cycles) expected product size: 242 bp (5' AGA ACA GCT GCA CCC ACT TC 3' and 5' CGC CTT GAT GTC TGG GTC TT 3'). Annealing temperature 56 °C: IL-6 (43 cycles) and IL-9 (40 cycles) [19]. Annealing temperature 55 °C: TGF β 1 (35 cycles) expected size product: 458 bp (5' GAG GTC ACC CGC GTG CTA ATG 3' and 5' AGC AGG AAG GGC CGG TTC 3'). Rigorous cycle analyses were performed with each primer set as previously [16] to ensure we had reached detection levels and that the reaction remained in the linear phase. In each case, PCR products of the expected size were obtained, as verified by analysis in 1.5% agarose gel and staining with ethidium bromide of a subset of samples and a positive control.

Dot-blot, hybridization and detection

The PCR products were analysed by dot-blot as described previously [16]. Briefly, double-stranded probes were produced by PCR reactions using biotin-16-dUTP (Boehringer Mannheim, Perth, Australia) at a ratio of 5 : 1, dTTP : dUTP. Templates for probe synthesis of β -actin, IL-4, IL-5 and IFN γ were as described in [16]. For the other cytokines, cDNA obtained from total adult peripheral blood mononuclear cells stimulated with PHA (1 µg/mL) for 24 h at 37 °C was used as template. Following overnight hybridization with biotinylated probes, the binding was visualized by chemiluminescence using ECL kit (Amersham, UK) and exposure to Hyperfilm (Amersham). The intensity of each dot was then determined using a densitometer (Scan Analysis 2.02, Biosoft, Cambridge, UK) and the results expressed as a ratio of cytokine to β -actin density.

ELISA assays for detection of cytokine protein

Production of IL-6, IL-13 and IFN γ protein was measured with commercial kits (Pelikine CompactTM CLB, Amsterdam, The Netherlands). The sensitivity of the assay was 5 pg/mL for IL-6, 2 pg/mL for IL-13 and 6 pg/mL for IFN γ . IL-4 kits from the same manufacturer were also used, but cytokine levels in our samples were below the

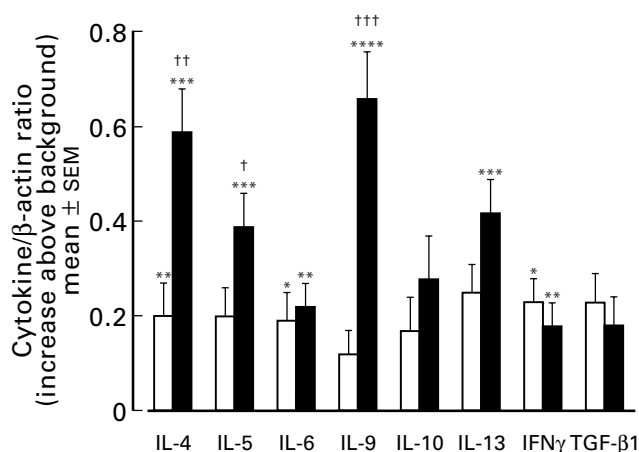


Fig. 1. HDM-specific cytokine mRNA production by HDM-SPT⁺ (black bars) and SPT⁻ (white bars) groups. 1 mL aliquots of PBMCs were cultured for 24 h in AIM-V only or with 10 μ g/mL HDM. Total RNA was extracted, transcribed to cDNA and amplified using primers for the respective cytokines. PCR products were analysed by dot-blot. The data obtained were initially expressed as arbitrary activity units by normalizing the scanned area value for each slot blot against the respective value for β -actin in the same culture. The data were finally expressed as mean increases above background controls ($\pm$ SEM), calculated individually by subtracting values for unstimulated control cultures (medium alone) from respective test cultures containing allergen. Statistical analysis of background control values for all cytokines showed no differences between SPT⁺ and SPT⁻ groups. Test > control cultures: **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05. SPT⁺ group > SPT⁻: ††† P < 0.0001, †† P < 0.01, † P < 0.05.

