

In utero priming of allergen-specific helper T cells

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Summary

Background Allergic diseases are major health problems in developed countries. Cord blood mononuclear cells (CBMC) at birth can proliferate after stimulation with allergen and this has led to the widespread view that the sensitization of the fetal immune system by allergens is a key determinant in establishing immunological bias towards allergy. However, the notion that the immune system can be primed by allergen *in utero* remains unproven. Determination of the CD45 isoform of responding T helper cells is an established method of determining the activation status of responding T helper cells because unsensitized cells express CD45RA^{high} and previously sensitized cells CD45RO^{high}.

Objective To determine if sensitization of allergen-specific T helper cells can occur *in utero* by determining the CD45 isoform of CBMC proliferating in response to allergen.

Methods CBMC proliferative responses were measured after stimulation in culture with a panel of allergens, mitogen and control antigen. To ascertain whether any responding T helper cells had been primed *in utero*, depletion experiments established whether they carried the CD45RO^{high} marker of previous activation or the CD45RA^{high} marker of unstimulated T cells.

Results CBMC from a high proportion of 223 randomly selected neonates were stimulated to proliferate *in vitro* by allergens, with 76% responding to timothy grass pollen. In 50% of such responses to timothy grass, the CD45 isoform of the T cells that proliferate indicated that they had been previously activated. However, the remaining 50% of responses to timothy grass were mediated by previously unstimulated T cells. Proliferative responses mediated by CBMC sensitized *in utero* tended to be greater in magnitude than those mediated by unsensitized cells ($P = 0.08$). Seventy-five per cent of CBMC samples proliferated after stimulation with mycobacterial PPD and, as in BCG-vaccinated adults, all such CBMC proliferative responses at birth were predominately mediated by sensitized cells.

Conclusion Allergen- and antigen-specific Th cells can be primed *in utero*.

Keywords: allergen, T helper cell, prenatal exposure, pregnancy, CD45

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Introduction

The continuing increases in the prevalence of the allergic diseases atopic asthma, eczema, hayfever and food allergy in developed countries are major health concerns. There

have been particularly marked increases in prevalence amongst children; for example, in countries such as the UK, 25% of children are now affected by asthma and 20% by eczema [1–5]. These allergic diseases are characterized by chronic immune mediated inflammation that is initiated and perpetuated by T helper (Th) cells [6]. The identification of the key interactions between the immune system and the environment, which are responsible for the increases in

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allergic disease, will be necessary for identification, design and implementation of primary preventative public health measures.

There is an emerging consensus that environmental factors influencing the developing immune system *in utero* substantially influence the subsequent risk of childhood atopic disease [7–10]. It has been proposed that one such factor is the priming of fetal Th cells by allergen and this is indirectly supported by a number of lines of evidence. First, allergen-specific IgE can be detected in neonatal cord blood, which must originate from the fetal immune system because maternal immunoglobulin of this class is not transported across the placenta. Secondly, many studies have also demonstrated that neonatal T cell proliferative and cytokine responses can be detected after stimulation with allergen *in vitro* [7–10]. Furthermore, cord blood mononuclear cell (CBMC) proliferative responses *in vitro* against birch pollen are greater if maternal exposure to this allergen occurred during certain periods of gestation [11,12]. It has been widely assumed that such proliferative and cytokine responses are a direct consequence of *in utero* priming; however, it has not been determined formally whether these are the responses of previously stimulated or unstimulated T cells. The possibility that these *in vitro* CBMC responses may not be the consequence of *in utero* sensitization has been raised by the demonstration that CBMC can proliferate after *in vitro* stimulation with non-recall antigens to which natural *in utero* exposure is extremely unlikely, such as keyhole limpet haemocyanin [13–15].

The aim of this study was to determine whether allergen-specific Th cells can be primed *in utero*. The approach was to use the well-established method for differentiating between the *in vitro* proliferation of previously stimulated and unstimulated Th cells, by determining the CD45 isoform of the Th cells that respond [13,15–18]. CD45 is a leucocyte membrane glycoprotein that exists in different isoforms determined by mRNA splicing [19]. Primary and recall T cell responses *in vitro* are mediated by T cells bearing CD45RA^{high} and CD45RO^{high}, respectively [13,15–18], enabling the question to be addressed as to whether neonatal T cells that proliferate *in vitro* against allergen or control antigen have been previously stimulated *in utero*.

Materials and methods

The culture conditions used in this study were based on those recommended by Plebanski and Burtles [20] for supporting optimal adult T cell proliferative responses, and adapted for CBMC culture as previously described [21]. It has been confirmed that these culture conditions are capable of supporting both primary and secondary *in vitro*

adult and cord blood T cell proliferative responses [13,14,16,17].

