

Antenatal determinants of neonatal immune responses to allergens

G. Devereux, R. N. Barker* and A. Seaton

*Departments of Environmental and Occupational Medicine and *Medicine and Therapeutics, University Medical School, Aberdeen, Scotland, UK*

Summary

Background The environmental factors responsible for recent increases in the prevalence of asthma and atopic disease have been assumed to act after birth. Their possible effects on fetal immune development *in utero* have not been investigated systematically, although sensitization to allergens may occur before birth.

Objective This prospective study determined whether the risk factors for asthma and atopic disease, namely family history of atopic disease, maternal smoking, birth order, or maternal dietary intake of antioxidant vitamins, exert antenatal effects on the fetal immune system that may predispose to childhood atopy.

Methods The T helper (Th) cell proliferative responses of cord blood mononuclear cells (CBMC) from a sample of 223 neonates, representative of children born to a cohort of 2000 pregnant women, were measured and related to family, maternal and environmental factors associated with the pregnancy.

Results The magnitude of CBMC-proliferative responses to allergens increased significantly in association with a family history of atopic disease or maternal smoking, and decreased significantly with increasing birth order and high maternal dietary intake of vitamin E. The epidemiological association between birth order and atopy may therefore be a consequence of antenatal influences rather than of protective effects of childhood infections. The association between maternal intake of vitamin E and CBMC responsiveness suggests that diet during pregnancy may influence the fetal immune system in such a way as to modulate the risk of childhood atopy.

Conclusion These results provide a new insight into the aetiology of atopic disease by demonstrating that the maternal environmental risk factors for atopy, diet, birth order and smoking, influence the development of the fetal immune system. This raises the prospect of preventative public health interventions during pregnancy.

Keywords atopy, T helper cells, antenatal influences, smoking, birth order, vitamin E

Submitted 29 June 2001; accepted 20 July 2001

Introduction

In recent years developed countries have experienced increases in the prevalence of the atopic diseases; asthma, eczema and hayfever. These have been particularly marked in children; for example in Aberdeen the prevalence of asthma in schoolchildren increased from 4.1% in 1964 to 19.6% in 1994 [1,2]. The rapidity of the increases suggests that they are due to changes in environment and/or lifestyle rather than in genetic predisposition. Epidemiological studies have identified a number of risk factors for asthma and atopy. A family history of atopic disease is the strongest known risk factor, and maternal smoking shows a weaker association [3]. It has also been proposed that childhood infections reduce the risk of atopic disease; this 'hygiene hypothesis' originally arose from the observation that atopic diseases exhibit a birth order effect, first-born children being at

the greatest risk [4]. The nutrition hypothesis attributes the increase in atopy to changes in the population's dietary intake of antioxidants [5] and/or lipids [6], and several studies in children and adults have shown an inverse association between dietary vitamin E/C intake and features of asthma [7,8] and atopy [9].

Atopic diseases are characterized by chronic immune-mediated inflammation initiated and perpetuated by T helper (Th) cells. Following the description of Th1 and Th2 clones, characterized by different patterns of Th cell cytokine secretion, including interferon (IFN)- γ and interleukin (IL)-4, respectively [10], it has become evident that atopic diseases are associated with Th2-biased responses [11]. In order to design rational primary preventative measures to reverse the increases in atopic disease, it is desirable to identify those interactions between the immune system and the environment that promote the development of atopy.

There is evidence that the immunological bias towards atopy can be established during the *in utero* development of the fetal immune system. Studies of neonates with a family history of atopy and neonates who subsequently develop atopic disease

Correspondence: Professor A. Seaton, Department of Environmental and Occupational Medicine, Medical School, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK. E-mail: a.seaton@abdn.ac.uk

have demonstrated that, at birth, cord blood mononuclear cells (CBMC)s demonstrate increased *in vitro* proliferative but reduced cytokine responses after stimulation with mitogens and allergens [12–18]. It has been shown that mite allergen can reach the fetal circulation [19]. It would thus appear that genetic susceptibility to atopy may influence *in utero* development of the fetal immune system. However, it is possible that other environmental factors may also be exerting *in utero* influences and that these may underlie the recent increase in atopic disease. Potential antenatal factors could include maternal diet during pregnancy, since it has been shown that childhood atopy is influenced by birth weight, length and head circumference, in part markers of fetal nutrition [20,21]. Maternal smoking increases the risk of childhood asthma but it has not been established whether this represents ante- or postnatal influences [3]. It is widely believed that the risk associated with birth order is a postnatal influence, but the possibility of *in utero* effects has not been investigated. The aim of the present study was to test the hypothesis that risk factors for asthma and atopy, namely family history, maternal smoking, birth order and dietary intake of antioxidants, can influence the developing fetal immune system, as reflected by *in vitro* CBMC-proliferative responses at birth.

Materials and methods

Subjects

Two thousand healthy pregnant women were randomly recruited at 16–20 weeks gestation during their first antenatal clinic visit, only excluding those attending certain disease-specific clinics. After obtaining written informed consent, a trained interviewer administered a standard respiratory symptom questionnaire that included detailed enquiries about respiratory symptoms, cigarette smoking and obstetric history. The presence of asthma, eczema and hayfever in the mother, father and siblings of the unborn child was also recorded and a family history of atopic disease was defined as asthma or eczema or hayfever in one of these relatives. The mothers were skin prick tested using ALK lancets (ALK, Hørsholm, Denmark) and the ALK solutions, saline (negative control), cat fur, timothy grass (*Phleum pratense*) and house dust mite (*Dermatophagoides pteronyssinus*). Skin weal diameters were measured after 15 min, a mean weal diameter 3 mm or more times greater than the negative control being considered significant [22].