limit of detection (5 pg/mL). IL-10 protein was measured by an in-house ELISA assay, using rat IgG₁ antihuman IL-10 monoclonal antibody (clone JES3-9D7, Pharmingen, San Diego, CA, USA) for coating and biotinylated rat IgG_{2a} antihuman IL-10 monoclonal antibody (clone JES3-12G8, Pharmingen) for capture. For the standard curve we used recombinant human IL-10 (Pharmingen). For detection, streptavidin-horseradish-peroxidase conjugate (Amersham, UK) was added, followed by substrate. The limit of detection was 200 pg/mL. IL-5 protein was also measured with an in-house assay, using rat IgG₁ antihuman IL-5 monoclonal antibody (clone TRFK5, Pharmingen) for capture and biotinylated rat IgG_{2a} anti-IL-5 monoclonal antibody (clone JES1-5A10, Pharmingen) for detection. The standard curve was generated using serial dilutions of recombinant human IL-5 (Pharmingen); the limit of detection was 13 pg/mL.

ELISA detection of IgG and IgE antibodies to Der p 1

Conventional ELISAs for quantification of IgG, IgG1 and IgG4 antibodies to the major HDM allergen Der p 1 were as

described in detail in [20]. Der p 1 (kindly provided by Dr W. Thomas, TVW Telethon Institute for Child Health Research, Perth, Australia), was prepared by affinity purification with the 4C1 monoclonal antibody. Quantification of IgG2 and IgG4 antibodies in samples below the detection level of the conventional ELISA were performed with a more sensitive enzyme-amplified ELISA [20]. The following monoclonal antibodies to the human IgG subclasses were used: IgG, clone HP6064, IgG1, clone HP6069, IgG2, clone HP6014, and IgG4, clone HP6011 (BioZac AB, Järfälla, Sweden). The reference sera were from patients undergoing house dust mite immunotherapy. The titres in the reference sera were: IgG1 1216 AU, IgG2 507 AU and IgG4 624 AU. IgE antibodies to Der p 1 were analysed via the high sensitivity protocol of Magic LiteTM, a commercial chemiluminometric method, according to the recommendations of the manufacturer (ALK, Hørsholm, Denmark).

Statistical analysis

Univariate analysis was based upon conventional methods [21]. These included the Mann-Whitney U -test for unpaired continuous responses, corrected for ties, and Wilcoxon matched-pairs signed rank test for paired responses. Correlation between continuous variables was assessed using the Spearman rank correlation coefficient. Categorical responses were analysed using the Fisher's exact test. The statistical package SPSS was used.

Multivariate analysis was based upon multiple linear regression and multiple logistic regression. Final models were subjected to standard tests of goodness-of-fit [22]. These included an analysis of standardized residuals, and an assessment of regression leverage and influence. All reported models were found to provide an acceptable fit to the observed data.

Results

Cytokine responses to HDM

Cytokine mRNA production Cytokine-specific mRNA production in cultures stimulated for 24 h with HDM is illustrated in Fig. 1. PBMCs from atopic HDM-SPT⁺ children upregulated production of mRNA specific for several cytokines, and statistically significant increases above control levels were detected for IL-4, IL-5, IL-6, IL-9, IL-13, and IFN γ . In the HDM-SPT⁻ group, production of IL-6 and IFN γ mRNA were observed at levels comparable with those in the SPT⁺ children; IL-4 production was also evident in the SPT⁻ group, but at much lower levels than in the atopics.

