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Characterization of early lymphoid precursor cells in the human fetus using monoclonal antibodies and anti-terminal deoxynucleotidyl transferase

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SUMMARY

Monoclonal antibodies (MoAbs) directed primarily against immature lymphoid cells (VIL-A1, BA-2, OKT10) or recognizing antigens associated with the B cell lineage (VIB-C5, OKI1) were used for the identification of lymphoid cells in liver, bone marrow, spleen and thymus of human fetuses between 8 and 20 weeks of gestational age. Many lymphocytes in liver, bone marrow and spleen reacted with the MoAbs used. In the fetal thymus, however, cells did not bind to the VIL-A1 and VIB-C5 MoAbs and only a few cells were BA-2$^+$ or OKI1$^+$. In the liver and bone marrow the VIL-A1, VIB-C5 and BA-2 MoAbs reacted almost exclusively with terminal deoxynucleotidyl transferase (TdT) containing cells, pre-B and B cells. TdT$^+$ cells were present in liver, bone marrow and thymus, but not in the spleen. In liver and bone marrow the relative numbers of TdT$^+$ cells decreased during gestation, in the thymus they increased. The antigenic make-up of the TdT$^+$ cells in liver and bone marrow was comparable to that of pre-B and B cells in these organs: most of them reacted with VIL-A1, VIB-C5 and OKT10 MoAbs and many were BA-2$^+$ and OKI1$^+$. TdT$^+$ cells in liver and bone marrow did not bind to T-cell-markers, i.e. OKT6 and WT-1. A few lymphoid cells in these organs contained TdT and μ heavy chains. TdT$^+$ cells in the thymus had a completely different phenotype: most of them were OKT6$^+$ and they did not react with the VIL-A1 and VIB-C5 MoAbs. These findings suggest that TdT$^+$ cells in fetal liver and bone marrow are precursors of the B cell lineage, whereas those in the thymus probably belong to the T cell lineage. In the fetal spleen almost all B cells displayed the VIB-C5 and OKI1 antigens. At 12 weeks of gestation $>80\%$ of splenic B cells were also VIL-A1$^+$ and BA-2$^+$; with ongoing gestation far less B cells in spleen expressed these antigens, however, indicating that these B cells are more mature than those in fetal liver and bone marrow, but still less mature than the B cells in postnatal blood and bone marrow, which do not display the VIL-A1 and BA-2 markers. These findings suggest that some further maturation of B cell stages takes place in the spleen during human fetal life.

Keywords TdT$^+$ lymphocyte pre-B lymphocytes B lymphocytes human fetus monoclonal antibodies

INTRODUCTION

Many lymphocytes in human fetal bone marrow (BM) and fetal liver (up to 16 weeks of gestation) are immature in that they have no surface immunoglobulins (sIg) or T-cell-specific-antigens, and

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most react with the OKT10 monoclonal antibody (MoAb) (Reinherz *et al.*, 1980) directed primarily against immature lymphoid cells (Asma, Langlois van den Bergh & Vossen, 1983; 1984). Many are pre-B cells ($c\mu^+/sIg^-$) but some could not be identified. In addition, many fetal B cells expressed the T10 antigen, indicating that they are phenotypically more immature than postnatal B cells.

Presumably some of these immature lymphoid cells contain the enzyme terminal deoxynucleotidyl transferase (TdT) (Brashem *et al.*, 1982; Bodger *et al.*, 1983) which is found only in immature lymphoid cells (Bollum, 1979). In postnatal man TdT is found in cortical thymocytes (Bollum, 1978) and in a small number of bone-marrow (BM) cells (Barr, Sarin & Perry, 1976). Few of the TdT^+ cells in BM also contain intracytoplasmic μ heavy chains (Janossy *et al.*, 1979) indicating that they belong to the B cell lineage. In rodents TdT^+ BM cells may contain cells of the T cell lineage (Silverstone *et al.*, 1976), but in man we do not know. TdT^+ cells can also be found in human fetal blood and liver (Bodger *et al.*, 1983; Bonati *et al.*, 1983). We have investigated further these immature lymphoid cells, using MoAbs directed primarily against immature lymphoid cells (OKT10, VIL-A1, BA-2) or cells of the B cell lineage (VIB-C5, OKI1). Also the presence of TdT in these cells was looked for.