At 30 weeks gestation a validated semiquantitative food frequency questionnaire (FFQ, referring to intake during the current pregnancy) was mailed to the mothers and the answers checked with her at the subsequent clinic [23]. The recruited women gave birth between April 1998 and December 1999. The study had the approval of the local Ethics Committee.

CBMC-proliferative responses

Within 30 min of delivery the attending midwife took cord blood samples by venepuncture of the umbilical vein and the cord blood was placed in sterile sodium heparin tubes. To obtain sufficient cells it was necessary to collect samples greater than 10 mL, which were processed within a median of 10 h (interquartile range [IQR] 5–15 h) after birth. CBMC isolation, the proliferation assays and their optimization have been described in detail elsewhere [24]. Briefly, CBMC were isolated by

density gradient centrifugation and cultured in 2 mL wells at a concentration of 1.25×10^6 cells/mL in the Alpha Modification of Eagle's Medium supplemented with 2.5% autologous serum. Cultures were stimulated with the mitogen con A (1 µg/mL), *Mycobacterium tuberculosis* PPD (5 µg/mL), timothy grass pollen extract (2500 IU/mL), or house dust mite extract (2500 IU/mL). 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 [24].

Statistical considerations

Data were analysed using SPSS® 8.0 (SPSS Inc., Chicago, IL, USA). CBMC proliferation was expressed as a stimulation index (SI); representing the ratio of stimulus-induced to unstimulated proliferation. Previous work has demonstrated that for this methodology an $\text{SI} \geq 3$ is statistically significant [24], a level that is comparable with other authors who quote either an $\text{SI} \geq 2$ or 3 as indicative of a significant proliferative response [12,14,15]. We have demonstrated that for all stimuli, SI values approximate to a log-normal distribution throughout the culture period [24], enabling SI values to be graphically represented by geometric mean values ($\pm$ 95% confidence intervals (CI)). General linear modelling for repeated measures (SPSS) was used to test for between-group differences, a technique that utilizes all available data and avoids multiple between-group *t*-testing. Maternal nutrient intakes were calculated from FFQ and food composition data, log-transformed, and then adjusted for maternal energy intake (log-transformed), the residual values being grouped into tertiles of intake [25].

Results

Representativeness of CBMC samples

In vitro CBMC-proliferative responses from 223 cord blood samples were characterized. Although it was not possible to recruit these 223 babies entirely randomly, their mothers did not differ significantly from the cohort of 2000 mothers with respect to age, obstetric history, smoking habit, social class or family history of atopic disease. The dietary intake of protein, carbohydrate, fat and energy by the mothers of the 223 neonates was also representative of the cohort of 2000 mothers. There was a non-significant trend for the mothers of the 223 neonates to take less vitamin supplementation during pregnancy; this resulted in significantly lower total vitamin E intake when compared with the remaining women of the cohort (7.70, 95% CI 7.24–8.19, mg/day vs. 8.28, 8.09–8.48, mg/day, $P=0.04$). It is probable that this was a chance association; all samples were processed 'blind' with no maternal or neonatal details known.

CBMC-proliferative responses

Absolute CBMC-proliferative counts ranged from 307 cpm (5th centile) to 7716 cpm (95th centile) for the unstimulated control cultures, and 504 cpm (5th centile) to 64 287 cpm (95th centile) for the stimulated cultures. Significant CBMC-proliferative responses on at least one of the days assayed were detected after stimulation with con A in 91% of samples, PPD 75%, timothy grass 76% and house dust mite 27%. It should be noted that responsiveness of CBMC to the four stimuli is

specific, since significant proliferation elicited by any one stimulus is not necessarily associated with responses to the others. Thus, 8.4% of samples responded to one of the stimuli, 28.9% to two, 45.3% to three, and 17.3% to all four of the stimuli.

Associations between known risk factors and CBMC-proliferative responses

Family history of atopic disease CBMC-proliferative responses after stimulation with con A, PPD, timothy grass and house dust mite tended to be greater in samples from neonates with a family history of atopic disease when compared with CBMC responses in samples from neonates without such a

family history. For example, there was a significant association between increased CBMC-proliferative response after stimulation with timothy grass and sibling history of atopic disease (Fig. 1). Figure 1(a) demonstrates CBMC SI values for 107 individual CBMC samples from neonates with siblings six days after stimulation with timothy grass. It can be seen that geometric mean CBMC-proliferative SI is higher if neonates had a sibling history of atopy when compared with responses in samples from neonates with non-atopic siblings ($P = 0.012$). In order to more clearly present the large numbers (> 1000) of CBMC-proliferative SI values for days 4–8 after stimulation, within-group SI values are expressed in the standard epidemiological fashion of geometric mean $\pm$ 95% CI and between