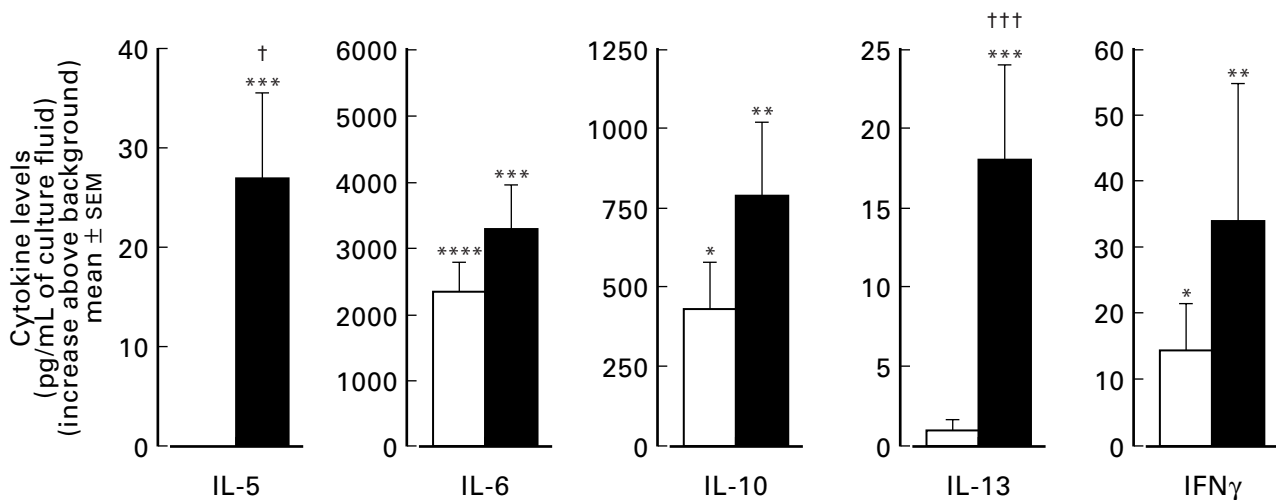


Fig. 2. HDM-specific cytokine protein production by HDM-SPT⁺ (black bars) and SPT⁻ (white bars) groups. PBMCs were cultured for 24 h in AIM-V only or with 10 µg/mL HDM. Supernatants were collected and cytokine protein content assayed by ELISA. The data shown represent mean ($\pm$ SEM) increases in cytokine protein content in allergen-stimulated (test) cultures above those of unstimulated (control) cultures. Cytokine levels in unstimulated cultures were low (in particular IL-5 which was undetectable) and did not differ significantly between SPT⁺ and SPT⁻ groups. Test > control cultures: **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05. SPT⁺ group > SPT⁻: † P < 0.0001.

Cytokine protein production

Supernatants from the cultures employed in the experiments in Fig. 1 were assayed for the presence of cytokine protein by ELISA. Levels of IL-5, IL-6, IL-10, IL-13 and IFN γ protein produced in response to HDM protein were within the detectable range (Fig. 2). The IL-5 and IL-13 responses to HDM clearly distinguished between SPT⁺/SPT⁻ groups, in particular IL-5 which was undetectable in SPT⁻ subjects, whereas production of IL-6, IL-10 and IFN γ was comparable.

Antibody production

This aspect of the study, illustrated in Fig. 3, focused upon responses to the Der p 1 component of HDM. Levels of specific IgE antibody in all SPT⁻ children were below detection levels, while 27 out of 30 SPT⁺ children had detectable antibody. For the IgG subclasses, seven SPT⁻ children exhibited detectable pan IgG to Der p 1 and five had IgG1, whereas the corresponding figures in the SPT⁺ group were 27/30 for pan IgG and 22/30 for IgG1 (frequency in SPT⁺ > SPT⁻; P < 0.0001 using Fisher's exact test). Only three children from the SPT⁻ group had detectable IgG4, in contrast with 26 children in the SPT⁺ group (P < 0.0001). Production of IgG subclass (except IgG2 with IL-9) and IgE antibodies against the major HDM allergen Der p 1 was strongly associated with IL-4 and IL-9 mRNA responses (P < 0.03– P < 0.0001 by Mann–Whitney), and with IL-5 and IL-13 protein responses (P < 0.005– P < 0.0001) against crude HDM. These findings contrast

with those for IgG2 antibody which was detected in the majority of both atopic and nonatopic subjects (Fig. 3). This may reflect in part the greater sensitivity of the IgG2 detection system; however, the mean titre in the SPT⁺ group was significantly elevated in comparison with their SPT⁻ counterparts (P < 0.002).

It is important to note that the crude HDM extract contains a range of allergens other than Der p 1, and a significant proportion of the overall T-cell response to the extract is likely to be directed against these other allergens.

In vitro cytokine production vs in vivo responses to HDM challenge

The univariate analyses in Fig. 1 indicate that at the population level, SPT reactivity to HDM is associated with positive *in vitro* cytokine responses to HDM extract comprising IL-4, IL-5, IL-9 and IL-3, together with IFN γ ; peak responses differed significantly between the SPT⁺ and SPT⁻ groups for IL-4, IL-5 and IL-9. Further analysis of these data by multiple logistic regression indicated that the most potent *in vitro* factor associated with a positive SPT response *in vivo* was the production of IL-9 mRNA (coefficient: 4.8334, SE: 1.504, t : 3.1377, P : 0.002).

