

Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation

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Blood samples were obtained from fetuses and premature babies ($n=51$) (15–34 weeks gestation) to determine at what stage the fetal immune system was able to produce a positive proliferative response to common allergens. Peripheral blood mononuclear cells (PBMC) were stimulated with the mitogen, phytohaemagglutinin (PHA), and the allergens, house dust mite, cat fur, birch tree pollen, β -lactoglobulin, ovalbumin and bee venom (mellitin). Results were expressed as ratios of stimulated to unstimulated ^3H thymidine incorporation, and as percent positive responders. There was an increase in proliferation ratio which correlated with increasing gestational age for PHA ($p<0.0001$), cat fur ($p=0.042$), birch pollen ($p=0.022$) and β -lactoglobulin ($p=0.006$). The point in gestation when cells from some individuals began responding to the allergens with a ratio of 2.0 was at approximately 22 weeks. PBMC proliferative response ratios were higher from samples from babies > 22 weeks gestation compared to < 22 weeks for the mitogen and all allergens, except mellitin. There was also a greater proportion of positive responders from samples > 22 weeks compared to < 22 weeks for the mitogen and all allergens, except mellitin. Maternal exposure to birch pollen, which has a discrete season, was assessed to determine whether exposure had occurred at 22 weeks gestation or beyond. Results showed a higher proliferative response in infant cells stimulated with birch pollen ($p=0.005$) and higher proportion of positive responders ($p=0.01$) in the group of babies whose mothers had been exposed to birch pollen beyond 22 weeks, compared to those whose mothers had not been so exposed. These results suggest that *in utero* fetal exposure to an allergen from around 22 weeks gestation may result in primary sensitisation to that allergen, leading to positive proliferative responses, at birth.

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The development of immune memory, at a cellular level, relies on the selective expansion of allergen-specific T lymphocytes. Therefore, a raised peripheral blood mononuclear cell (PBMC) proliferative response to an allergen *in vitro* represents a previously exposed and primed T lymphocyte. There have been several studies of inhalant allergen, and more recently food allergen induced proliferation of PBMC from patients with allergic disease. Rawle et al. demonstrated a higher PBMC proliferative response to the major house dust mite allergen, Der p 1, in patients with asthma or atopic dermatitis with symptoms triggered by the house dust mite, com-

pared to non-allergic control patients (1). Kondo et al. also showed that PBMC proliferations to ovalbumin or BSA were raised in children with food associated atopic dermatitis compared to healthy children or to children with food-associated disease resulting in the immediate symptoms of acute urticaria or angioedema (2).

Recently, PBMC proliferative responses from umbilical cord blood have been demonstrated to common inhalant allergens (Der p 1 and Lol p 1 (the major allergen from Rye grass pollen)) and food allergens (β -lactoglobulin and ovalbumin) (3–7). A raised cord blood proliferative response to BSA (5)

has been shown to have a predictive capacity for the subsequent development of allergic disease in infants. In addition, we have demonstrated that infants who subsequently develop eczema, associated with milk and egg ingestion, have a higher PBMC proliferative response and lower interferon- γ production upon stimulation with β -lactoglobulin and ovalbumin at birth, compared to babies with non food sensitive eczema or those with no eczema (6). We have suggested that T cells may be primed to environmental allergens *in utero*.

However, there is controversy about the functional maturity of T cells obtained from umbilical cord blood. Hayward, 1981, showed a positive proliferative response to the mitogen, phytohaemagglutinin, from fetal thymus tissue as early as 10 weeks gestation (8), and it has been shown that the response of newborn cells upon stimulation with PHA is comparable to the response from adult cells (9,10). On the other hand, Holt et al. have shown that neonatal T cells are immature compared to adults in relation to interferon- γ production, particularly in children from atopic families (11), and Miles et al. have suggested that cord blood from babies who subsequently develop allergic disease have lower numbers of activated (CD25 +ve) T cells compared to babies who did not develop allergy (12). Nevertheless, the ability of PBMC to proliferate develops during antenatal life and as T cells respond to specific allergens at birth, these responses must have been acquired during gestation. This study investigated the fetal proliferative responses to PHA, house dust mite, cat fur, birch pollen, β -lactoglobulin and ovalbumin in PBMC obtained at the delivery of premature babies and fetuses.