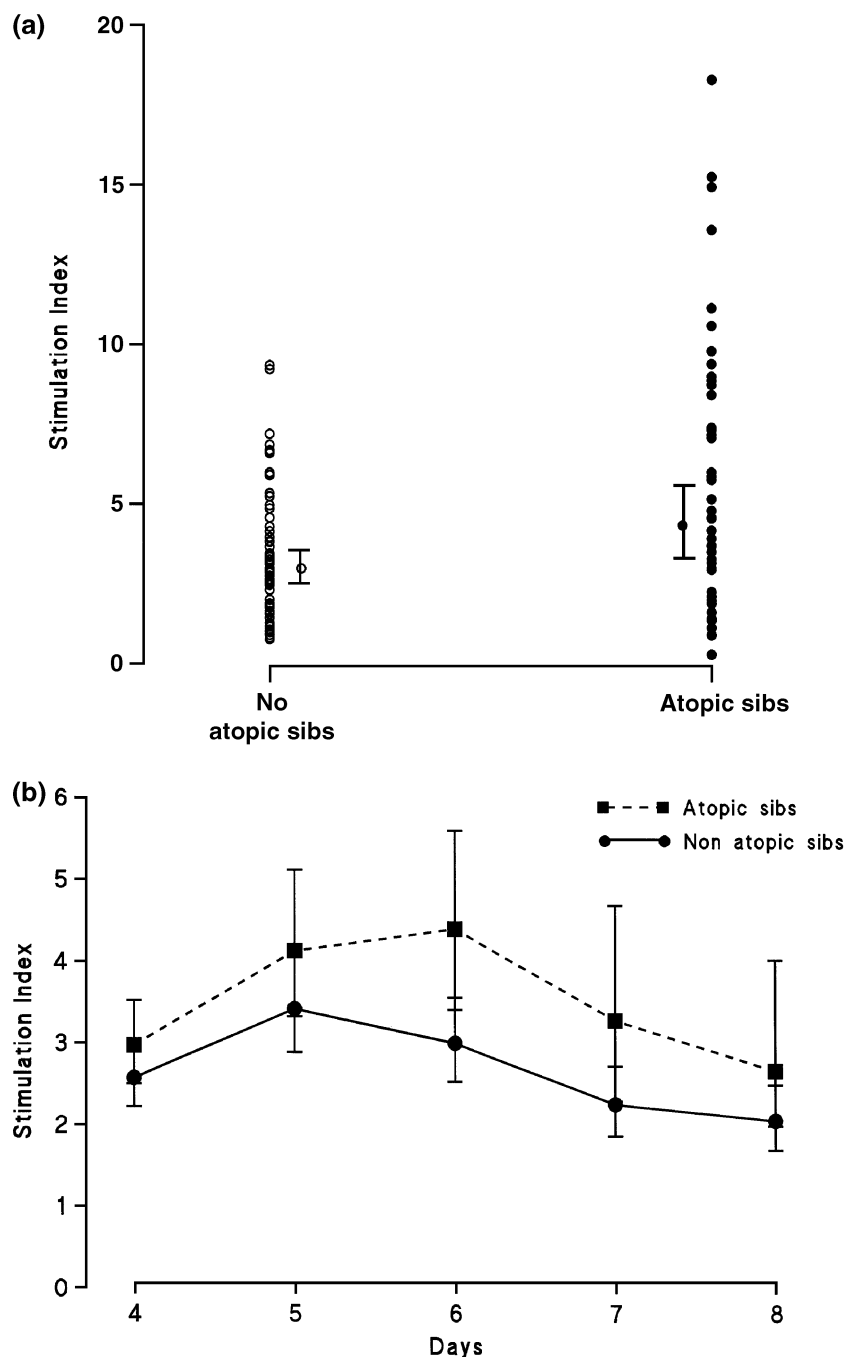


Fig. 1. Proliferative responses of CBMC after stimulation with timothy grass pollen extract in samples from neonates with ($n = 45$) and without ($n = 62$) atopic siblings. (a) Individual CBMC-proliferative responses six days after stimulation, with geometric mean SI $\pm$ 95% CI. Between-group difference $P = 0.012$. (b) CBMC-proliferative responses for each of the five days when proliferative responses assayed expressed as geometric mean proliferative response (SI $\pm$ 95% CI). Between-group difference $P = 0.04$.

group differences determined by linear modelling for repeated measures (Fig. 1b). There were no associations between results of maternal skin prick tests and CBMC-proliferative responses.

Maternal smoking Maternal cigarette smoking at 16–20 weeks gestation was associated with a trend for increased CBMC-proliferative responses after stimulation with con A, PPD, timothy grass and house dust mite. This association was strongest and highly significant ($P=0.008$) for CBMC-proliferative responses after stimulation with the allergen house dust mite (Fig. 2).

