

Fetal exposure to intact immunoglobulin E occurs via the gastrointestinal tract

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Summary

Background Consideration of the evolutionary significance of IgE might provide insight into the immunological interactions occurring *in utero* and during early post-natal life that regulate later atopic disease.

Objective We postulated that the fetal gut is exposed to intact amniotic fluid IgE that might interact with local IgE receptors.

Methods IgE levels in matched maternal blood and amniotic fluid ($n=47$) or breast milk ($n=15$) collected from pregnant women in the UK (Southampton) and Brazil (Sao Paulo) were studied. Expression of IgE receptors, FcεRI and FcεRII (CD23), in fetal gastrointestinal tract ($n=19$) and skin ($n=11$) was examined immunohistochemically.

Results Human amniotic fluid at 16–18 weeks' gestation contained intact IgE at levels that increased as maternal circulating levels increased (Spearman's $\rho=0.897$; $P<0.001$). Circulating IgE levels from women in Sao Paulo, Brazil, associated positively not only with term (>37 weeks' gestation) amniotic fluid ($\rho=0.993$; $P<0.001$) but also breast milk IgE levels ($\rho=0.785$; $P=0.001$). Maternal levels of IgE did not change significantly over pregnancy and fetal circulating levels of IgE were very low (<0.6 IU/mL). Low-affinity IgE receptors (CD23) were expressed in lymphoid follicles of the fetal gut from 16 weeks of gestation (6/8), but not from 11 to 16 weeks (0/11) or in the skin.

Conclusion Amniotic fluid contains intact IgE that might bind to CD23⁺ cells within the lymphoid follicles of the fetal gastrointestinal tract. The evolutionary significance of these interactions might be to prepare the immune system for helminthic parasite exposure at birth via IgE-mediated antigen focusing, or 'education' of the developing immune system about the prevailing extrauterine environment. However, at present in societies where helminthosis is not a significant health issue, this pathway may still be operational and associated with the development of atopic disease.

Keywords amniotic fluid, breast milk, CD23, fetal gut, IgE, *in utero*

Submitted 21 January 2002; revised 24 August 2002; accepted 16 October 2002

Introduction

Many studies have demonstrated that maternal atopy exerts a greater influence than paternal atopy on the development of atopy by the offspring [1, 2] so IgE-mediated allergies may have their origins in early life. A number of mechanisms might contribute to the detrimental effect of maternal atopy on the development of atopy by her offspring including: the T helper type-2 (Th2)-biased cytokine milieu that maintains her own atopic status affecting the developing immune system, and exposure of the fetus to maternally derived IgE that facilitates IgE-mediated antigen-focusing, or provides less specific 'education' of the developing immune system about the prevailing extrauterine environment.

Very small amounts of antigen may be insufficient to induce humoral immune responses but high local concentrations of

antigen-specific IgE enable the capture and focusing of antigen via IgE receptors on antigen-presenting cells and thereby presentation to T cells. IgE-mediated antigen focusing relies on the expression of IgE receptors (CD23 or FcεRI) by antigen-presenting cells [3, 4]. Human (and murine) B cells present antigen in complex with IgE more efficiently than antigen alone, a process mediated by specific IgE receptors, with IgE-dependent antigen focusing and presentation to T cells 100-fold more efficient in the presence compared to the absence of IgE [3, 4]. Two IgE receptors have been described – a high affinity (FcεRI) and a low affinity receptor (FcεRII; CD23) that has two isoforms (a: constitutively expressed, predominantly on B cells; b: inducible, many cell types including eosinophils, B cells, monocytes and Langerhans cells) [5].

