

Polyclonal and allergen-induced cytokine responses in children with elevated immunoglobulin E but no atopic disease

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Summary

Background Reduced Th1 and elevated Th2 cytokine responses are considered to be a principal mechanism in the generation of the inflammation leading to the manifestations of atopic disease in the skin of atopic dermatitis and in the airways of asthma. If reduced Th1 and elevated Th2 responses are principal determinants of the manifestation of atopic disease it might be expected that subjects with established disease would exhibit differences in their cytokine profiles as compared with atopic patients without clinical disease.

Objective To determine whether asymptomatic atopic children exhibit a cytokine imbalance similar to that seen in patients with established atopic disease or if they behave like non-atopic controls. Cytokine responses in a group of children with elevated IgE but no clinical manifestations of disease, atopic children with established disease and non-atopic controls were compared.

Methods We examined allergen-induced (house dust mite, HDM, rye grass pollen and RYE) cytokine responses in parallel with polyclonal (staphylococcal enterotoxin B, SEB) cytokine responses in a group of children with elevated serum IgE levels without current or past evidence of atopic disease (median age 6.6 years) and compared these with a non-atopic control group (median age 6.5 years) and a group of children with atopic disease (median age 6.7 years).

Results Symptomatic atopic children had reduced SEB-induced IFN- γ and increased SEB-induced IL-4 and IL-5 as compared with non-atopic controls. In contrast, SEB-induced IFN- γ , IL-4 and IL-5 production in asymptomatic atopics was not significantly different from the non-atopic control subjects. Allergen-induced Th1 (IFN- γ) and Th2 (IL-5 and IL-13) cytokine production was increased in both symptomatic atopics and asymptomatic atopics when compared with non-atopic controls.

Conclusion The defect in polyclonally induced IFN- γ production was associated with the clinical manifestation of atopic disease but not the atopic state *per se*. This suggests that the global reduction in IFN- γ is the key determinant of the development of overt atopic disease. In contrast, elevated allergen-induced Th2 cytokine responses in children related to the atopic state *per se* irrespective of the presence of clinical atopic disease.

Keywords allergens, atopy, childhood, cytokines, house dust mite, interferon- γ , interleukins-4/-5/-13, rye grass pollen, superantigen

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Introduction

A cytokine imbalance involving reduced T helper 1 (Th1), i.e. interferon-gamma (IFN- γ), and increased Th2, i.e. interleukin-4, -5 and -13 (IL-4, IL-5, IL-13), cytokines has been postulated to be a major factor determining the inflammatory response that leads to the development of atopic disease [1–3]. Cytokine imbalances in atopic disease can be evaluated in two ways. Allergen-induced T cell responses provide information on cytokine production by sensitized T cells that may be influenced by prior antigen exposure, whereas stimulation of T cells by

non-specific mitogens examines production of cytokines from a wide range of T cells. A defect in polyclonally induced IFN- γ production has been previously demonstrated by ourselves and others in the cord blood of patients who went on to develop atopic disease [4, 5]. Persistence of neonatal Th2 skewed allergen-induced cytokine responses beyond the first year of life has been shown to be associated with the subsequent development of atopic disease [6]. It is considered that allergen-driven Th2 cytokine responses are a principal mechanism in the generation of the inflammation leading to the manifestation of atopic disease in the skin of atopic dermatitis and in the airways of asthma [7–14]. If reduced Th1 and elevated Th2 allergen-induced responses are principal determinants of the manifestation of atopic disease it might be expected that subjects with established disease would exhibit differences in their cytokine profiles as compared with atopic patients without clinical disease.

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Most studies of cytokine production in atopic disease have compared children with atopic disease with a healthy control group. To date there have been no reports investigating asymptomatic atopic children with elevated total and specific IgE who had no clinical manifestations of atopic disease. Thus the evaluation of asymptomatic atopic children provides an opportunity to identify key factors determining the manifestations of clinical atopic disease. Furthermore, it allows comparison of polyclonal and allergen-induced cytokine responses. We have examined allergen-induced and polyclonal cytokine responses in a group of children with elevated serum IgE levels without a current or past history of atopic disease and compared these with a group of children with elevated serum IgE and atopic disease, and a group of non-atopic controls.