Relationships between the magnitude of individual SPT responses (as measured by weal diameter) and cytokine responses were examined in more detail in Table 1. Of the 30 HDM-SPT⁺ children in the study, 29 exhibited HDM-induced weals $\geq$ 3 mm, and 15 of the group exhibited weals

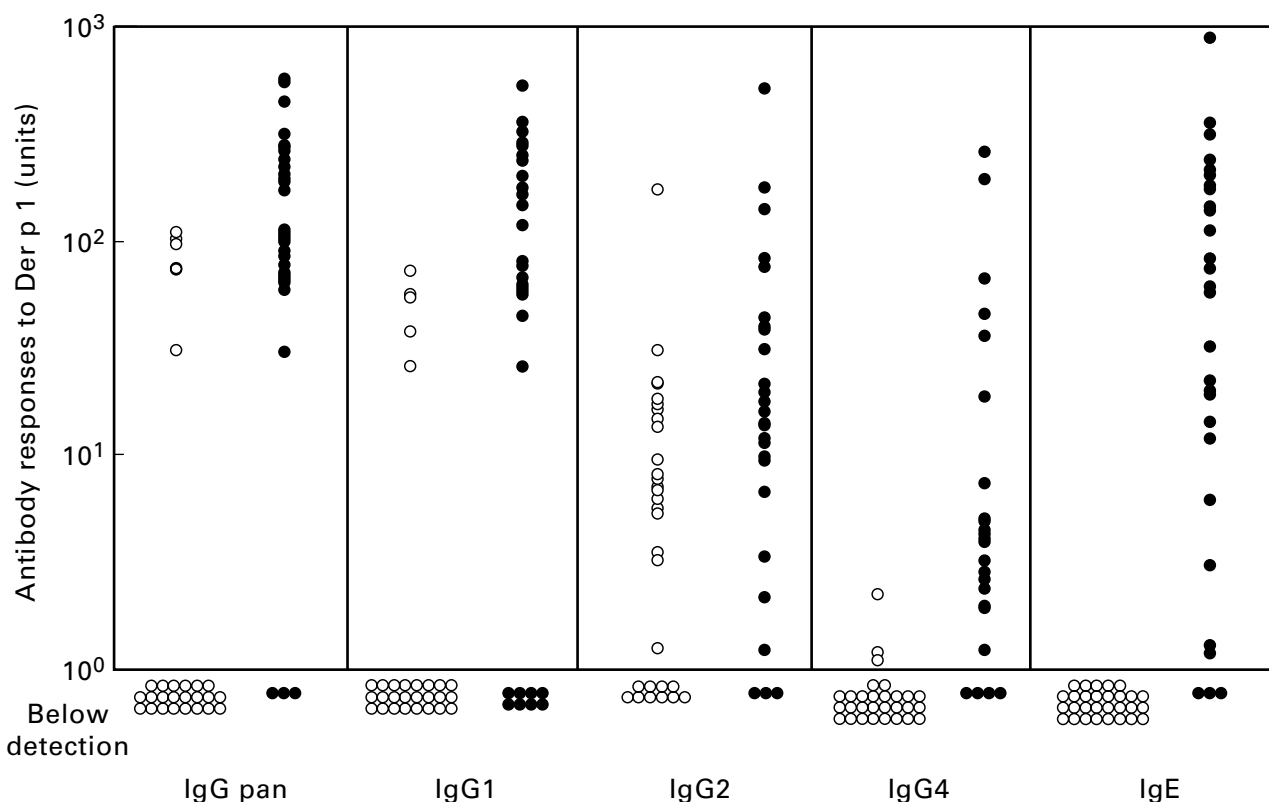


Fig. 3. Antibody titres specific for the HDM allergen Der p 1 in SPT⁺ (black circles) and SPT⁻ (white circles) children. Levels of antibodies were determined by ELISA as detailed in Methods. IgG subclasses are expressed in arbitrary units (AU) and IgE in SU/mL. The limits of detection were 60 AU for IgG pan, 50 AU for IgG1, 2.4 AU for IgG2, 0.4 for IgG4 and 1 SU/mL for IgE.

