

Cytokine production by cord blood mononuclear cells stimulated with cows milk proteins *in vitro*: interleukin-4 and transforming growth factor β -secreting cells detected in the CD45RO T cell population in children of atopic mothers

A. C. Hauer*, M. Riederer*, A. Griessl*, H. Rosegger* and T. T. MacDonald†

*Universitätsklinik für Kinder- und Jugendheilkunde, Karl-Franzens-Universität, Graz, Austria and †Division of Infection, Inflammation and Repair, University of Southampton School of Medicine, Southampton, UK

Summary

Background Food antigens from the maternal circulation may sensitize fetal T cells *in utero* and be an important determinant in the development of food allergy.

Methods Here we have examined the spontaneous and recall response to cow's milk proteins of cord blood mononuclear cells (CBMC) of newborn children, using single cell ELISPOT assays.

Results In term newborns, confirming previous studies, the spontaneous cytokine response of CBMC is dominated by IL-4, IL-5, IL-10, and as shown here for the first time, TGF- β . For TGF- β only, the response of samples from infants of atopic mothers was significantly lower than samples from infants of non-atopic mothers. *In vitro* stimulation of CBMC with bovine serum albumin, casein and β -lactoglobulin resulted in a significant increase of all cytokine-secreting cells, again dominated by T helper type 2 (Th2) cytokines. There was a clear tendency for samples from infants of atopic mothers to have lower Th2 responses than samples from infants of non-atopic mothers, which was particularly significant for both IL-4 and TGF- β . Spontaneous cytokine secreting cells were virtually absent in cord blood from infants < 34 weeks gestation, as were cows milk protein-induced responses, although they were readily detectable in samples from infants aged > 34 weeks. To explore whether the cytokine secreting cells were in the naive CD4⁺ CD45RA population or memory CD4⁺ CD45RO T cells, these subsets were purified by positive and negative selection and tested for spontaneous and cows milk protein-induced cytokine responses. Strikingly, although the responses were small, the CD45RO⁺ cells from children of atopic mothers showed significant spontaneous and antigen-specific IL-4 and TGF- β responses, whereas the same population from infants of non-atopic mothers showed virtually no response. In addition CD45RA⁺ cells from infants of mothers with maternal atopy showed decreased IL-4 and TGF- β responses, especially the latter.

Conclusions The cows milk antigen-specific IL-4 and TGF- β responses preferentially seen in the memory cell subset of infants with a maternal history of atopy strongly suggests Th2 skewing to dietary antigens *in utero*.

Keywords atopy, ELISPOT, interleukin-4, memory T cells, newborns, transforming growth factor-beta

Submitted 2 January 2002; revised 12 June 2002; accepted 24 January 2003

Introduction

The epidemic of atopy and asthma in children in the developed world has focused attention on the determinants of allergic sensitization early in life, with particular focus on the role of T helper type 1 (Th1) cells and Th2 cells and fetal sensitization. Studies using cord blood mononuclear cells (CBMC) stimulated with polyclonal activators such as phytohaemagglutinin (PHA) or cows milk protein antigens, have clearly demonstrated that neonates who develop atopy show reduced

secretion of IFN- γ at birth compared to neonates who do not subsequently develop atopy [1, 2]. Further studies have examined antigen-specific responses of CBMC and showed not only was it possible to reproducibly achieve weak antigen-specific responses to house dust mite, egg albumen, β -lactoglobulin (β -LG) and cat allergen but that these responses were markedly Th2-skewed [3, 4]. Longitudinal studies have now shown that continuing allergen-specific Th2 responses during the first 2 years of life are a feature of the development of atopic disease [4], as well as continuing impairment of IFN- γ production [5], and the persistence of Th2 responses into early adolescence [6].

Antigen-specific responses of CBMC strongly suggest sensitization of the fetus *in utero* and transfer of allergens into amniotic fluid from maternal circulation is now established

Correspondence: A. C. Hauer, Universitätsklinik für Kinder- und Jugendheilkunde, Auenbruggerplatz, A-8036, Graz, Austria.
E-mail: almuthe.hauer@kfunigraz.ac.at

[7–9]. It is still unclear as to why fetal priming leads to a Th2-biased immune response, although successful completion of pregnancy will depend on down-regulated cell-mediated immunity to prevent the fetus being rejected as an allograft. Clearly based on present knowledge of the factors which control Th1 versus Th2 differentiation, it would suggest either a deficiency of Th1-inducing cytokines such as IL-12 or IL-18, or overexpression of Th2-inducing factors such as IL-4 in the fetal environment [10]. Because IL-12 is produced by dendritic cells and macrophages in response to bacteria [11], the sterile environment *in utero* will limit this pathway and perhaps allow Th2 responses to develop as a default pathway.

Functional T cell subsets in addition to Th1 and Th2 lineages have now been defined. Feeding low doses of antigens, especially in mice, is a particularly effective way of generating T cells capable of making TGF- β [12, 13]. In addition, by growing CD4 T cells *in vitro* with antigen and IL-10, it is possible to generate cell lines which make large amounts of IL-10, and lesser amounts of Th2 cytokines [14]. There have been no studies to date in which TGF- β cells have been examined in the neonate. Likewise if Th2 sensitization to food antigens occurs *in utero* in the infants of atopic mothers, then one might expect that these cells would have the phenotypic markers of antigen-activated T cells, such as CD45RO ([15–18])

In this work we have used single-cell cytokine ELISPOTs to examine the response of CBMC to three antigens of cows milk-casein, β -LG and bovine serum albumin (BSA). Our aim was to extend the profile of non-Th1 cytokines expressed in the neonate and so we examined TGF- β 1 responses as well as interferon- γ , IL-4, IL-5 and IL-10. In addition, we examined the ontogeny of antigen-specific responses in CBMC, the relationship between maternal history of atopy and cytokine responses, and whether the antigen-specific ELISPOT responses of CBMC were present in the naive (CD45RA) or memory (CD45RO) cell subsets.