Materials and methods

Reagents

Reagents used: AIM-V medium (Gibco, Grand Island, NY, USA); B-mercaptoethanol (B-ME) (ICN, Costa Mesa, CA, USA); fetal calf serum (FCS) (Gibco); staphylococcal enterotoxin B (SEB) (Sigma Chemical Co., St Louis, MO, USA); avidin-peroxidase (Sigma Chemical Co.); Ficoll-Hypaque (Pharmacia, Uppsala, Sweden); monoclonal antibodies (MoAbs), anti-IFN- γ , biotinylated anti-IFN- γ , anti-IL-4, biotinylated anti-IL-4, anti-IL-5, biotinylated anti-IL-5, anti-IL-13, biotinylated anti-IL-13, recombinant human (rh)-IFN- γ , rh-IL-4, rh-IL-5, rh-IL-13 (Pharmingen, San Diego, CA, USA).

Patients

In a study comparing children with atopic disease with non-atopic children we recruited a group of 53 children selected as potential control subjects who were admitted for elective surgery (circumcisions, herniotomies, orchidopexies, squint repairs or orthopaedic procedures) and had no history of atopic disease. In this group we identified 23 children who had raised total and allergen-specific IgE but no current or past history of atopic disease when assessed by parental interview and questionnaire specifically inquiring into current or past symptoms or signs of eczema, asthma, rhinitis, and food or drug allergy. We called these children asymptomatic atopsics and age matched them with an equal number of non-atopic healthy children and children with established atopic disease.

Heparinized venous blood samples were obtained from:

- Twenty-three children (asymptomatic atopsics) with no evidence of atopic disease (median age 6.6 years, range 8 months to 16 years) who had elevated IgE levels for age (median IgE 175 kU/L) and a positive radioallergosorbent test (RAST) to one or more of egg white ($n = 4$), house dust mite ($n = 18$) and rye grass pollen ($n = 6$).
- Twenty-three healthy children (non-atopsics) were selected on the basis of nearest birth date to the subjects in the asymptomatic atopic group (median age 6.5 years, range 9 months to 15 years 4 months). These were patients who were admitted for elective surgery (circumcisions, herniotomies, orchidopexies, squint repairs or orthopaedic procedures) at the Royal Children's Hospital. These children had no history of atopy, plasma IgE levels within the normal range for

age (median IgE 24 kU/L) and negative allergen-specific IgE for egg white, house dust mite and rye grass pollen. Blood was obtained immediately on anaesthetic induction.

- Twenty-three children with atopic disease (symptomatic atopsics) were selected from a larger group of 71 atopic children, who had studies of cytokine secretion and attended the allergy clinics of the Royal Children's Hospital, on the basis of nearest birth-date to the subjects from the asymptomatic atopic group (median age 6.7 years, range 9 months to 16 years 3 months). These patients had elevated IgE levels for age (median IgE 428 kU/L) and a positive radioallergosorbent test (RAST) to one or more of the three allergens tested, i.e. egg white ($n = 8$), house dust mite ($n = 21$) and/or rye grass pollen ($n = 18$). Manifestations of atopic disease in these patients were eczema ($n = 8$), asthma/rhinitis ($n = 8$) or both ($n = 7$).

Children with atopic disease were matched according to IgE levels with asymptomatic atopic children giving 15 in each group. Subjects with total IgE levels < 200 kU/L were matched to a mean of plus or minus 6 kU/L (range 0–20) and those > 200 kU/L were matched to plus or minus 13% (range 4–22%). The mean IgE levels in the symptomatic group were 340 kU/L (SD 329 kU/L), and in the asymptomatic group they were 334 kU/L (SD 315 kU/L). The mean age in the symptomatic group was 6.4 yrs (SD 3.6 yrs), and in the asymptomatic group it was 7.4 yrs (SD 3.6 yrs).

