

T-cell “priming” against environmental allergens in human neonates: sequential deletion of food antigen reactivity during infancy with concomitant expansion of responses to ubiquitous inhalant allergens

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The study below comprises prospective analysis of patterns of allergen-specific T-cell reactivity in a cohort of 23 children bled at regular intervals from 6–10 weeks to 2 years of age, together with cross sectional studies on panels of cord and adult blood samples. The results indicate reciprocal patterns of responses to dietary and inhalant allergens, the former being frequent in infancy but rare in adults, whereas the latter are preserved and expand between infancy and adulthood. These findings are consistent with a recently proposed model for the development of immunity to environmental allergens which involves allergen-driven T-cell “selection” during early life leading to deletion of food allergen-specific T-cells via the induction of specific anergy, with concomitant selection and ultimately expansion of mutually exclusive TH-1-like or TH-2-like reactivity to inhalant allergens via Immune Deviation mechanisms.

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The immune responses which underlie the expression of allergic symptoms in adulthood, are now believed to be initiated in infancy and early childhood (1, 2). The most direct evidence in support of this suggestion comes from prospective studies on the kinetics of postnatal appearance of allergen-specific serum antibodies, which have unequivocally demonstrated that presence of both IgE (3, 4) and IgG subclass antibodies (5, 6) to both dietary and inhalant allergens, by the age of 1–2 yrs. These responses which (in the case of IgE) are often transient (4), occur to varying degrees in infants both with and without family history of atopy, and provide a clear indication of underlying allergen-specific T-helper (TH) cell reactivity to these allergens, during early postnatal life (2).

The biphasic (sometimes seesawing) nature of these serum IgE responses (3, 4), which closely parallel those seen in experimental animal models of primary immune responses to mucosally-delivered allergens (7), strongly suggest an underlying process of cross-regulation between competing TH-cell populations (2). Based upon recent findings from adult human T-cell cloning studies, which demonstrate that both normal and atopic subjects contain allergen specific TH cells (albeit from different functional subsets) in PMBC (8–10), it has been proposed that the T-cell responses to these allergens during infancy are part of an on-going process of TH-cell “selection” which is driven by normal environmental allergen exposure (2). Moreover, it is hypothesised that the result of this selection process,

Table 1. Allergen-specific T-cell responses in cord blood. Data shown are mean CPM from replicate cultures, in a sample of 7 randomly selected cord bloods (CB). The individual allergens employed (and their respective concentrations are shown in the body of the Table

	Dose µg/ml	CB1	CB2	CB3	CB4	CB5	CB6	CB7
Background		815	1233	783	1973	868	4479	3146
HDM	10	1009	*3465	*3010	2164	*3067	3471	4098
WRE	30	*2439	1995	1429	2984	535	6025	5772
OVA	100	*6846	*5049	*2274	*6467	*3025	1754	5714
DerP1	10	1038	738	*2248	1321	890	*9765	5028
DerP1	30	1159	1450	*1611	1478	*4067	2025	*6720
DerP2	10	811	917	*2572	1907	*4291	*9525	3882
DerP2	30	*7622	*6563	*2923	*11073	*3227	7143	3876
LolP1	10	1182	2072	*2871	2084	*2909	*12366	2894
LolP1	30	823	461	1760	1255	363	1959	3166
TT	# 0.5	907	890	934	1897	890	3236	4134

* response (Δ cpm > 1000; SR > 2.0)

Lf/ml

in the form of the functional phenotype of the TH-cell subset that eventually establishes dominance of immunological "memory" for individual allergens, ultimately determines allergen-responder phenotype throughout later life (2).

At present, the key aspects of this hypothesis which relate to an allergen-specific immune responsiveness during the neonatal period, rest almost exclusively upon indirect evidence i.e. the downstream effects of the TH-cell activity, manifesting as antibody titres in serum. The study reported below constitutes the first attempt to systematically study the kinetics of development of TH-cell reactivity *per se* to individual allergens, in groups of subjects spanning from the neonatal period through to adulthood. These experiments have been made feasible by recent improvements in the efficiency of antigen-specific T-cell proliferative methodology, achieved by the use in our laboratory of a culture system based upon serum-free medium, which markedly increases capacity to detect TH-cell responses at the low precursor frequencies expected in neonates (11).