≥ 8 mm. The association between *in vitro* HDM-specific cytokine mRNA production and the HDM-SPT weal diameter within this group was analysed by multiple linear regression. The magnitude of the IL-4 response was strongly associated with weal diameter. Allergen-specific IL-13 and IL-5 responses were significantly but less strongly associated with weal diameter, and an inverse relationship was observed with respective IL-10 responses. In this model, IL-9 contributed only a small effect which was not statis-

tically significant. It should be noted that as with IL-9, the evidence for IL-4 production here is limited to mRNA, and these findings accordingly require verification at the protein level.

Discussion

Recent studies from our laboratory indicate that responsiveness to ubiquitous inhalant allergens typified by HDM is demonstrable in >85% of CB samples [12,15,23]. The strong TH2 polarity of these cytokine responses, which mirrors that of adult atopics, begs the question of why allergy in later life is the exception as opposed to the rule, within the population.

On the basis of patterns of allergen-specific lymphoproliferation and accompanying IL-4/IFN γ mRNA expression during infancy [15,16,23], we have suggested that the allergen responder phenotype expressed in late childhood and adulthood is determined principally by the changes occurring within these foetally primed T-cell responses during early childhood, which are driven by direct allergen exposure. The present study focuses in more detail on the

Table 1. Association between size of HDM-induced weal (in mm) and HDM-induced *in vitro* cytokine mRNA response using Multiple Linear Regression

	Value	SE	t-value	P-value
IL-4	4.93897	1.1032	4.477	0.0008
IL-5	2.54399	1.1862	2.145	0.05
IL-9	0.51699	2.2875	0.226	0.8269
IL-10	-3.4994	1.3881	-2.521	0.03
IL-13	3.72415	1.5755	2.364	0.04

status of these T-cell responses at the end of the preschool years.

Examination of responses to HDM reveals two distinct response patterns. Firstly, it should be noted that despite the presence of frank HDM-specific TH2 reactivity in PBMCs from a high proportion of infants [12], the frequency of HDM-specific SPT reactions under 6 months of age is usually zero (data not shown); we assume this is a reflection of the low precursor frequency of reactive cells in these early responses. However, by age 6 years, 30% of children exhibit HDM-SPT reactivity, with accompanying IgE and IgG subclass antibody production against Der p 1 (Fig. 3), and allergen-challenged PBMCs from these subjects exhibit the TH0 cytokine pattern comprising IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IFN γ . In contrast, the majority of the population (70%) remain HDM-SPT⁻ and have detectable IgG2 antibody to Der p 1 over a wide concentration range which overlaps that of their HDM-SPT⁺ counterparts. Specific cytokine responses in the SPT⁻ subjects involves IFN γ , IL-6 and IL-10 production at levels comparable to the SPT⁺ group, together with very low levels of IL-4 mRNA. It is pertinent to note in this context that IgG2 antibody production has been shown to be IFN γ dependent [24,25].

It is conceivable that the differences between SPT⁺ and SPT⁻ groups may stem in part from variations in the relative frequencies of HDM-specific T cells. However, parallel analyses of HDM-specific lymphoproliferative responses in the two groups indicated comparable levels of reactivity (SPT⁺: 4917 ± 1078 d.p.m./culture; SPT⁻: 4403 ± 1530), suggesting that the most biologically relevant differences between the groups are at the level of cytokine production patterns.

It is additionally possible that some of the differences in *in vitro* cytokine production between the groups may be related to the short sampling time (24 h in culture), which theoretically favours demonstration of 'early' as opposed to 'late' cytokines such as IFN γ . The restricted availability of cells precluded direct testing of this possibility in the present study.

Detailed statistical analysis of cytokine response patterns and SPT reactivity revealed additional new information relating to atopy expression in this age group. Of particular interest are the data in Fig. 1 on IL-9, which represents the first indirect evidence in humans of an association between allergen-specific production of this cytokine and *in vivo* allergen responsiveness.

A potentially important role for IL-9 in the pathogenesis of atopic disease is suggested by several recent publications. Notably, murine studies have implicated IL-9 in regulation of mast cell proliferation and maturation [26,27], and this cytokine synergizes with IL-4 in upregulation of IgE production by human [28] and murine [29] B cells. Interest in

the potential role of IL-9 in human atopy has also been fuelled by recent studies seeking genetic markers of disease. In particular, the human IL-9 gene lies in the 5q31.1-q33 region, adjacent to the IL-4/IL-5/IL-13 gene cluster, and linkage between markers in this region and a gene controlling total serum IgE concentration has been described [30]. There is also a recent claim of polymorphism in the promoter region of the IL-9 gene associated with variations in IgE levels [31], and in a mouse model of bronchial hyperresponsiveness IL-9 has been suggested as a potential risk factor for asthma [32].