Informed consent was obtained from a parent/guardian of all subjects. This study was approved by the Ethics Committee of the Royal Children's Hospital, Melbourne.

Allergen preparation

House dust mite (HDM) (*Dermatophagoides pteronyssinus*) (CSL Australia) was prepared according to the method of Stewart et al. [15]. Briefly, crude dried house dust mite (HDM) was homogenized in PBS, clarified by centrifugation, dialysed in 0.05M ammonium bicarbonate overnight, freeze dried and stored in aliquots at -70°C . Aliquots were reconstituted in PBS, filter sterilized (pore size $2\mu\text{m}$) and stored at -70°C until use in cell culture.

Rye grass pollen extract (RYE) (*Lolium perenne*, Greer Laboratories, Lenoir, NC, USA) was prepared by C. Suphioglu (Melbourne, Australia) according to previously published methods [16].

Plasma IgE & RAST

Total IgE and allergen-specific IgE levels were determined by UniCAP total IgE and specific IgE fluoroenzyme immunoassays (Pharmacia & Upjohn Diagnostics Uppsala, Sweden). Levels of total IgE are expressed in kU/L with the normal ranges for age: 0.5 to 0.7 years, 0–20 kU/L; 0.7 to 1 year, 0–25 kU/L; 1 to 2 years, 0–35 kU/L; 2 to 4 years, 0–130 kU/L; 4 to 7 years, 0–150 kU/L; > 7 years, 0–200 kU/L [17]. Allergen-specific IgE levels are expressed as kU/L, with levels above 0.7 kU/L considered positive.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. The PBMC layer was washed three times with

sterile PBS. PBMC (1×10^6 cells) were cultured in a volume of 1000 μ L in AIMV supplemented with B-ME (5×10^{-6} M) at 37 °C in a humidified atmosphere with 5% CO₂. PBMCs were stimulated with SEB (1 μ g/mL), purified allergen (HDM 10 μ g/mL, RYE 20 μ g/mL) or left unstimulated. Preliminary experiments using a range of allergen concentrations (1, 10, 100 and 1000 μ g/mL) determined that these concentrations were optimal for cytokine production.

Cytokine production

Time course experiments revealed SEB induced maximal IL-4, IL-5, IL-13 and IFN- γ production at 48 h and allergens induced maximal IL-5, IL-13 and IFN- γ production at 6 days. Cell cultures were centrifuged and cell free supernatants were collected at these time points and stored at -70 °C. INF- γ , IL-4, IL-5 and IL-13 were assayed by sandwich ELISA as previously described [18]. Briefly, high binding ELISA plates were coated with purified capture MoAb (anti-IFN- γ , anti-IL-4, anti-IL-5 or anti-IL-13) at a concentration of 1 μ g/mL in carbonate buffer pH 9.6 and incubated overnight at 4 °C. Plates were blocked with PBS-Tween-10% fetal calf serum (FCS) for 2 h. Samples were incubated at room temperature for 4 h. After washing, biotinylated MoAb (anti-IFN- γ , anti-IL-4, anti-IL-5 or anti-IL-13) (0.5 μ g/mL) was added and incubated at room temperature for 45 min, followed by avidin-peroxidase (1:400 of 1 mg/mL) for 30 min. After final washing, TMB substrate (KPL, Gaithsburg, MD, USA) was added and reaction stopped by addition of H₂SO₄. Plates were read at 450 nm on a spectrophotometer.

All standards and samples were performed in duplicate, and data were analysed using Biomek 1000 data reduction software (Beckman Fullarton, CA, USA). Minimum detection levels were calculated by Biomek 1000 software and were IFN- γ 10 pg/mL, IL-4 2 pg/mL, IL-5 10 pg/mL and IL-13 5 pg/mL.

Statistical analysis

SEB- and allergen-induced cytokine levels are presented as boxplots displaying medians, interquartile ranges, adjacent values and outliers. As SEB- and allergen-induced cytokine levels were not normally distributed the significance of the difference in the data was examined by the Mann-Whitney non-parametric *U*-test using Stata statistical software: release 5 (StataCorp 1997, College Station, Texas, USA).