Obstetric history CBMC-proliferative responses after stimulation with PPD, timothy grass and house dust mite tended to be higher in samples from first-born children, but this trend only approached statistical significance ($P=0.1$) for CBMC responses to the allergen house dust mite. There were much stronger associations with the mother's total number of previous pregnancies (including miscarriages). Thus CBMC-proliferative responses after stimulation with con A, PPD, timothy grass and house dust mite tended to be greatest in samples from neonates born of first pregnancies, with CBMC-proliferative responses from subsequent pregnancies being

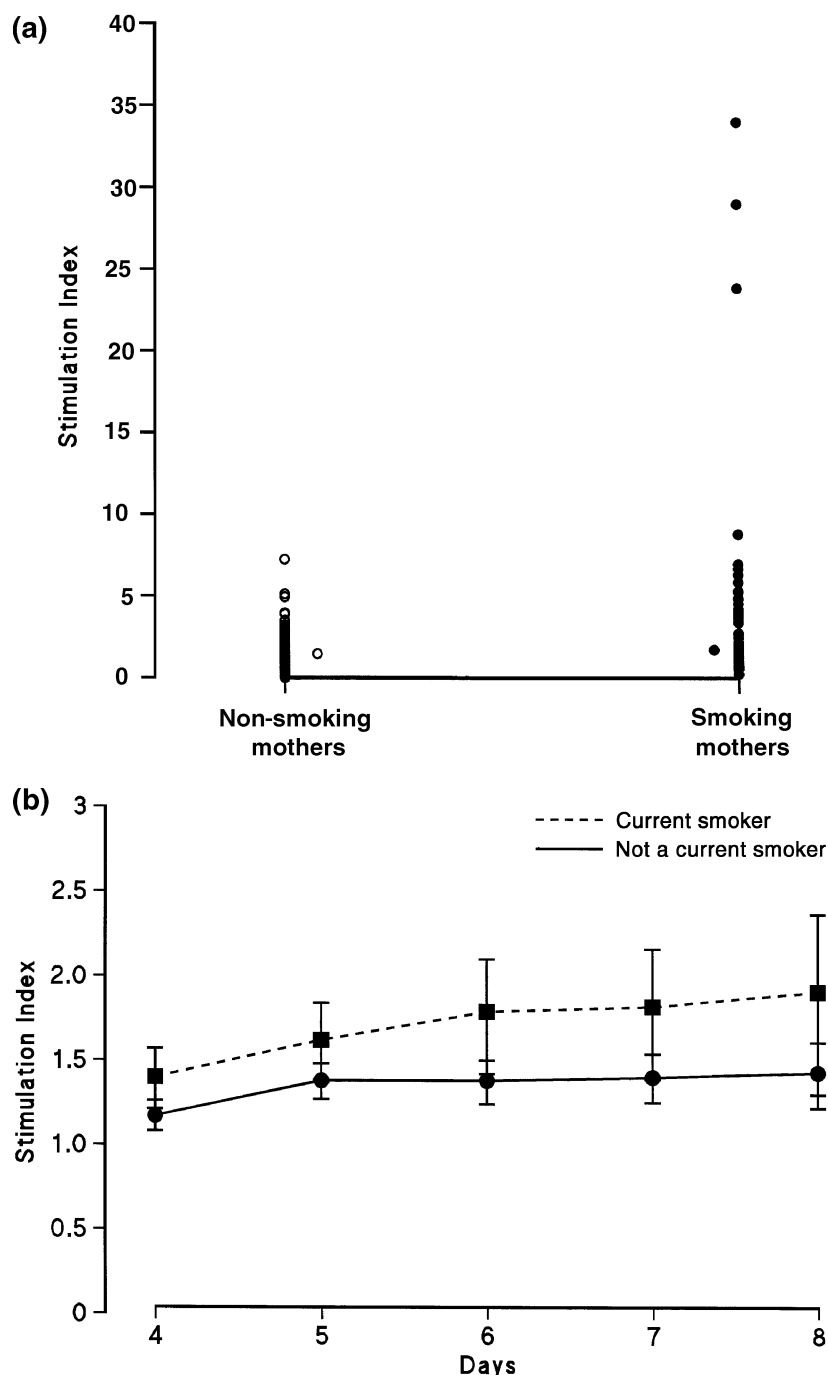


Fig. 2. Proliferative responses of CBMC after stimulation with house dust mite extract in samples from neonates whose mothers smoked ($n=55$) and neonates whose mothers did not smoke ($n=166$). (a) Individual CBMC-proliferative responses eight days after stimulation, with geometric mean SI $\pm$ 95% CI. Between-group difference $P=0.04$. (b) CBMC-proliferative responses for each of the five days when proliferative responses assayed expressed as geometric mean proliferative response (SI $\pm$ 95% CI). Between-group difference $P=0.008$.