In relation to the possible site(s) of action of IL-9 in this process, it is pertinent to note that despite the very strong association between *in vitro* HDM-induced IL-9 production and HDM-SPT positivity, the magnitude of the HDM-induced weal was not directly related to the size of the IL-9 response (Table 1). Instead, the key cytokine linked to weal diameter in these subjects was IL-4, and secondarily IL-5 and IL-13. These findings are consistent with the general hypothesis that the principal role of IL-9 may be in the inductive phase of atopic disease. It will be of interest to further test this hypothesis in different groups of atopics, particularly at later ages. It will also be important to formally demonstrate the production and secretion of IL-9 protein, once appropriate assay systems are developed, and until these data are available the physiological relevance of IL-9 will remain speculative.

A noteworthy additional finding from this analysis was the inverse relationship between weal diameter and HDM-specific IL-10 responses. This finding is consistent with recent reports indicating a generalized anti-inflammatory role for IL-10 (e.g. [33–35]), which includes inhibition of T-cell proliferation [36,37]. The situation in adult atopy is not yet clear, but at least one report has suggested deficient IL-10 production in the lung of atopic asthmatics [38]. It is also pertinent to note the presence of the IL-10 component in the response of the HDM-SPT⁻ subjects in this study; it is feasible that this may have a function in inhibiting inflammation which may ensue from production of IFN γ at challenge sites.

The results of this cross-sectional study, taken together with recent small prospective surveys [39,40], suggest that patterns of TH cell memory which determine allergen-responder phenotype in later life are developed during the period between birth and the commencement of primary school. Studies in adults [3,4] and recent data (above and [12]) from young children strongly suggest that 'normality' (viz. lack of atopy) is associated with capacity to upregulate TH1-polarized immunity post-natally, in order to avoid consolidation of allergen-specific TH memory in the TH2 compartment. Accumulating evidence (reviewed in [41]) suggests that atopic children may have a specific maturational defect in TH1 function, in particular capacity to

produce IFN γ , which underlies their relatively frequent failure to successfully immune deviate their foetally primed TH2 responses, instead boosting them to a level sufficient for expression of SPT reactivity. Further studies along the lines of those reported here, in particular prospective studies tracking the postnatal development of allergen-specific T-cell immunity in individual children, are required to further elucidate this process. Of particular interest in such studies is the question of the spectrum of SPT reactivity within individual atopic children, which is usually restricted to only a small subset of the allergens present within their immediate environment.

Acknowledgements

This work was supported by GlaxoWellcome and The National Health & Medical Research Council. The authors thank Lee Clohessy and Dr Kevin Blake, and the staff of the Western Australian Pregnancy Cohort Study for information relating to the phenotypes, and Jenny Tizard and Kristina Warstedt for skilled technical assistance in the lab programme.