Material and methods

Subjects

Blood samples were collected after obtaining informed consent from subjects or their parents. Three subject panels were employed, comprising (i) a group of randomly selected cord blood samples (no information available on history); (ii) a cohort of 22 infants with a family history (≥ 1 first degree relative) of atopy; and (iii) a group of 30 adults who were part of an ongoing study on the relationship between serum IgE levels and TH-cell reactivity in PBMC, of which approximately half were allergic by RAST or skin test to one or more inhalant allergens. The infant cohort were bled at ages 6–10

weeks, 6 months, 1 year and 2 years; samples were obtained from $\geq 75\%$ of the group on each occasion.

Antigens

Crude house dust mite (HDM) and whole rye (WRE) extracts were gifts from Dr G Stewart, and were prepared respectively from *Dermatophagoides pteronyssinus* and rye pollen obtained from CSL Limited (Melbourne, Australia). Native DerP1 and DerP2 were prepared by affinity chromatography as per (12). Affinity purified LolP1 was obtained from Dr B Knox (University of Melbourne, Australia). Ovalbumin was purchased from Sigma Chemicals (St Louis, USA), and tetanus toxoid (TT) was obtained from CSL Limited. On the basis of preliminary screening experiments with PBMC from normal and atopic subjects, the antigens were used at the concentrations shown in Table 1.

T-cell stimulation *in vitro*

Blood was collected into an equal volume of RPMI medium containing preservative free heparin. Peripheral blood mononuclear cells (PBMC) were isolated on a Lymphoprep density gradient (Nycomed, Norway), and washed three times in RPMI containing 2% fetal calf serum. PBMC from the cohort subjects were cryopreserved by standard methodology in DMSO/fetal calf serum, and analysed in batches at the end of the collection period; in our hands, cryopreservation of this nature has only marginal effects ($\leq 25\%$ reduction) on *in vitro* allergen-specific T-cell responses. PBMC from cord samples and from adults were analysed fresh.

Cultures were established in triplicate, comprising 2×10^5 cells in 200 μ l AIM-V serum free medium (Gibco, USA), supplemented with 2×10^{-5} M 2-

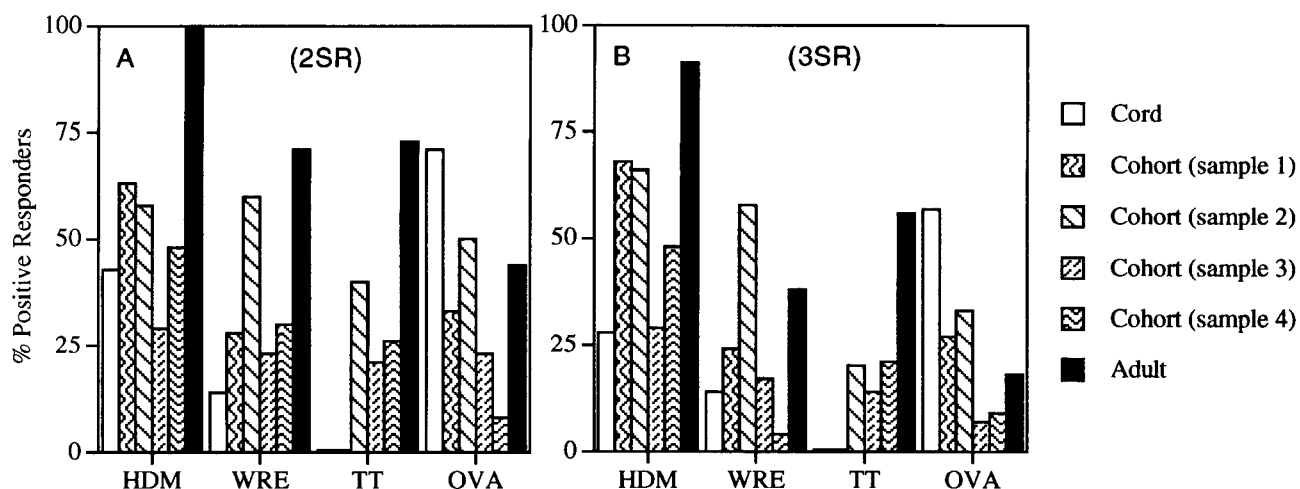


Fig. 1. Age related variations in frequency of T-cell reactivity to a panel of allergens. PBMC from a group of randomly selected cord bloods, and from a cohort of infants bled repeatedly over a two year period (sample #1 at 6–10 weeks age, #2 at 6 months, #3 at 1 year, #4 at 2 years), plus 30 adults, were stimulated *in vitro* with antigens, as detailed in Material and methods. Data shown represent the % positive responders in each age group employing a cutoff of $SR \geq 2.0$ (panel A) or $SR \geq 3.0$ (panel B).