Results

Superantigen-induced cytokine production

The symptomatic atopics had significantly reduced SEB-induced Th1 (IFN- γ) and increased Th2 cytokine (IL-4 and IL-5) production as compared with non-atopics (Figs 1a, b and c). Cytokine production: symptomatic atopic vs. non-atopic, median (pg/mL); IFN- γ , 6808 vs. 12300, $P < 0.001$ (Fig. 1a); IL-4, 73 vs. 36, $P < 0.001$ (Fig. 1b); IL-5, 385 vs. 205, $P = 0.04$ (Fig. 1c). There was no significant difference in IL-13 production (757 vs. 379, $P > 0.05$) (Fig. 1d). Cytokine production in the asymptomatic atopics was not significantly different from

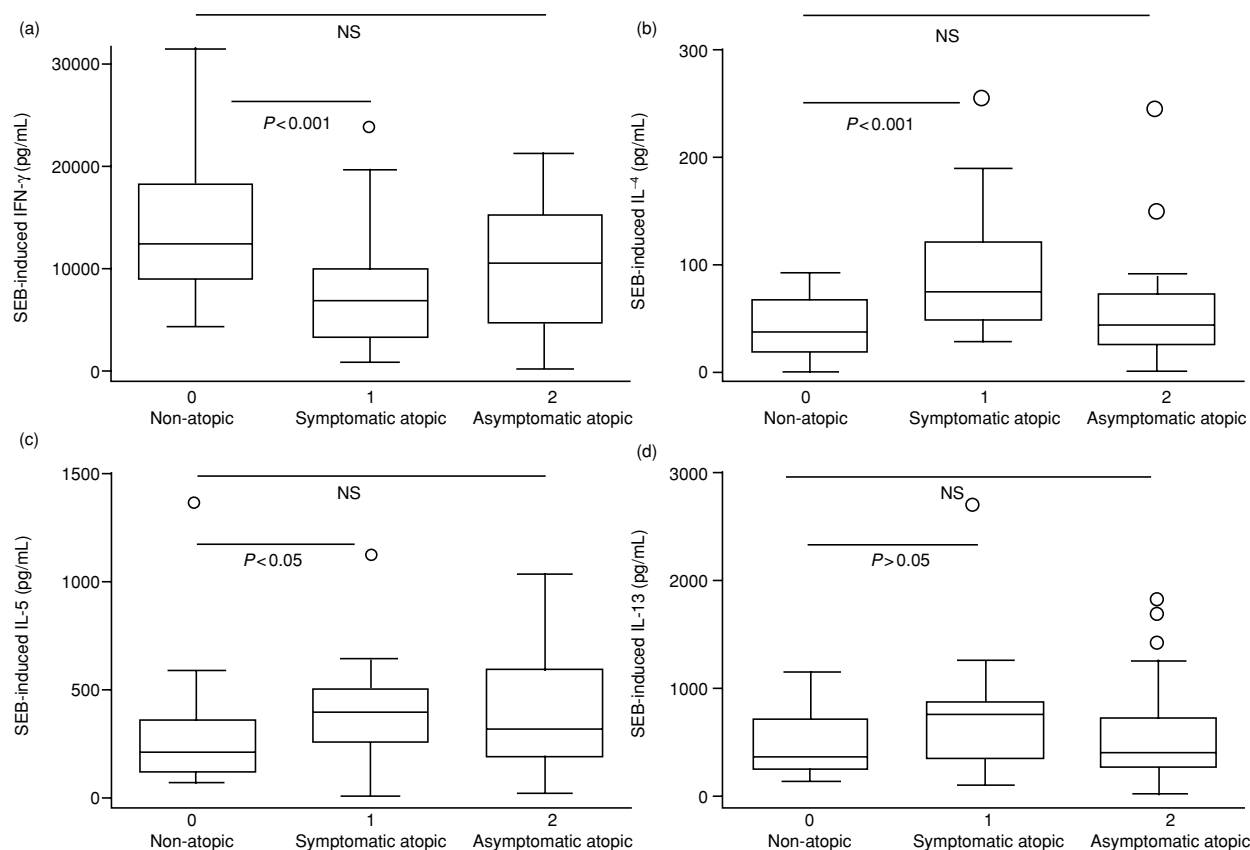


