Clin. exp. Immunol. (1984) **56**, 407–414.

Development of pre-B and B lymphocytes in the human fetus

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(Accepted for publication 15 December 1983)

SUMMARY

Cell suspensions from human fetal liver, bone marrow and spleen were systematically studied at between the fetal ages of 8 and 20 weeks by the direct immunofluorescence technique for the presence of pre-B and B cells. Pre-B cells were characterized as lymphoid cells containing cytoplasmic μ heavy chains but lacking surface IgM. Based on their size and morphological appearance, these cells were subdivided into large and small pre-B cells. In the livers of 8 week old fetuses, more than 90% of the total pre-B plus B cell population consisted of pre-B cells; the relative number of liver pre-B cells gradually decreased with increasing gestational age and, after the 14th week, B cells outnumbered pre-B cells. At 20 weeks, the ratio of pre-B to B cells was only 0·25. In contrast, the number of pre-B cells in fetal bone marrow (12–20 weeks) was always greater than that of the B cells. Large and small pre-B cells were present in the liver and bone marrow. Small pre-B cells outnumbered the large ones in both organs and with increasing gestational age the ratio of small to large pre-B cells increased four-fold. In fetal spleen (12–20 weeks), no large pre-B cells were seen and the small ones comprized only a minor fraction of the total B-cell population. It can be concluded from these data that during early human fetal life the liver is an important site of pre-B cell production. From 12 weeks onwards, this function is gradually taken over by the bone marrow. During the second half of pregnancy, pre-B cell production in fetal liver becomes very much less as compared with the bone marrow. No generation of pre-B cells takes place in the fetal spleen, but a certain amount of maturation of cells of the B cell line may take place in this organ.

Keywords pre-B lymphocytes B lymphocytes human fetus ontogeny

INTRODUCTION

In several mammalian species (the rabbit, the mouse and man), the first recognizable cell of the B cell lineage, the pre-B cell, is a large lymphoid cell containing a scant number of intracytoplasmic μ heavy chains and lacking surface immunoglobulins (sIg) and intracytoplasmic light chains detectable by immunofluorescence (Raff *et al.*, 1976; Gathings, Lawton & Cooper, 1977; Hayward *et al.*, 1978).

In vivo and *in vitro* kinetic studies in mice using ^{3}H-thymidine labelling (Owen *et al.*, 1977), elimination of pre-B and B cells by cyclophosphamide (Landreth, Rosse & Clagett, 1981), anti-μ suppression (Burrows *et al.*, 1978; Freitas *et al.*, 1982; Fulop, Gordon & Osmond, 1983) and cell and organ culture studies (Owen, Raff & Cooper, 1975; Phillips & Melchers, 1976; Paige, 1983), have shown that large pre-B cells are rapidly dividing ones. These give rise to small non-dividing pre-B cells which differentiate into sIg^+ cells.

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In the human fetus, pre-B cells are first found in the liver at 7·5 weeks of gestation; sIg^+ cells appear at 1–2 weeks later (Gathings *et al.*, 1977; Kamps & Cooper, 1982). Large and small pre-B cells are found in human fetal liver and bone marrow (BM). Only small pre-B cells are occasionally observed in other fetal organs.

We report here on a systematic investigation of the relationship between large pre-B cells, small pre-B cells and B cells in human fetal liver, BM and spleen between the gestational ages of 8 and 20 weeks. Cell suspensions from these tissues were investigated by immunofluorescence techniques to identifiy cytoplasmic μ^+/surface μ^- ($c\mu^+/s\mu^-$) large and small pre-B cells and surface μ^+ ($s\mu^+$) B cells.

MATERIALS AND METHODS

Tissue samples. Human fetuses of 8–20 weeks gestational age (ga) were obtained by interruption of pregnancy on non-medical grounds. The use of this material for research purposes was approved by the Human Ethical Research Committee of the University Hospital of Leiden. Single cell suspensions were made from the liver, BM and spleen as previously described (Asma, Langlois van den Bergh & Vossen, 1983). Cell viability as determined by trypan blue dye exclusion was always more than 90%.

Conventional antisera and monoclonal antibodies (MoAbs). For the detection of membrane bound IgM, a fluorescein isothiocyanate (FITC) labelled IgG fraction of a rabbit anti-human IgM isotype specific serum (RaHu/IgM(Fc)/FITC; dilution 1:20; Dako Immunoglobulins AS, Copenhagen, Denmark) was used.