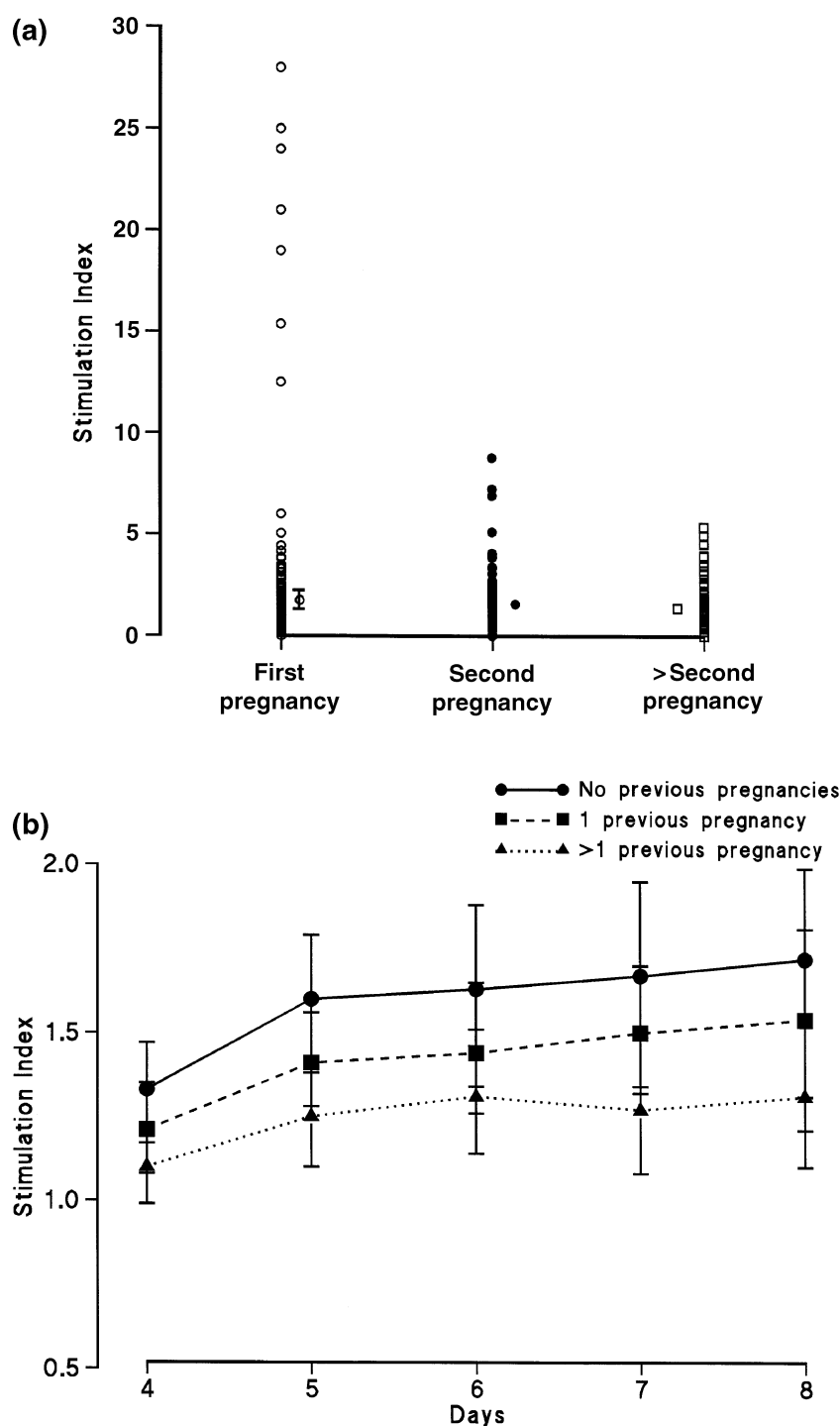


Fig. 3. Proliferative responses of CBMC after stimulation with house dust mite extract in samples from neonates who were born of first ($n = 81$), second ($n = 77$) and third or more pregnancies ($n = 62$). (a) Individual CBMC-proliferative responses eight days after stimulation, with geometric mean SI $\pm$ 95% CI. Between-group differences $P < 0.001$. (b) CBMC-proliferative responses for each of the five days when proliferative responses assayed expressed as geometric mean proliferative response (SI $\pm$ 95% CI). Between-group differences $P = 0.021$.

sequentially reduced. The association between CBMC proliferation and birth order was strongest and statistically significant for responses to house dust mite ($P = 0.021$) (Fig. 3), but approached statistical significance for responses to PPD ($P = 0.07$).

Maternal dietary intake of vitamin E Dietary intake data were available for 208 (93%) of the mothers. CBMC-proliferative responses after stimulation with PPD, timothy grass and house dust mite tended to be reduced in samples

from neonates whose mothers had the highest intake of vitamin E during pregnancy. The association between CBMC proliferation and maternal vitamin E intake was significant for CBMC-proliferative responses to timothy grass and house dust mite (between group P -values = 0.01 and < 0.001 , respectively, Fig. 4). The lowest tertile of intake was associated with significantly greater CBMC-proliferative responses than the highest tertile, but the difference between the lowest and middle tertiles was not significant. Repeating the analyses with serum levels of vitamin E from maternal blood taken at 16–20 weeks