Material and methods

Cord blood samples were collected from babies if they were delivered between 24 and 34 weeks of gestation (n=38), or aborted fetuses (for social reasons or due to a fetal abnormality which did not affect the immune system) between 15 and 23 weeks gestation (n=13). Umbilical cord blood was obtained by aspiration from the umbilical vein of the placenta following the birth of the premature babies, and blood was obtained from either the umbilical cord or by heart puncture from the aborted fetuses. All abortions were induced using prostin. Of the 32 mothers who delivered premature babies, 26 received dexamethasone injections prior to delivery to aid maturation of the infants' lungs. Premature babies were delivered for a variety of reasons including, premature rupture of membranes (n=4), pre-eclampsia in the mother (n=3), fetal distress (n=3), intrauterine growth retardation (n=2), breech deliv-

ery (n=2), multiple fetuses (n=1), and in one case, suspected maternal sepsis. The remaining babies were delivered prematurely for unknown reasons. Eighteen of the mothers delivered their babies by normal vaginal delivery and the remaining fourteen by caesarian section. In the first few days of the babies lives, the majority (n=23) received antibiotics, although only two had a positive culture test and raised C-reactive protein indicative of infection, and one had probable sepsis (none of these three babies were born to the mother with suspected sepsis which suggests that the infections developed following delivery).

The study was approved by the Joint Ethics Committee of Southampton University Hospitals, and fulfilled the requirements of the Polkinghorne Committee report on the research use of fetuses and fetal tissue.

Isolation of PBMC

PBMC were isolated from the heparinised blood samples within 6 h of collection. Cells were separated by density gradient centrifugation using Histo-paque (Sigma, UK). Cells were cultured in RPMI-1640 medium supplemented with 10% autologous plasma (filtered through 0.22 μ m filter (Flowpore, ICN)), 2mM glutamine, 100 μ g/ml streptomycin and 100U/ml penicillin.

Proliferation assay

A total of 1×10^5 PBMC per 100 μ l culture medium were stimulated with 1 μ g/ml PHA (Sigma, Poole UK), 2000U/ml house dust mite (HDM) (National Institute for Biological Standards and Controls - 1st International Standard, Potters Bar, Herts, UK), 2000U/ml cat fur extract (NISBC), 2000U/ml Birch tree pollen (NISBC), 10 μ g/ml β -lactoglobulin (Sigma), 10 μ g/ml ovalbumin (Sigma), 0.5 μ g/ml mellitin (bee venom) (Sigma) in triplicate in a 96-well-round bottomed microtitre plate (Costar, Northumbria Biologicals, UK). After 3 days culture for PHA stimulation, and 6 days for the allergens at 37°C in a humidified incubator with 5% CO₂, thymidine incorporation was assessed by pulsing with 1 μ Ci [³H] thymidine (Amersham, Bucks, UK) per well for the last 16 h. Cells were then harvested onto glass fibre mats and [³H] thymidine incorporation assessed by liquid scintillation counting. Mitogen and antigen concentrations were optimised as described previously (6). Mitogenic responses to allergens were investigated by stimulation of the PBMC from 5 cord blood samples with the optimum doses of allergen for both 3 and 6 days. No positive proliferative responses (2.0 $\times$ background) were seen in 3 day cul-

tures, demonstrating that the allergens did not have a mitogenic effect.