There is a significant positive correlation between maternal circulating IgE and IgE levels in amniotic fluid at 16–18 weeks' and >37 weeks' gestation [6]. It is self-evident that the fetal skin will be exposed to components of the amniotic fluid but as fetal swallowing of amniotic fluid begins early in gestation, with the fetus swallowing 700 mL of amniotic fluid per day by term, the gastrointestinal tract is also exposed [7, 8]. Thus amniotic fluid

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IgE might exert antigen-specific (and non-specific) effects on various fetal tissues. Therefore, the aim of this study was to determine if amniotic fluid IgE is intact and if IgE receptors are expressed within the fetal gut and skin.

Materials and methods

Tissue, blood, amniotic fluid and breast milk samples

Human fetal gut ($n = 19$) and skin ($n = 11$) was obtained from the Medical Research Council Tissue Bank from terminations performed at 11–24 weeks' gestation. Fetal blood was collected by cardiac puncture or venepuncture of the umbilical vein from terminated fetuses of 16–22 weeks' gestation ($n = 8$) or by umbilical vein puncture of pre-term (26–37 weeks' gestation; $n = 21$) or term deliveries (> 37 weeks' gestation; $n = 17$). Amniotic fluid and matched maternal blood was obtained from women undergoing diagnostic amniocentesis at 16–18 weeks' gestation at Princess Ann Hospital, Southampton ($n = 20$). Only samples from women without fetal abnormality and who continued with the pregnancy were included in the analysis. Prospective blood samples for the preparation of serum were collected at approximately 17, 24 and 32 weeks' gestation and immediately post-partum from women with a healthy, singleton pregnancy in Southampton. Their atopic status was assessed at recruitment by skin prick testing to common allergens [*Dermaphagoides pteronyssinus*, *Felix domesticus*, grass pollen mix, tree pollen mix, cow's milk, hen's egg, a negative control (allergen diluent) and a positive control (10 mg/mL histamine); all ALK, Hørsholm, Denmark]. All samples were obtained with the informed consent of the mother and in accordance with the requirements of the Polkinghorne Report on the use of human fetal tissue in research. The Southampton and SW Hants Joint Research Ethics Committee approved this study. Matched maternal plasma, amniotic fluid and breast milk ($n = 27$; although all samples were not collected from all mothers) were collected from women undergoing elective caesarean section at term at the Amparo Maternal Hospital, Sao Paulo, Brazil, with the approval of the local research ethics committee.

IgE assay

IgE levels in fetal blood, maternal blood, amniotic fluid and breast milk samples were measured with a high sensitivity immunoassay (sensitivity 0.05 IU/mL; MagicLite, Chiron, Denmark). For the measurement of IgE in fractions eluted from the Superose column described below (donors with > 0.9 IU IgE/mL amniotic fluid were used), an 'in-house' enzyme-linked immunosorbent assay (ELISA) was utilized. Briefly, the 96-well plate (Greiner, Stonehouse, UK) was coated with anti-IgE (HB121; American Type Culture Collection, Rockville, MD, USA) and then blocked with 3% bovine serum albumin (BSA; Sigma, Poole, UK) in phosphate-buffered saline (PBS). After incubation with sample (50 μ L of neat eluate), biotinylated monoclonal anti-IgE (250 ng/mL, mIgG1; BD Pharmingen, Oxford, UK) was used as the detecting antibody with subsequent incubation with streptavidin–horseradish peroxidase (1/1000; Sigma) followed by colour development with tetramethylbenzidine (substrate reagent set; BD Pharmingen). After stopping the reaction with 2.5 M H_2SO_4 the plate was read at 450 nm. A standard curve was prepared with World

Health Organization standardized IgE [National Institute for Biological Standards and Controls (NIBSC); Potters Bar, UK] and the assay had a sensitivity of 0.156 IU/mL.