The following reagents were used for the intracytoplasmic staining of heavy and light Ig chains. Antibodies specific for μ and α chains were prepared from rabbit anti-IgM and goat anti-IgA antisera (a kind gift of Dr J. Radl, Institute for Experimental Gerontology, Rijswijk, The Netherlands), respectively, by affinity chromatography and conjugated to tetramethylrhodamine isothiocyanate (TRITC); the RaHu/IgM/TRITC antibody was used in a dilution of 1:48, the GaHu/IgA/TRITC antibody in a dilution of 1:80. GaHu/IgG(Fc)/TRITC (dilution 1:80) and GaHu/IgD (Fc)/TRITC (dilution 1:32) conjugates were obtained from Kallestad Laboratories, Chaska, Minnesota, USA. A TRITC conjugated mouse anti-human λ light chain MoAb (dilution 1:250) was a kind gift of Dr J. Radl and Dr J. Haaijman, Institute for Experimental Gerontology, Rijswijk. A TRITC conjugated mouse anti-human κ light chain MoAb (dilution 1:50) was obtained from the Ortho Pharmaceutical Laboratories. These anti-human heavy and light chain conjugates reacted only with the appropriate isotypes of immunoglobulins as tested by cytoplasmic immunofluorescence on monoclonal bone marrow preparations (Hijmans, Schuit & Klein, 1969).

Immunofluorescence staining and microscopic identification of pre-B and B cells. Viable cells ($1–10 \times 10^5$ in 25 μl 1% bovine serum albumin [BSA] solution in phosphate-buffered saline [PBS]) were incubated with RaHu/IgM(Fc)/FITC as previously described (Asma *et al.*, 1983). Cytocentrifuge preparations of these cells were made and fixed at −20°C for 15 min in a solution of 5% glacial acetic acid and 95% ethanol (vol./vol.). The preparations were then rinsed three times in PBS at 4°C for 10 min and stained with TRITC conjugated anti-μ antibodies at room temperature for 30 min. In some experiments, the fixed preparations were stained with the anti-α, γ, δ heavy chain or anti-κ and λ light chain conjugates. The slides were washed in PBS at 4°C for 24 h and mounted under coverslips in a mixture of 90% glycerol and 10% PBS (vol./vol.). Microscopic examination was done with a Zeiss Standard 18 microscope equipped with a Ploem type vertical illuminator IV F1 and containing a combination of filters suitable for discriminating between TRITC and FITC fluorescence. When fixed cells are stained for immunoglobulins, not only intracytoplasmic Ig but also surface Ig will be detected. Moreover, the morphology and fluorescence pattern of fixed small pre-B cells and B cells are almost identical. For the discrimination between the two cell types, a combined staining for sIg on vital cells and for cIg after fixation (using two different fluorochromes) has to be done (Gathings *et al.*, 1977; Pearl *et al.*, 1978). With this combined staining, small $s\mu^+/c\mu^+$ B cells, small $s\mu^-/c\mu^+$ pre-B cells and large $s\mu^-/c\mu^+$ pre-B cells were identified in the fetal organs. The mean diameter of small pre-B and B cells was $9·9 \pm 0·6$ μm. The mean diameter of large

pre-B cells was $14{\cdot}4 \pm 1{\cdot}3$ μm and they had the typical deeply indented or convoluted nucleus described by Gathings *et al.* (1977). The numbers of pre-B and B cells are given per 100 '$c\mu^+$' cells. The relative numbers of large and small pre-B and B cells in preparations of BM cells suspensions were also determined per 1,000 nucleated cells using phase contrast microscopy.

Anti-IgM-rosetting of B cells. In addition to the detection of sIg^+ cells in BM cell suspensions by immunofluorescence, $sIgM^+$ lymphoid cells were also determined by rosette formation with ox red blood cells (OxRBC) coated with immunosorbent purified goat anti-human μ antibodies. The anti-IgM coated OxRBC were a kind gift of Dr G.C. de Gast, Department of Immunohaematology, University Hospital, Utrecht, The Netherlands. The coating of the OxRBC with anti-IgM and the rosetting procedure were essentially the same as previously described (de Gast & Platts-Mills, 1979). The number of cells binding two or more OxRBC per 200 lymphoid cells was determined.

