

Altered T lymphocyte phenotype at birth in babies born to atopic parents

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Flow cytometry was used to analyse the cord blood T cells of 33 babies at high risk 'HR' for developing allergy (born to at least one atopic, asthmatic parent), and 10 low risk 'LR' babies (born to non-atopic parents), following normal term deliveries. Significantly lower numbers of CD25⁺, (activated) T cells ($p < 0.005$) were seen in the cord blood of the HR babies who had developed both allergic symptoms and positive skin prick tests by one year of age when compared with the LR group. CD45RO⁺ (memory) T cells were detected in both HR and LR babies with a trend for lower numbers of memory cells to be detected in HR infants who later developed allergic symptoms and/or positive skin prick tests. Significantly lower numbers of CD4⁺/CD45RO⁺ were seen in the cord blood of HR babies who developed allergic symptoms compared to HR babies who showed no sign of allergy by one year and to the LR babies ($p < 0.05$ and $p < 0.005$). The presence of activated and memory T cells at birth implies intra-uterine priming. The significantly lower numbers of memory T cells in the HR babies suggests a suppression of T cell activation or lack of antigenic priming in this group. This prenatal influence on babies born to atopic parents may have important implications with regard to the mechanisms underlying atopic sensitisation.

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There is considerable controversy regarding the functional maturity of neonatal T cells. It has been established that T cells can be isolated from fetal thymus tissue and proliferate in response to phytohaemagglutinin at 10 weeks of gestation. Between weeks 12 and 23 migration from the thymus takes place with increasing numbers of lymphocytes detected in the spleen and liver (1). These studies however do not necessarily imply that the lymphocytes are immunocompetent.

A more recent study looking at cord blood T cells found a similar percentage of CD3⁺ cells in cord and adult blood but found impaired proliferative responses of the cord T cells when cultured with anti CD3 monoclonal antibody. It was suggested that the limited antigenic exposure which cord blood T cells have undergone during intrauterine development might be responsible for this immaturity (2).

Phenotypic studies of T cells have shown lower numbers of CD3⁺, CD4⁺ and CD8⁺ T cells in cord blood than in adult peripheral blood samples (3–6). The numbers of CD45RA⁺ cells, indicative of im-

munological naivety, have been found to be higher in cord blood than in adults. (3–9). Conversely, numbers of CD45RO⁺ T cells were lower than in adults (7–9). The CD45RO receptor, the low molecular weight isoform of the leucocyte common antigen, is considered to be a feature of primed T cells (10). These cells, thought to be the resting memory T cell population, show a far greater proliferative response to recall antigen and enhance IgE production (11, 12). It has been suggested that the high CD45RA⁺ low CD45RO⁺ T cells in cord blood is due to lack of immunological stimulation during intra-uterine development (7).

Alterations in the T cell population in patients with established allergy have been recorded. Peripheral blood in allergic asthmatics has been shown to have higher numbers of activated (CD25⁺) T cells (13, 14). Skin biopsies taken from allergen-induced late-phase skin reactions (a convenient model for studying the pathophysiology of atopic inflammation) contained greater numbers of T helper cells (CD4⁺) which were predominantly CD45RO⁺ (15).

Few studies have investigated T cell phenotype prospectively in infants before they have developed allergic problems.

Two such prospective studies looking at the T cells of high risk infants prior to any signs of allergy found a significantly lower number of T cells in infants born to allergic parents than those born to non-atopic parents (6, 16).

We studied two populations of infants at birth, those at high risk of development of allergy (HR), born to parents at least one of whom had allergic asthma; and those born to non-atopic parents at low risk of development of allergy (LR). To elucidate possible T cell differences in HR babies, compared with LR babies we investigated T cell subpopulations and activation markers at birth.

Material and methods

Sample collection

Cord blood samples were obtained from 33 HR babies born to at least one asthmatic atopic parent (established by clinical history, bronchial challenge with a concentration of histamine producing a 20% fall in lung function (PC20) of less than 8mg/ml and at least one positive skin prick test to common allergens) and 10 LR babies born to non-atopic parents (no history of allergic disease and no positive skin prick tests).

