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Analysis of lymphocyte phenotypes in cord blood from early gestation fetuses

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SUMMARY

Using cord blood samples obtained from fetuses between 16 and 40 weeks gestation, we have used a lysed whole blood flow cytometric technique to study the natural history of lymphocyte phenotypes known to be highly represented in cord blood at birth. The majority (51·0 ± 14·7%) of lymphocytes expressed CD45RA, a marker of 'virgin' cells and there was a correlation between increasing percentages of CD45RA$^+$ lymphocytes and gestational age ($r = 0{\cdot}44$, $P < 0{\cdot}01$). Few cells (8·5 ± 4·2%) expressed the CD45RO marker of primed lymphocytes and very few (1·0 ± 0·7%) co-expressed CD45RA and RO, indicating little traffic between the two maturation markers. The percentage of B lymphocytes co-expressing CD5 was high in the fetal circulation (55·5 ± 10·5%) compared with healthy adults (23·2 ± 14·3%; $P < 0{\cdot}00001$) and the level of CD5$^+$ B cells declined with gestational age in an exponential manner ($r = -0{\cdot}45$, $P < 0{\cdot}05$). Similarly, levels of T lymphocytes expressing the $\gamma\delta$ T cell receptor (TCR) declined exponentially ($r = -0{\cdot}59$, $P < 0{\cdot}005$). These results demonstrate that lymphocytes remain almost entirely unprimed before birth. In addition, CD5$^+$ B lymphocytes and TCR-$\gamma\delta^+$ T lymphocytes decline exponentially towards birth, in a manner suggesting that they may be seeding peripheral sites such as the spleen, skin and mucosae.

Keywords fetal blood fetal lymphocytes CD45R isoforms B-1 cells TCR-$\gamma\delta^+$ lymphocytes

INTRODUCTION

Analysis of cord blood has shown that at birth the human neonate has a complement of circulating cells expressing mature lymphocyte markers, including CD3$^+$, CD4$^+$ or CD8$^+$ T lymphocytes and CD20$^+$, CD19$^+$, and surface immunoglobulin-positive B lymphocytes [1–4]. In addition, several lymphocyte sub-populations are found at much higher levels in the circulation at birth than during adult life. At birth, T lymphocytes express the CD45RA isoform of CD45 almost exclusively [1,5] and the majority of B lymphocytes express CD5 [6,7] (now termed B-1 cells) [8]. These, along with T lymphocytes expressing T cell receptors (TCR) comprising γ and δ chains, the genes for which are the first to be rearranged during thymic ontogeny [9], are often referred to as 'fetal' phenotypes [10].

In pathological conditions associated with autoimmune reactions, expansions in CD45RA$^+$ T lymphocytes, CD5$^+$ B lymphocytes and TCR-$\gamma\delta$-expressing T lymphocytes have been described [10–12], though the relationship between these changes and the development of autoimmunity is unclear. Our knowledge about the ontogeny of these sub-populations in man has relied largely on extrapolation from animal studies [13] and the analysis of cord blood obtained from healthy newborn babies [14] and fetal spleen [6]. None of these approaches gives any indication of the dynamics of lymphocyte development within the fetal circulation and until recently it has not been possible to study the natural history of 'fetal' lymphocyte phenotypes in peripheral blood due to lack of fetal blood samples. In specialist centres, however, cordocentesis has become an established technique for the early diagnosis of fetal abnormalities. In the present study, we investigated lymphocyte phenotypes in cord blood from as early as 16 weeks gestation through to term.

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MATERIALS AND METHODS

Human fetal blood samples

Umbilical cord blood samples were obtained with informed maternal consent from a total of 36 fetuses of between 16 and 40 weeks gestation undergoing prenatal diagnosis of genetic disease or following elective caesarean section or normal delivery (Table 1). In 34 cases a normal karyotype was established and haematological values were normal for gestational age based on our previously established data [15], with the exception of two cases of fetal anaemia due to rhesus isoimmunization. Fetal blood samples were also obtained from twins who had toxoplas-

Table 1. Gestational age, diagnosis and mean leucocyte count of 36 fetuses studied