Materials and methods

Subjects and samples

This study received approval from the ethical committee of the University of Graz, Austria. Venous blood samples were taken in heparinized tubes from the fetal site of the umbilical cord of 141 term (66 male, 75 female) and 58 pre-term infants (34 male, 24 female, 26–34 weeks' gestation) immediately after delivery. The history of maternal atopy was based on a validated questionnaire [19, 20] presented to the mothers by one of the authors (HR) not earlier than 48 h post-delivery and before the investigators were aware of the assay results.

Questions included a detailed history of clinical manifestations of atopy, focusing on immediate (< 60 min) reactions to inhalent (pollen, house dust mite, mould, cat, dog, horse) and food allergens (cows milk, fish, eggs). Questions were also included about symptoms suggestive of bronchial hyper-reactivity, occurrence of cough and/or wheezing and/or dyspnoea during the pollen season, at night or at contact with animals. For perennial rhinoconjunctivitis, questions included nasal blocking and/or discharge and/or eye oedema or pruritus during the pollen season. For atopic dermatitis, questions included the occurrence of chronic itching eczema, particularly of the flexural folds. Mothers were also asked to provide the

authors with medical records including radioimmunosorbent (RIST) and radioallergosorbent (RAST) tests to relevant antigens, but documentation of these tests was not an essential inclusion criterion. Mothers who had had symptoms of atopy as described above for at least 24 months, also if earlier in life, were classified as atopic. Those who had never had any of these symptoms were classified as non-atopic. Overall, 60 mothers had a clinically relevant history of atopy. Thirty-nine had immediate reactions to pollen and/grass and three to cat dander. The remaining 18 had suffered from atopy early in life, including eight with recurrent bronchial hyper-reactivity to pollen, seven with atopic eczema, two with cows milk allergy and one with an anaphylactic episode to shellfish. These details were confirmed by medical records ($n = 49$) and/or unequivocal RIST (> 100 KU/L) and RAST ($> +++$) values ($n = 18$). Full blood cell counts and assessment of mononuclear cell subpopulations was also carried out in the term born infants.

It is important to emphasize that this is not a cohort study. We collected sequential samples of cord blood and accumulated data on ELISPOTs so that we could be sure of differences between samples from atopic versus non-atopic mothers, then we then went on and analysed other parameters, such as the response of CD45 subsets. At no time were any samples included or excluded for analysis and there has been no selection in the presentation of the data.

Isolation of CBMC and lymphocyte culture

Umbilical cord blood mononuclear cells (UCBC) were obtained from 0.5 to 2.5 mL of venous heparinized cord blood layered on a Ficoll-Hypaque density gradient using standard procedures. The cells in the buffy coat were collected and washed three times in RPMI-1640 medium (Sigma, Poole, UK) containing sodium bicarbonate, mercaptoethanol, penicillin, streptomycin, gentamicin and supplemented with 10% heat-inactivated fetal calf serum (FCS, Life Technologies, Paisley, UK). After resuspension in 1 mL of medium, cell numbers were determined using a haemocytometer. Viable cells were identified by trypan blue (0.1%) exclusion and were always more than 95% alive. Cells were immediately used for the ELISPOT assay at 100 000 cells/well.

Cow's milk proteins [BSA, β -LG, casein (Cas)] and keyhole limpet haemocyanin (KLH) were purchased from Sigma. Aliquots (200 μ L) containing $0.7\text{--}1.25 \times 10^5$ mononuclear cells were applied per well in round-bottomed 96-well microtitre plates (Greiner, Frickenhausen, Germany). Cow's milk proteins (BSA, β -LG, Cas) were added per well to final concentrations of 100 μ g/mL (BSA) or 500 μ g/mL (β -LG; Cas, KLH). Negative controls consisted of mononuclear cell suspensions in culture medium only. After culture for 24 h at 37° and 5% CO₂, the cells were washed with RPMI/10% FCS and counted.

Enumeration of IFN- γ , IL-4, IL-5, IL-10 and TGF- β 1-secreting cells

Antibodies to IFN- γ were purchased from Chromogenix AB (Molndal, Sweden), IL-4 antibodies from Mabtech AB (Stockholm, Sweden) and IL-5 and IL-10 antibodies from Pharmin-gen (San Diego, CA, USA). ELISPOT assays for detection of these cytokines were carried out exactly as described elsewhere [21]. TGF- β 1 antibodies were purchased from R & D Systems (Minneapolis, MN, USA). All steps of the TGF- β assay were

performed as for the above-mentioned cytokines, but optimal concentrations of the antibodies as well as the optimal buffer solutions and cell media had to be determined in preliminary experiments. Thus the capture antibody was used at 3 µg/mL (in 100 µL of PBS/well) and the biotin-labelled detecting antibody at 70 ng/mL (in 100 µL of TBS/0.1% BSA/well). The RPMI cell medium used was supplemented by serum replacement 2 (Sigma). All assays were performed in duplicate. The ELISPOT assay was carried out overnight.

The number of spots in each well was enumerated using a dissecting microscope ($\times 25$ magnification). The frequency of spot-forming cells per 10^5 input mononuclear cells was then calculated. The spontaneous cytokine response was expressed as the number of spot-forming cells/ 10^5 mononuclear cells (SFC/ 10^5 MNC) in the absence of antigen. In the stimulation assays, the antigen-elicited response was expressed as Δ -ELISPOTS/ 10^5 MNC (i.e. number of antigen elicited SFC minus the number of spontaneous SFC/ 10^5 MNC). All counts were checked by two of the authors (AG and ACH, or MR and ACH).

Positive selection for CD14⁺ CBMC

Mononuclear cell suspensions were prepared and then incubated with Anti-CD14 MicroBeads (MACS, Miltenyi Biotec Inc., Auburn, CA, USA), diluted 1:5 in phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 0.5% BSA for 15 min at 6 °C. Cells were washed, resuspended in buffer and positively selected according to the manufacturer's instructions. The purity of CD14⁺ cells in the various cell fractions was quantified by FACS analysis and was > 95%.