RESULTS

B cells identified by surface IgM fluorescence vs *anti-IgM OxRBC rosette formation* (Table 1)
Suspensions of fetal BM were subjected to either membrane fluorescence with the FITC conjugated RaHu/IgM(Fc) or rosette formation with OxRBC coated with anti-IgM antibodies. Independently of each other, two investigators examined the fluorescence and rosette preparations. Table 1 shows that, on the average, the number of lymphocytes which formed rosettes with the anti-IgM OxRBC was the same as that of the number of lymphocytes which was stained with the RaHu/IgM(Fc)/FITC conjugate. We found no significant difference between the two methods used for the detection of sIg on B cells (Wilcoxon's signed ranked test, two tailed $\alpha = 0{\cdot}01$).

Pre-B and B cells in fetal liver (Figs 1a & 2)
Fig. 1a gives the results obtained in liver cell suspensions from 8–20 week old fetuses. sIg^+ B cells were seen in livers of fetuses as early as 8 weeks ga. From 8 to 14 weeks, the $sIg^-/c\mu^+$ pre-B cells outnumbered the sIg^+ B cells. More small than large pre-B cells were seen at all ages. The percentages of both small and large pre-B cells gradually decreased during gestation and, after week 14, the relative numbers of B cells were greater than those of pre-B cells. The ratios of small to large pre-B cells and of all pre-B cells to B cells in relation to gestational age are given on a semi-logarithmic scale in Fig. 2. The ratio of small to large pre-B cells increased from 3·4 to 16 with increasing ga. At the age of 8 weeks, 11·5 times more pre-B cells than B cells were present in fetal liver and, at the age of 20 weeks, only one pre-B cell for every five B cells was found.

Pre-B and B cells in fetal BM (Figs 1b, 2 & 3)
The ratio of pre-B cells to B cells in fetal BM decreased from 4 to 1·7 in 12–20 week old fetuses (Figs 1b & 2). In this organ as in fetal liver, there were always more small than large pre-B cells. The proportional increase of small pre-B cells versus large ones during gestation was comparable in BM and liver (Fig. 2).

1. $sIgM^+$ lymphocytes in the human fetal bone marrow

Gestational age (weeks)	Anti-IgM OxRBC rosettes	Direct immunofluorescence*
12–18 ($n = 13$)	$14{\cdot}9 \pm 5{\cdot}3$† (6·5–24)	$14{\cdot}8 \pm 4{\cdot}6$ (8–20·5)

* RaHu/IgM(Fc)/FITC.
† Mean of reactive lymphocytes/100 lymphocytes $\pm$ 1 s.d.
Numbers in parenthesis indicate the range.