Indication	Gestation (weeks)	Number	Mean leucocyte count ($\times 10^9/l$)	Mean lymphocyte count ($\times 10^9/l$)
Normal	16	2	1·8	1·6
Normal	20	6	4·4	3·7
Normal	21	4	3·8	3·4
Normal	22	3	3·8	3·4
Normal	24	1	6·2	4·7
Normal	25	1	3·3	2·8
Normal	26	1	2·6	2·2
Normal	28	1	4·7	3·8
Normal	32	2	6·8	4·3
Normal	33	1	9·4	4·2
Normal	34	1	6·9	4·4
Normal	35	1	10·6	3·8
Normal	37	1	12·3	7·9
Normal	38	1	8·3	5·1
Normal	40	6	14·5	5·8
Toxoplasmosis	26	2*	2·3	1·8
69XXY karyotype	19	1	12·2	11·2
46XX/45X karyotype	37	1	12·4	4·6

* Twin pregnancy, both fetuses affected.

mosis. In addition, samples were obtained from two fetuses with chromosomal abnormalities (69XXY and 46XX/45X). Samples were collected into 10 mM ethylenediaminetetraacetic acid and analysed within 3 h. The fetal origin of the blood was confirmed by the acid elution (Kleihauer) method.

For comparative purposes, blood samples from 25 healthy adults (11 males) were studied (mean age $35·4 \pm 6·6$ years).

Lymphocyte phenotypic analysis by flow cytometry

Lymphocyte subsets were analysed by two-colour direct immunofluorescence and flow cytofluorimetry with a FACScan (Becton Dickinson, Oxford, UK) using a lysed whole blood technique. Fifty μl blood were incubated with saturating amounts of MoAb, using the following combinations: phycoerythrin (PE) conjugated anti-CD45RA (2H4, Coulter Electronics, Luton, UK) and fluorescein isothiocyanate (FITC) conjugated anti-CD45RO (UCHL1, Dako, High Wycombe, UK); PE-conjugated anti-CD20 and FITC-conjugated anti-CD5 (Becton Dickinson); and PE-conjugated anti-CD5 with either FITC-conjugated anti-TCR $\gamma\delta$ or FITC anti-TCR $\alpha\beta$ (Becton Dickinson). After 15 min incubation at room temperature in the dark, erythrocytes were lysed by addition of 2 ml of lysis solution (FACSlyse, Becton Dickinson) and incubated for a further 10 min. Following centrifugation at 250 ***g***, cells were washed once in phosphate buffered saline, resuspended in 300 μl sheath fluid (FACSFlow; Becton Dickinson) with 1% paraformaldehyde and kept on ice until analysis.

Lymphocytes were identified by forward and 90° light scatter properties and a gate set to exclude contaminating debris. Fluorescence signal overlap was subtracted by electronic compensation set using lymphocytes stained with a single fluorochrome. A minimum of 5000 events were acquired for each sample and analysed using FACScan Research software (Becton Dickinson). Quadrant markers were set using preparations of lymphocytes stained with MoAbs conjugated with either FITC or PE alone or in combination, such that cells in quadrant 1 stain with PE-conjugated antibody alone, in quadrant 2 with both fluorochromes, in quadrant 3 with neither and in quadrant 4 with FITC-conjugated antibody alone. Non-specific binding was assessed using isotype- and fluorochrome-matched MoAbs directed against an irrelevant target (anti-keyhole limpet haemocyanin, Becton Dickinson) and was subtracted from all results.

Statistical analysis

Lymphocyte subsets were normally distributed according to the Kolmogorov–Smirnov goodness of fit test and mean values were compared using the Student's *t*-test. Correlations between variables and gestational age were studied by regression analysis. Abnormal fetuses were excluded from all statistical analyses. Differences between variables and correlations were considered significant if $P < 0·05$.