Positive and negative selection for CD4⁺ CD45RA⁺ and CD4⁺ CD45RO⁺ UBM

As expected, 87.6% of CBMC CD4 cells were CD45RA⁺ and 12.3% of them CD45RO⁺ (not shown). First, CD4⁺ T cells were isolated by negative selection using CD4⁺ T cell isolation kit (MACS, Miltenyi Biotec Inc., Auburn, CA, USA). Briefly, cells were incubated with a hapten-antibody cocktail (consisting of hapten-conjugated CD8⁺, CD11b⁺, CD16⁺, CD19⁺, CD36⁺ and CD56⁺ mABs), diluted 1:5 in PBS supplemented with 2 mM EDTA and 0.5% BSA for 15 min at 6 °C. After two washing steps, cells were resuspended in buffer and incubated with MACS anti-hapten microbeads diluted 1:5 in buffer for 15 min at 6 °C. Cells were again washed twice and the purity of the CD4⁺ fraction evaluated by flow cytometry. From these pre-selected CD4⁺ cells, CD45RA⁺ and CD45RO⁺ cell fractions were then isolated by incubation with MACS CD45RA and MACS CD45RO MicroBeads, respectively, diluted 1:5 in PBS supplemented with 2 mM EDTA and 0.5% BSA for 15 min at 6 °C. After a final wash cells were positively selected for the CD45RA and CD45RO subfractions according to the manufacturer's instructions. Both efficiency of the magnetic separation and quantification of these subfractions was then assessed by FACS analysis. The selected cell populations were > 95% CD4⁺ and CD45RA⁺ or CD45RO⁺. The negatively selected CD45RA and R0 cells were also analysed in some children.

Statistical analysis

Data were first tested to determine whether they conformed to a sample from a normally distributed population. If they did, comparison between the control groups was done by a *t*-test;

if the normality test failed, then a two-tailed Mann-Whitney test was used. A paired *t*-test was used to determine if the response elicited by antigen was greater than that seen in cells cultured in media alone. All *P*-values shown have been corrected for multiple comparison errors.

Results

Spontaneous cytokine secretion in term infants

ELISPOTS for spontaneous secretion of all five cytokines were performed on blood samples of 78 neonates > 38 weeks' gestation. Forty-seven of these infants had no history of maternal atopy and in 31, maternal atopy was known. In both groups, the same hierarchy of cytokine secretion was found, with the highest frequency for IL-4-secreting cells, followed by TGF-β1, IL-10, IL-5 and IFN-γ (Fig. 1). Although there was a clear tendency for samples from children with atopic mothers to have lower values than those of non-atopic mothers, only for TGF-β1-secreting cells did this achieve significance (*P* < 0.005).

TGF-β1 can be made by many different cell types and so we positively selected CD4 T cells and CD14 cells (macrophages) from 10 samples and analysed TGF-β1 ELISPOTs. In unseparated cells, there was a median of 16 (3–68)/ 10^5 cytokine secreting cells. In the CD14⁺ population this was reduced to a median of 5 (3–8)/ 10^5 SFC. However, in the CD4⁺ population the frequency increased to 27 (6–112) SFC/ 10^5 input cells. These results show that the majority of spontaneous TGF-β-secreting cells in CBMC are CD4 T cells.

Cow's milk protein induced cytokine secretion in term infants

In vitro stimulation of CBMC with BSA, β-LG and casein resulted in a significant increase in the numbers of all cytokine-secreting cells, in particular upon stimulation with casein (*P* < 0.01). Again, frequencies of Th2 cytokines were significantly higher than those of IFN-γ. In infants with a history of

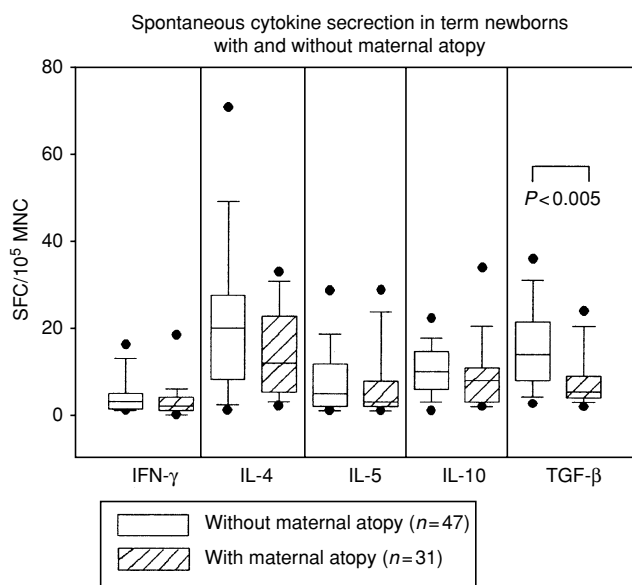


Fig. 1. Spontaneous cytokine production in CBMC of 47 children without a history of maternal atopy and 31 with a history of maternal atopy.

maternal atopy, overall frequencies tended to be lower (Fig. 2) and achieved significance for the IL-10 casein response, the β -LG and casein IL-4 response and the TGF- β response to all milk proteins.

We also investigated responses to KLH, an antigen to which Austrian mothers should not have been exposed, in 17 babies of non-atopic mothers and in 13 babies of atopic mothers. The spontaneous SFC results for the non-atopics were-IFN- γ : median of 3 (range 2–13), IL-4: median of 20 (range 4–43), IL-5: median of 5 (range of 2–29), IL-10: median of 7 (range

of 2–22); TGF- β : median of 18 (range of 3–38). For the 13 atopics, the results were-IFN- γ median of 1 (range 1–12), IL-4: median of 16 (range of 2–37); IL-5: median of 4 (range of 2–27), IL-10: median of 5 (range of 2–21); TGF- β : median of 7 (range of 2–23). When the cells were stimulated with KLH at a concentration of 500 μ g/mL, an increase of 1–2 SFC was seen in two CBMC samples from children of atopic mothers and one sample from a child of non-atopic mothers. We also used 100 μ g/mL KLH in 10 patients and observed no increase over the spontaneous production.