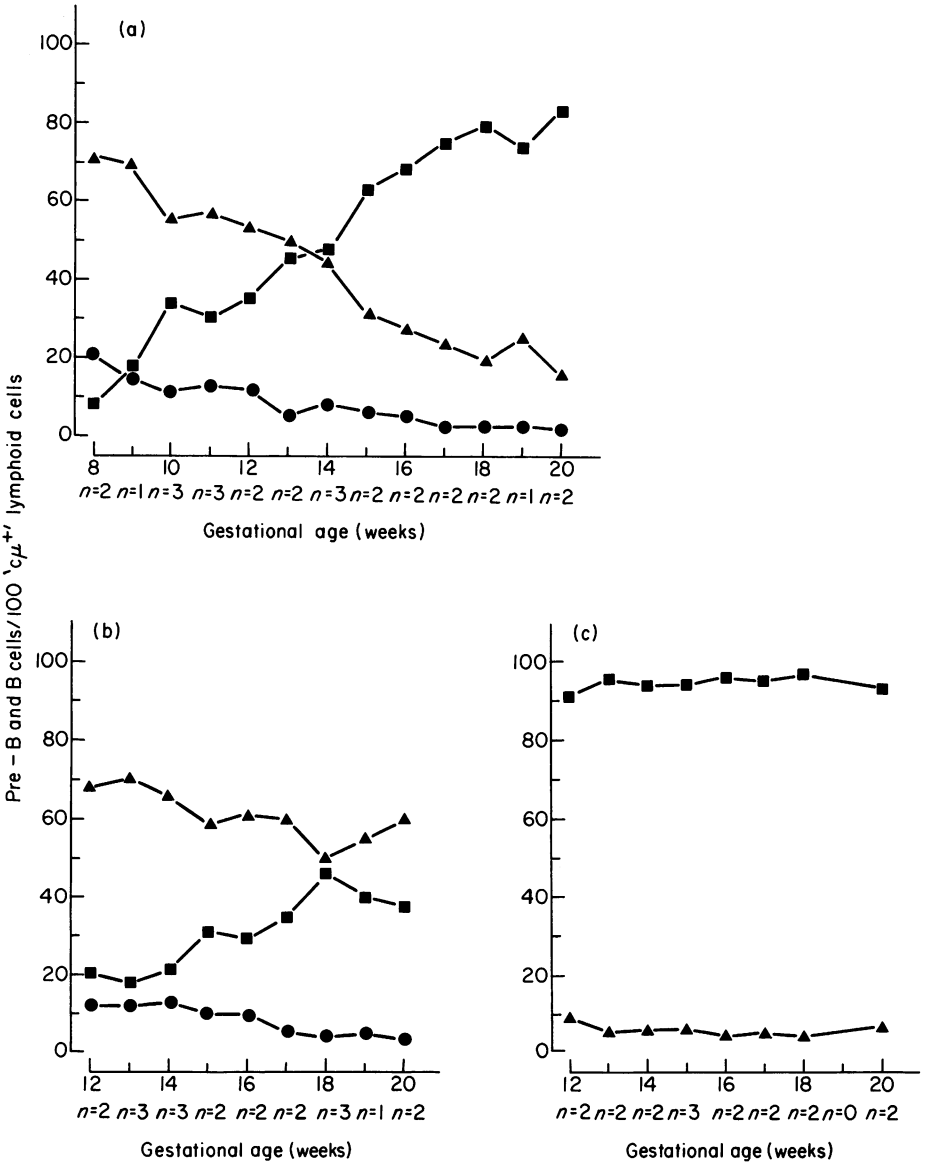

Fig. 1. Large ($c\mu^+/s\mu^-$) pre-B cells (●–●), small ($c\mu^+/s\mu^-$) pre-B cells (▲—▲) and B ($s\mu^+$) cells (■—■) in (a) human fetal liver, (b) bone marrow and (c) spleen expressed as % of '$c\mu^+$' cells ('$c\mu^+$' cells include cytoplasmic and surface μ positive cells). The data represent the mean value found in the number of experiments given for each gestational age. No large pre-B cells were seen in fetal spleen.

When pre-B and B cells per 1,000 nucleated BM cells were counted, the numbers of small pre-B cells and B cells showed sharp increases during the age period investigated: from 48 to 172 and 14 to 106, respectively, (Fig. 3), whereas the number of large pre-B cells remained constant: ± 10 in 1,000 nucleated BM cells.

We also tested for the presence of intracytoplasmic light chains and α, γ and δ heavy chains (Table 2). For the detection of intracytoplasmic light chains, a combined staining for $s\mu$ and cytoplasmic κ or λ chains was done. Almost all $c\kappa^+$ or $c\lambda^+$ cells were also $s\mu^+$. Occasionally, a small

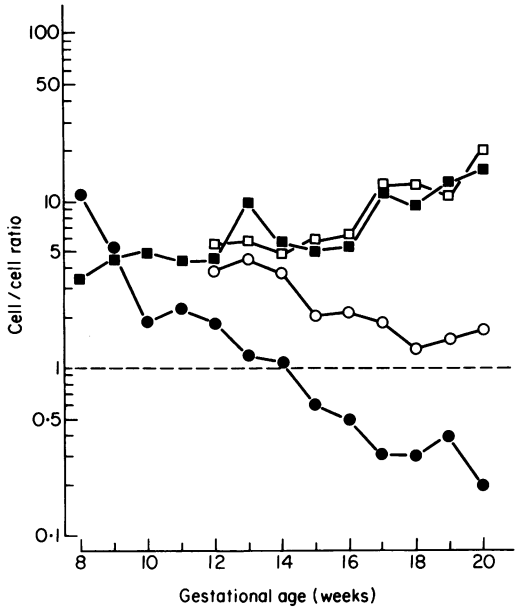

Fig. 2. Ratio of pre-B ($c\mu^+/s\mu^-$): B ($s\mu^+$) cells in human fetal liver (●—●) and bone marrow (○—○) and of small pre-B: large pre-B cells in human fetal liver (■—■) and bone marrow (□—□).