Results were expressed as counts per minute (cpm) and as the ratio of [^3H] thymidine incorporation of PHA or allergen stimulated to unstimulated cells incubated in medium alone for the same time period (stimulation index (S.I.)). Proliferative responses were considered to be positive if $\text{S.I.} \geq 2.0$. Twenty two weeks gestation was used as the dividing point because this was the first time point at which we saw a $> 2 \times$ background proliferative response to any allergen.

Assessment of exposure

As mothers were enrolled into this study at the time of termination or delivery it was not possible to assess their exposure to perennial inhalant allergens (HDM and cat) in their homes during pregnancy nor to obtain accurate food diaries relating to their intake of milk and egg. Perennial allergens give no indication of the importance of the timing of exposure, however, due to the discrete season of the birch pollen (the beginning and end of which can be obtained from pollen counting centres) the mothers could be divided into two groups 1) those who were exposed to birch pollen at 22 weeks of pregnancy or later and 2) those who had no exposure to birch pollen after 22 weeks of gestation. The proliferative responses to birch pollen allergen were compared in the infants born to the two groups of mothers.

Statistical analysis

Spearman's rank correlation test was used to correlate gestational age with proliferative response. The non-parametric Mann-Whitney U test was used to compare between proliferative responses from samples greater and less than 22 weeks gestation, and between babies of mothers who had and had not been exposed to birch pollen from the 22nd week of pregnancy. The Yates corrected χ^2 test was used to compare positive and negative responders in samples of < 22 and > 22 weeks, and between mothers who had and had not been exposed to birch pollen.

Results

Blood samples were obtained from 12 fetuses terminated at < 22 weeks of pregnancy, one fetus terminated at 23 weeks and 38 premature babies born at > 22 weeks gestation (24–34 weeks).

The counts per minute achieved for each of the samples tested are shown in Table 1. The proliferative response ratio to stimulation with the mitogen PHA correlated significantly with increasing gesta-

tional age of the fetus, as shown in Fig. 1a ($p < 0.0001$). This was also the case when cells were stimulated with β -lactoglobulin ($p=0.006$) (Fig. 1b), birch pollen ($p=0.022$) (Fig. 1c), and cat allergen ($p=0.042$). There was a trend for this association of increasing responsiveness with increasing gestational age when cells were stimulated with HDM ($p=0.113$) and ovalbumin ($p=0.108$) but not with the bee venom allergen (mellitin) ($p=0.566$) (Fig. 1d).

There were four samples where the PHA responses were not considered to be positive (suggestive that the cells were dead), however, when data was analysed including and excluding this group of samples the statistically significant results were not changed. The cpm from unstimulated cells (background) obtained from the three babies who subsequently developed infections were compared to babies of the same gestational age who had not developed infection. The background proliferative responses of the infected babies were within the range of data obtained from uninfected babies indicating that the proliferative responses were not unusually raised due to the presence of an infection. It has been suggested that dexamethasone can suppress the proliferative responses from lymphocytes (13). To investigate this possibility, the PHA response from the six babies whose mothers who did not receive dexamethasone before delivery were compared to babies of the same gestational age who did receive dexamethasone. There was no statistically significant difference between the two groups, and therefore administration of dexamethasone was not considered to have affected the results.

The point in gestation when the cells from some individuals began responding to the allergens with a stimulation index of ≥ 2.0 was at approximately 22 weeks. The proliferative responses obtained from blood samples of less than and greater than 22 weeks of gestation are shown as absolute stimulation ratio (Fig. 2a,b) and as % positive responders whereby a positive response was classified as $\text{S.I.} > 2.0$ (Fig. 3). There were significantly higher proliferative responses obtained from babies > 22 weeks compared to < 22 weeks when cells were stimulated with PHA: $p < 0.0001$, and the allergens, β -lactoglobulin: $p < 0.0001$, ovalbumin: $p=0.03$, (Fig. 2a), HDM: $p=0.019$, cat: $p=0.003$ and birch pollen: $p=0.004$ (Fig. 2b), but not when cells were stimulated with the allergen from bee venom (mellitin: $p=0.13$, data not shown). There were higher proportions of positive responders from babies > 22 weeks gestation compared to < 22 weeks when cells were stimulated with all allergens, except bee venom and this was significant for PHA: $p=0.002$, β -lactoglobulin: $p=0.009$ and birch pollen: $p=0.003$ (Fig. 3). The data was also analysed taking a posi-