RESULTS

Lymphocyte expression of CD45RA and CD45RO in normal fetuses

Percentage levels of lymphocytes expressing CD45RA (mean $\pm$ s.d.; $51·0 \pm 14·7\%$) were higher than levels of CD45RO$^+$ lymphocytes ($8·5 \pm 4·2\%$; $P < 0·0001$) in the normal fetuses. Levels of lymphocytes simultaneously co-expressing both CD45RA and CD45RO were low but detectable ($1·0 \pm 0·7\%$) (Fig. 1). There was a significant correlation between increasing percentages of CD45RA$^+$ lymphocytes and gestational age ($r = 0·44$, $P < 0·01$), whilst the median fluorescence intensity of cells expressing CD45RA declined with increasing gestation ($r = -0·41$, $P < 0·05$) from a mean level of 533 arbitrary units (AU) at 16 weeks gestation to a mean level of

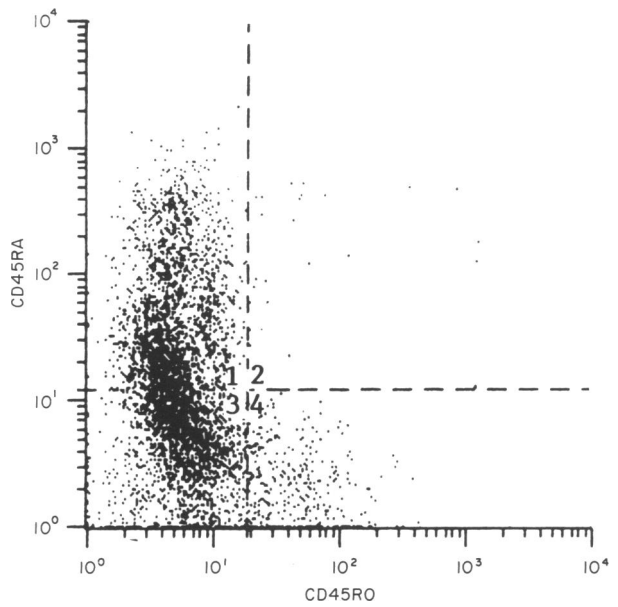

Fig. 1. Typical dual colour flow cytometric analysis of lymphocytes from a 32-week fetus stained with FITC-conjugated anti-CD45RO and PE-conjugated anti-CD45RA. The majority of lymphocytes are CD45RA$^+$ (quadrant 1). Few cells are CD45RO$^+$ (12%; quadrant 4) or CD45RA$^+$RO$^+$ (1·2%; quadrant 2), indicating a lack of transition from CD45RA$^+$ 'naive' cells to CD45RO$^+$ 'primed' cells. Cells in quadrant 3 comprise lymphocytes lacking CD45RA or RO as well as contaminating nucleated red blood cells which have similar light scatter properties to lymphocytes.

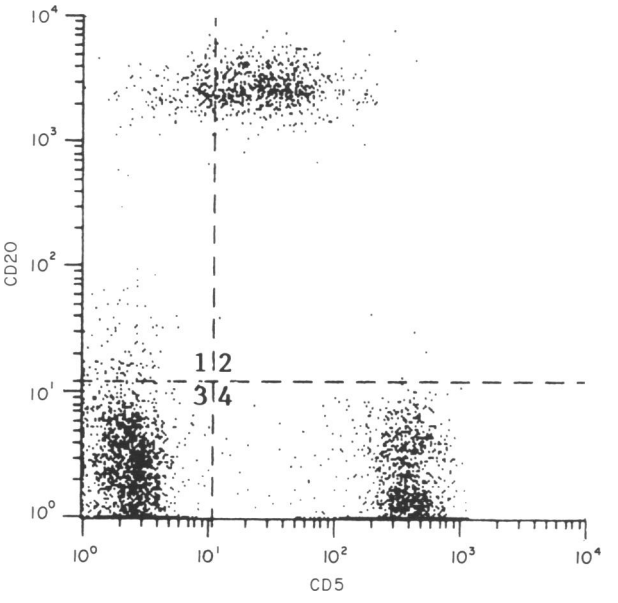

Fig. 2. Typical dual colour flow cytometric analysis of fetal lymphocytes stained with FITC-conjugated anti-CD5 and PE-conjugated anti-CD20. Two populations of B lymphocytes expressing CD20 alone are identified in quadrant 1, with either high (CD20^{+high}CD5$^-$) or low (CD20^{+low}CD5$^-$) surface expression. The majority of all CD20$^+$ B lymphocytes (CD20^{+high}CD5$^-$, CD20^{+low}CD5$^-$ and CD20^{+high} CD5^{+low}, quadrants 1 and 2) co-express CD5 (66·1%; CD20^{+high} CD5^{+low}, quadrant 2). Cells negative for both markers are in quadrant 3, and quadrant 4 contains T lymphocytes expressing high levels of CD5.