Fig. 1. SEB-induced cytokine responses. SEB-induced production of (a) IFN- γ , (b) IL-4, (c) IL-5 and (d) IL-13 was measured in non-atopics, symptomatic atopics and asymptomatic atopics. *P*-values for comparison of symptomatic and asymptomatic atopics with controls are shown. $n = 23$ in each group.

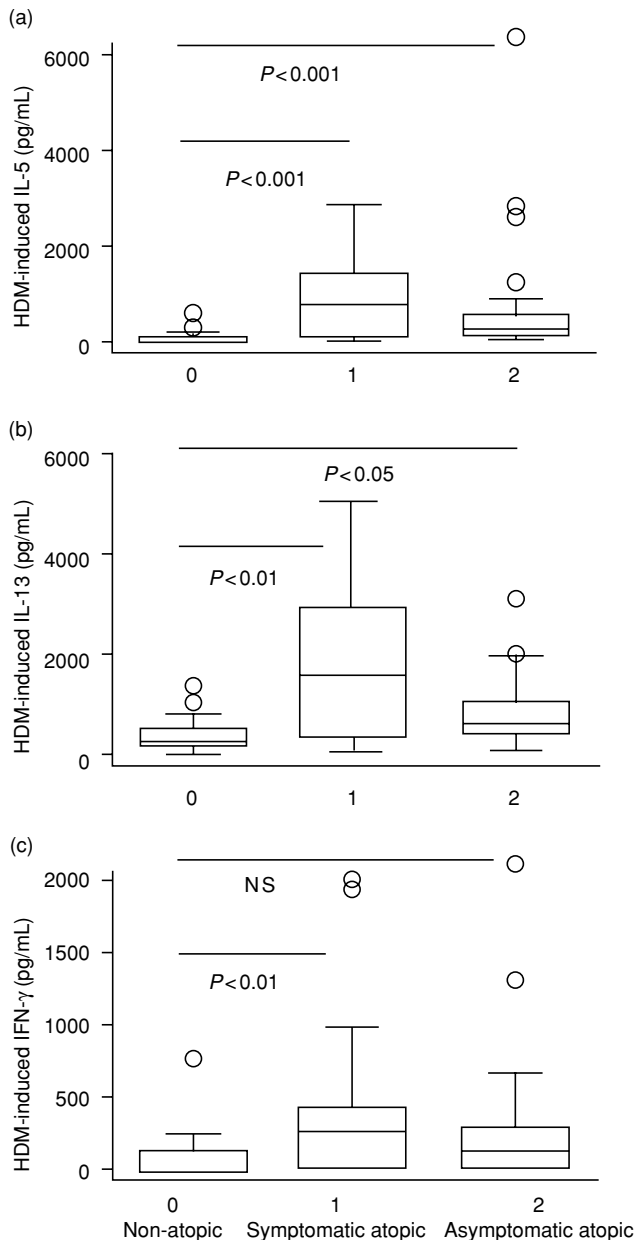


Fig. 2. HDM-induced cytokine responses. HDM-induced production of (a) IL-5, (b) IL-13 and (c) IFN- γ was measured in non-atopics, symptomatic atopics and asymptomatic atopics. *P*-values for comparison of symptomatic and asymptomatic atopics with controls are shown. $n = 23$ in each group.

the non-atopics: IFN- γ , 10362 vs. 12300, $P = 0.08$ (Fig. 1a); IL-4, 42 vs. 36, $P = 0.5$ (Fig. 1b); IL-5, 302 vs. 205, $P = 0.1$ (Fig. 1c); IL-13, 382 vs. 379, $P > 0.05$ (Fig. 1d).