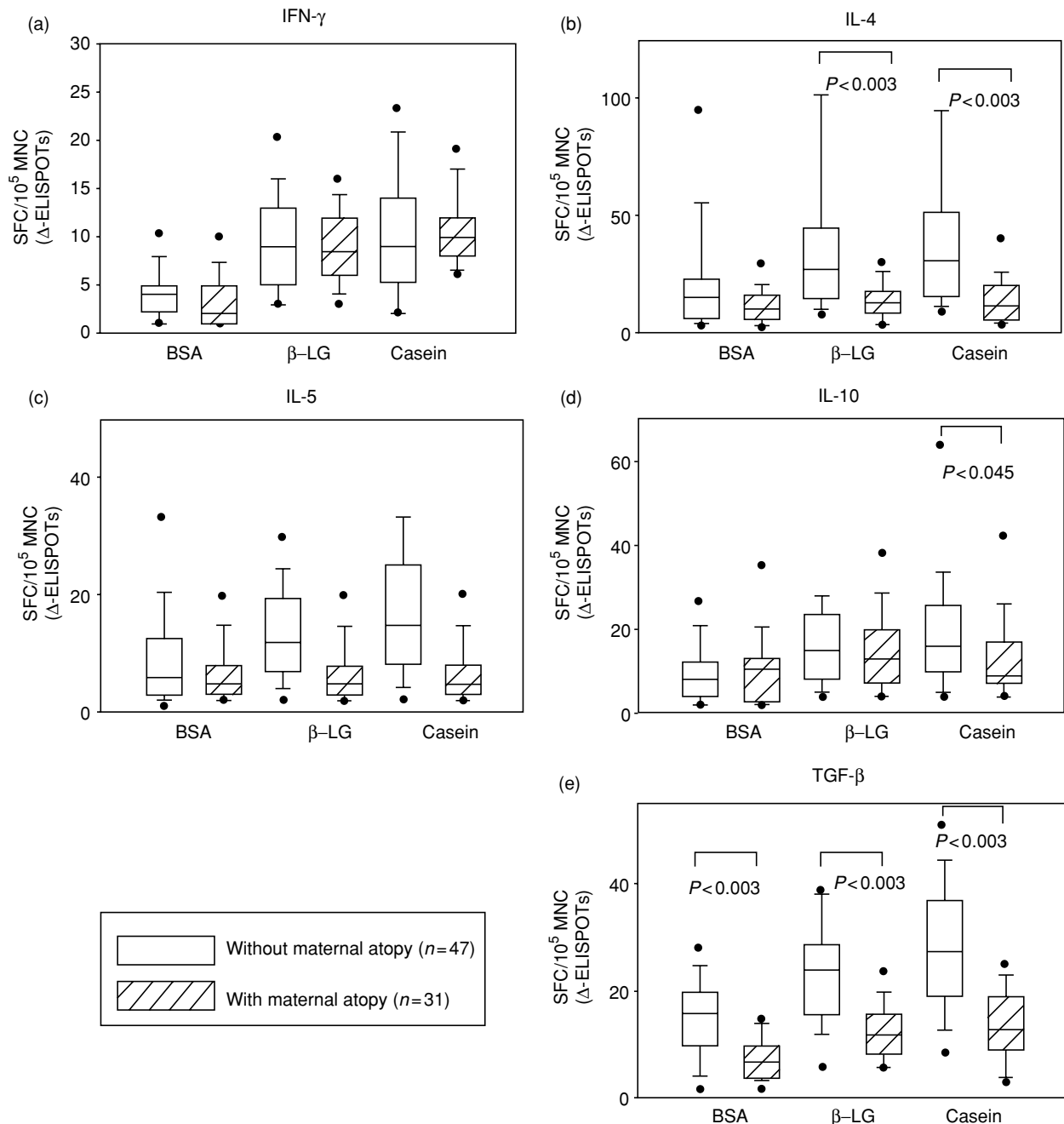


Fig. 2. Antigen-specific cytokine responses in CBMC of term newborns with ($n = 31$) or without maternal atopy ($n = 47$). All values are the increase in the number of ELISPOTS over the spontaneous production.

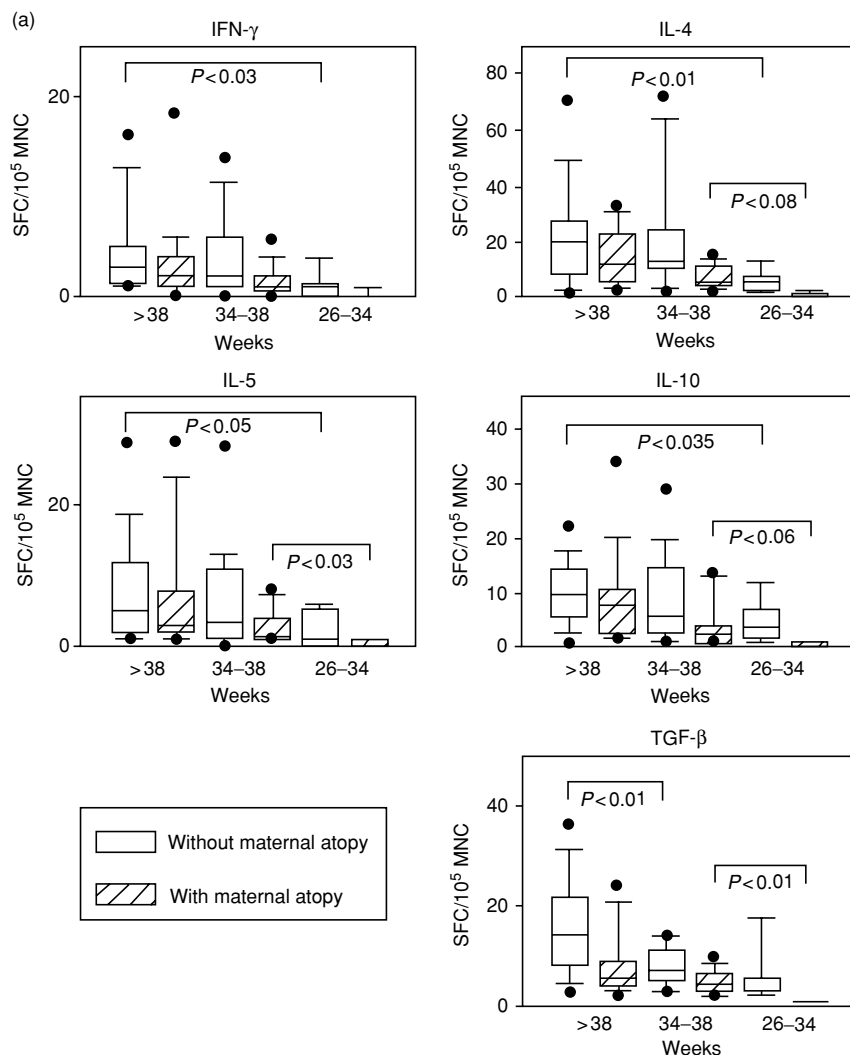
Spontaneous cytokine secretion in preterm infants