MATERIALS AND METHODS

Tissue samples. Presumed healthy human fetuses of 8 to 20 weeks gestational age (g.a.) were obtained from abortions done by suction and curettage. Gestational age was estimated by the length of the feet (Moore, 1973). The study of the fetal material was approved by the Ethical Research Committee of the University of Leiden. Single cell suspensions were made from liver, thymus, BM and spleen as previously described (Asma *et al.*, 1983).

Monoclonal antibodies and conventional antisera. The characteristics of the MoAbs used in this study are summarized in Table 1. For the development of the MoAbs, fluorescein- or rhodamine labelled IgG fractions of goat antisera directed against mouse Ig and Ig subclasses were used (Nordic Immunological Laboratories, Tilburg, The Netherlands). Surface and intracytoplasmic μ chains were detected with either a RaHu/IgM/TRITC antibody (Asma *et al.*, 1984) or a mouse-anti-human μ chain MoAb (Ortho Pharmaceutical Laboratories, Raritan, NJ, USA). Nuclear terminal deoxynucleotidyl transferase (TdT) was detected by a TdT immunofluorescent assay kit (Bethesda Research Laboratories GmbH, Neu Isenburg, FRG).

Immunofluorescence staining and microscopic analysis. Cells were stained in suspensions as previously described (Asma *et al.*, 1983). Lymphoid cells were identified by phase contrast microscopy as described by Vossen & Hijmans (1975).

To detect intracytoplasmic μ and nuclear TdT, viable cells were incubated with the MoAbs, cytocentrifuged and either stained with the RaHu/IgM/TRITC antibody as previously described (Asma *et al.*, 1984), or fixed in methanol at 4°C for 30 min and stained for nuclear TdT with rabbit anti-TdT antibody, followed by $F(ab')_2$ goat anti-rabbit IgG-FITC. In some experiments, cytocentrifuge slides fixed in the same way were stained with the mouse-anti-μ chain MoAb, followed by the GAM/Ig/TRITC conjugate, washed in phosphate-buffered saline (PBS) at 4°C for 24 h, and then stained for nuclear TdT.

When fixed cells are stained for immunoglobulins, not only intracytoplasmic Ig but also surface Ig are detected (Pearl *et al.*, 1978). The '$c\mu^+$' population, therefore, consists of $s\mu^+$ B cells and $s\mu^-/c\mu^+$ pre-B cells. Microscopy was performed as previously described (Asma *et al.*, 1983).

RESULTS

Fetal liver

From 8 to 20 weeks of gestation, more than 50% of fetal liver lymphocytes reacted with the VIL-A1 and VIB-C5 MoAbs (Table 2). Approximately 30–40% of lymphocytes stained with the BA-2 MoAb. The percentage of lymphocytes with Ia antigen increased progressively from $\pm$ 19% in 8- to 11-week-old-fetuses to $>$ 50% in 16- to 20-week-old-livers. More than 80% of the '$c\mu^+$' cells in liver