Immunohistochemistry

Biopsies of human fetal gut and skin were frozen in liquid nitrogen and 8- μ m sections cut at the cryostat. Standard immunohistochemical procedures were employed using anti-CD23 (MHM6, mIgG1; Dako, Cambridgeshire, UK), and anti-CD21 (BU33, mIgG1; Ancell, Alexis Corporation, Nottingham, UK), and anti-Fc ϵ RI (clone 15.1, mIgG1, a kind gift of Professor Kinet). Briefly, after blocking potential non-specific binding of secondary antibody with 10% rabbit serum and of endogenous biotin with methanol/ H_2O_2 , primary antibody, isotype control or no primary antibody was applied at pre-optimized concentrations to the tissue sections. Biotinylated rabbit anti-mouse immunoglobulins (Dako) were applied to thoroughly washed slides and, subsequently, 3-aminoethyl carbazole (red colour) or 3,3'-diaminobenzidine tetrahydrochloride (brown colour) was used as the chromogen and the slides were counterstained with haematoxylin. Slides were mounted in DPX mounting media (BDH, Merck, Poole, UK) prior to viewing. The appropriate mouse isotype control (mIgG1; BD Pharmingen) was always included as a negative control and sections of human tonsil were included as a positive control.

Gel filtration chromatography

Amniotic fluid from four donors (500 μ L of each) identified as having high levels of IgE (> 0.9 IU/mL) was concentrated twofold using 10 000 Da molecular weight cut-off filters (Millipore, Watford, UK) centrifuged at 3000 g for 15 min at 4 °C. Sample (250 μ L) was then applied to a pre-equilibrated (PBS) Superose 6 10/30 column (Pharmacia, Amersham, UK). Fractions (250 μ L) eluted from the column in PBS were assayed for IgE as described above. The molecular weight of the positive fractions was determined by comparison to a series of standards of known molecular weight (Bio-Rad, Hemel Hempstead, UK) run on the same column under the same conditions. The standards (molecular weight in Da) were bovine thyroglobulin (670 000), bovine γ -globulin (158 000), chicken ovalbumin (44 000), equine myoglobin (17 000) and vitamin B12 (1350).

Statistical analyses

The Kruskal–Wallis, Mann–Whitney U -test and Spearman correlation (SPSS Version 8, SPSS, Chicago, IL, USA) were used to statistically evaluate the data.

Results

Amniotic fluid, fetal and breast milk IgE

Amniotic fluid IgE levels at mid gestation (16–18 weeks) were significantly higher in samples collected from women with circulating IgE levels of > 100 IU/mL compared to those with circulating levels of < 100 IU/mL ($P < 0.01$, Mann–Whitney U -test; Fig. 1; Spearman's $\rho = 0.897$; $P < 0.001$).

Prospective peripheral blood samples were obtained from women ($n = 10$) at approximately 17, 24, and 32 weeks' gestation and immediately post-partum. There were no significant

changes in serum IgE levels over gestation irrespective of whether a mother was atopic or non-atopic (Fig. 2).

Circulating levels of IgE in the fetus were extremely low to non-detectable throughout much of gestation even when using an assay with a sensitivity of 0.05 IU/mL (MagicLite; Fig. 3).

The association of elevated amniotic fluid IgE levels with high maternal circulating levels was confirmed using matched amniotic fluid and maternal plasma samples collected from women in Sao Paulo, Brazil who were undergoing elective caesarean section at term (>37 weeks' gestation). This series of samples reveals that amniotic fluid IgE levels continue to increase with maternal IgE (Fig. 4a, $P = 0.0001$, Kruskal–Wallis; Spearman's $\rho = 0.933$; $P < 0.001$). Furthermore, matched breast milk samples collected within 5 days of the birth were also available, and increasing breast milk IgE as maternal circulating levels increased was also found (Fig. 4b, $P = 0.03$, Kruskal–Wallis; Spearman's $\rho = 0.785$; $P = 0.001$).