Following normal deliveries of term babies from uncomplicated pregnancies, cord blood samples were taken into heparin by needling from the placental end of the umbilical vein after cleaning with a swab (Steret, Seton Prebbles Ltd., Oldham).

Clinical assessment

The babies were clinically assessed routinely at 6 months and 1 year of age by one of us (JOW). Additionally parents were encouraged to bring the infants for extra assessments if there was any persistent skin, bowel or chest problem. All evaluations were performed blind to the results of immunological tests. Asthma was diagnosed only if infants had greater than either 2 separate wheezing episodes or 2 separate episodes of paroxysmal nocturnal cough with sleep disturbance for 3 consecutive nights. All such patients were also deemed to require anti-asthma therapy. Eczema was diagnosed in infants with erythematous, papular, macrovesicular chronic skin eruptions lasting for longer than 4 weeks and affecting either face (excluding nose and lips), torso and extensor surfaces of limbs or skin flexures. The babies were skin prick tested at one year to a positive histamine control (1%), negative control (phenol/saline 0.9%), grass pollen (2.5%), tree pollen (2.5%), cat dander (150%), dog dander (150 %),

house dust mite – *Dermatophagoides pteronyssinus* (1.2%), *Cladosporium cladosporioides* (10%), *Aspergillus fumigatus* (5%), *Alternaria alternata* (10%), milk (50%), whole egg (6%) and wheat (10%) (SmithKline Beecham, Welwyn Garden City, Herts, UK). These data were used to divide the infants into four groups. Group 1, babies who had both allergic symptoms and positive skin prick tests to one or more substance, Group 2, those who had allergic symptoms but no positive skin prick tests by one year, Group 3, infants with positive skin prick tests but no apparent allergic symptoms and Group 4, babies with no positive skin prick tests or allergic symptoms by one year.

Immunostaining

A two-colour immunofluorescence method was employed (17) to analyse the lymphocytes using the following combinations of monoclonal antibodies: 1) fluorescein isothiocyanate (FITC) conjugated anti-CD3 ('inhouse') and R-phyco-erythrin (RPE) conjugated anti-CD25 (Dako, High Wycombe, Bucks.); 2) FITC conjugated anti-CD3 ('inhouse') and RPE conjugated anti-CD45RO (UCHL1, Dako); 3) FITC conjugated anti-CD4 and RPE conjugated anti-CD45RO (Dako); 4) FITC conjugated anti-CD8 (Dako) and RPE conjugated anti-CD45RO (Dako) and 5) a negative control (mouse IgG 1) conjugated to both FITC and RPE (Dako).

10 µl of antibody conjugated to FITC or RPE was added to 100 µl of heparinised blood and incubated for 15 min on ice. The red blood cells were then lysed with 2 mls of FACS lysing solution (formaldehyde 3.75 mls, ethylene glycol 4.5 mls, 0.2 M TRIS 1.75 mls, dH₂O 90 mls) for 5 min at room temperature. The remaining leucocytes were pelleted at 200 g for 5 min, washed once in PBS with azide and resuspended in 500 µl of PBS containing 1% para-formaldehyde. A FACScan (Becton Dickinson, Cowley, UK) flow cytometer was used to count 10,000 cells. The Becton Dickinson 'Lysis II' software was used to analyse the data. The lymphocytes were initially gated using side (90°) and forward laser light scatter to select for lymphocytes by cell size and granularity. Lymphocytes within this gate were then analysed for FITC and RPE fluorescent staining. Quadrants were set dividing the gated lymphocytes into cells stained by the RPE conjugated antibody, cells stained by the RPE and the FITC conjugated antibodies, cells stained by the FITC conjugated antibody alone and lastly those cells unstained by either antibody.

Table 1. Medians and interquartile ranges of the percentage of umbilical cord blood lymphocytes staining for CD3, CD25, CD45RO, CD4 and CD8 receptors in LR babies born to non-atopic parents and HR babies born to atopic parents subdivided into those who develop allergic symptoms (SYMP⁺) and/or positive skin prick tests (SPT⁺) by 1 year of age.