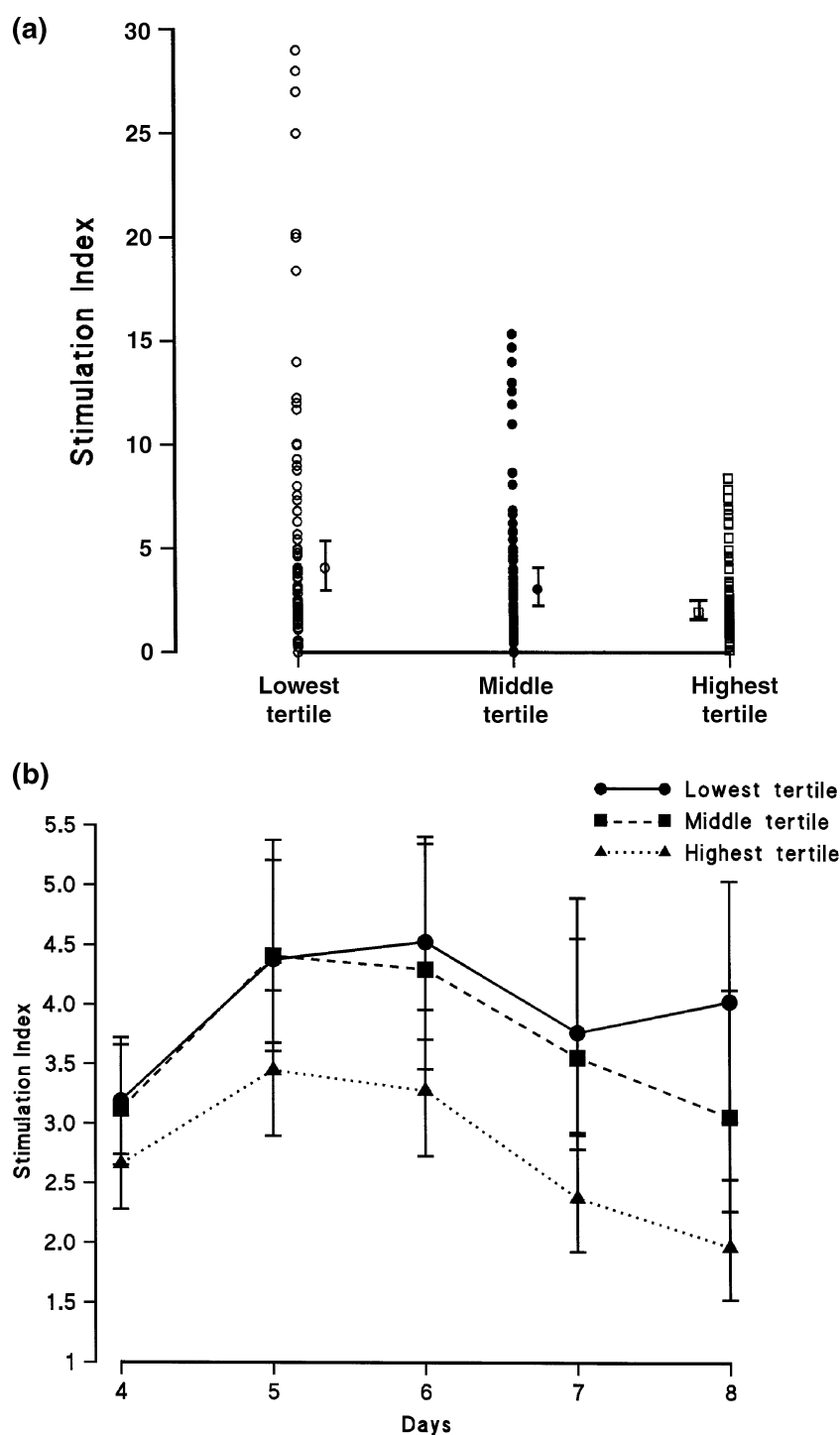


Fig. 4. Proliferative responses of CBMC after stimulation with timothy grass pollen extract in samples from neonates whose mothers' intake of vitamin E during pregnancy was in the lowest ($n = 69$), middle ($n = 69$) and highest ($n = 69$) tertile. (a) Individual CBMC-proliferative responses eight days after stimulation, with geometric mean SI $\pm$ 95% CI. Between-group differences $P = 0.002$. (b) CBMC-proliferative responses for each of the five days when proliferative responses assayed expressed as geometric mean proliferative response (SI $\pm$ 95% CI). Between-group differences $P = 0.01$, for difference between lowest and highest tertiles $P = 0.003$; and for difference between middle and highest tertiles $P = 0.02$.

gestation revealed no evidence of association between serum vitamin E (unadjusted or adjusted for serum cholesterol and serum total lipid) and CBMC-proliferative responses to any of the stimuli.

There were no consistent associations between CBMC-proliferative responses to any of the stimuli used and baby gender, maternal age, parental social class or maternal dietary intake of vitamin A, vitamin C, selenium, magnesium, manganese, iron copper or zinc. Multivariate analysis indicated that the associations between CBMC-proliferative

responses and family history of atopy, maternal smoking, obstetric history and maternal vitamin E intake were all independent.

Discussion

This is the first demonstration that *in vitro* CBMC-proliferative responses are positively associated with the established epidemiological risk factors for asthma and atopic disease, namely

family history of atopy and maternal smoking [3]. Not only were CBMC-proliferative responses to allergenic stimuli negatively associated with the established risk factor of birth order [4], but there was also a negative association with maternal dietary intake of vitamin E, the reduced intake of which is being increasingly associated with atopic disease in children and adults [7–9]. This study demonstrates that epidemiological risk factors for asthma and atopy, which are conventionally considered to be postnatal influences, do in fact exert antenatal influences. In addition to emphasizing the importance of the *in utero* environment, the results also suggest that maternal diet during pregnancy may influence the risk of childhood atopy and that immunological mechanisms underlie these associations.

Nearly all previous studies of CBMC-proliferative responses have selected, on the basis of parental atopy, groups of neonates and related their CBMC-proliferative responses to family history, and the subsequent development, of atopic disease [12–18]. The present study is the largest of its kind to date and has characterized a general, rather than a sample, population selected by parental atopic status. A possible consequence of the selection bias of previous work is that the reported associations between atopy and CBMC responses will have been biased towards the effects of genetic predisposition rather than to the environmental influences investigated in our study.