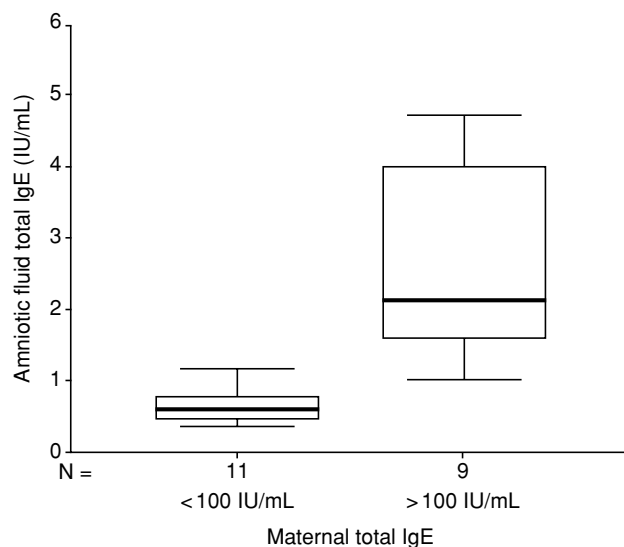


Fig. 1. IgE levels in matched amniotic fluid and maternal plasma. Amniotic fluid from mothers with IgE levels >100 IU/mL had significantly more IgE than that from women with IgE levels <100 IU/mL ($P < 0.01$; Mann–Whitney U -test).

Gel filtration chromatography was used to fractionate amniotic fluid on the basis of molecular weight and each of the fractions was assayed for IgE by ELISA. By comparison to standards of known molecular weight run on the same column, amniotic fluid IgE was found to be of approximately 200 000 Da, indicating that it is intact ($n = 4$; data not shown).

IgE receptor expression in fetal tissues

The expression of FcεRI and FcεRII receptors within fetal tissues, particularly the skin and gastrointestinal tract that are exposed constantly to amniotic fluid, was examined. CD23 immunoreactivity was detectable in samples of human fetal gut from 16 to 24 weeks of gestation (6/8) but it was not detectable at 11–16 weeks of gestation (0/11). Later time points were not studied. Within positive tissues, CD23-positive cells could be detected in the rudimentary lymphoid follicles (Fig. 5); in the

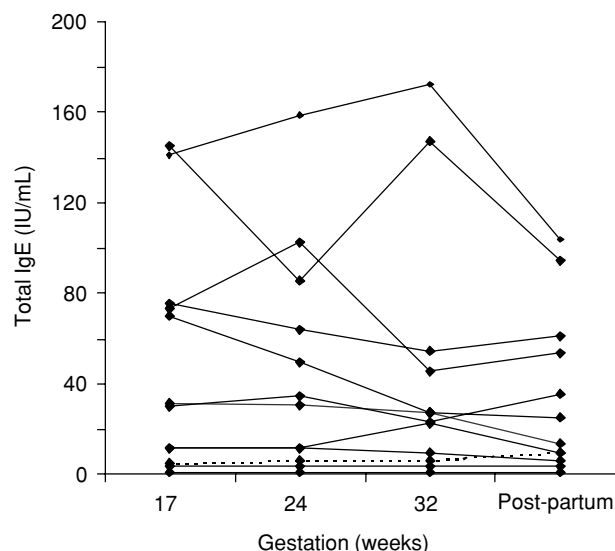


Fig. 2. IgE levels in the maternal circulation at various time points over gestation. Matched samples were collected at 17, 24, 32 weeks' gestation and immediately post-partum for the assessment of IgE levels. There were no significant differences in levels at different time points in women with low (<100 IU/mL), or high (>100 IU/mL) IgE levels.