T cell stimuli

CBMC cultures were stimulated with the following final concentrations of mitogen and control antigens: concanavalin A 1 µg/mL (Sigma, Poole, Dorset, UK), *Mycobacterium tuberculosis* PPD 5 µg/mL (Statens Serum Institut, Copenhagen, Denmark), keyhole limpet haemocyanin (KLH) 5 µg/mL (Calbiochem-Novabiochem, La Jolla, CA, USA). The allergenic stimuli were timothy grass pollen extract 2500 IU/mL and house dust mite extract 2500 IU/mL, obtained from NIBSC (Potters Bar, UK), preparations which have previously been widely used [8,10,11] and shown to be free of non-specific CBMC stimuli.

Antibodies

Tissue culture supernatants containing the murine monoclonal antibodies UCHL-1, specific for human CD45RO, and SN-130, specific for human CD45RA, were kind gifts from Professors P. C. L. Beverely and G. Janossy (London, UK), respectively. Monoclonal antibodies specific for CD4 and red cell glycoporphin, and isotype controls, were purchased from Becton Dickinson (Oxford, UK). Optimal concentrations of these antibodies were determined by previous titration experiments.

Cord blood samples

Cord blood samples obtained by umbilical cord venepuncture were taken at birth from healthy, randomly selected neonates born in the Aberdeen Maternity Hospital between September 1998 and October 1999. The local ethics committee approved the study and mothers gave informed written consent.

Cord blood mononuclear cell culture

CBMC isolation and proliferation assays have been described previously [21]. Briefly, after CBMC isolation, 1-mL wells containing 1.25×10^6 cells in the Alpha Modification of Eagles Medium supplemented with 2.5% autologous serum were stimulated with Con A, PPD, timothy grass pollen extract or house dust mite extract. Unstimulated negative control cultures were also set up. Cell proliferation was assessed on days 4–8 after stimulation by the incorporation of ³H-thymidine into newly synthesized DNA in triplicate 100-µL aliquots withdrawn from the wells.