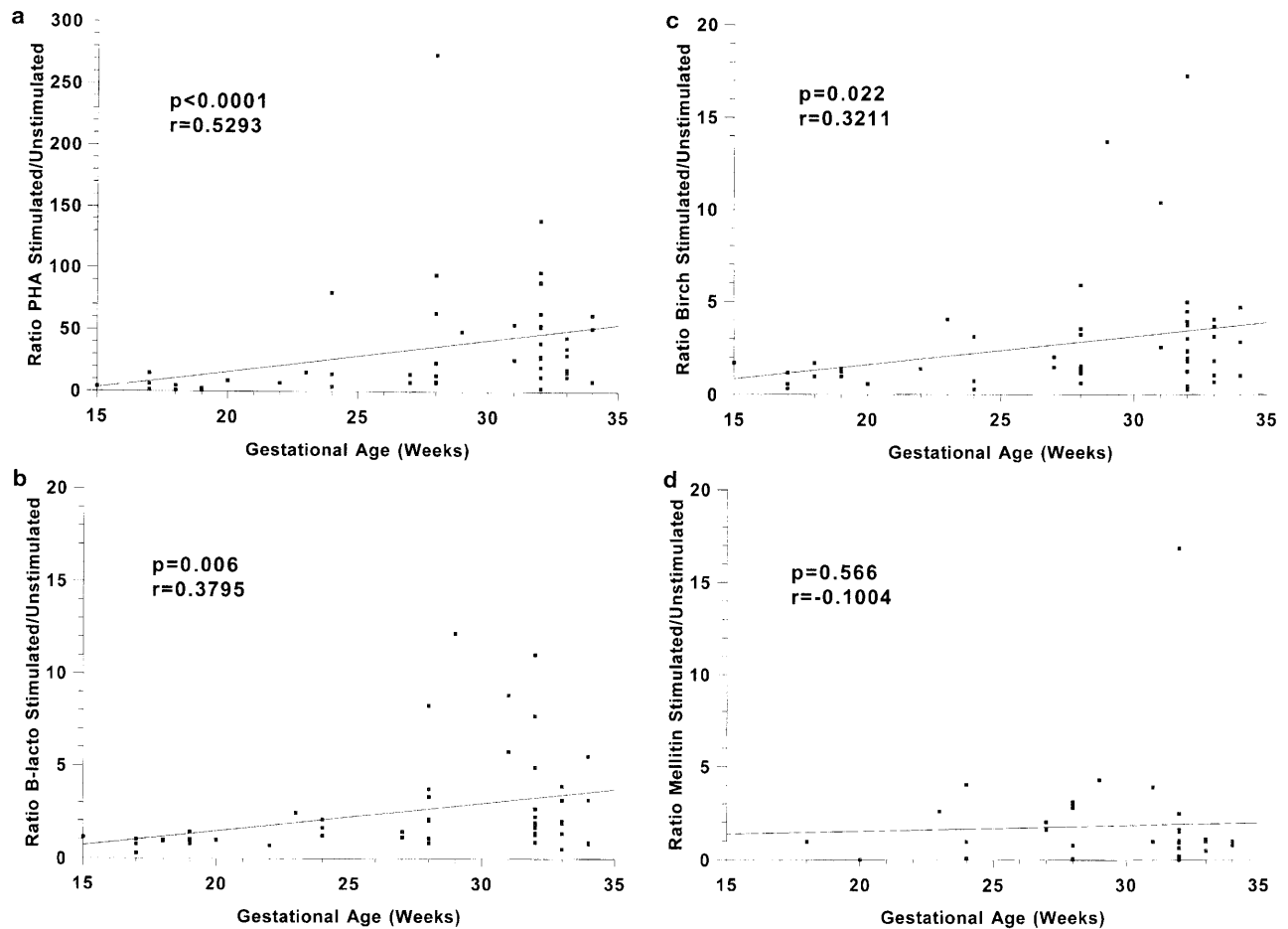


Fig. 1a, b, c, d. Graphs showing correlation between gestational age and stimulation ratio for cells stimulated with a) PHA, b) β -lactoglobulin, c) birch pollen, d) bee venom (mellitin).

tive response to be $S.I. > 3.0$ and showed that the percent positive responders from cells > 22 weeks gestation were higher when stimulated with PHA $p = 0.0002$, β -lactoglobulin $p = 0.02$ and birch pollen $p = 0.05$.

Using information from a pollen counting centre it was possible to determine which of the pregnant mothers had been exposed to birch pollen during their pregnancy, when the developing fetus was > 22 weeks of gestation. Eighteen mothers had been exposed to birch pollen after the 22nd week of their pregnancy and 22 mothers had no exposure from 22 weeks onwards until the birth of their baby. The remaining 11 blood samples were obtained from aborted fetuses of < 22 weeks gestation and were therefore not included in this analysis. There was a significantly higher proliferative response and more positive responders, when cells were stimulated with birch pollen, in blood samples obtained from babies whose mothers had been exposed to tree pollen after 22 weeks gestation compared to those obtained from babies whose mothers had not been exposed to tree pollen at this time ($p = 0.005$ stimulation index (Mann Whitney U test), and $p = 0.01$ per-

cent positive responders (Yates corrected χ^2 test, Fig. 4). There was no difference on comparing the gestational ages of the babies born to the two groups of mothers, thus removing the effect of bias due to gestational age on the lower proliferative responses from the mothers not exposed to pollen from 22 weeks of pregnancy.

Discussion

It has been suggested for several years that babies are 'at risk' of being sensitised to environmental allergens if they are exposed to high concentrations at a very early age (14,15), and that the first year of life appears to be the most important. It has been suggested that the immune responses which give rise to allergic symptoms in adult life are actually initiated in early infancy (16). Much of this indirect evidence is from the many studies which have been performed detailing the appearance of dietary and inhalant allergen-specific antibodies (both IgE and IgG subclasses) in the serum of both normal and atopy prone individuals in the first 1–2 years of life (17–19). These antibody responses (particular IgE)