	HR n=33	HR Group 1 SYMP ⁺ /SPT ⁺ n=13	HR Group 2 SYMP ⁺ /SPT ⁻ n=6	HR Group 3 SYMP ⁻ /SPT ⁺ n=7	HR Group 4 SYMP ⁻ /SPT ⁻ n=7	LR CONTROLS n=10
CD3 ⁺	42.6 (27.44–50.21)	37.8 (20.08–49.33)	37.55 (27.95–46.75)	45.2 (36.1–46.6)	50.4 (24.7–67)	48.13 (30.79–57)
CD3 ⁺ /CD25 ⁺	7.08 (5.13–9.7)	5.16 (4.89–7.43)	8.49 (6.58–12.6)	7.57 (5.03–9.3)	6.33 (5.48–31.17)	9.05 (6.95–11.8)
CD3 ⁺ /CD45 ⁺	52.93 (29.84–63.14)	52.93 (33.17–73.23)	45.09 (12.57–71.25)	37.6 (26.79–63.43)	59.28 (47.56–66)	62.18 (46.51–75.97)
CD4 ⁺ /CD45 ⁺	17.26 (8.88–23.15)	19.01 (7.19–20.9)	14.2 (7.27–19.16)	17.48 (2.09–28.65)	23.91 (13.84–28.57)	21.65 (20.53–36.08)
CD8 ⁺ /CD45 ⁺	2.85 (1.57–6.39)	3.45 (1.78–6.07)	5.24 (2.78–10.5)	2.85 (1.33–7.39)	2.77 (1.6–6.45)	4.61 (3.09–7.13)

T cell counts

Peripheral blood mononuclear cells were separated from whole blood by density gradient centrifugation. The cells were stained with equal volumes of trypan blue (0.4% in 0.85% saline, Flow Laboratories, Irvine, Scotland) and loaded onto a haemocytometer. Lymphocytes were distinguished by size and morphology and counted. Total T cell numbers were derived by calculating the percentage of these lymphocytes which were CD3⁺ by flow cytometric analysis.

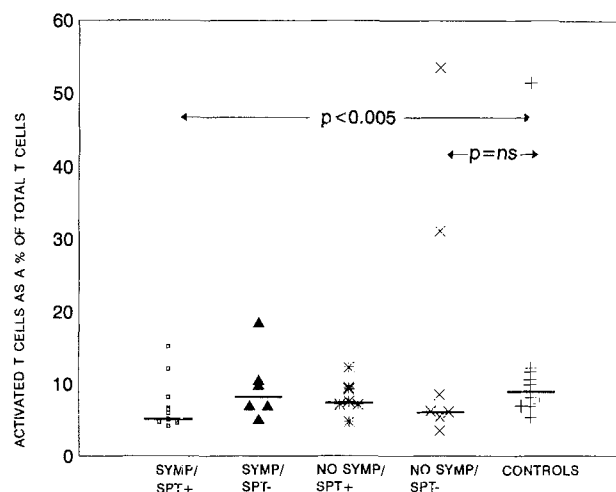


Fig. 1. CD25⁺ T cells as a percentage of total cord blood T lymphocytes in 33 'high' atopy risk babies, divided into those babies with allergic symptoms and positive skin tests (SYMP⁺/SPT⁺) those with symptoms but no positive skin tests at 1 year (SYMP⁺/SPT⁻), asymptomatic infants with positive skin prick tests (SYMP⁻/SPT⁺), asymptomatic babies with no positive skin tests by 1 year (SYMP⁻/SPT⁻) and 10 control 'low' atopy risk babies.

Statistical analysis

The data were analysed using non-parametric methods (Mann-Whitney U test).

Results

The LR group consisted of 10 babies born to non-atopic parents. The 33 babies in the HR group were born to families where at least one parent was an allergic asthmatic. These HR babies were divided into four groups. Group 1 babies had both allergic symptoms and positive skin prick tests by 1 year of age. This included 13 babies, 9 with eczema, 1 with asthma and 3 infants with both asthma and eczema. Group 2, HR babies with allergic symptoms but no positive skin prick tests by 1 year, included 6 infants, 1 with eczema, 3 with asthma and 2 children with both asthma and eczema. The Group 3 HR babies had positive skin prick tests but no allergic symptoms by one year (n=7). Group 4 included the 7 HR babies who did not develop allergic symptoms or positive skin prick tests by one year. The total T cell numbers in the umbilical cord blood samples were lower than the quoted normal adult levels of 65–96% (18–20), with a median value of 43% of gated cells in cord blood from HR babies born to at least one atopic parent and 48% in cord blood from LR babies born to non-atopic parents with the lowest values being seen in the HR babies in Groups 1 and 2, i.e. who presented with allergic symptoms by one year of age (p=ns). (Table 1).