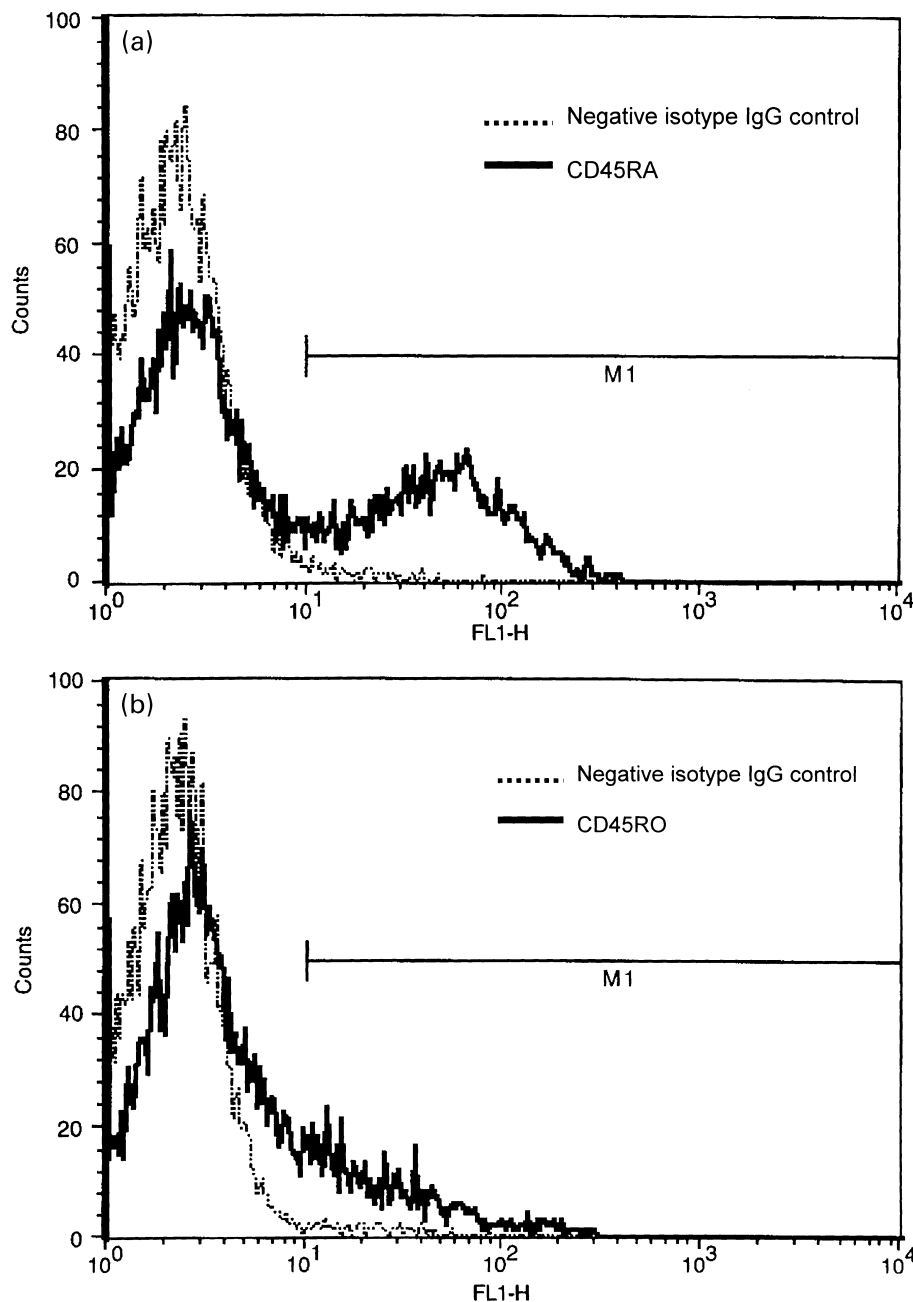


Fig. 1. Flow cytometric analysis of a typical unfractionated CBMC sample ($n = 16$) stained for CD45RA (a) and CD45RO (b). The bar labelled M1 indicates specific labelling of cells of greater intensity than non-specific negative isotype IgG control staining. The majority (mean 79%, SD 8.6) of CD4⁺ CD45⁺ CBMC were CD45RA^{high} and a minority (mean 21%, SD 8.6) CD45RO^{high}.

Flow cytometric analysis of CD45RA and CD45RO expression by CBMC

Flow cytometry quantified the CD45RA^{high} and CD45RO^{high} CBMC Th cells labelled with SN-130 and UCHL-1, respectively (Becton Dickinson FACSVantage). SN-130 and UCHL-1 bound to Th cells were detected by FITC-conjugated goat anti-mouse IgG. Figure 1 demonstrates that a significant proportion (mean 47%, SD 21) of the unfractionated CBMC preparation was negative for both CD45RA and CD45RO. Dual staining with antibodies specific for the Th

cell marker CD4 and red cell glyophorin indicated that these CD45 negative cells were contaminating fetal erythrocytes. The relative proportions of CD45RA^{high} and CD45RO^{high} cells in CBMC samples are therefore expressed as proportions of CD4⁺ Th cells only.

Depletion of CD45RO^{high} and CD45RA^{high} cells

Techniques used for fractionation of adult peripheral blood mononuclear cells [13,16–18] were modified for use with