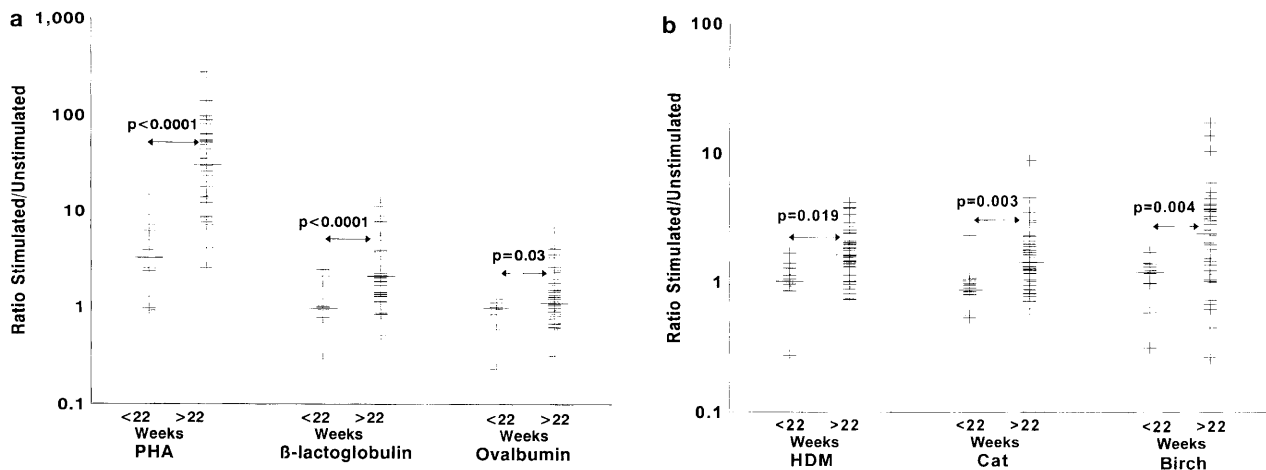


Fig 2a,b. Graphs showing differences in PBMC proliferative response ratios from samples less than and greater than 22 weeks gestation, to mitogens and allergens, PHA, β -lactoglobulin and ovalbumin (Fig. 2a) and house dust mite (HDM), cat allergen and birch tree pollen (Fig. 2b).

can be transient. They occur in both normal children and children with positive family history for atopy. It seems likely, therefore, that there is specific T helper cell activity to these allergens resulting in this early antibody production.

More recently, several laboratories, including our own, have reported positive proliferative responses by PBMC from umbilical cord blood stimulated with both dietary and inhalant allergens (3–7). This has been suggested by some authors as evidence of *in utero* exposure of the fetus to allergens which results in primed T cells enabling a proliferative response on subsequent *in vitro* exposure after birth. Piastra et al. however, discussing the results in relation to fetal exposure to β -lactoglobulin, suggested that prenatal exposure was an unlikely explanation due to the inability to detect milk proteins in adult serum, even following milk challenge (7). They hypothesised that T cells from the individuals which responded to allergen seemingly before *in vivo* priming had occurred, behaved differently compared to T cells from the individuals who did not respond, due to the allergen acting as a mitogen. However, a possible alternative interpretation of these results was that the milk proteins in the serum were complexed with antibody and as such were not detectable as free protein.

Piccinni et al. have shown that PBMC from all samples of umbilical cord blood (irrespective of the atopic status of the parents) are capable of responding to certain allergens e.g. the perennial house dust mite allergen Der p 1, but that not all samples respond to seasonal allergens e.g. Lol p 1 (3). The explanation offered for this is the possibility of fetal sensitisation *in utero* due to the allergen being inhaled by the mother during pregnancy, and the differences observed between the two allergens tested being dependent on the exposure of the mother to

these substances which was throughout the entire pregnancy for Der p 1, and relatively short lived for Lol p 1.

However, if we consider the data available on T cell expression of the isoforms of CD45 in umbilical cord blood T cells, the interpretation of prenatal exposure is less clear. It is well established that CD4+ve T cells which have acquired memory for an antigen, express the CD45RO phenotype, while naive unprimed T cells express CD45RA. There have been reports of 5–10% of umbilical cord blood T cells expressing CD45RO (20,21), however, we have shown (12), more recently, that ~50% of umbilical cord blood T cells from babies express the memory phenotype. In addition, flow cytometric data on this cohort of premature babies and fetuses, (not yet published), shows that the proportion of CD4+ve T cells expressing the CD45RO phenotype

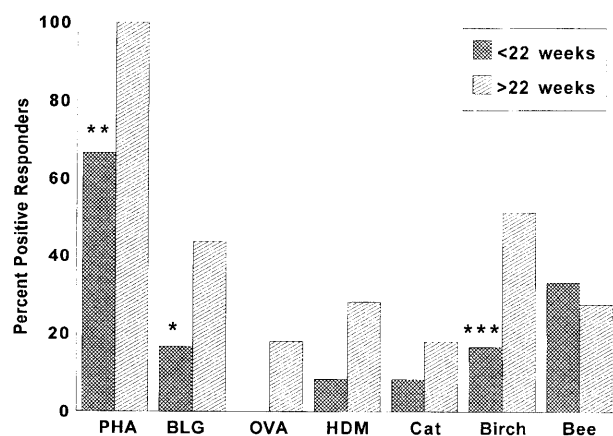


Fig. 3. Graph showing the percent of positive responders from samples less than and greater than 22 weeks gestation when cells were stimulated with the mitogens and the allergens. Responses were considered to be positive if the stimulation index was >2.0 . * $p=0.002$, ** $p=0.003$, *** $p=0.009$.