ELISPOTS for spontaneous secretion of all five cytokines were performed on blood samples of 58 pre-term (< 38 weeks) born infants (Fig. 3a). Samples from infants aged less than 38 weeks had lower numbers of cytokine secreting cells than infants greater than 38 weeks gestation. Indeed, in samples from infants born at 26–34 weeks' gestation, responses were minimal, and for infants of non-atopic mothers the responses at ≥ 38 weeks and ≤ 34 weeks differed significantly for all cytokines.

Antigen-specific responses were also measured (Fig. 3b) and showed the same age-dependency as spontaneous cytokine secreting cells, with infants born over 38 weeks showing the largest responses. For brevity, only casein responses are shown, but the same results were obtained with BSA and β -LG. Responses in infants less than 34 weeks' gestation were minimal. Again, there was markedly smaller IL-4 and TGF- β responses in infants older than 38 weeks born of atopic mothers, but the differences disappeared in younger infants, probably due to the weak responses detected.

Frequency of cytokine-secreting cells in $CD4^+ CD45RA^+$ and $CD4^+ CD45RO^+$ T cells of term infants with and without maternal atopy

Positive selection for $CD4^+ CD45RA^+$ and $CD4^+ CD45RO^+$ T cell subsets was performed in another unselected 34 term infants and the frequency of IFN- γ , IL-4 and TGF- β 1-SFC in the various subsets assessed. Limited number of cells were available after purification, therefore the number of different cytokines analysed was also limited to IFN- γ , IL-4 and TGF- β 1. In 22 of these infants, there was no history of maternal atopy and in the remaining 12 maternal atopy was known. Spontaneous cytokine secretion was seen in purified CD4 cells, and again the IL-4 and TGF- β response was markedly higher than the IFN- γ response (Table 1). The TGF- β 1 response of CD4 cells from children of atopic mothers was markedly lower than infants from non-atopic mothers. In the $CD45RA^+$ cells, IL-4 and TGF- β responses were greater than the IFN- γ responses, and for TGF- β 1 the response was also lower in infants of atopic mothers compared to infants from