CD3⁺/CD25⁺ cells were present in a range of 5–9%. HR babies who developed allergic symptoms and positive skin prick tests by one year of age (Group 1) had significantly lower numbers of activa-

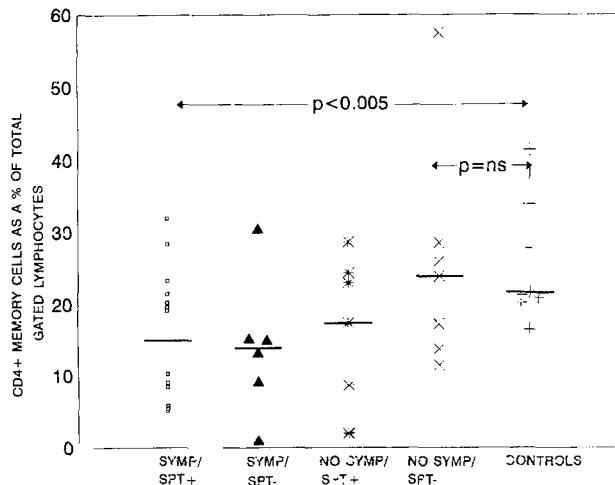


Fig. 2. CD45RO⁺/CD4⁺ T cells as a percentage of total cord blood lymphocytes in 33 'high' atopy risk babies, divided into those babies with allergic symptoms and positive skin tests (SYMP⁺/SPT⁺) those with symptoms but no positive skin tests at 1 year (SYMP⁺/SPT⁻), asymptomatic infants with positive skin prick tests (SYMP⁻/SPT⁺), asymptomatic babies with no positive skin tests by 1 year (SYMP⁻/SPT⁻) and 10 control 'low' atopy risk babies.

ted T cells (median 5%) when compared with the LR babies (median 9%, $p < 0.005$) (Fig. 1).

CD3⁺/CD45RO⁺ cells accounted for 62% of the T cells in the LR group and 53% in the HR group with the lowest numbers being seen in HR Groups 1 and 2 who developed allergic symptoms either with or without positive skin prick tests by one year of age ($p < 0.005$, $p < 0.09$ respectively).

The CD45RO marker was seen on 22% of the CD4 subset in the LR infants and 17% in the HR babies as a whole. The asymptomatic HR babies (Group 4) were not significantly different from the LR group at birth with a median value of 24% CD4⁺/CD45RO⁺ cells. The HR infants who later developed allergic symptoms and positive skin prick tests (Group 1) had 19% CD4⁺/CD45RO⁺ cells and those babies who developed allergic symptoms but no positive skin prick tests (Group 2) had 14% CD4⁺/CD45RO⁺ cells at birth, both significantly less than the LR infants ($p < 0.005$ and $p < 0.006$ respectively). The symptomatic HR babies with positive skin prick tests (Group 1) also had significantly fewer CD4⁺/CD45RO⁺ cells than the asymptomatic HR babies (Group 4) ($p < 0.05$). Although a trend of lower numbers of CD4⁺/CD45RO⁺ cells was apparent when the symptomatic babies who did not de-