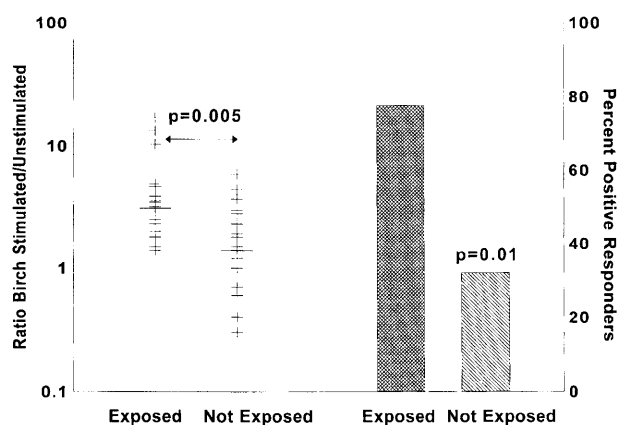


Fig. 4. Graph showing both ratios and percent positive responders to cells stimulated with birch pollen divided regarding whether the mothers were exposed or not exposed to birch pollen from 22 weeks gestation.

was significantly higher when delivery was at > 22 weeks compared with < 22 weeks gestation ($p = 0.0006$). This increase in the proportion of CD4+ve T cells expressing CD45RO complements the proliferative response results, and supports the idea of intra-uterine antigen priming.

All previous studies of prenatal exposure have been performed on umbilical cord blood from full term babies (40 weeks gestation). This study, however, has addressed the question of when during the 40 week gestational period of the human fetus does T cell priming occur. Our results clearly demonstrate the maturation of the fetal immune system in responsiveness to the mitogen, PHA and the specific allergens, notably, β -lactoglobulin, extracts of cat fur and birch tree pollen.

Furthermore, a positive PBMC proliferation stimulation index (classified as ≥ 2.0) was seen to some of the allergens in fetuses and premature babies from 22 weeks gestation onwards. Two $\times$ background is a reasonable artificial threshold suggested by Holt et al. but lower ratios may be indicative of sensitisation. Nevertheless, these data suggest that *in utero* fetal exposure to an allergen from around 22 weeks gestation may result in primary sensitisation to that allergen, leading to positive PBMC proliferative responses, at birth. This is reinforced by our demonstration of the stimulation of PBMC with birch tree pollen allergen where it was possible to accurately determine allergen exposure of the mother during pregnancy. Whether the exposure of the fetus is directly to the allergen via the maternal blood stream or via allergen-antibody complexes which cross the placenta, is as yet, not clear. Holt et al. has postulated that *in utero* priming may occur via the transplacental passage of both antibody

(IgG) and allergen fragments, which are subsequently presented to the fetus as antigen-antibody complexes (4) alternatively anti-idiotypic antibodies may act as antigen and thus stimulate the fetal immune cells. This may not necessarily be a mechanism which discriminates between atopic and non atopic mothers since there is a high IgG reactivity to ovalbumin and house dust mite in the general adult population.

We and others have shown that a higher PBMC proliferative response at birth to particular allergens predicts a higher probability of subsequent allergic disease and type 1 hypersensitivity to those particular allergens (5, 6). Therefore, primary sensitisation of the fetus to environmental allergens occurring from around 22 weeks of gestation has implications for the development of allergic sensitisation, however, more detailed analysis of cytokine profiles from prenatal lymphocytes is required to elucidate these mechanisms.