References

- 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.
- Finkelman FD, Holmes J, Katona IM et al. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu Rev Immunol* 1990; 8:303–33.
- Wierenga EA, Snoek M, de Groot C et al. Evidence for compartmentalization of functional subsets of CD2⁺ T lymphocytes in atopic patients. *J Immunol* 1990; 144:4651–6.
- Romagnani S. Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? *Immunol Today* 1992; 13:379–81.
- Essayan DM, Han W-F, Xiao H-Q, Kleins-Tebbe J, Huang S-K. Clonal diversity of IL-4 and IL-13 expression in human allergen-specific T lymphocytes. *J Allergy Clin Immunol* 1996; 98:1035–44.
- 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.
- 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.
- Imada M, Estelle F, Simons R, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon- γ production in primary culture: responses of subjects with allergic rhinitis and normal controls. *Immunology* 1995; 85:373–80.
- Borish L, Rosenwasser L. TH1/TH2 lymphocytes: doubt some more. *J Allergy Clin Immunol* 1997; 99:161–4.
- Thepen T, Bihari IC, Wichen DF, van Reijssen FC, Mudde GC, Bruijnzeel-Koomen CAFM. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial Th2 to a Th1 response in situ: an immunohistochemical study. *J Allergy Clin Immunol* 1996; 97: 828–37.
- Holt PG. Primary allergic sensitisation to environmental antigens: perinatal T-cell priming as a determinant of responder phenotype in adulthood. *J Exp Med* 1996; 183:1297–301.
- Prescott SL, Macaubas C, Holt BJ et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T-cell responses towards the Th-2 cytokine profile. *J Immunol* 1998; 160:4730–7.
- Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; 14:353–6.
- Roth I, Corry DB, Locksley RM, Abrams JS, Litton MJ, Fisher SJ. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med* 1996; 184:539–48.
- 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.
- Yabuhara A, Macaubas C, Prescott SL et al. Th-2-polarised immunological memory to inhalant allergens in atopics is established during infancy and early childhood. *Clin Exp Allergy* 1997; 27:1261–9.
- Nakamura T, Kamogawa Y, Bottomly K, Flavell RA. Polarization of IL-4- and IFN- γ -producing CD4⁺ T cells following activation of naive CD4⁺ T cells. *J Immunol* 1997; 158:1085–94.
- Shaw G, Kamen R. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 1986; 46:659–67.
- Zhou L-J, Tedder TF. A distinct pattern of cytokine gene expression by human CD83⁺ blood dendritic cells. *Blood* 1995; 86:3295–301.
- Jenmalm MC, Holt PG, Björkstén B. Maternal influence of IgG subclass antibodies to Bet v 1 during the first 18 months of life as detected with a sensitive ELISA. *Int Arch Allergy Immunol* 1997; 114:175–84.
- Armitage P, Berry G. *Statistical Methods in Medical Research* 3rd edn. Oxford: Blackwell Scientific Publications, 1994.
- McCullagh P, Nelder JA. *Generalised Linear Models* 2nd edn. London: Chapman & Hall, 1989.
- 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.
- Inoue R, Kondo N, Kobayashi Y, Fukutomi O, Orii T. IgG2 deficiency associated with defects in production of interferon- γ ; comparison with common variable immunodeficiency. *Scand J Immunol* 1995; 41:130–4.
- Kondo N, Inoue R, Kasahara K et al. Reduced expression of the

- interferon-gamma messenger RNA in IgG2 deficiency. *Scand J Immunol* 1997; 45:227–30.
- 26 Hultner L, Druetz C, Moeller J et al. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur J Immunol* 1990; 20:1413–6.
- 27 Eklund KK, Ghildyal N, Austen KF, Stevens RL. Induction by IL-9 and suppression by IL-3 and IL-4 of the levels of chromosome 14-derived transcripts that encode late-expressed mouse mast cell proteases. *J Immunol* 1993; 151:4266–73.
- 28 Dugas B, Renauld JC, Pene 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.
- 29 Petit-Frere C, Dugas B, Braquet P, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. *Immunology* 1993; 79:146–51.
- 30 Marsh DG, Neely JD, Breazeale DR et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 1994; 264:1152–6.
- 31 Rosenwasser LJ, Klemm DJ, Dresback JK et al. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. *Clin Exp Allergy* 1995; 25:74–8.
- 32 Nicolaides NC, Holroyd KJ, Ewart SL et al. Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci USA* 1997; 94:13175–80.
- 33 de Vries JE. Immunosuppressive and anti-inflammatory properties of interleukin 10. *Ann Med* 1995; 27:537–41.
- 34 Zuany-Amorim C, Hailé S, Leduc D, Dumarey C, Huerre M, Vargaftig BB. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. *J Clin Invest* 1995; 95:2644–51.
- 35 Borish L. IL-10: evolving concepts. *J Allergy Clin Immunol* 1998; 101:293–7.
- 36 Del Prete G, De Carli M, Almerigogna F, Guidizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 1993; 150:353–60.
- 37 Groux H, Bigler M, de Vries JE, Roncarolo M-G. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4⁺ T cells. *J Exp Med* 1996; 184:19–29.
- 38 Borish L, Aarons A, Rumblyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996; 97:1288–96.
- 39 Piccinni M-P, Beloni L, Giannarini L et al. Abnormal production of T helper 2 cytokines interleukin-4 and interleukin-5 by T cells from newborns with atopic parents. *Eur J Immunol* 1996; 26:2293–8.
- 40 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.
- 41 Holt PG, Macaubas C. Development of long term tolerance versus sensitisation to environmental allergens during the perinatal period. *Curr Opin Immunol* 1997; 9:782–7.