mercaptoethanol, in round bottom microplate wells (11). ^3H -DNA synthesis was determined after 7 days in culture, and expressed as mean counts per minute (CPM) per culture $\pm$ sd. A figure for *stimulation ratio* (SR), defined as the ratio of CPM test culture (containing allergens) $\div$ CPM background control (antigen free), was then derived for each allergen; test replicates expressing $SR \geq 2.0$ (or in some cases

≥ 3.0), accompanied by a nett Δ response (i.e. in excess of respective background control) $\geq 1,000$ CPM, were taken as indicative of positive responses.

Results

Frequency of allergen-specific T-cell responses in populations of varying ages

The percentage positive responders to each allergen/antigen are shown in Fig. 1; these data were derived employing the conventional cut-off of $SR \geq 2.0$ (Fig. 1A), and also the more conservative criterion of $SR \geq 3.0$. Responses to the vaccination antigen TT were absent in the cord and the 6–10 week old bleed, appearing first in the 6 month sample, and were highest (frequency approx. 75%) in adult samples.

In contrast, responses to the inhalant allergen HDM was present at relatively high frequency in the cord samples, fluctuated between 30–60% positive between 6 months – 2 years, and was present in all adults. WRE responses were present in a small number of cords, and the frequency in the infant cohort peaked at the 6 month bleed (60%), declining to 30% at the 2 year old bleed; rye responses were detected in 70% of adult samples.

The reverse pattern was observed for responses to the food allergen OVA. The peak response (by frequency) was observed in cord samples, declining thereafter throughout childhood, and reactivity was relatively infrequent in adults.

Plotting the data from infants in the cohort individually (not shown) revealed that responses in some children fluctuated between the positive and negative range during the course of the study, particularly for HDM and WRE.

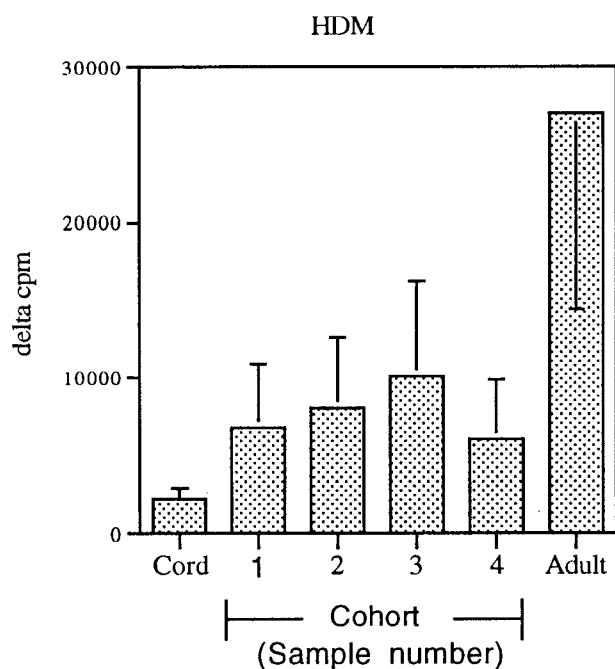


Fig. 2. Age related variations in the magnitude of T-cell responses to HDM. The positive HDM responders from Fig. 1 were segregated from the overall population, and analysed as a group. Data shown are mean $\pm$ sd of positive responses to HDM at each age. Results are expressed as delta (Δ) CPM from replicate cultures, as detailed in Material and methods.