Table 1. Monoclonal antibodies used

Monoclonal antibody	Reactivity*	Molecular weight (kD)	Mouse subclass	References
OKT10	thymocytes lymphoid precursors plasma cells activated T cells	45	IgG1	Reinherz *et al.*, 1980 Hercend *et al.*, 1981 Janossy *et al.*, 1981 van Camp *et al.*, 1982
VIL-A1	CALL antigen present on B precursors	95	IgM	Knapp *et al.*, 1982
BA-2	5–20% of BM cells, including 50% of TdT^+ cells and pre-B cells 10–20% of thymocytes < 5% of PBL activated T cells	24	IgG3	Le Bien *et al.*, 1981 Melink & Lebien, 1983
VIB-C5	pre-B cells B cells	—	IgM	Knapp *et al.*, 1981
OKI1	Ia antigen framework present on pre-B and B cells, TdT^+ BM cell activated T cells	29/34	IgG2	Janossy *et al.*, 1979 Reinherz *et al.*, 1979
OKT3	medullary thymocytes peripheral T cells	19	IgG2a	Kung *et al.*, 1979
OKT6	cortical thymocytes	49	IgG1	Reinherz *et al.*, 1980

* Reactivity with nonlymphoid or malignant cells is not shown.

Table 2. Reactivity of human fetal lymphocytes with the VIL-A1, BA-2, VIB-C5 and OKI1 MoAbs

	Fetal Tissues								
	Liver			Bone marrow		Spleen		Thymus	
MoAb	8–11* ($n=4$)	12–15 ($n=4$)	16–20 ($n=5$)	12–15 ($n=5$)	16–20 ($n=5$)	12–15 ($n=8$)	16–20 ($n=9$)	11–15 ($n=5$)	16–20 ($n=5$)
VIL-A1	58·0±9·2†	52·8±4·8	59·0±13·0	81·5±4·4	76·8±5·3	27·3±5·6	17·7±5·1	0	0
BA-2	31·3±8·3	35·2±6·1	39·2±3·0	55·0±8·6	60·5±4·8	39·0±9·2	28·3±6·3	8·5±0·7	8·0±4·0
VIB-C5	57·0±9·9	67·6±10·0	68·6±7·3	95·0±2·5	94·0±2·1	50·7±13·8	58·3±6·6	0	0
OKI1	19·2±15·4	21·4±12·7	55·1±12·2	59·4±19·3	71·8±8·8	41·8±16·0	47·2±7·0	3·2±1·9	1·2±0·8

* Gestational age in weeks

† Mean ± s.d. expressed as % of positive lymphocytes

cell suspensions reacted with the OKT10, VIB-C5, VIL-A1 and OKI1 MoAbs (Table 3), whereas fewer cells reacted with the BA-2 MoAb. These numbers were not related to gestational age. Results were similar for the TdT^+ cells except that fewer TdT^+ cells were Ia-antigen positive.

At all ages most of the cells reacting with the MoAbs were '$c\mu^+$' (Table 4) and the proportion increased with age. Fewer cells contained TdT and their relative number decreased with fetal age.

A few lymphoid cells were positive for both '$c\mu$' and TdT, especially in the younger fetuses. We never found TdT^+ cells in fetal liver and BM lymphocytes which were sIg^+ ($n=8$, data not shown),

Table 3. Reaction of TdT+ cells and 'cμ+'* cells in human fetal liver and bone marrow with monoclonal antibodies

	Liver						Bone marrow			
	8–11† (n=4)		12–15 (n=4)		16–20 (n=4)		12–15 (n=5)		16–20 (n=6)	
MoAb	TdT+ cells	'cμ+' cells	TdT+ cells	'cμ+' cells	TdT+ cells	'cμ+' cells	TdT+ cells	'cμ+' cells	TdT+ cells	'cμ+' cells
OKT10	92·5±3·5‡	92·8±6·0	90·5±1·7	86·8±2·1	91·5±5·3	89·3±3·8	92·0±6·8	92·9±4·7	87·0±7·2	90·6±5·7
VIL-A1	83·0±1·4	91·7±4·0	77·5±7·0	85·0±5·3	82·0±11·6	81·3±4·7	79·8±7·9	86·3±5·6	74·0±5·6	89·3±3·2
BA-2	73·0±0	75·3±4·9	68·8±10·2	78·0±3·2	69·3±4·5	73·0±5·3	56·0±7·2	64·2±8·1	34·8±10·3	75·2±6·3
VIB-C5	85·3±6·8	87·0±2·8	81·8±8·0	92·3±7·0	89·5±12·5	90·2±5·8	94·0±4·8	97·0±2·7	88·8±7·2	95·0±2·0
OKI1	63·5±6·4	83·0±7·0	60·3±12·8	89·5±1·3	51·8±10·4	91·3±1·3	37·5±9·7	94·1±2·8	17·2±3·3	91·8±3·7