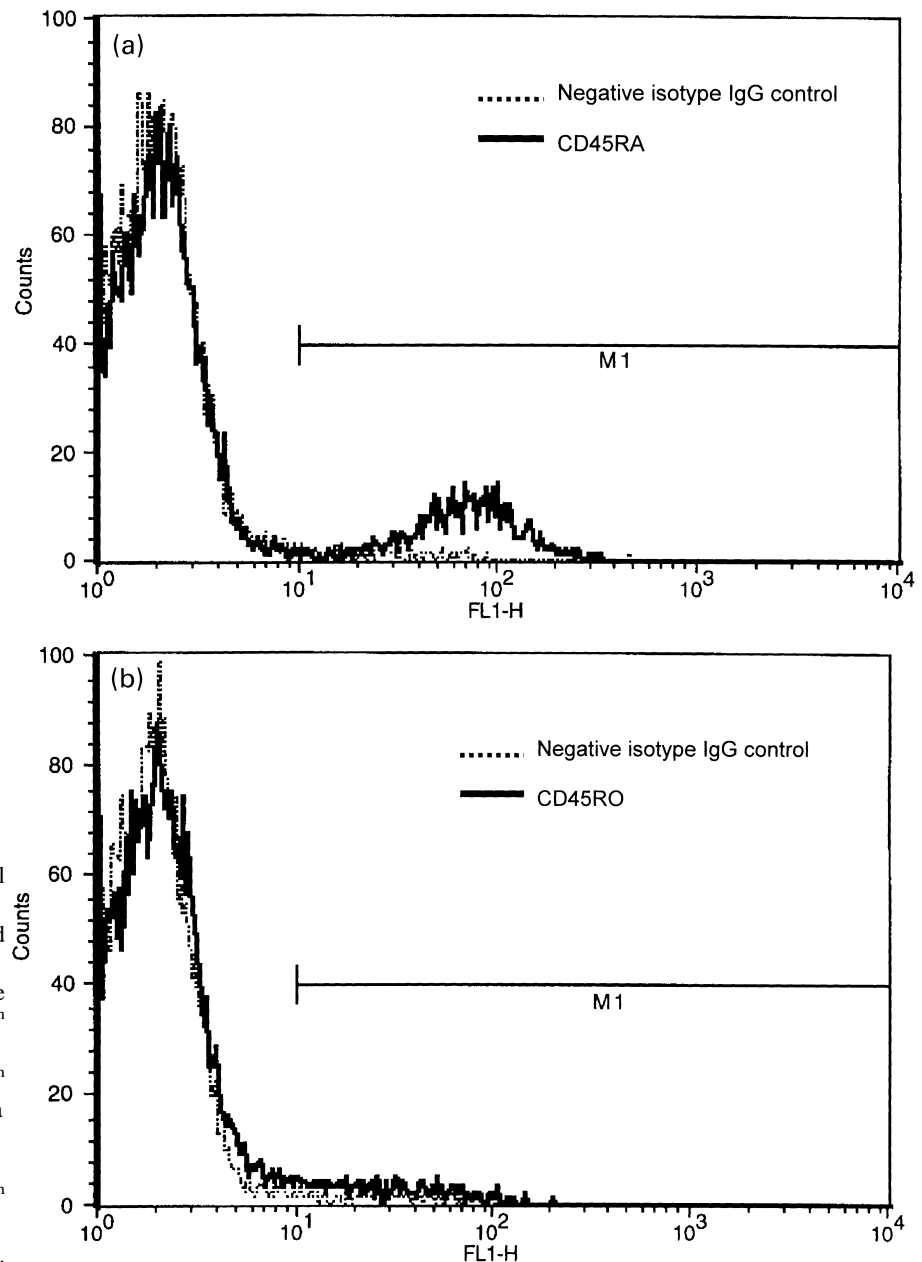


Fig. 2. Flow cytometric analysis of typical CBMC samples depleted of CD45RO^{high} cells (a and b) or CD45RA^{high} cells (c and d), and stained for CD45RA (a and c) or CD45RO (b and d). Prior to depletion the mean ratio of CD45RA^{high} to CD45RO^{high} was 3.8 : 1. After CD45RA^{high} enrichment the mean ratio of CD45RA^{high} to CD45RO^{high} was 6.63 : 1, indicating a 1.8-fold CD45RA^{high} enrichment (2a and 2b). After CD45RO^{high} enrichment the mean ratio of CD45RA^{high} to CD45RO^{high} was 1 : 1.8, indicating a 6.7-fold enrichment in CD45RO^{high} cells (c and d).

cord blood samples. CBMC samples to be depleted of CD45RO^{high} and CD45RA^{high} cells were incubated for 1 hour on ice with UCHL-1 and SN-130, respectively. Excess antibody was washed off before the CBMC were incubated with anti-mouse IgG coated magnetic beads as recommended by the manufacturers (Biomag, PerSeptive Biosystems, Framingham, MA, USA) for 30 min on ice. CBMC bound by UCHL-1/SN-130 and magnetic beads were then removed with a magnetic separator. To enhance the effectiveness of the depletion, this incubation and removal of magnetic beads was performed a total of three times. The purity of the enriched populations was tested as

described previously [13,15–17]. Briefly, enriched CBMC samples were incubated with SN-130 or UCHL-1 and stained with FITC-conjugated goat anti-mouse IgG prior to flow cytometry. This use of indirect staining enables the same set of monoclonal antibodies to be used both for depletion and testing purity.

Statistical considerations

Data were analysed using SPSS[®] 8.0 (SPSS Inc., Chicago, IL, USA). CBMC proliferation was expressed as a stimulation index (SI); the ratio of stimulus-induced to