To examine whether the cytokine differences related solely to the higher mean IgE levels in the symptomatic group we matched 15 of the symptomatic and asymptomatic atopics with respect to IgE levels (mean IgE symptomatic 340 kU/L (SD 329 kU/L), asymptomatic 334 kU/L (SD 315 kU/L)) and compared these with 15 age-matched controls. The findings were the same as that observed for the entire cohorts with significantly reduced SEB-induced Th1 (IFN- γ) and increased Th2 cytokine (IL-4 and IL-5) production as compared to

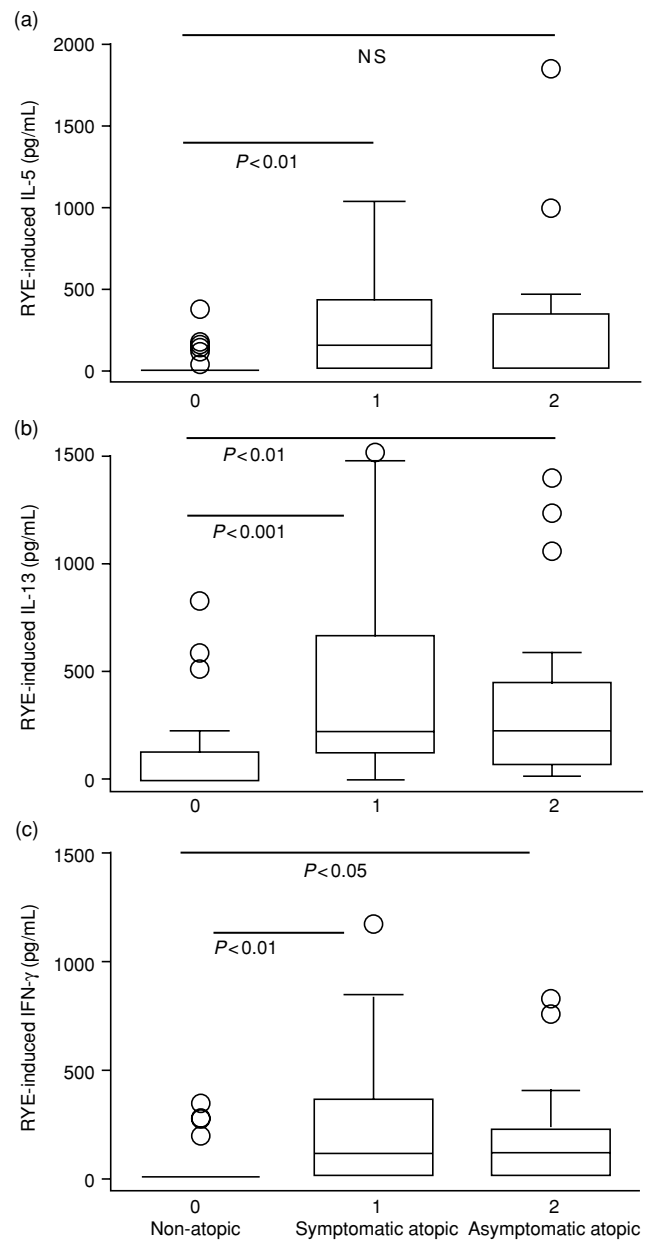


Fig. 3. RYE-induced cytokine responses. RYE-induced production of (a) IL-5, (b) IL-13 and (c) IFN- γ was measured in non-atopics, symptomatic atopics and asymptomatic atopics. *P*-values for comparison of symptomatic and asymptomatic atopics with controls are shown. $n = 23$ in each group.

non-atopics for the symptomatic group (symptomatic atopic v non-atopic, IFN- γ : $P = 0.007$; IL-4: $P = 0.004$; IL-5: $P = 0.04$ but no significant differences between controls and the asymptomatic group (IFN- γ : $P = 0.2$; IL-4: $P = 0.4$; IL-5: $P = 0.6$).