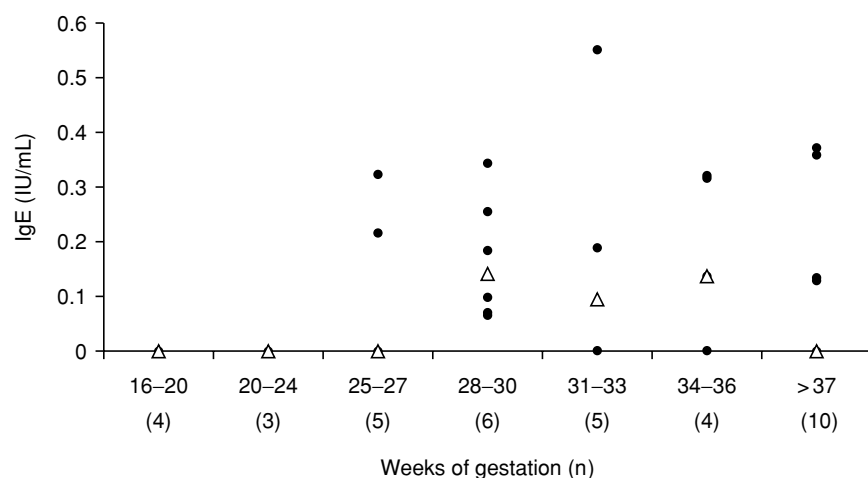


Fig. 3. IgE levels in the fetal circulation from 16 weeks' gestation to term. Total IgE levels in plasma samples prepared from blood obtained from fetuses and newborns of varying gestational ages were measured with a high sensitivity assay. ●, Individual results; △, median.

two samples of greater than 16 weeks' gestation in which CD23 immunoreactivity was not detectable, lymphoid follicles could not be found. CD23 immunoreactivity was not observed in any

of the skin samples examined (12–20 weeks' gestation; $n = 11$; data not shown).

FcεRI-positive cells were not detected within the fetal skin but were seen in 5/19 fetal gut tissues. Expression did not show any variation with gestational age (the youngest being 11 weeks of gestation and the oldest 24 weeks) and only two or three isolated positive cells were identifiable within tissues (data not shown).

Human CD23 has ligands other than IgE. The best characterized of these is CD21 [9]; therefore, CD21 expression was examined. CD21 immunoreactivity showed the same staining pattern as seen for CD23 being virtually restricted to lymphoid follicles and thereby being only detectable in samples in which these follicles were discernible (6/8 samples of 16–24 weeks' gestation).

Discussion

Herein we confirm [6] that as maternal circulating levels of IgE increase so too do levels detectable in matched amniotic fluid samples collected at mid and late gestation. This relationship also occurs in breast milk and existed in samples collected from women dwelling in a region where parasitosis can still occur and maternal IgE levels can be very much higher than those of women from Southampton, UK. However, examination of stool samples from the women who lived and delivered their babies in Sao Paulo, Brazil revealed that none of the mothers were infected currently with any parasites due to the introduction of a clean water supply in the preceding 2 years.

Endogenous production of IgE by the fetus occurs from the second trimester [10, 11]; however, circulating levels are very low (this study) and unless IgE is preferentially produced into the lumen of the fetal gastrointestinal tract and thereby the amniotic fluid, it is likely that amniotic fluid IgE is of maternal origin although this remains to be confirmed. However, during helminth infection of the gastrointestinal tract, IgE is abundant in the gut, and serum levels are only 0.5–1% of the total IgE [12], so local production in the fetal gut may not be mirrored by elevated circulating levels. Although there is no evidence of IgE production in the fetal gastrointestinal tract at the time when amniotic fluid IgE is first detectable [10, 13] this does warrant further consideration.

As amniotic fluid IgE is likely to be of maternal origin, we then considered whether, because of the postulated predominance