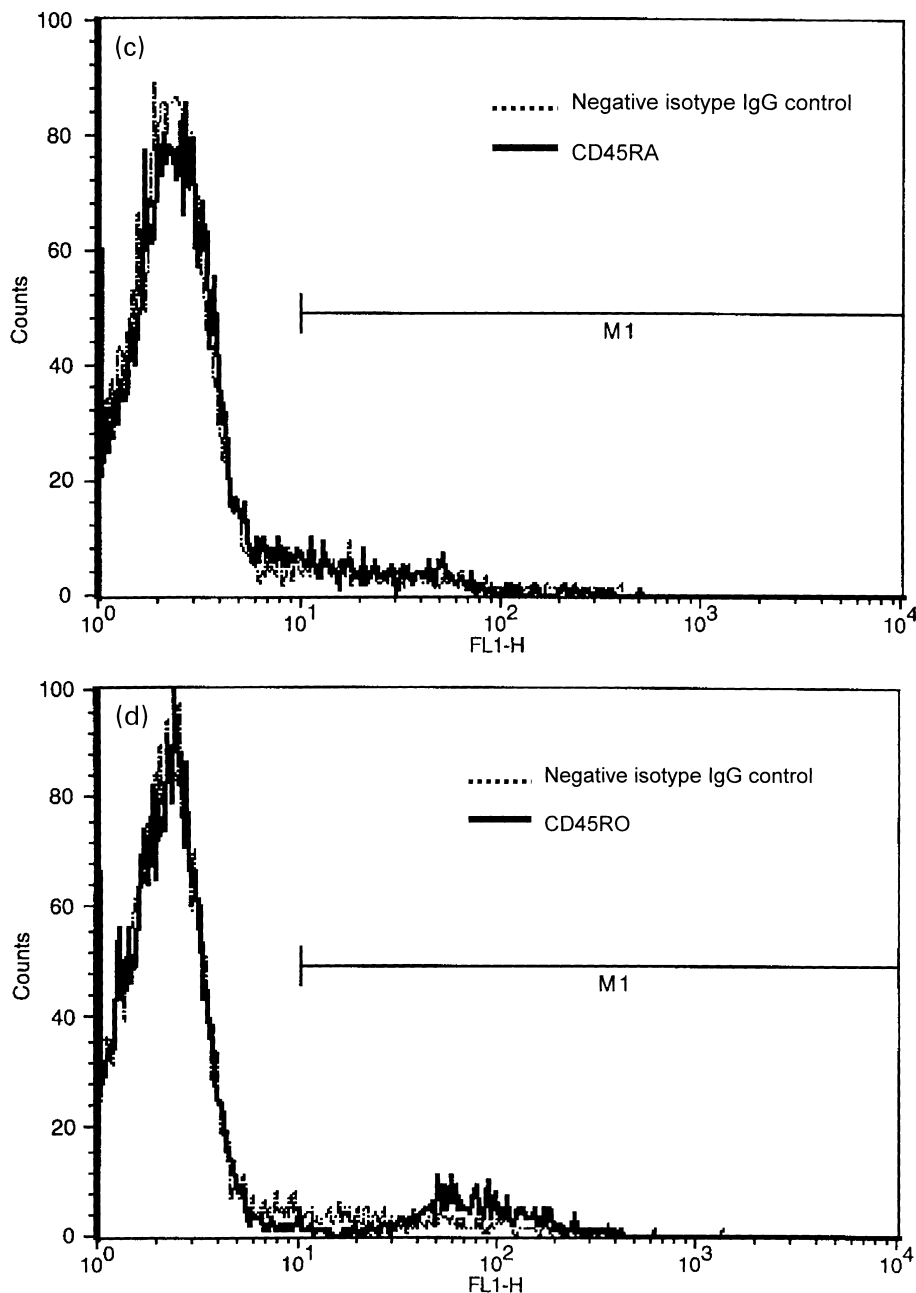


Fig. 2. Continued

unstimulated proliferation. Analysis of inter- and intra-assay repeatability has demonstrated that an SI ≥ 3 is statistically significant [21], a level that is comparable with other investigators who quote SI values of ≥ 2 or ≥ 3 as indicators of significant proliferation [7–10]. For individual samples proliferation is expressed as SI ± 1 SD but between-group proliferative responses are expressed as geometric mean values ($\pm 95\%$ confidence intervals, CI), this is because previous work has demonstrated that using this methodology, for all stimuli and days tested, stimulation indices approximate to a log-normal distribution [21].

General linear modelling for repeated measures (SPSS) was used to test for between-group differences.

Results

Analysis of cord blood mononuclear cells (CBMC)

To determine in the general population the frequency with which CBMC T cells respond to specific and non-specific stimuli, CBMC samples from 223 randomly selected neonates were stimulated with a panel of mitogen, control antigen and

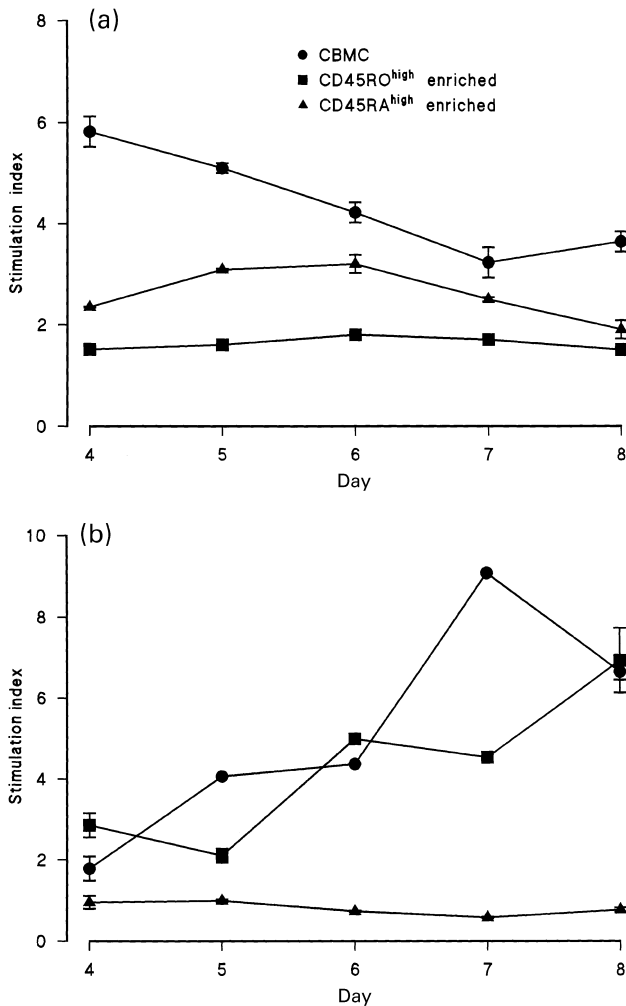


Fig. 3. Proliferative responses of CBMC and CD45RA^{high} or CD45RO^{high} enriched CBMC stimulated by the allergen timothy grass pollen. Representative example of CD45RA^{high} (3a, $n = 5$) and CD45RO^{high} (3b, $n = 5$) mediated responses. Proliferation expressed as stimulation index (± 1 SD) with significant responses being those with a stimulation index ≥ 3 .

allergens. Although 76% of CBMC samples proliferated in response to the allergen timothy grass pollen and 27% to house dust mite extract, 75% responded to PPD and 91% responded to the mitogen Con A. It should be noted that responsiveness of CBMC to each of the four stimuli is specific, as significant proliferation elicited by any one stimulus is not necessarily associated with responses to the others. Thus, 10% of samples responded to one of the stimuli, 28% to two, 44% to three, and 18% to all four of the stimuli.