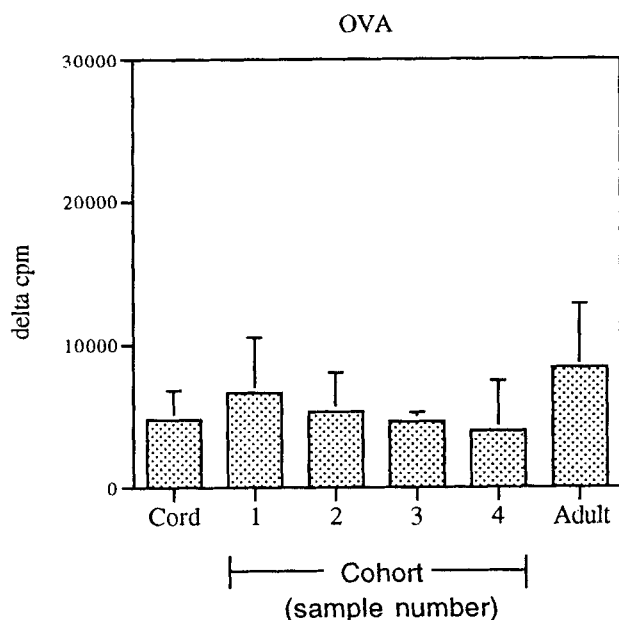


Fig. 3. Age-related changes in the magnitude of T-cell responses to OVA. Data shown are mean $\pm$ sd of positive responses to OVA, derived from Fig. 1 as described in the legend to Fig. 2.

Magnitude of allergen-specific T-cell responses at different ages

The data of Figs 2 and 3 were derived from analysis of pooled data from the positive responders to HDM and OVA within each age group, employing the cutoff of $SR \geq 2.0$ (expression of the data based on a $SR \geq 3.0$ cutoff yielded essentially identical results). It can be seen that for house dust mite (Fig. 2), the magnitude of the positive responses within the PBMC samples followed a strict hierarchy as follows: adult $\gg$ infant $\gg$ cord. It is noteworthy that no such hierarchy was found within the cohort samples, comparing the various ages. Fig. 3 shows responses to OVA; in contrast to HDM, the magnitude of the responses to the food allergen remained within a relatively narrow (and low) range, which did not expand in adulthood.

Cord blood T-cell responses

Data relating to cord blood responses are shown in detail in Table 1, to facilitate direct comparison with published results obtained employing conventional T-cell activation systems (discussed in detail below). Of particular note are the consistency and magnitude of responses to OVA and mite antigens, in particular DerP2, and the complete absence of responses to TT.

Discussion

Research into the aetiology of allergic respiratory diseases has progressed rapidly in the last 5 years, following the demonstration that aeroallergen-reac-

tive T-cells can be cloned from both atopic and normal adults (8–10, 13). The cytokine profiles of the clones are skewed towards a murine TH-2-like pattern (IL-4/IL-5) in atopics, as opposed to a TH-1-like pattern (dominated by IFN γ) in normals (8–10, 14).

Despite these findings, the generation of allergen-specific T-cell clones from normal subjects has proven extremely difficult in most laboratories; even more so, it has proven difficult to demonstrate reliable allergen-specific T-cell responses in short term bulk cultures in comparison to conventional "antigens" such as TT. A recent technical advance in our lab, notably the improvement of the sensitivity of the standard lymphoproliferation assay based upon the use of serum-free medium, now permits demonstration of unequivocal T-cell reactivity to ubiquitous allergens such as HDM in virtually 100% of adult subjects (Fig. 1, 11). A major impetus for the development of this methodology has been the need for a test system to study primary immune responses in normal and atopic infants. The available data on the kinetics of postnatal appearance of allergen-specific IgE (3, 4) and IgG (5, 6) antibodies to inhalants predict that T-cell responses should be initiated during this period, and further that they should ultimately proceed in subtly different directions in atopic and normal subjects. However, formal proof of this hypothesis is lacking, and this study represents an initial attempt to obtain direct information relating to this important issue.

The experiments reported above provide a general picture of the overall kinetics of the primary T-cell sensitisation process during infancy. Firstly, it should be noted that the use of the "vaccine" antigen TT provides internal validation of the specificity of the assay system i.e. it is clear that the infant T-cell system does not respond to TT until ≥ 6 months of age, a pattern which is predictable based upon standard vaccination practices. In stark contrast, T-cell responses to many environmental allergens (both dietary and inhalant) are demonstrable in the same infants by 6–10 weeks of age, a time at which their relevant allergen-specific antibodies are predominantly (probably exclusively) of maternal origin (5).

The responses observed for cord blood PBMC merit particular comment. A number of laboratories have recently reported presumptive evidence of *in utero* priming of fetal T-cells to environmental allergens, using conventional assay systems based on lymphoproliferation (15–18). These claims have not attracted the attention they may well merit, as the magnitude of the responses are small and usually close to the limits of detection with conventional lymphoproliferation assays. For this reason, data for cord blood responses obtained with the present assay

system are shown here (Table 1) in underderived form, to facilitate comparison with the wider published literature on adults. Certain of these responses, notably to DerP2 and OVA, are well within the adult range (data not shown).