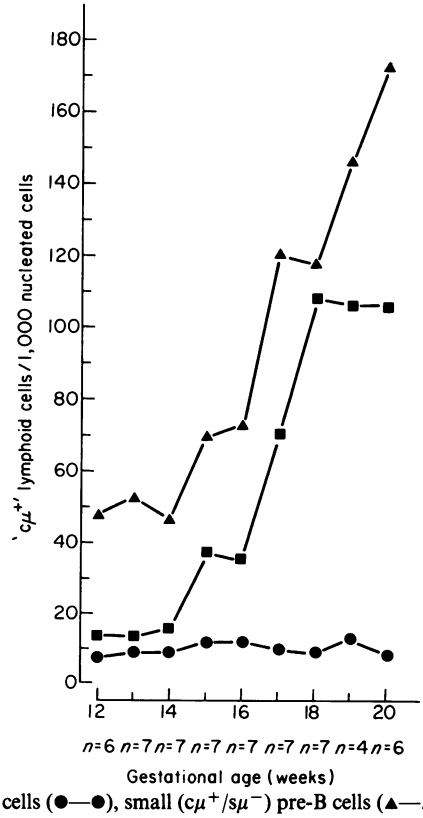

Fig. 3. Large ($c\mu^+/s\mu^-$) pre-B cells (●—●), small ($c\mu^+/s\mu^-$) pre-B cells (▲—▲) and B ($s\mu^+$) cells (■—■) per 1,000 nucleated cells in human fetal bone marrow. The data represent the mean value found in the number of experiments given for each ga.

Table 2. Cytoplasmic immunoglobulin isotypes (other than μ) in human fetal bone marrow lymphoid cells

Gestational age (weeks)	$c\kappa^+/s\mu^-$*	$c\lambda^+/s\mu^-$*	$c\alpha$†	$c\gamma$†	$c\delta$†
12	1‡	0	0	0	0
13	0	0			
14	0	1	0	0	0
15	0	0			
16	0	0	0	0	
17	1	1			
18	0	0	0		
20	4	2	0	0	0

* In 100 $c\kappa^+$ or $c\lambda^+$ cells.

† In $0{\cdot}5 \times 10^5$ nucleated BM cells.

‡ $c\kappa^+/s\mu^-$ and $c\lambda^+/s\mu^-$ cells were always small lymphocytes.

$c\kappa^+$ or $c\lambda^+$ cell which did not express $s\mu$ was seen, indicating that about 1% or less of the small pre-B cells contained intracytoplasmic light chains. No large pre-B cells with $c\kappa$ or $c\lambda$ were seen.

Also, no lymphoid cells with intracytoplasmic α, γ or δ heavy chains were found in the 5 BM samples studied.

Pre-B and B cells in fetal spleen (Fig. 1c)
More than 90% of '$c\mu^+$' lymphoid cells in fetal spleen expressed $s\mu$. Less than 10% of the '$c\mu^+$' lymphoid cells were small $s\mu^-/c\mu^+$ pre-B cells. No large pre-B cells were seen in this organ. This pattern was consistent during the entire fetal age period (from 12 to 20 weeks ga) investigated.

DISCUSSION

Although most investigators have been unable to demonstrate surface IgM on pre-B cells (reviewed in Cooper & Lawton, 1979), Melchers, Von Boehmer & Phillips (1975), using radioiodination, and Rosenberg & Parish (1977), applying a rosette assay, reported that murine pre-B cells may express small amounts of membrane IgM with rapid turnover. In our study, an immunofluorescence assay was used for the detection of surface IgM on human fetal lymphocytes. Immunofluorescence has been reported to be a relatively insensitive technique for the detection of sIg as compared with, for instance, antibody rosette assays (Dhaliwal *et al.*, 1978). To exclude the possibility that we would miss cells with low densities of sIgM, we compared the capacity of fetal BM lymphocytes to bind anti-IgM OxRBC with their reactivity with an FITC conjugated RaHu/IgM(Fc) antiserum. The two methods were found to be equally sensitive for the detection of IgM on the surface of lymphocytes. Moreover, we never observed large lymphocytes which either bound the anti-IgM OxRBC or reacted with the fluorescent anti-IgM antiserum and thus were unable to demonstrate sIgM on human fetal pre-B cells by these methods.

In earlier studies, it was found that pre-B cells are produced in the liver and BM of the human fetus; others found that there were twice as many pre-B cells as B cells in the livers of 7–14 week old fetuses, while the two cell types were found in equal quantities in the livers and BM of older fetuses (up to 17 weeks) (Gathings *et al.*, 1977; Kamps & Cooper, 1982).