Flow cytometric analysis of 16 CBMC samples confirmed that, in contrast to the results obtained from adults, the majority (mean 79%, SD 8.6) of neonatal T cells with the CD4⁺ helper phenotype bear the CD45RA^{high} marker of unstimulated cells, and a minority (mean 21%, SD 8.6) are

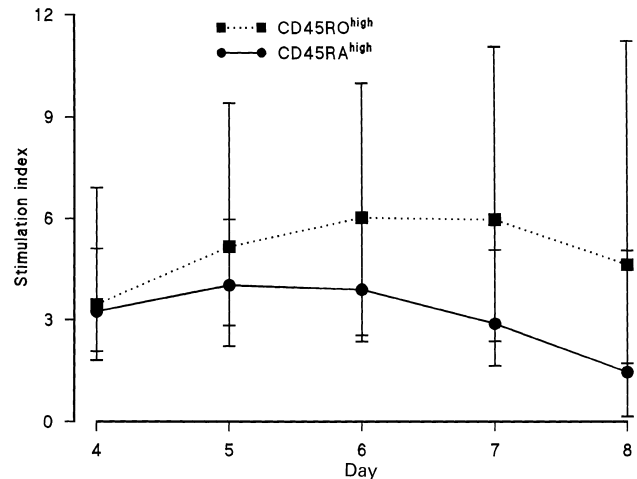


Fig. 4. Proliferative responses of unfractionated CBMC stimulated by timothy grass from neonates with CBMC responses subsequently demonstrated to be mediated by CD45RA^{high} Th cells ($n = 5$) or CD45RO^{high} Th cells ($n = 5$). Between-group difference, $P = 0.08$. Results expressed as geometric mean proliferative response (stimulation index $\pm 95\%$ confidence interval) for each of the 5 days when proliferative responses were assayed.

of the previously stimulated CD45RO^{high} subset (Fig. 1). This raises the question of whether the CD45RO^{high} subset contains Th cells specific for allergen.

Analysis of fractionated CBMC

In order to determine whether the observed CBMC

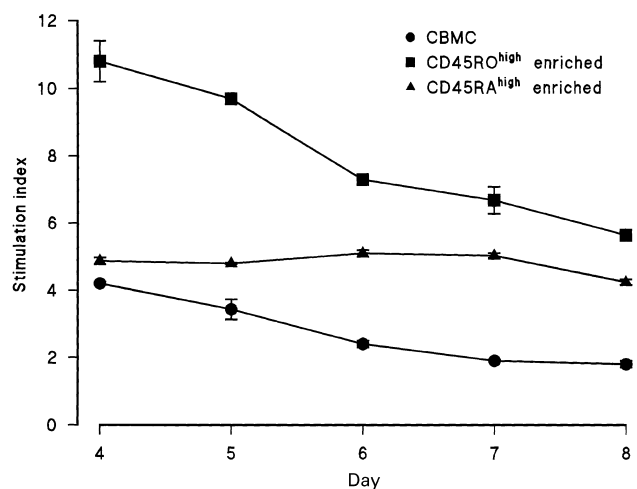


Fig. 5. Representative example ($n = 10$) of proliferative responses of CBMC and CD45RA^{high} or CD45RO^{high} enriched CBMC stimulated by PPD. Proliferation expressed as stimulation index (± 1 SD) with significant responses being those with a stimulation index ≥ 3 .

responses to allergens and control antigen are mediated by previously stimulated or unstimulated Th cells, the CD45 isoform of the cells that proliferate was identified by depletion experiments. The effectiveness of the depletion of CD45RO^{high} or CD45RA^{high} was confirmed as in previous studies [13,15–17] by flow cytometric analysis and an example is shown in Fig. 2. As previously demonstrated with adult cells [13,15,16], further work (not shown here) with the control non-recall antigen KLH also demonstrated that all proliferative responses to this antigen were mediated by CD45RA^{high} cells, indicating that any contamination of the CD45RO^{high} enriched preparations by CD45RA^{high} cells has no detectable effect in the proliferation assays.