Allergen-induced cytokine responses

Allergen-induced Th2 (IL-5 and IL-13) cytokine production was increased in both symptomatic atopics and asymptomatic atopics when compared with non-atopics for HDM and RYE (Figs 2a, b and 3a, b). Cytokine production: symptomatic atopic vs. non-atopic, median (pg/mL); HDM-IL-5, 745 vs. 0,

$P < 0.001$ (Fig. 2a); HDM IL-13, 1544 vs. 255, $P = 0.003$ (Fig. 2b); RYE-IL-5, 156 vs. 0, $P = 0.002$ (Fig. 3a); RYE-IL-13, 222 vs. 0, $P < 0.001$ (Fig. 3b); asymptomatic atopic vs. non-atopic HDM-IL-5, 210 vs. 0, $P < 0.001$ (Fig. 2a); HDM IL-13, 541 vs. 255, $P = 0.01$ (Fig. 2b); RYE-IL-5, 0 vs. 0, $P = 0.09$ (Fig. 3a); RYE-IL-13, 220 vs. 0, $P = 0.003$ (Fig. 3b). In the group of subjects matched for IgE levels allergen-induced Th2 (IL-5 and IL-13) cytokine production was also significantly increased in both symptomatic atopics and asymptomatic atopics when compared with non-atopics. (Cytokine production: symptomatic atopic vs. non-atopic, HDM-IL-5; $P < 0.001$) HDM IL-13 ($P = 0.02$); RYE-IL-5 ($P = 0.01$); RYE-IL-13 ($P = 0.001$); asymptomatic atopic vs. non-atopic HDM-IL-5 ($P = 0.002$); HDM-IL-13 ($P = 0.02$); RYE-IL-5 ($P = 0.5$); RYE-IL-13 ($P = 0.05$). Comparison of allergen-induced cytokine production between the symptomatic and asymptomatic atopics showed there was no significant difference in IL-5 or IL-13 production between these two groups. IL-4 production was generally very low or below the lower limit of detection.

Allergen-induced IFN- γ production was increased in the symptomatic atopics when compared with non-atopics for both HDM and RYE. Cytokine production: symptomatic atopic vs. non-atopic, median (pg/mL); HDM-IFN- γ , 253 vs. 0, $P = 0.003$ (Fig. 2c); RYE-IFN- γ , 103 vs. 0, $P = 0.007$ (Fig. 3c). In the asymptomatic atopics there was increased IFN- γ production to RYE and HDM, with the increase in RYE-induced IFN- γ being significantly higher than controls. Cytokine production: asymptomatic atopic vs. non-atopic, median (pg/mL); HDM-IFN- γ , 99 vs. 0, $P = 0.07$ (Fig. 2c); RYE-IFN- γ , 99 vs. 0, $P = 0.03$ (Fig. 3c). No significant difference in allergen-induced IFN- γ production was apparent between the two atopic groups.

Discussion

The principal findings of this study are: (i) the allergen-induced cytokine profile of a group of atopic children without atopic disease was similar to that of children with established atopic disease; and (ii) the cytokine profile in response to superantigen in atopic children without atopic disease was not significantly different from control subjects. This suggests that a Th2 response to allergen is associated with the atopic state but does not necessarily lead to the development of atopic disease. In contrast a global skewing of cytokine responses, with reduced Th1 and increased Th2, was only evident here with the presence of established disease. It is possible that the asymptomatic atopics and the symptomatic atopics represent two distinct patient groups, with the latter group being defined by the presence of a global skewing of cytokine responses that determines the manifestation of disease. This is supported by the demonstration that infants who develop atopic disease had a global defect of IFN- γ production at birth prior to the onset of disease [4, 5]. The decreased Th1 and increased Th2 responses to the polyclonal stimulus seen in the symptomatic but not asymptomatic group were not merely a reflection of the somewhat higher median IgE levels in the symptomatic group as these differences remained after comparison of the two groups matched for IgE levels.