We also found that pre-B cells outnumbered B cells in the livers of fetuses younger than 14 weeks ga; however, in contrast to the findings of Gathings *et al.* (1977) and Kamps & Cooper (1982), a

gradual decrease of the pre-B to B cell ratio from 14 to 1 was found at between 8 and 14 weeks. In the livers of older fetuses, this ratio further decreased to 0·25 at 20 weeks. In BM of fetuses of 12–20 weeks ga, the ratio of pre-B to B cells also progressively decreased, but to a lesser extent than in the fetal liver and was always greater than one.

In accord with the recently reported observations of Kubagawa *et al.* (1982), we found that pre-B cells in human fetal BM do not express intracytoplasmic heavy chains other than μ, whereas light chains are expressed only occasionally and never in large pre-B cells. As suggested by Kubagawa *et al.* (1982), the small pre-B cells containing both μ and light chains in their cytoplasma may represent the most mature stage of pre-B cells, just before the conversion to sIg^+ B cells. Although both small and large pre-B cells were seen in the liver and BM at all times of gestation investigated, the small pre-B cells by far outnumbered the large ones in both organs. Moreover, the ratio of small pre-B cells to large ones was the same in fetal liver and BM at comparable ga and considerably increased with ongoing gestation.

Another noteworthy observation was that the number of large pre-B cells (per 1,000 nucleated BM cells) remained constant over the entire period investigated, whereas the numbers of small pre-B cells and B cells considerably increased during the same period.

These findings are suggestive for a distinct relationship between large and small pre-B cells and are in accord with the hypothesis that large pre-B cells are dividing cells giving rise to small pre-B ones which in turn can further differentiate into sIg^+ B cells (reviewed in Cooper, 1981). In fetal liver preparations, we were unable to establish the relative numbers of pre-B and B cells for two reasons: firstly, the percentage of lymphoid cells was very low (less than 4% of nucleated cells) and, secondly, after incubation with antisera, the liver parenchyma cells showed a tendency to clump and this made it impossible to count the total number of cells with any degree of accuracy. In view of the small variation in the percentage of liver lymphoid cells between weeks 12 and 20 of gestation and the observed decrease in the pre-B to B cell ratio during this period of gestation, it can be assumed that, in contrast to the findings in fetal BM, the numbers of small and large pre-B cells per nucleated cells in fetal liver decrease between 12 and 20 weeks of gestation. Our data confirm the observation of other investigators (Gathings *et al.*, 1977; Kamps & Cooper, 1982) and indicate that the liver is the major and probably only site for the production of pre-B cells in younger fetuses, whereas this function is progressively taken over by the BM from 12 weeks onwards. Ultimately, the production of pre-B cells in liver completely ceases (Kamps & Cooper, 1982).

When the results of this study are taken together with the data on the generation of the T cell line in the human fetus, which we reported earlier (Asma *et al.*, 1983), it seems justified to assume that in the second half of pregnancy, i.e. from about 16 weeks ga, the liver plays only a minor role in the generation of lymphoid precursor cells.

These findings correlate well with the experiences in fetal liver transplantation. O'Reilly, Kapoor & Kirkpatrick (1980) and Touraine (1983) reported that patients suffering from severe combined immunodeficiencies and transplanted with fetal liver derived haematopoietic cells only showed successful engraftment when the liver cells were obtained from fetuses younger than 13–14 weeks ga. Our results predict that after that time of gestation fetal BM will be a better source of lymphoid precursor cells than fetal liver. Moreover, BM in older fetuses contains much less 'mature', that is $OKT3^+$, T lymphocytes than does the liver (Asma *et al.*, 1983), which could have a favourable influence on the prevention of graft versus host disease (Van Bekkum, Löwenberg & Vriesendorp, 1978).

No large pre-B cells were ever found by us and Gathings *et al.* (1977) in the fetal spleen. This indicates that this organ is not a site of production of B cell precursors, although the presence of a relatively small number of small pre-B cells in this organ could indicate that B cell maturation can take place to a certain extent in the spleen during human fetal life, as was also found for T cell maturation (Asma *et al.*, 1983).

We wish to thank Dr J. Radl, Institute for Experimental Gerontology, Rijswijk (ZH), The Netherlands, for his careful review of the manuscript. We are grateful to Dr D. van Lith, Center for Human Reproduction, Leiden, The Netherlands, for his kind assistance in obtaining the fetal material.

The investigations were supported by the Foundation for Medical Research FUNGO (grant No. 13-40-74).

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