Having demonstrated the effectiveness of CD45 isoform depletion, the CD45 fraction responding to allergen was determined. Access to samples from many (> 500) neonates enabled us to select the 15 cord blood samples of sufficient size (> 60 × 10⁶ CBMC) for depletion of CD45RO^{high} or CD45RA^{high} cells. In view of the limited CBMC available from even these samples, we chose to investigate responses to only one representative allergen, and timothy grass pollen was selected because it commonly elicits CBMC responses, is of clinical importance in the UK and is widely inhaled by the population. Figure 3 illustrates that proliferative responses to the allergen timothy grass pollen can be mediated by either CD45RA^{high} (Fig. 3a) or CD45RO^{high} (Fig. 3b) Th cells in CBMC samples from different neonates. Fractionation of eight CBMC samples that responded to timothy grass pollen revealed that the proliferating CD4⁺ T cells were drawn from the CD45RA^{high} subset in three neonates and from the CD45RO^{high} sub-population in five neonates. In a further two neonates, there were only sufficient cells to set up CD45RO^{high} enriched cultures, and these responded to control stimuli but not to the timothy grass pollen, leading to the inference that the observed proliferative responses of unfractionated CBMC stimulated by allergen were mediated by CD45RA^{high} CD4⁺ Th cells. Thus, there is direct evidence that timothy grass pollen specific Th cells can be stimulated before birth, and the results indicate that 50% of the neonatal Th cell proliferative responses to the allergen *in vitro* are mediated by such primed cells.

Figure 4 compares the unfractionated CBMC proliferative responses against timothy grass that were shown by subsequent depletion experiments to be mediated by previously stimulated CD45RO^{high} cells (*n* = 5), or by unstimulated CD45RA^{high} cells (*n* = 5). There was a trend (*P* = 0.08) for unfractionated CBMC proliferative responses mediated by Th cells that had been primed *in utero* to be higher than those mediated by unstimulated Th cells.

Our routine use of PPD as a control stimulus provided an

opportunity to determine whether Th cells can be sensitized *in utero* to antigens other than timothy grass pollen. Strikingly, in all 10 neonates whose unfractionated CBMC responded to PPD, proliferating cells were drawn from the CD45RO^{high} subset. In four of these cases there were enough CBMC to set up separate cultures enriched with cells bearing each CD45 isoform, and these showed that CD45RA^{high} T cells were also able to proliferate in response to PPD, albeit less vigorously than CD45RO^{high} cells (Fig. 5).

The CD45 isoform analysis of the 10 CBMC samples responding to timothy grass and the 10 CBMC samples responding to PPD was extrapolated to the study of 223 neonates. Thus, 76% of 223 CBMC samples proliferated *in vitro* after stimulation with timothy grass, but only 50% were mediated by CD45RO^{high} cells, implying that 38% of the general neonatal population have been sensitized *in utero* by timothy grass. Similarly, although 75% of 223 CBMC samples proliferated *in vitro* after stimulation with PPD, all of these were mediated by CD45RO^{high} cells, suggesting that this antigen had sensitized 75% of the general population of neonates *in utero*.

Discussion

We have used CD45 isoform fractionation of CBMC to demonstrate that timothy grass allergen specific fetal Th cells can be sensitized *in utero*, but that this priming does not occur in all individuals, even if their CBMC are able to respond to the allergen. Such priming of fetal Th cells is not limited to allergens because our use of control antigens demonstrated that PPD-specific fetal Th cells can also be sensitized *in utero*.

This work is dependent on CD45 isoform differentiating between Th cells that mediate primary and recall responses, a dichotomy that is well established in adults [13–18], although CD45RA^{high} and CD45RO^{high} are probably respective markers for reversible resting and activated states rather than for distinct naïve and memory Th cell populations. Several lines of evidence indicate that reliance on this relationship for distinguishing between primary and secondary responses is also justified in neonates. First, there are relatively few CD45RO^{high} cells in neonates compared with adults, as shown here and elsewhere [22,23], consistent with the expectation that neonates have a largely inactive T cell repertoire. Secondly, as demonstrated by ourselves (unpublished) and others [15,16], cultures of neonatal CD45RA^{high} Th cells increasingly express CD45RO^{high} after stimulation with mitogens, antigens including the non-recall antigen KLH and allergens such as timothy grass. Thirdly, the CD45RA^{high} to CD45RO^{high} transformation of neonatal T cells is associated with the loss of T cell receptor rearrangement

deletion circles, which characterize naïve T cells [24]. Fourthly, in adults, proliferation elicited by the non-recall antigen keyhole limpet haemocyanin is abrogated by depletion of Th cells bearing the CD45RA^{high} isoform, but not by removing CD45RO^{high} Th cells; we have also demonstrated comparable results with cord blood [15,16]. Finally, all of the timothy grass pollen responses we analysed were only mediated by one or other CD45 isoform cell subset.

Analysis of the *in vitro* CBMC responses of 223 neonates representative of the general population demonstrated that, with this methodology, there was great variation in CBMC proliferation kinetics. CBMC proliferation kinetics, unlike those for adult T cells [13,15–17,20], cannot be clearly divided into slow responses that are obviously primary, and faster secondary responses, and it was this observation that prompted the present study. Figures 3 and 4 illustrate the lack of a simple dichotomy in the speed of responses, confirming that CBMC proliferation kinetics are not reliable indicators of whether individual proliferative responses are mediated by *in utero* primed or naïve Th cells. The inability to differentiate CBMC responses on the basis of kinetics may reflect less consistently fast secondary proliferation than is seen for adult T cells, and is probably compounded by minor changes in cell numbers, unidentified *in utero* influences, differences in autologous serum constituents and genetic influences. Previous studies by others have not followed *in vitro* CBMC response kinetics, and instead have relied on determining proliferative and cytokine responses at a single time point [7–10]. The advantage of using CD45 isoform fractionation to determine whether *in vitro* CBMC proliferative responses are mediated by previously sensitized or naïve T cells is that this approach obviates the need to interpret unreliable proliferation kinetics.