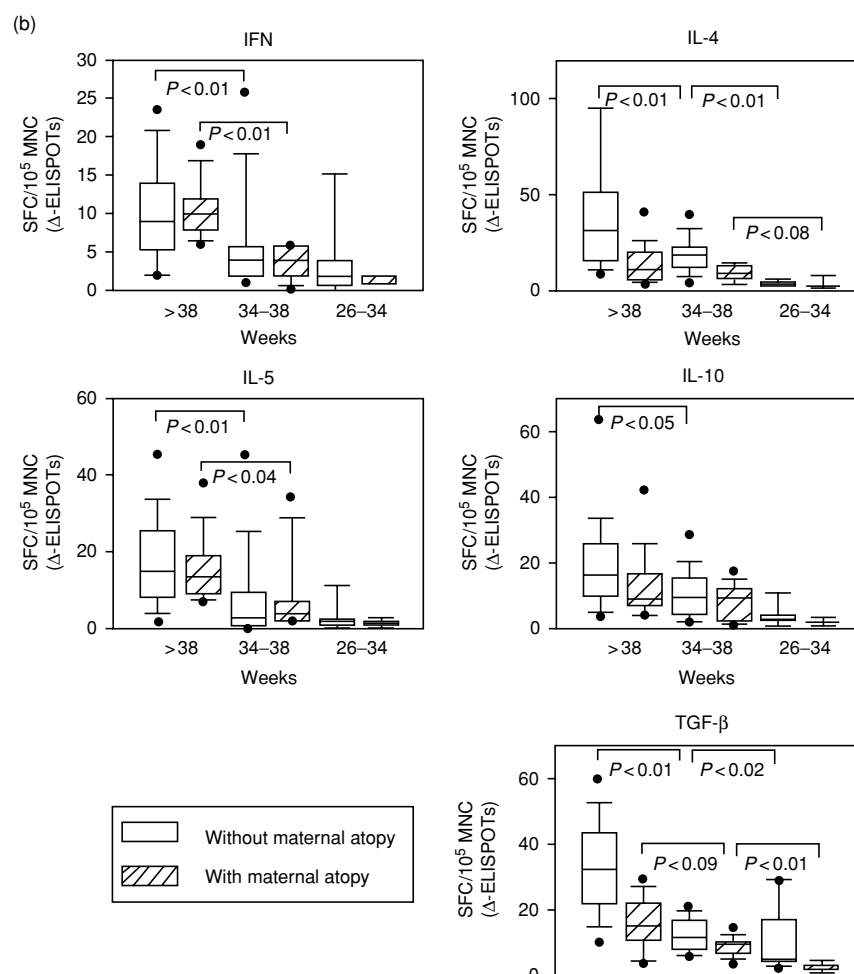


Fig. 3. (a) Spontaneous cytokine production in CBMC of infants born before 38 weeks' gestation with and without a history of maternal atopy. For comparison the data in term children born over 38 weeks' gestation from Fig. 1 is also shown. In the 34- to 38-week age group, 30 children were born of mothers with no history of atopy and 12 were born of mothers with maternal atopy. In the 26- to 34-week age group, 10 children were born of mothers with no history of atopy and six were born of mothers with maternal atopy. The box and whisker show the 95% confidence intervals and the solid dots show the range. (b) Casein-induced cytokine responses in the same children as shown in (a). The box and whisker show the 95% confidence intervals and the solid dots show the range. All values are the increase in the number of ELISPOTs over the spontaneous production.

Table 1. Spontaneous ELISPOTs in positively selected CD4⁺, CD45RA⁺ and CD45RO⁺ cord blood T cells from children with atopic ($n = 12$) or non-atopic mothers ($n = 22$)

	Spontaneous ELISPOTs/10 ⁵ cells (median and range)		
	CD4 ⁺	CD45RA ⁺	CD45RO ⁺
IFN-γ			
Non-atopic	5 (2–17)	5 (1–15)	0 (0–1)
Atopic	4 (1–24)	3 (1–12)	1 (0–1)*
IL-4			
Non-atopic	31 (11–187)	25 (9–164)	1 (0–3)
Atopic	26 (10–156)	20 (8–125)	5 (2–14)†
TGF-β			
Non-atopic	28 (6–112)	25 (4–98)	2 (0–3)
Atopic	15 (4–45)*	12 (3–41)*	4 (2–6)†

* $P < 0.03$, atopic versus non-atopic; † $P < 0.003$, atopic versus non-atopic.

non-atopic mothers. Interestingly, in the CD45RO⁺ cells, for all cytokines, significantly greater responses were seen in infants with atopic mothers, than in samples from babies with non-atopic mothers. However, it must be emphasized that the responses of the CD45RO⁺ cells were extremely low, and in the case of samples from children of non-atopic mothers, the majority gave no response.

Antigen-elicited responses were also examined (Table 2). Generally, this response was approximately twice as large as the number of spontaneous SFC's for the CD4⁺ and the CD4⁺ CD45RA⁺ T cells, and was highly significantly different ($P \leq 0.001$), with casein and β -LG eliciting larger responses than BSA. However, there was considerable interindividual variability. For CD4 cells, the IL-4 and TGF- β response was higher than the IFN- γ response to all antigens. Antigen-elicited responses were also extremely low in the CD45RO cells and for all cytokine/antigen combinations, the response of cells from non-atopic mothers was not significantly greater than

Table 2. Antigen-specific ELISPOTs in positively selected CD4⁺, CD45RA⁺ and CD45RO⁺ cord blood T cells from children with atopic (*n* = 12) or non-atopic mothers (*n* = 22)

		ΔAntigen-specific ELISPOTs/10 ⁵ cells (median and range)								
		CD4 ⁺			CD45RA ⁺			CD45RO ⁺		
		BSA	β-LG	Casein	BSA	β-LG	Casein	BSA	β-LG	Casein
IFN-γ										
Non-atopic		7 (3–15)	12 (4–35)	13 (3–33)	6 (1–9)	12 (4–31)	12 (5–32)	0	0	0
Atopic		3 (2–14)	9 (2–29)	9 (4–27)	4 (1–8)	11 (3–24)	10 (3–24)	0	0	0
IL-4										
Non-atopic		19 (7–161)	29 (6–180)	35 (4–190)	17 (5–154)	27 (3–183)	32 (4–201)	0	1	0
Atopic		14 (4–23)	16 (5–27)	16 (4–29)	12* (3–21)	15 (5–23)	14 (5–19)	1† (0–2)	4* (3–8)	4* (2–9)
TGF-β										
Non-atopic		18 (6–120)	26 (8–150)	29 (5–148)	16 (4–132)	26 (6–135)	28 (3–141)	0	0	0
Atopic		10 (4–118)	16 (6–20)	17 (6–21)	8† (4–13)	13† (5–19)	13 (4–20)	2* (0–3)	4* (2–9)	5* (2–9)

**P* < 0.03, atopic versus non-atopic; †*P* < 0.003, atopic versus non-atopic.

the spontaneous number of ELISPOTs. Only a single patient gave a single IL-4 SFC to β-LG. However, in the CD45RO⁺ cells from infants with atopic mothers, small antigen-specific responses were obtained from most patients, which were significantly greater than the spontaneous SFC's (*P* ≤ 0.01). The IL-4 and TGF-β response of the CD45RA⁺ cells was significantly lower in the infants of atopic mothers compared to infants of non-atopic mothers. It should be emphasized, however, that by far the greatest antigen-specific responses were seen in the CD45RA⁺ cells from all samples.

To determine if the positive selection procedure was responsible for the weak responses in the RO population and the strong responses in the RA population, we also examined the

antigen-specific responses of the negatively selected cells in seven patients with a history of maternal atopy and seven without a history of maternal atopy (Table 3). Results were identical to that seen with the CD45RO positively selected cells in that the non-RA⁺ cells from children with atopic mothers gave significantly greater responses than the non-RA⁺ cells from children of non-atopic mothers.

Discussion

To our knowledge this is the first study in which ELISPOTs have been used to analyse the numbers of cells secreting cytokines in CBMC. When we measured the spontaneous production of cytokines by CBMC it was clear that IFN-γ responses were lower than IL-4, IL-5, IL-10 and TGF-β. These results are therefore consistent with others and show that the T cell response of CBMC is Th2-dominated [3, 4]. To our knowledge, however, TGF-β has not been studied before so we can add this cytokine to the list of non-Th1 cytokines overexpressed at birth. An interesting feature of the spontaneous TGF-β response was that it was markedly lower in infants of atopic mothers than in infants of non-atopic mothers.