An alternative to be considered is that the asymptomatic atopic patients are in a pre-symptomatic phase of their disease

and represent the same population as the symptomatic atopics. However, this would seem unlikely in the light of previous evidence that a global defect of IFN- γ production identified by polyclonal stimulation predates the development of atopic disease [4, 5] and this was not found in the asymptomatic atopic group. Longitudinal cohort studies indicate that the great majority of subjects who develop symptoms will do so by the age of 7 years [19, 20]. As the mean age of the asymptomatic atopic group was 6.6 years it might be expected that these subjects would be likely to demonstrate some manifestations of allergic disease at the time of testing if they were going to develop atopic disease in childhood. Whilst there is a considerable overlap in total IgE levels between atopic and non-atopic children [21], the detection of specific IgE to common allergens is widely used to define a subject as atopic [21]. Other studies have suggested that there is a significant group of atopic children who do not develop atopic symptoms. Saarinen, in a prospective study from birth to 5 years, found that 20% of asymptomatic children at 5 years of age had laboratory features (eosinophilia) or a positive skin prick test suggestive of atopy [22]. Sigurs found that 17 to 18% of children with evidence of specific IgE antibodies in early childhood had not developed any atopic disease when followed to 15 years of age [20]. These studies describe a cohort of children with serological features of atopy who have not developed atopic disease by the age of 5 years and remain free of atopic disease in later childhood. We conclude that it is likely that our asymptomatic atopic group is a similar cohort.

There are a number of studies comparing children with atopic disease with non-atopic controls that demonstrate increased allergen-induced Th2 cytokine production [7–9, 11–14, 23]. From such studies it has often been considered that this increased Th2 response results in inflammation leading to atopic disease. However, none of these studies have examined asymptomatic atopic children without atopic disease. The observation that the asymptomatic atopic children exhibited increased Th2 cytokine secretion to inhalant allergens raises the possibility that increased allergen-induced Th2 responses are not the principal factor that determines the development or maintenance of symptoms. However, the critical factor(s) that determine the presence or absence of clinical disease are not known. Consistent with these present findings we have recently demonstrated that resolution of asthma in a prospectively followed cohort of asthmatics from childhood into adulthood was not associated with a reduction in allergen-induced Th2 cytokine production [24]. A recent study that found that a monoclonal antibody against IL-5 did not change asthma symptoms while successfully reducing eosinophilia would also suggest that factors in addition to a Th2 inflammatory response are required for the development and maintenance of symptoms [25].

We have demonstrated a dichotomy between the allergen-induced and polyclonally induced cytokine responses in the same individuals. Asymptomatic atopics demonstrated abnormal allergen-induced cytokine responses but normal polyclonally induced cytokine responses. Furthermore, symptomatic atopics demonstrated increased allergen-induced IFN- γ production but reduced polyclonally induced IFN- γ production. Previous studies have reported a similar finding with increased allergen-induced IFN- γ production in childhood atopics with clinical disease [8, 23, 26, 27]. This highlights the notion that allergen-induced T cell responses provide information on cytokine production by sensitized T cells that may be influenced

by prior antigen exposure, whereas stimulation of T cells by non-specific mitogens or superantigens examines production of cytokines from a wider range of T cells and may reflect an underlying predisposition not necessarily influenced by allergen exposure. It is likely that the increased allergen-induced IFN- γ production reflects an expansion of allergen-specific T cell populations in atopic patients, with activation of greater numbers of T cells following allergen stimulation. As a reduced polyclonally induced IFN- γ response in the cord blood is associated with the subsequent development of atopic disease [4, 5] the polyclonal stimulus appears to reveal a more fundamental defect necessary for the development of atopic disease.

In conclusion, in a population of children with elevated IgE but without manifestations of atopic disease we have demonstrated increased allergen-induced Th2 and Th1 cytokine responses similar to those seen in atopic children with established allergic disease. However, this same group of patients did not exhibit a similar alteration in their polyclonal cytokine responses, with their responses being similar to the non-atopic controls. Thus, allergen-induced Th2 cytokine responses relate to the atopic state (i.e. the presence of elevated serum total and specific IgE) *per se* and not necessarily to the presence of atopic disease. In contrast, the polyclonally induced defect in IFN- γ was associated with the clinical manifestations of atopic disease, suggesting that this defect is in some way fundamentally related to their development.

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