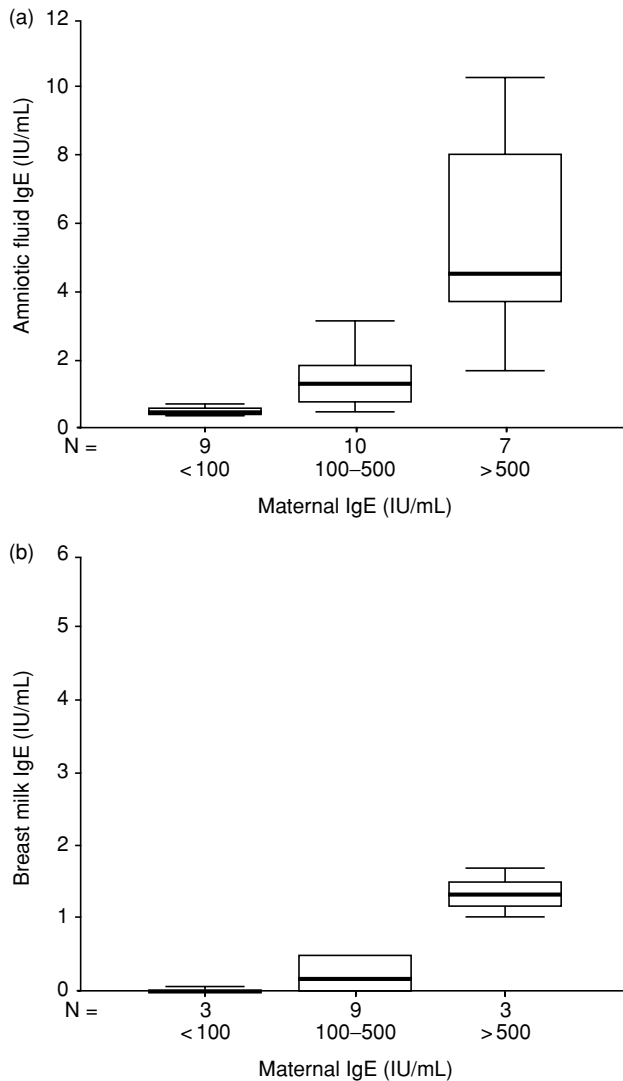


Fig. 4. IgE levels in matched maternal plasma and amniotic fluid or breast milk. Levels of IgE were measured in maternal plasma from women delivering by elective caesarean section in Sao Paulo, Brazil and levels compared to that in matched (a) amniotic fluid and (b) breast milk.

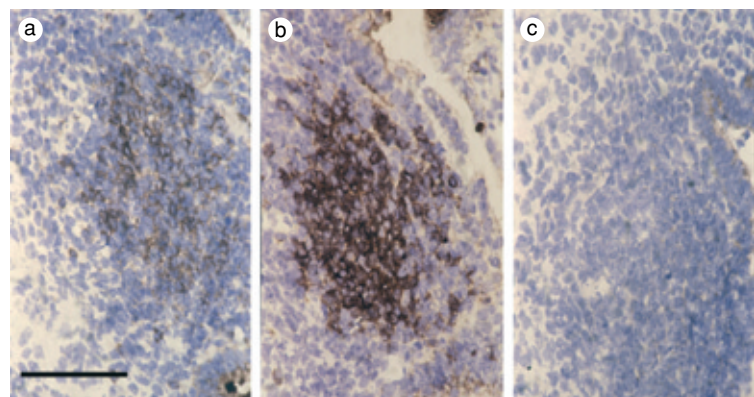


Fig. 5. Low-affinity IgE receptor expression in the fetal gut. Immunoreactivity in the fetal gut with (a) anti-CD23 (b) anti-CD21 and (c) isotype control was observed in the absence of immunoreactivity in fetal skin.

of Th2 reactivity during pregnancy [14], maternal IgE levels increase during pregnancy. Unfortunately we did not have a pre-pregnant sample from any of these women, making it impossible to state unequivocally that IgE levels do not increase with pregnancy. However, as women with very low IgE levels maintain such levels over gestation, it is unlikely that IgE levels are increasing significantly as a consequence of pregnancy.