Antigen-specific responses to cow's milk proteins were also examined and responses were obtained in all infants. A striking feature of these responses was the fact that for all three proteins, there was a clear and significant reduction in the number of Th2 SFC (especially IL-4 and TGF-β) in samples from infants of atopic mothers compared to infants of non-atopic mothers. This at first might seem counter-intuitive in that one would expect these infants to have higher responses, if *in utero* sensitization to cow's milk proteins is priming Th2 cells in a genetically susceptible baby. There could, however, be a number of reasons for this difference. For example, we are sampling

Table 3. Antigen-specific ELISPOTs in negatively selected CD45RO⁺ cord blood T cells from children with atopic (*n* = 7) or non-atopic mothers (*n* = 7)

		Antigen-specific ELISPOTs/10 ⁵ cells (median and range)			
		Spont	BSA	β-LG	Casein†
IL-4					
Non-atopic	1 (0–3)	0	0	0	0
Atopic	4* (2–10)	1 (0–2)	3* (1–5)	3* (1–7)	3*
TGF-β					
Non-atopic	2 (1–11)	0	0	0	0
Atopic	4* (2–7)	2* (2–5)	2* (2–4)	3* (1–4)	3*

**P* < 0.03, atopic versus non-atopic; †For each sample, the antigen-specific response is the number of ELISPOTs over and above that seen in medium alone.

CBMC and it may be that the *in utero*-sensitized Th2 cells in infants of atopic mothers are in the lymph nodes, spleen, liver, gut or lung. However, the same result has been seen in CBMC of children who later went on to develop atopic disease [4]. It has been also suggested that this might be due to delayed maturation of the Th2 response in individuals destined to become atopic [3].

There have been a number of other studies along these lines and they fall into two groups. First, CBMC responses to allergens have been measured and then the infants followed to determine if they develop allergy or not. The response of CBMC is then retrospectively analysed between clearly atopic and non-atopic children. Using this kind of analysis, Hill et al. [1] showed that PHA-induced IFN- γ responses were lower in atopic children than non-atopic children at birth, and Warner et al. [2] showed decreased IFN- γ production to β -LG in CBMC of children who developed atopy by 12 months of age. Prescott et al. [3], however, showed that the allergen-induced IL-4 and IL-5 mRNA responses of CBMC of children who developed atopy were lower than those who did not develop atopy, which is consistent with our observations on children at risk rather than those with atopic disease. The second type of study, including this one, examines the response of CBMC and tries to relate the responses to the maternal atopic status. We found no differences in IFN- γ production between the two groups, but again this is confounded by the fact that we analysed children at risk of atopy because of maternal atopy, rather than clinically defined atopy. Similar results have recently been obtained by Kopp et al. [22] who found identical β -LG induced IFN- γ production in CBMC from infants whose mothers had proven allergy and those who did not. IL-5 was also measured in this study and again there was no difference. On the other hand, Liao et al. [23], using cytokine enzyme-linked immunosorbent assays (ELISAs) showed that the PHA-induced IFN- γ responses in infants of atopic mothers were lower than the responses of infants from non-atopic mothers. However, using intracellular cytokine staining, Yang et al. [24] could see no difference in IL-4 or IFN- γ production between infants of non-atopic mothers and infants of atopic mothers. Overall, there are conflicting data in that some studies suggest that CBMC responses can be related to maternal atopy and others do not, but the different methodologies used make it hard to make any firm conclusion.

The most interesting result from this study, however, came when we analysed spontaneous and antigen-specific responses of CD4 cell subsets. For CD4 cells, the pattern of responses were the same as for CBMC in that the spontaneous and antigen-specific responses were much higher for IL-4 and TGF- β than IFN- γ . Likewise the spontaneous TGF- β SFC response was lower in infants with atopic mothers than non-atopic mothers. The same trend was seen in the antigen-specific responses but did not achieve significance after we corrected for multiple comparison errors. In the RA and RO subsets, the largest number of antigen-specific SFC was seen in the naive population, which is difficult to reconcile with the notion that this is a population which has not been exposed to antigen [15–18]. Nonetheless, when we measured CBMC responses to KLH, an antigen to which Austrian mothers are unlikely to be exposed, we did not manage to generate an antigen-specific response. Therefore the responses to cow's milk proteins do appear to be recall responses and not primary responses, and

yet paradoxically, they are obtained from the CD45RA population. We have no explanation for this, although we are reassured that Devereux and colleagues [25] were also able to demonstrate proliferative responses by CBMC CD45RA⁺ cells to grass pollen.

In the CD45RO populations, however, although the numbers were small, it was clear that the majority of spontaneous and antigen-specific responses were in the infants with atopic mothers. Indeed, the vast majority of samples from non-atopic mothers failed to show a response. The most obvious explanation for this is that sensitization has resulted in activation and the switch from RA to RO occurred *in utero*. Given the fact that it is now clear that antigens and allergens can cross the placenta into amniotic fluid and the fetal circulation [7–9], the fact that the memory cell response was virtually only seen in the children of atopic mothers (and it seems probable that antigen will reach the fetal circulation in non-atopic mothers), means that the sensitization *in utero* may have a genetic basis, rather than just being due to exposure to antigen.

We also had the opportunity to examine spontaneous and antigen specific responses in CBMC from infants as young as 26 weeks' gestation. There was a clear age-related effect in that the responses at 26–34 weeks of age were markedly lower than older babies. This is consistent with earlier studies using antigen-induced proliferation as a readout which showed that these also increased with age [26]. However, the same study also showed that very weak proliferative responses were seen in a minority of infants at 23 weeks of age. It is a matter of interpretation as to whether these proliferative responses are biologically significant [27]. For example, in our experiments, infants born near term had a spontaneous IL-4 response which ranged from 0 to 70 SFC/10⁵ cells. When we added casein, the response more than doubled in most patients and was clearly obvious in the assay. However, in the youngest specimens analysed at 26–34 weeks' gestation, the spontaneous production was less than 10 SFC/10⁵ cells and with casein the numerical increase was in single figures. One could argue that these small increases are significant, but we are cautious in making any suggestion based on these data, that *in utero* sensitization does not occur until the third trimester.