Consideration of the cord, infant and adult data collectively, the following salient points emerge:

- (i) T-cell responses to both dietary and inhalant allergens are frequently initiated *in utero* or during very early infancy;
- (ii) responses to inhalant allergen (exemplified by HDM) increase with age both in frequency and intensity (i.e. cpm per culture), comparing the infant and adult panels;
- (iii) the reverse pattern is seen for food allergen (exemplified by OVA), with responses being more frequent during neonatal life, and those surviving into adulthood remain within the low (intensity) range.

The differing patterns of responses observed comparing inhalant and food allergens is consistent with a general model we have recently proposed for the "priming" of the human immune system to environmental allergens (2). This model envisages that specific T-cell responses to inhalant allergens are initiated in very early life, and are "refined" postnatally by a process of allergen-driven T-cell selection involving cross-regulation between competing T-cell subsets (2). In normals, this process is postulated to result in positive selection for TH-1 immunity, thus excluding TH-2-dependent IgE responses; this protective process is proposed to have a significant failure rate in atopics, resulting in the occasional development of TH-2 immunity and hence prolonged allergen specific IgE responses (2). An essential prediction of the model is that at the completion of the "selection" process, both atopics and normals should express T-cell reactivity to the allergens, which is demonstrable by such assays as *in vitro* lymphoproliferation (2). The findings presented above, which clearly show T-cell reactivity to inhalant allergens in virtually all adults, are consistent with this suggestion.

In addition, the restriction of responses in the infant cohort within a range which (particularly for HDM) is well below that of the adults, suggests that inhalant allergen-specific T-cell populations do not undergo final expansion to adult-equivalent levels until after the age of 2 years. This may be expected if the evolving T-cell responses involved cross-regulation between competing, mutually antagonistic T-cell subsets (viz. TH-1-like *versus* TH-2-like), which should result in the establishment of a form of "equilibrium" until one of the competing T-cell phenotypes establishes dominance; once the latter is

achieved, the dominant TH-cell population would then be free to expand under the continuing drive of environmental allergen exposure. The findings from a number of labs indicating that the steep rise in IgE titres to inhalant allergen and development of accompanying SPT reactivity is usually delayed until after the age of 2 years (3, 4, 19, 20) is compatible with such a process.

A related but subtly different process may regulate responses to food allergens, based upon recent findings in the Oral Tolerance literature. The latter (reviewed in 2) indicate that in addition to TH-1/TH-2 cross-regulation which is driven by low-level allergen exposure, the higher dosages of food allergens are sufficient to induce specific T-cell "anergy", resulting in the deletion of relevant food-allergen-specific T-cells. High levels of the TH-2-inhibitory cytokine IFN γ are also generated during this process, and hence any allergen-specific T-cells that escape deletion will tend to be of the TH-1 phenotype (2). The model thus predicts that unlike T-cell responses to inhalants, those to foods should demonstrate an approximately inverse relationship to age. The present findings, which indicate that responses to OVA decline in frequency throughout childhood before stabilising at a relatively low level in adulthood, are also consistent with the model.

The significance of *in utero* T-cell priming to some of these allergens is unknown. However, it is noteworthy that the frequency of cord responses to DerP2 was particularly high (Table 1), and it is of interest in this context that recent Western Blotting data indicate that IgE responses to HDM in young children are commonly dominated by this specificity (21). Additionally, IgE responses to OVA are very common in early childhood (3), and were also common in the cord samples (Table 1). Given the high frequency IgG reactivity to these allergens in the adult population and the perennial presence of both allergens in the environment, it is conceivable that *in utero* priming may occur via the transplacental passage of both antibody and allergen fragments, thus presenting the fetal immune system with immunogen in the form of antigen-antibody complexes. This suggestion is consistent with the TT data, where maternal IgG-anti-TT is present, but the antigen is usually absent.

Finally, the responses to WRE merit individual comment. The cohort here are all from "high risk" atopic families, and all children were born within the local rye pollen season. Interestingly, the frequency of T-cell responses to WRE in the cohort did not show a rapid postnatal rise, but instead peaked transiently coincident with the 6-month bleed i.e. following their first seasonal exposure. The significance of this finding will be further explored in follow-up studies employing other seasonal aller-

gens. In particular, it will be necessary to perform detailed longitudinal studies on individual subjects, from cord blood through to the period of late childhood where serum IgE titres in atopics usually stabilise, in order to resolve the issues raised in the present study.

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