* 'cμ+' cells comprise sμ+ B plus sμ⁻/cμ+ pre-B cells

† Gestational age in weeks

‡ Mean±s.d. expressed as percentage of TdT+ cells or 'cμ+' cells

Table 4. Fractions of TdT$^+$ cells and 'cμ^+'* cells within the VIL-A1$^+$, BA-2$^+$ or VIB-C5$^+$ lymphoid population in human fetal liver and bone marrow

	Liver			Bone Marrow	
	8–11† ($n=4$)	12–15 ($n=4$)	16–20 ($n=5$)	12–15 ($n=5$)	16–20 ($n=6$)
Within VIL-A1$^+$ cells					
TdT$^+$	29·0±12·8‡	27·0±9·6	13·8±7·8	32·3±12·7	20·0±5·6
'cμ^+'	51·0±7·0	80·7±5·1	80·3±3·8	69·7±7·5	80·3±1·2
Within BA-2$^+$ cells					
TdT$^+$	29·5±3·5	18·3±3·3	4·8±1·5	29·5±7·8	12·4±2·7
'cμ^+'	66·3±3·5	79·5±1·3	86·0±6·1	73·8±3·3	80·2±4·8
Within VIB-C5$^+$ cells					
TdT$^+$	34·3±6·4	20·8±4·6	9·0±3·2	32·8±2·2	15·3±3·4
'cμ^+'	74·5±4·9	82·0±4·3	88·4±6·3	62·8±8·3	75·6±4·0

* 'cμ^+' cells comprise sμ^+ B cells plus sμ^-/cμ^+ pre-B cells
† Gestational age in weeks
‡ Mean ± SD expressed as percentage of MoAb$^+$ cells

so we presume that the 'cμ^+' TdT$^+$ cells contain intracytoplasmic μ chains and therefore are pre-B cells. The percentage of TdT$^+$ cells which contained cμ were: 8·0±1·4, 5·0±1·4 and 3·0±1·4 in 8- to 11-, 12- to 15- and 16- to 20-week-old-livers, respectively.

Fetal BM

Almost all BM lymphocytes (12- to 20-weeks g.a.) reacted with the VIB-C5 MoAb (Table 2) at all ages, and most also bound to the VIL-A1 MoAb and about 60% to BA-2. The relative number of Ia$^+$ lymphocytes increased with age: from ±60% in 12- to 15-week-old-BM to ±72% in the older age group.

BM 'cμ^+' cells reacted with the MoAbs similarly to liver 'cμ^+' cells (Table 3). About 90% of BM 'cμ^+' cells stained with the VIB-C5, VIL-A1, OKT10 and OKI1 reagents and about 70% was BA-2$^+$. Fewer BM TdT$^+$ cells than liver TdT$^+$ cells were BA-2$^+$ and Ia$^+$, and the proportion fell during gestation.

As in the liver, most of the VIL-A1$^+$, VIB-C5$^+$ and BA-2$^+$ cells were 'cμ^+' (Table 4) and the proportion increased with gestational age, whereas the fraction which was TdT$^+$ decreased. Combined staining for 'cμ' and TdT detected less than 3% of TdT$^+$ cells in BM which contained cμ, irrespective of gestational age.

The proportion of TdT$^+$ cells within the lymphoid cell population decreased from 36% at 12 weeks to 11% at 20 weeks and that of 'cμ^+' cells increased from 56 to 70%. The proportion of nucleated BM cells which were TdT$^+$ cells remained fairly constant i.e. 4–6%.