479 AU at 40 weeks gestation, a reduction of 10·1%. There was no relationship between the percentage levels or intensity of CD45RO expression and gestational age.

Lymphocyte expression of CD5 and CD20 in normal fetuses

Analysis of CD5 and CD20 expression on lymphocytes in the fetus demonstrated four distinct populations (Fig. 2, Table 2). Two populations expressed CD20 alone, either at high (CD20^{+high}CD5$^-$) or low (CD20^{+low}CD5$^-$) levels of surface expression (Fig. 2, quadrant 1). A third population of cells which co-expressed high levels of CD20 and low levels of CD5 (CD20^{+high}CD5^{+low}) are the CD5$^+$ B cell population previously described and now known as B-1 cells [8]. The fourth population constitutes T lymphocytes expressing CD5 at a high level in the absence of CD20.

The physical characteristics of the population of CD5$^+$ B cells was examined using forward and 90° light scatter. CD5$^+$ B lymphocytes had forward and side scatter between 2·5 and 5% higher than CD20^{+high}CD5$^-$ B lymphocytes.

Analysing the relationships between populations expressing CD5 and CD20 and age of the fetus, there was a tendency towards a correlation between gestation and declining levels of CD5$^+$ B lymphocytes (i.e. CD20^{+high}CD5^{+low}) as a percentage of total lymphocytes which failed to reach conventional levels of significance ($r=-0{\cdot}36$, $P=0{\cdot}052$). Log transformation of the percentage of CD5$^+$ B lymphocytes improved the relationship ($r=-0{\cdot}45$, $P=0{\cdot}011$) indicating that the decline in CD5$^+$ B cells with gestation is best described by an exponential relationship. The percentage of all CD20$^+$ B lymphocytes (i.e. CD20^{+high}CD5$^-$ plus CD20^{+high}CD5^{+low} plus CD20^{+low}CD5$^-$ cells) decreased with gestational age ($r=0{\cdot}39$, $P<0{\cdot}05$). In contrast, the percentage of CD5$^+$ T lymphocytes (CD20$^-$CD5^{+high} cells) increased with gestation ($r=0{\cdot}47$, $P<0{\cdot}01$).

The proportion of B lymphocytes expressing CD5 (CD20^{+high}CD5^{+low}) in fetal blood was $55{\cdot}5\pm10{\cdot}5\%$ (mean $\pm$ s.d.), which was significantly different from that in healthy adults ($23{\cdot}2\pm14{\cdot}3\%$, $P<0{\cdot}00001$). Absolute numbers of CD5$^+$ B lymphocytes were also significantly higher in the fetal circulation ($418{\cdot}1\pm337{\cdot}8$ cells/μl) compared with adult blood ($53{\cdot}8\pm56{\cdot}6$, $P<0{\cdot}00001$). There was no correlation between absolute number of CD5$^+$ B lymphocytes and gestation.

Lymphocyte expression of TCR-$\gamma\delta$ and TCR-$\alpha\beta$ in normal fetuses

In a representative number of normal fetuses, sufficient blood was available for analysis of TCR-$\gamma\delta$ ($n=21$) and TCR-$\alpha\beta$ ($n=7$) expression. The mean ($\pm$ s.d.) percentage of CD5$^+$ T lymphocytes expressing TCR-$\gamma\delta$ was $7{\cdot}1\pm7{\cdot}7\%$. There was a tendency for the proportion of T cells expressing TCR-$\gamma\delta$ to decline with increasing gestation which did not reach statistical significance ($r=-0{\cdot}40$, $P=0{\cdot}075$). Log transformed values of TCR-$\gamma\delta$ expressing T cells showed a significant negative correlation with gestation, however, indicating that the decline is best described by an exponential relationship ($r=-0{\cdot}59$, $P=0{\cdot}0049$) (Fig. 3). In the seven cases studied, the median level of TCR-$\alpha\beta$ expression by CD5$^+$ T lymphocytes was 94·6% (range 73·8–99·4%). There was no apparent relationship between TCR-$\alpha\beta$ expression and gestation when studying this small number of cases.