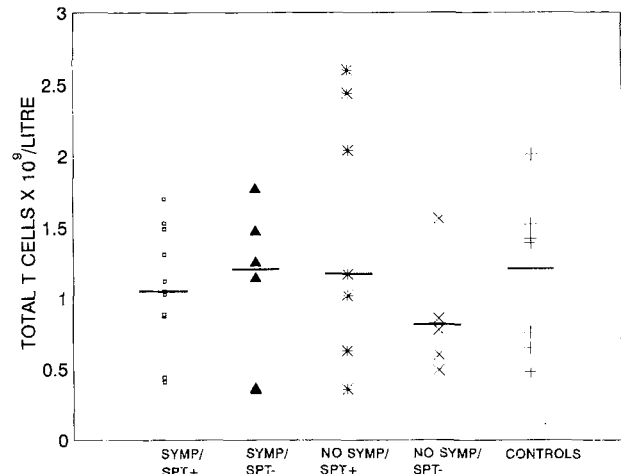


Fig. 3. Total T cell numbers at birth in 33 'high' atopy risk babies, divided into those babies with allergic symptoms and positive skin tests (SYMP⁺/SPT⁺) those with symptoms but no positive skin tests at 1 year (SYMP⁺/SPT⁻), asymptomatic infants with positive skin prick tests (SYMP⁻/SPT⁺), asymptomatic babies with no positive skin tests by 1 year (SYMP⁻/SPT⁻) and 10 control 'low' atopy risk babies.

velop positive skin prick test responses by one year (Group 2) were compared with the asymptomatic HR babies (Group 4). This was not significant. There were no significant differences seen in CD4⁺/CD45RO⁺ cell numbers between the symptomatic HR babies (Groups 1 and 2) and those HR infants who developed positive skin prick tests (Group 3) (Fig. 2). Approximately 5% of gated lymphocytes were CD8⁺/CD45RO⁺ in the LR group and 3% of cells in the HR group. This was not significantly different.

Total T cell numbers did not differ significantly between any of the HR groups or the LR group having a median of $1.05 \times 10^9/l$ and $1.21 \times 10^9/l$ in the symptomatic HR babies groups (1 and 2 respectively) $1.17 \times 10^9/l$ in those infants with positive skin tests but no symptoms (Group 3) $0.81 \times 10^9/l$ in the asymptomatic HR babies (Group 4) and $1.21 \times 10^9/l$ in the LR group (Table 2) (Fig. 3).

Discussion

Early work investigating cord blood T cells compared with adult T cells suggested that differences seen might reflect the immature 'virgin' unstimulated immune system at birth (21). Our study suggests that the degree of immaturity varies and in-

Table 2. Median and interquartile ranges of total T cell numbers at birth.

	HR Gp 1	HR Gp 2	HR Gp 3	HR Gp 4	LR
Cells $\times 10^9/litre$	1.05 0.88–1.49	1.21 0.37–1.56	1.17 0.63–2.44	0.81 0.57–1.04	1.21 0.68–1.5

fluences the potential of the infant to become allergic.

In this study, as in previous studies, neonatal T cell numbers were considerably lower than in adults, being approximately 50% of lymphocytes (20, 21, 22). There was no significant difference in total T cell numbers between the HR and LR babies in contrast to studies showing that a family history of allergy, particularly asthma, coincided with lower numbers of T cells (6, 16). However, once the HR group was divided into those babies who subsequently developed allergic symptoms and/or positive skin prick tests by one year of age and those who did not, there was a trend for lower numbers of T cells to be seen in those babies who later developed allergic symptoms (HR Groups 1 and 2).

Numbers of CD25⁺ T cells in HR and LR babies were approximately 50% those of adults, as documented by others (5) with greater numbers seen in the LR group when compared with the HR babies. This was significant when comparing HR babies who subsequently developed both allergic symptoms and positive skin prick tests by one year of age (Group 1) with the LR group ($p < 0.005$), however, there was no significant difference between the HR babies who did not develop allergies by one year (Group 4) and the LR babies. The discrepancies seen between groups in T cell activation, which are small, may reflect the result of variations in recent antigenic exposure rather than an innate difference between babies who subsequently developed allergic disease and those who did not.

The T cell activation suggests intrauterine exposure to antigens which is supported by the detection of CD45RO⁺ cord blood T cells. There was a trend of higher numbers of CD45RO⁺ T cells to be found in the LR infants and HR babies who did not develop allergic symptoms or positive skin prick tests by one year (Group 4) when compared with HR infants who had allergic symptoms and/or positive skin prick tests (Groups 1 and 2). Previously reported figures of 5 and 10% for T cell memory at birth are somewhat lower than our results (8, 9), however, this may be explained by small sample numbers and the use of T cell separation techniques in preference to a whole blood staining technique which could alter T cell subpopulations (23, 24). Horgan showed that in some cord blood samples a significant proportion of cells had a dull rather than bright expression of CD45RA which is not typical for naive cells and suggests that *in utero* antigen exposure may be causing this phenotypic change (25).