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Table 1.

Gestation (Weeks)	Unstim 3 days	PHA	Unstim 6 days	HDM	Cat Fur	Birch Pollen	B-lact	Ovalb	Bee
15	30932	120474	7013	11680	7517	11897	7908	7766	ND
17	2793	17149	836	951	749	974	847	823	ND
17	216	273	536	139	123	165	158	122	ND
17	7205	105681	12501	12287	6686	7214	9513	7432	ND
18	156	150	165	157	157	162	162	156	158
18	5630	27177	3157	4397	3300	5335	2932	3858	ND
19	149	372	1814	2315	1842	2548	1848	1821	ND
19	79	68	74	63	63	91	63	62	ND
19	85	77	89	83	75	86	69	89	ND
19	9581	22374	820	858	803	1071	1147	792	ND
20	9389	84283	14015	15667	11148	8122	13675	14595	114
22	7171	50768	1705	464	946	2344	1202	1124	ND
23	6712	103905	4044	8049	9088	16330	9858	4347	10486
24	4161	59233	1269	1976	1562	918	1537	1678	115
24	1785	7259	2114	5657	3754	6545	4392	1443	8562
24	1131	90185	1219	1102	1432	316	1989	743	1182
27	1941	59817	8358	13304	16408	16694	11902	10591	16730
27	12467	172670	58	80	98	84	66	71	92
28	289	78930	180	134	153	109	151	137	126
28	15898	209104	6106	15371	27678	35816	50484	8642	18918
28	124	11676	18	29	32	58	60	63	50
28	1954	15582	3414	3007	5312	5179	7205	3971	124
28	2092	48444	2685	7707	3538	9484	10045	4742	8062
28	3631	229356	3521	6363	2708	4763	7087	2352	248
28	2138	15084	947	1901	1176	1093	1012	856	ND
29	1687	81393	341	820	779	4663	4157	2285	1467
31	1081	27788	575	831	821	1450	5081	864	556
31	1476	80015	557	2116	910	5773	3207	791	2171
32	1509	79018	3435	11548	6038	15243	9160	5090	5417
32	139	12288	62	96	78	307	138	203	97
32	142	19735	59	92	77	1016	80	154	38
32	633	61025	630	2363	981	2470	3096	561	127
32	3393	41012	6215	7047	5435	14524	11323	3864	86
32	1376	73290	5053	6617	4091	15085	8374	8563	7470
32	1655	4189	3801	6706	7169	7350	7239	3235	ND
32	1974	53784	2358	3818	4005	8713	2050	2660	2170
32	2944	115604	2021	6590	5884	3597	15572	4838	2042
32	4115	259516	8373	8492	10575	2112	10895	6763	465
32	1708	149878	4331	8639	3547	1914	5545	1332	654
32	1204	34273	1211	5007	10641	1483	13379	4883	20389
32	4882	96497	16509	21689	16686	37669	30325	20044	40658
33	3730	161783	14029	10301	9894	43444	27787	13758	6883
33	2083	24956	2886	5970	3666	11624	11235	1928	ND
33	3391	117643	7102	13064	24763	25838	22279	16281	7058
33	1580	28038	7792	7473	7534	8072	10343	9842	8486
33	5121	79335	2798	3991	3979	4969	5293	3974	2817
33	4089	120382	6965	6990	4338	4633	3552	4120	ND
34	4936	41602	13509	10892	12658	13628	10698	13987	13273
34	920	46964	416	812	867	1167	1308	807	ND
34	4713	40974	18073	16659	18803	17927	15693	16801	13885
34	795	49195	2084	4939	6275	9737	11477	2145	ND

Table showing counts per minute (cpm) for proliferative responses of PBMC stimulated with PHA and the allergens (PHA = phytohaemagglutinin, HDM = house dust mite, β -lacto = β -lactoglobulin, Ovalb = ovalbumin, Bee = mellitin, ND = not done).

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