The cell culture conditions used in the present study were based on the optimized culture conditions recommended by Plebanski and Burtles [26] for supporting adult T cell proliferative responses, but adapted and optimized for CBMC-proliferative responses [24] in this study. Previous studies have characterized CBMC proliferation 6 [12,14,27–30] or 7 [15] days after allergenic stimulation. In the present study the kinetics of the CBMC-proliferative responses were characterized 4–8 days after stimulation but antenatal subgroup-associated differences were already apparent by days 4, 5 and 6 and the results of days 7 and 8 did not change any conclusions from the results of CBMC proliferation characterized on days 4, 5 and 6. CBMC-proliferative responses after stimulation with PPD have been demonstrated previously [31]. Several studies have shown significant positive associations between CBMC-proliferative responses and parental atopy [12,13,15]. However, a similar number of studies [27,29,30,32] have failed to demonstrate such an association. The present study found a relatively weak, but significant, positive association between parameters of family atopic (asthma, eczema and hayfever) history and CBMC-proliferative responses.

A major finding was the demonstration of a negative association between birth order and CBMC-proliferative responses. The association between birth order and atopy is widely accepted; hayfever and eczema are most prevalent in first-born children, with subsequent siblings becoming sequentially less likely to develop atopic disease [4]. The 'hygiene' hypothesis has been proposed as an explanation for this association, attributing the reduction in susceptibility associated with later birth order and family size to increased childhood infections that promote Th1 differentiation [4]. In contrast, our study suggests that the inverse relationship between atopy and birth order is a consequence of *in utero* influences on the fetal immune system rather than postnatal childhood infections. The nature of these influences is speculative. There is evidence that specific T cell tolerance against paternal major histocompatibility complex (MHC) alloantigens is induced during pregnancy in mice, and

that such immunological recognition and tolerance of the fetus is essential for a successful pregnancy [33]. The associations between birth order and CBMC-proliferative responses may reflect the development of increasing maternal immune tolerance with successive pregnancies. Another possibility is that the essential fatty acid (EFA) status of women during child-bearing years is precarious. Each pregnancy markedly depletes EFA stores and neonates of successive pregnancies have decreasing levels of EFA [34]. It is known that EFA can influence *in vitro* Th cell responses [6], and thus birth order-related changes in fetal EFA status and maternal dietary intake of EFA may underlie our observations.

The association between maternal smoking during pregnancy and CBMC-proliferative responses is consistent with epidemiological data showing an association between parental smoking and the development of childhood wheezy illness and asthma [3]. Maternal smoking appears to be more deleterious to infant respiratory health than paternal smoking. The assumption has been that this is a consequence of a mother being more likely to smoke in the presence of her child than a father, but our results and recent epidemiological data [35] indicate that smoking may exert an important *in utero* effect on the fetal immune system. Maternal smoking has the potential to do this because peripheral blood mononuclear cells from smokers secrete significantly greater IL-4 *in vitro* [36].

We believe that the demonstration of an association between maternal dietary intake of vitamin E and CBMC-proliferative responses to allergens is an important advance in the identification of the environmental/lifestyle factors contributing to the recent rise in atopy. The association between maternal dietary intake of vitamin E during pregnancy is similar in magnitude to the effects of family history of atopy, maternal smoking and birth order. The failure to demonstrate an association between maternal serum levels of vitamin E and CBMC responses may be a consequence of maternal serum levels being measured at 16–20 weeks gestation and CBMC responses at birth. It should also be noted that the FFQ has been validated in a standard manner against a four-day weighed intake of food and not serum levels [23]. Thus, maternal vitamin E intake may be a marker of other more influential dietary components, such as dietary lipids, which are hypothesized to be one of the reasons for the increased prevalence of atopy [6].

This research focuses attention on the importance of the antenatal environment in the aetiology of asthma and atopic disease. It also provides new insight into the immunological mechanisms associated with the risk factors of family history of atopy, maternal smoking and birth order in the aetiology of asthma and atopy. The demonstration of an association between maternal dietary intake of vitamin E and the development of the fetal immune system suggests that manipulation of maternal diet during pregnancy should be investigated further as the basis of public health measures designed to reduce the prevalence of atopic disease.

Acknowledgements

This work is supported by the Scottish Hospitals Endowment Research Trust (SHERT)/Mrs Jean V Baxter Research Fellowship, the Grampian University Hospitals Endowment Fund and the National Asthma Campaign. We acknowledge

help and support from Professor A. J. Rees and Drs Doris Campbell and George Russell. We would also like to thank the midwifery staff of the Aberdeen Maternity Hospital, Mr George Henderson, Mrs Alison Scaife and Mrs Gillian Moir for collecting and processing the cord blood samples.