The activity of Th1 and Th2 cells can be regulated by Th3 cells making TGF- β or Tr cells making IL-10 [12–14]. Because we only measured individual cytokine-secreting cells, we cannot make any conclusions about the functional role of these cells. Nonetheless, it was striking that no major differences were seen in either the spontaneous or antigen-specific responses of IL-10-secreting cells in children of atopic or non-atopic mothers. In contrast, the cells secreting TGF- β were consistently lower in children of atopic mothers, both in terms of spontaneous and antigen-specific responses. This would suggest that there may be some deficiency in Th3 cells from children of atopic mothers, but further functional studies need to be carried out.

Acknowledgements

This study was supported by a grant from the Rudolph-Chaudoire-Wissenschaftsförderung. We thank Dr Michael Mokry for help in preparing the illustrations and Dr Judith Holloway and Dr Cathy Jones for general advice.

References

- 1 Tang ML, Kemp AS, Thornburn J, Hill DJ. Reduced interferon- γ secretion in neonates and subsequent atopy. *Lancet* 1994; 344:983–6.
- 2 Warner JA, Miles EA, Jones AC et al. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy* 1994; 24:423–30.
- 3 Prescott SL, Macaubas C, Holt BJ et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998; 160:4730–7.
- 4 Prescott SL, Macaubas C, Smallacombe T et al. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; 353:96–200.
- 5 Jujo K, Renz H, Abe J et al. Decreased interferon-gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. *J Allergy Clin Immunol* 1992; 90:323–31.
- 6 Jenmalm MC, Van Snick J, Cormont F, Salman B. Allergen-induced Th1 and Th2 cytokine secretion in relation to specific allergen sensitisation and atopic symptoms in children. *Clin Exp Allergy* 2001; 31:1528–35.
- 7 Van Duren-Schmidt K, Pichler J, Edbner C et al. Prenatal contact with inhalant allergens. *Pediatr Res* 1997; 41:128–31.
- 8 Holloway JA, Warner JO, Vance GHS et al. Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. *Lancet* 2000; 356:1900–2.
- 9 Szepefalusi Z, Loibichler C, Pichler J et al. Direct evidence for transplacental allergen transfer. *Pediatr Res* 2000; 48:404–7.
- 10 Maldonado-Lopez R, Moser M. Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin Immunol* 2001; 13:275–82.
- 11 Thoma-Uszynski S, Kiertcher SM, Ochoa MT et al. Activation of toll-like receptor 2 on human dendritic cells triggers induction of IL-12 but not IL-10. *J Immunol* 2000; 165:3804–10.
- 12 Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today* 1997; 18:335–43.
- 13 Strobel S, Mowat AM. Immune responses to dietary antigens: oral tolerance. *Immunol Today* 1998; 19:173–81.
- 14 Groux H, O'Garra A, Bigler M et al. A CD4⁺ T-cell subset inhibits antigen-specific T cell responses and prevents colitis. *Nature* 1997; 389:737–42.
- 15 Clement T. Isoforms of the CD45 leukocyte common antigen family: markers for human T cell differentiation. *J Clin Immunol* 1992; 12:1–10.
- 16 Beverly PC. Functional analysis of human T cell subsets defined by CD45 isoform expression. *Semin Immunol* 1992; 4:35–41.
- 17 Young JL, Ramage JM, Hill Gaston JS et al. *In vitro* responses of human CD45R0brightRA- and CD45R0-RAbright T cell subsets and their relationship to memory and naive T cells. *Eur J Immunol* 1997; 27:2383–9.
- 18 Plebanski M, Saunders M, Burbles SS et al. Primary and secondary human *in vitro* T-cell responses to soluble antigens are mediated by subsets bearing different CD45 isoforms. *Immunology* 1992; 75:86–92.
- 19 Bergmann RL, Bergmann KE, Lau-Schadensdorf S et al. Atopic diseases in infancy. The German multicenter atopy study (MAS-90). *Ped Allergy Immunol* 1994; 5:19–25.
- 20 ISAAC (International study of asthma and allergy in childhood) manual, 2nd edn. Münster, Germany: Institut für Epidemiologie und Sozialmedizin Universität, 1993.
- 21 Hauer AC, Breese EJ, Walker-Smith JA, MacDonald TT. The frequency of cells secreting interferon- γ , IL-4, IL-5, and IL-10 in the blood and jejunal mucosa of children with cow's milk hypersensitivity. *Ped Res* 1997; 42:629–38.
- 22 Kopp MV, Zehle C, Pichler J et al. Allergen-specific T cell reactivity in cord blood: the influence of maternal cytokine production. *Clin Exp Allergy* 2001; 31:1536–43.
- 23 Liao SY, Liao TN, Chiang BL et al. Decreased production of IFN gamma and increased production of IL-6 by cord blood mononuclear cells of newborns with a high risk of allergy. *Clin Exp Allergy* 1996; 26:397–405.
- 24 Yang YH, Chen MC, Tsai MJ et al. Costimulatory molecules expression and cytokine profiles of cord blood mononuclear cells in newborns with low and high risk of developing atopic disease. *J Microbiol Immunol Infect* 2000; 33:159–64.
- 25 Devereux G, Seaton A, Barker RN. *In utero* priming of allergen-specific helper T cells. *Clin Exp Allergy* 2001; 31:1686–95.
- 26 Jones AC, Miles EA, Warner JO et al. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Pediatr Allergy Immunol* 1996; 7:109–16.
- 27 Jones CA, Holloway JA, Warner JO. Does atopic disease start early in fetal life. *Allergy* 2000; 55:2–10.