Table 2. Percentages (mean (s.d.)) of lymphocyte populations expressing CD5 and CD20 in 32 normal, two chromosomally abnormal fetuses and twin fetuses with *Toxoplasma gondii* infection

Lymphocyte population	Normal foetuses mean % (s.d.)	Chromosomal abnormality 69XXY	Chromosomal abnormality 46XX/45X	Twin 1	Twin 2
$CD20^{+high}CD5^{-}$	8·8 (4·4)	0·1%	10·2%	7·2	5·7
$CD20^{+low}CD5^{-}$	2·2 (3·0)	0·9	0·4	0·7	1·4
$CD20^{+high}CD5^{+low}$	11·2 (5·9)	0·0	7·2	17·4	12·2
All $CD20^{+}$ B lymphocytes*	22·1 (9·7)	1·0	17·8	25·3	19·3
All $CD5^{+}$ T lymphocytes†	38·9 (16·6)	3·3	73·8	41·3	52·9

* $CD20^{+high}CD5^{-}$ plus $CD20^{+high}CD5^{+low}$ plus $CD20^{+low}CD5^{-}$ cells.
† $CD20^{-}CD5^{+high}$ cells.

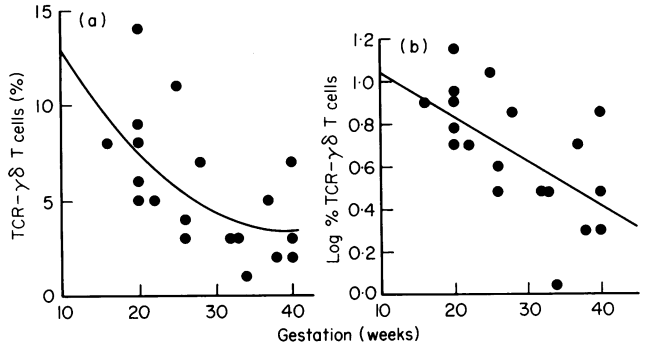

Fig. 3. (a) Graph demonstrating the relationship between levels of TCR-$\gamma\delta$ T cells and gestation. The value of the regression coefficient r is higher following log transformation of the levels of TCR-$\gamma\delta$ T cells ($r = -0{\cdot}59$, $P < 0{\cdot}005$), indicating that the relationship is best described by an exponential curve (b).

Lymphocyte subsets in abnormal fetuses

Of the 36 blood samples obtained, four were from abnormal fetuses (Table 1). A fetus of 19 weeks gestation with a 69XXY karyotype had a markedly raised leucocyte count of $12{\cdot}2 \times 10^9/l$, but very low levels of lymphocytes expressing CD45RA (0·6%) and CD45RO (2·0%) and an abnormal distribution of CD20 and CD5 expressing lymphocytes (Table 2). A fetus with a different chromosomal abnormality (46XX/45X) analysed at 37 weeks had a high leucocyte count but demonstrated a different distribution of lymphocyte phenotypes, with high levels of expression of CD45RA (92·2%), low levels of CD45RO expression (2·2%) and levels of CD20 and CD5 expression similar to the mean values obtained for the normal fetuses (Table 2). In the twin fetuses with toxoplasmosis, the leucocyte count was similar to normal fetuses of a similar gestation (Table 1) and expression of CD45RA (41·6% and 47·7%), CD45RO (10·1% and 11·8%), CD20 and CD5 (Table 2) were similar to the normal fetuses.

DISCUSSION

In this study we examined lymphocyte sub-populations typically found at high levels in the cord blood of full-term babies. Using blood obtained from fetuses with a range of gestations from 16 to 40 weeks, we have shown that the percentage of lymphocytes expressing CD45RA, a marker of 'naive' or 'virgin' lymphocytes [16,17] increases with gestation, although the level of surface expression of this molecule has an inverse relationship with increasing fetal age. The percentage of $CD5^{+}$ B cells declines exponentially with gestation, as does the percentage of T lymphocytes expressing TCR-$\gamma\delta$, while B lymphocyte and T lymphocyte levels increase with gestational age.