Memory within the CD4 subset (CD45RO⁺/CD4⁺) was seen in 22% of lymphocytes in the LR babies and 17% in the HR babies. These numbers are broadly in agreement with data published showing that the reciprocal CD45RA⁺/CD4⁺ (considered to

mark naive T cells) constitutes between 75–91% of cord blood T cells (5, 26, 27). Again the numbers were higher in the LR babies than the HR babies. When the HR babies were divided into groups according to their allergic symptoms, those symptomatic babies who did not develop positive skin prick responses (Group 2) had significantly lower numbers of CD4⁺/CD45RO⁺ than the LR group ($p < 0.006$). The HR symptomatic group with positive skin prick tests (Group 1) had significantly lower numbers of CD4⁺/CD45RO⁺ cells than those HR babies who had not developed symptoms or positive skin prick tests (Group 4) and the LR group ($p < 0.05$ and $p < 0.005$ respectively). Conversely, there was no significant difference in CD45RO⁺/CD4⁺ cell numbers between the HR babies with positive skin prick tests (Group 3) and the asymptomatic HR group (Group 4) compared with the LR group.

No significant differences were seen in CD4⁺/CD45RO⁺: CD8⁺/CD45RO⁺ ratios between HR and LR groups in contrast with previous studies showing an increase in CD4⁺/CD8⁺ ratio in established atopic subjects.

Total T cell numbers were in keeping with published data, with no significant differences seen between the HR groups and the LR babies (17). These numbers were not included in the original study design, but due to there being some controversy over the expression of flow cytometry results as percentages of total lymphocytes rather than actual numbers of cells per litre (references in this paper are variable in their methods), they have subsequently been calculated.

The significantly lower T cell memory seen at birth in the babies who subsequently develop allergic disease is perhaps of greater importance than the similar trend in T cell activation. T cell memory is a reflection of cumulative antigenic exposure which in this case is *in utero*. There are three possible explanations for this discrepancy in T cell memory at birth. The first is a T cell suppression either innate to these infants or influenced by the mother which is supported by the differences seen in T cell activation. Rinas showed that a family history of atopy was associated with a reduced T cell production of interferon-gamma (IFN- γ) (a potent downregulator of immunoglobulin E) in a recent study investigating cord blood lymphocytes (28). He suggested that there was an impairment of naive T cells to become activated T cells in newborns with a high-risk of developing allergy. The second explanation is that in infants who develop allergies, intrauterine exposure to antigens is limited by the mother. If T cell memory is a prerequisite to immune tolerance this lack of primed T cells may result in an altered outcome to postnatal antigen exposure and

development of allergic disease. The third possibility suggested by the results of another study of infants with a family history of atopic disease is that these children are born with a less mature immune system. Impaired post-natal maturation of the immune system in infants with a family history of atopy was proposed as an inherited defect by Holt who showed a reduced number of immunocompetent T cells in these children (29). The T cell clones cultured from these high risk infants produced demonstrably less IFN- γ than clones from children with no family history of allergy.

The lower numbers of activated T cells seen in those babies born to atopic parents who also developed allergic symptoms by one year might also suggest that intra-uterine events could be an additional factor in the 'heritability' of atopy. A recent study found maternal atopy to be the only significant risk factor in the development of atopic dermatitis in children (30). There were insufficient numbers of babies born to atopic fathers and non-atopic mothers (91% had atopic mothers) in our study to analyse the effect of this on T cell characteristics.

In conclusion, we have shown the neonatal immune system to be activated and primed at birth suggesting intra-uterine exposure to antigens. Significantly lower numbers of activated T cells, as well as lower numbers of memory T cells seen in those babies born to atopic parents who subsequently developed allergic symptoms implies that this exposure could be different in these babies due to maternal influence or that there is some alteration in T cell immunocompetence in these babies. This may be an important clue to the mechanism of inheritance of atopy.

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