Faecal IgE occurs as a 40 000 Da fragment corresponding to the Fc region as a consequence of degradation by proteolytic enzymes within the gastrointestinal tract [15]. Such activity is not found in the fetal gut [16], nor has it been observed in amniotic fluid [7] and, as shown herein, amniotic fluid IgE is intact. Thus a lack of proteolytic activity at this site and constant replenishment of any IgE ingested by the fetus from the maternal circulation, if this is indeed the source, will ensure ongoing exposure of the fetus to intact IgE. Fetal swallowing of amniotic fluid begins early in gestation and by the third trimester the fetus swallows 700 mL of amniotic fluid per day and may obtain as much as 10% of its calorie and protein requirements by this route [7, 8]. So even when amniotic fluid IgE levels are 0.5 IU/mL, the fetus will be exposed to 350 IU of intact IgE every day. As the fetal gut is very leaky, closure not being complete until after birth [16], specific transport of IgE across the gut epithelium as has been described [17] will not be required for access to the lymphoid follicles. However, the expression of IgE receptors within the fetal gut will be necessary if IgE is to exert any biological effect on the fetus.

CD23 was the predominant IgE receptor expressed in fetal gut but was observed only when lymphoid follicles were evident (after 16 weeks' gestation in our sample set). Transcripts for both CD23a and CD23b are abundant from the first trimester [11] and our observation confirms a previous observation of CD23⁺ cells within the fetal gut on cells within lymphoid follicles [18]. CD23 immunoreactivity was localized to the same area of tissue as CD20 expression, a marker for B lymphocytes (data not shown); however, expression of CD23 by non-B cell populations such as dendritic cells cannot be ruled out at this stage. Patient confidentiality prohibits us obtaining information on racial origins of the samples and atopic status of the parents.

The abundance of CD23 in comparison to FcεRI within the fetal gut implies that CD23-dependent IgE-mediated antigen focusing would be the preferred pathway if such a phenomenon is occurring in the human fetal gut. The ability of CD23 to mediate IgE-dependent antigen focusing is one of its most consistently observed functions. Human (and murine) B cells present antigen in complex with IgE more efficiently than antigen alone, a process mediated by CD23, with IgE-dependent antigen focusing and presentation to T cells 100-fold more efficient in the presence compared to the absence of IgE [3, 4]. In the human, CD23 has ligands other than IgE, including CD21 [9]. We observed CD21 expression also within the lymphoid follicles. The cellular distribution and functional significance of possible CD23–CD21 interactions in this tissue remains to be determined.

In conclusion, we postulate that, in the absence of IgE passage across the placenta [19, 20], *trans*-amniotic transfer from the maternal circulation may have a role in preparing the fetus for the post-natal environment, historically helminth

parasitosis. In particular, the initiation of antigen-specific reactivity via IgE-mediated antigen focusing within the fetal gut via fetal swallowing of IgE (and antigen), initially in the amniotic fluid and subsequently in the breast milk, would localize the antigen-specific response to the gastrointestinal tract, the site at which it is required during helminth infection. This pathway may have evolved to prepare the neonate for immediate post-natal parasite infection, however, rudiments of this pathway may now be associated with the development of atopy. Additional to the postulated antigen-specific effects of IgE via antigen focusing, IgE itself or the environment that favours elevated IgE production (e.g. IL-4 and/or IL-13) might also 'educate' the developing immune system about the post-natal environment. Whether the responses arising from this intrauterine interaction are transient or permanent may be determined by post-natal modification as the commensal flora is acquired and immunological tolerance is established. The development of sensitive methods to detect specific IgE and allergen in amniotic fluid combined with ongoing clinical follow-up of our amniocentesis cohort will enable evaluation of this hypothesis.

Acknowledgements

We extend our gratitude to Dr Les Wong at the MRC Tissue Bank, Professor Tom MacDonald and Dr Sylvia Pender for the samples of fetal tissue. We are grateful to the staff in the Department of Fetal Medicine, Princess Anne Hospital, Southampton for their help in collecting samples of amniotic fluid as well as all the staff on the delivery suite who assisted in the collection of cord blood samples. This work was supported by NIH grant number HL-61858 and a Hope grant from the Wessex Medical Trust, Southampton General Hospital.

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