References

- Ninan TK, Russell G. Respiratory symptoms and atopy in Aberdeen schoolchildren: evidence from two surveys 25 years apart. *Br Med J* 1992; 304:873–5.
- Omran M, Russell G. Continuing increase in respiratory symptoms and asthma in Aberdeen schoolchildren. *Br Med J* 1996; 312:34.
- Cook DG, Strachan DP. Parental smoking and prevalence of respiratory symptoms and asthma in school age children. *Thorax* 1997; 52:1081–94.
- Strachan DP. Family size, infection and atopy: the first decade of the 'hygiene hypothesis'. *Thorax* 2000; 55 (Suppl. 1):S2–S10.
- Seaton A, Godden DJ, Brown KM. Increase in asthma: a more toxic environment or a more susceptible population? *Thorax* 1994; 49:171–4.
- Black PN, Sharpe S. Dietary fat and asthma: is there a connection? *Eur Resp J* 1997; 10:6–12.
- Hijazi N, Abalkhail B, Seaton A. Diet and childhood asthma in a society in transition; a study in urban and rural Saudi Arabia. *Thorax* 2000; 55:775–9.
- Bodner C, Godden D, Little J, Ross S, Brown K, Seaton A. Antioxidant intake and adult-onset wheeze: a case-control study. *Eur Respir J* 1999; 13:22–30.
- Fogarty A, Lewis S, Weiss S, Britton J. Dietary vitamin E, IgE concentrations and atopy. *Lancet* 2000; 356:1573–4.
- Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol* 1989; 7:145–73.
- Robinson DS, Hamid Q, Ying S et al. Predominant Th2 like bronchoalveolar T-lymphocyte population in atopic asthma. *New Engl J Med* 1992; 326:298–304.
- Miles EA, Warner JA, Jones AC, Colwell BM, Bryant TN, Warner JO. Peripheral blood mononuclear cell proliferative responses in the first year of life in babies born to allergic parents. *Clin Exp Allergy* 1996; 26:780–8.
- Piastra M, Stabile A, Fioravanni G, Castagnola M, Pani G, Ria F. Cord blood mononuclear cell responsiveness to beta-lactoglobulin: T-cell activity in 'atopy prone' and 'non-atopy prone' newborns. *Int Arch Allergy Immunol* 1994; 104:358–65.
- Warner JA, Miles EA, Jones AC, Quint DJ, Colwell BM, Warner JO. Is deficiency of interferon gamma production by allergen triggered cord blood cells a predictor of atopic eczema? *Clin Exp Allergy* 1994; 24:423–30.
- Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; 353:196–200.
- Liao SY, Liao TN, Chiang BL et al. Decreased production of IFN γ 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.
- Tang MLK, Kemp AS, Thorburn J, Hill DJ. Reduced interferon- γ secretion in neonates and subsequent atopy. *Lancet* 1994; 344:983–5.
- Rinas U, Horneff G, Wahn V. Interferon- γ production by cord blood mononuclear cells is reduced in newborns with a family history of atopic disease and is independent from cord blood IgE levels. *Ped Allergy Immunol* 1993; 4:60–4.
- Holloway JA, Warner JO, Vance GHS, Diaper ND, Warner JA, Jones CA. Detection of house dust mite allergen in amniotic fluid and umbilical cord blood. *Lancet* 2000; 356:1900–2.
- Godfrey KM, Barker DJP, Osmond C. Disproportionate fetal growth and raised IgE concentration in adult life. *Clin Exp Allergy* 1994; 24:641–8.
- Olesen AB, Ellingsen AR, Olesen H, Juul S, Threstrup-Pedersen K. Atopic dermatitis and birth factors: historical follow up by record linkage. *Br Med J* 1997; 314:1003–8.
- Devereux G, Ayatollahi T, Ward R et al. Asthma and airway responsiveness in two Health Districts of Northern England. *Thorax* 1996; 51:169–74.
- Tomany J. The influence of maternal diet on orofacial clefting: a case-control study. MSc Thesis. University of Aberdeen, 1999.
- Devereux G, Hall AM, Barker RN. Measurement of T-helper cytokines secreted by cord blood mononuclear cells in response to allergens. *J Immunol Meth* 2000; 34:13–22.
- Willett WC, Stampfer MJ. Implications of Total energy Intake for Epidemiological Analyses. In: Willett WC, ed. *Nutritional Epidemiology*. New York: Oxford University Press, 1998:273–301.
- Plebanski M, Burtles S. In vitro primary responses of human T-cells to soluble protein antigens. *J Immunol Meth* 1994; 170:15–25.
- Szepfalusi Z, Nentwich I, Gerstmayr M et al. Prenatal contact with milk proteins. *Clin Exp Allergy* 1997; 27:28–35.
- Jones AC, Miles EA, Warner JO, Colwell BM, Bryant TN, Warner JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. *Ped Allergy Immunol* 1996; 7:109–16.
- v Duren Schmidt K, Pichler J, Ebner C et al. Prenatal contact with inhalant allergens. *Ped Res* 1997; 41:128–31.
- Chan-Yeung M, Ferguson A, Chan H et al. Umbilical cord blood mononuclear cell proliferative response to house dust mite does not predict the development of allergic rhinitis and asthma. *J Allergy Clin Immunol* 1999; 104:317–21.
- Piccinni MP, Mecacci F, Sampognaro S et al. Aeroallergen sensitization can occur during fetal life. *Int Arch Allergy Immunol* 1993; 102:301–3.
- 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.
- Kohut KG, Anthony MN, Salafia CM. Decidual and placental histologic findings in patients experiencing spontaneous abortions in relation to pregnancy order. *Am J Reprod Immunol* 1997; 37:257–61.
- Al MDM, van Houwelingen AC, Hornstra G. Relation between birth order and the maternal and neonatal docosahexaenoic acid status. *Eur J Clin Nutr* 1997; 51:548–53.
- Gilliland FD, Li YF, Peters JM. Effects of maternal smoking during pregnancy and environmental tobacco smoke on asthma and wheezing in children. *Am J Respir Crit Care Med* 2001; 163:429–36.