This is the first study to have used fractionation of CBMC CD45 isoforms to formally demonstrate that allergen-specific fetal Th cells can be sensitized *in utero*. Although it has been accepted that the neonatal T cell repertoire includes a minority of previously stimulated CD45RO^{high} cells, their specificities were hitherto unknown [19,25]. Previous studies have demonstrated that increased CBMC proliferative responses are associated with an increased risk of childhood atopic disease [8–10]. Thus, our demonstration that CBMC proliferative responses mediated by T cells sensitized *in utero* tend to be greater in magnitude than CBMC proliferative responses mediated by unsensitized T cells suggests that the process of *in utero* sensitization of allergen specific T cells will be associated with increased risk of childhood atopy. Since there is no evidence to suggest that maternal allergen exposure has increased markedly in recent years [26], any changes in *in utero* sensitization must be due to other environmental factors.

There is a growing body of evidence that factors such as the dietary intake of antioxidants and fatty acids have changed recently and influence the risk of expression of asthma in children and adults [24–30]. It is possible that interactions between *in utero* allergen sensitization and such environmental factors may have contributed to the recent increase in atopic disease. These factors may increase fetal immune responsiveness and the likelihood of sensitization to a given level of allergen exposure: additionally or alternatively, they may influence the immunological *milieu* in which sensitization occurs and critically determine the subsequent Th cell bias of the response. This leads us to propose that the maternal diet during pregnancy influences the allergic potential of *in utero* sensitization, and we are actively investigating this hypothesis.

Another potentially important finding was that neonatal CBMC proliferative responses to PPD are predominantly mediated by CD45RO^{high} Th cells with a minor contribution by CD45RA^{high} Th cells. An identical pattern is seen in the *in vitro* responses to PPD of Th cells from BCG immunized adults, whilst proliferation of CD45RA^{high} T cells alone is seen exclusively in adults not immunized with BCG [15,16]. The mixed response in immunized adults may be due to either presentation of epitopes *in vitro* that are cryptic *in vivo*, or reversion of memory Th cells from CD45RO^{high} to CD45RA^{high} expression. This latter explanation seems unlikely in newborn infants. Irrespective of the explanation, our results indicate that sensitization to antigens other than allergens can occur *in utero*.

Previous studies have demonstrated CBMC proliferative responses after stimulation with PPD or mycobacterial heat shock protein [31,32] and it has also been suggested that CBMC proliferative responses to PPD may be a consequence of cross-reactivity between human and mycobacterial heat shock proteins [31]. This seems unlikely because *in vitro* adult Th cell proliferative responses to human hsp60 are mediated exclusively by CD45RA^{high} cells, whilst the responses to mycobacterial hsp60 are mediated by both CD45RA^{high} and CD45RO^{high} cells [13]. Exposure to such mycobacterial antigens *in utero* could potentially influence the Th cell bias of the developing immune system and the subsequent development of allergy. For example, in the past, natural mycobacterial sensitization may have been more effective in promoting Th1 differentiation and reducing the risk of childhood allergy, when compared with maternal BCG vaccination, which has been the major source of mycobacterial exposure in the UK in the last 40–50 years [33,34].

This study indicates that cell-mediated immunity may, in effect, be vertically transmitted, challenging the accepted belief that humoral, but not cell-mediated, immunity is placentally transferred from mother to fetus. The mechanism

by which such transmission of cell-mediated immunity occurs remains to be elucidated, but, given that IgG antibodies are actively transported across the placenta, one possibility is that antigen is also transferred in the form of immune complexes. This suggestion is supported by the recent report confirming the presence of the cat allergen fel d 1 complexed with IgG [35] and house dust mite antigen in cord blood [36]. Thus, such antigens would be available to prime fetal T cells. The ability of the neonatal immune system to respond to antigens is now accepted [37] and this ability is illustrated by the high frequency of proliferative responses observed in the current study. Another important question raised is how extensive a range of antigens can prime the fetal immune system, since this has important implications for the study and manipulation of immunity to food, allergens, self-antigens, microorganisms and vaccines in neonates and subsequently adults.

The transfer of advantageous maternal environmental adaptations to offspring may be a common biological phenomenon, possibly reflecting some form of evolutionary advantage [38]. However, although the vertical transmission of antigen to stimulate cell-mediated immunity may be advantageous under most circumstances, we propose that recent environmental changes have adversely influenced the process of fetal allergen sensitization in such a way as to predispose to allergic disease. Thus, we hypothesize that changes, not in the prevalence of *in utero* allergen sensitization but in the conditions under which it occurs, are responsible for the current epidemic of childhood allergy.

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