The cell surface antigens CD45RA and CD45RO are considered to delineate two functionally distinct populations of lymphocytes. $CD45RA^{+}$ cells are considered 'virgin', or immunologically naive cells and following antigen stimulation become $CD45RO^{+}$ 'primed' or memory cells, presumably through a period of co-expression of both markers [17–21]. Until recently it was thought that this maturation was both unidirectional and irreversible, although this notion has been challenged [22,23]. In functional terms, naive cells are unresponsive to specific antigen stimulation and suppress rather than promote antibody production, whereas memory cells are capable of proliferative responses and providing help in lymphocyte functions [17]. High levels of $CD45RA^{+}$ lymphocytes have been described in cord blood of full-term infants [1,5] and in the present study we demonstrate that these levels are achieved following a linear increase throughout gestation. In addition, we demonstrate that there appears to be little transition to the memory status, with few cells expressing CD45RO, or co-expressing CD45RA and RO and no increase in these populations during gestation. This presumably reflects the lack of immunological stimulation during normal intrauterine development. The decline in density of CD45RA molecules on the cell surface during fetal development may be part of a lymphocyte maturation process which is independent of antigen.

High levels of $CD5^{+}$ B lymphocytes have been described in the circulation of healthy full-term babies [2,7], and also appear to be a feature of both non-organ specific and organ specific autoimmune disease such as rheumatoid arthritis, Sjögren's syndrome [10] and Type 1 diabetes [24]. $CD5^{+}$ B lymphocytes produce polyreactive, low affinity antibodies which often have rheumatoid factor activity [25] and it has been proposed that they have an important role in the primary immune response, when polyreactive antibodies may be a useful first-line defence [26]. The present study shows that as a percentage of all lymphocytes, levels of these cells show an exponential decline with gestation. Early gestation could be interpreted, therefore, as a period during which $CD5^{+}$ B cells are seeded into the circulation, possibly in order to populate the secondary lymphoid organs such as the spleen and lymph nodes. The

possibility that some of the apparent reduction is a dilutional effect as total lymphocyte numbers increase cannot be excluded. In physical terms, $CD5^+$ B lymphocytes have been described as having a higher level of forward and 90° light scatter than $CD5^-$ B cells in mice [27], suggesting that they are larger, activated cells, and this was confirmed for human fetal B-1 cells in our study. In addition, studies on mice have indicated that $CD5^+$ B cells appear very early in fetal development and that their absolute numbers rapidly reach adult levels [28]. Our own data indicate that absolute numbers of $CD5^+$ B lymphocytes are almost ten times higher in the fetus and in this study there is no suggestion that they increase with gestational age.

There have been few previous studies of lymphocyte subsets in early fetal life. Thilaganathan *et al.* [29] found an increase in absolute numbers of $CD5^+$ B lymphocytes with gestation but did not report these as a proportion of all B lymphocytes. In addition, they used MoAb to CD19 to identify B lymphocytes, and the earlier appearance of this marker than CD20 in B cell ontogeny could explain the discrepancy.

The population of T lymphocytes expressing TCR-$\gamma\delta$ has also been described as being a 'fetal' phenotype, and it is known that the δ and γ chain genes are the first to be rearranged during thymic ontogeny [9]. In the present study, the proportion of T lymphocytes expressing TCR-$\gamma\delta$ declined exponentially with increasing gestational age. Again, this pattern would be consistent with a population of cells released early during T lymphocyte ontogeny to seed peripheral sites. There is evidence to suggest that in the mouse, a TCR-$\gamma\delta$ population of T lymphocytes matures extra-thymically in the intraepithelial environment in the intestine [30] but similar findings have yet to be made in man.

Blood samples obtained from chromosomally abnormal fetuses revealed lymphocyte abnormalities affecting both T and B cell development. Interestingly, fetuses with intrauterine infection with *Toxoplasma gondii* had leucocyte counts and lymphocyte sub-populations similar to normal fetuses of the same gestation. Further studies will be needed to establish whether patterns of lymphocyte phenotypic abnormalities can be of use in the diagnosis of abnormal fetuses.

The combination of samples obtained by cordocentesis from early fetuses and immunophenotyping using a lysed whole blood technique and flow cytometry has provided a powerful and accurate approach to the study of fetal blood. It has enabled the demonstration of large shifts in lymphocyte populations during fetal development. Functional studies are required to establish the roles of some of these populations in the development of the immune system.

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