Fetal spleen

About 50% of spleen lymphocytes reacted with the VIB-C5 MoAb and about 45% were Ia$^+$ (Table 2). Before gestational week 16, more cells were BA-2$^+$ (±40%) and VIL-A1$^+$ (±27%) than after week 16 (±28% & 17%, respectively).

Throughout the age period investigated >80% of sIg$^+$ spleen lymphocytes bound the VIB-C5 and Ia reagents (Fig. 1). Moreover, at 12 weeks, >80% of sIg$^+$B lymphocytes was also BA-2$^+$ and VIL-A1$^+$. During gestation, the relative number of B lymphocytes which reacted with either the BA-2 or VIL-A1 MoAb decreased to 53% and 29%, respectively, at 20 weeks.

TdT$^+$ cells were not found in the fetal spleen (12- to 20-weeks, $n=5$).

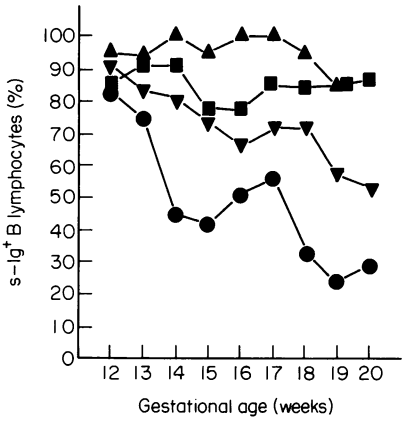

Fig. 1. Percentage of sIg$^+$ lymphocytes in the human fetal spleen reacting with the monoclonal antibodies VIB-C5 (▲), OKI1 (■), BA-2 (▼) and VIL-A1 (●).

Fetal thymus

Between 11 and 20 weeks of gestation, no thymocytes which reacted with the VIB-C5 or VIL-A1 MoAb were seen (Table 2); < 10% were BA-2$^+$ and about 3% were Ia antigen positive.

In one 12-week- and one 14-week-old-fetus 4% of thymocytes were TdT$^+$. In the four fetuses between 17- to 20-week-old 24–34% were TdT$^+$. These TdT$^+$ cells did not react with the VIL-A1 MoAb and less than 1% were VIB-C5$^+$; > 80% were OKT6$^+$ and 3–16% were BA-2$^+$ and/or T3$^+$.

DISCUSSION

Greater than 70% of the lymphoid cells in the human fetal liver (up to 16-weeks of gestation) and in BM (12- to 20-weeks of gestation) displayed neither T-cell-specific antigens, as defined by OKT MoAbs, nor sIg (Asma *et al.*, 1983). Presumably these cells are heterogeneous and include lymphoid stem cells, TdT$^+$ cells (Bodger *et al.*, 1983; Bonati *et al.*, 1983) and pre-B cells (Gathings, Lawton & Cooper, 1977; Asma *et al.*, 1984). Our aim was to characterize further this sIg$^-$/T-antigen$^-$ lymphoid population, by their reactivity with MoAbs, known to react mainly with immature lymphoid cells (BA-2, VIL-A1, OKT10) and cells of B lineage (VIB-C5, OKI1). We also studied the proportion of TdT$^+$ cells and pre-B cells.

Many lymphoid cells in the liver (9- to 20-weeks) and most lymphoid cells in the BM (12- to 20-weeks) bound the CALLA (VIL-A1) and VIB-C5 reagents, and many bound the BA-2 and OKI1 MoAbs, especially in the BM. These MoAbs thus stained most sIg$^-$/T-antigen$^-$ lymphoid cells in BM. In the liver it can be calculated that many sIg$^-$/T-antigen$^-$ lymphoid cells must react with VIL-A1 and VIB-C5, and probably also with BA-2 and OKI1 MoAbs.

TdT$^+$ lymphoid cells were found in the liver, BM and thymus, but not in the spleen. In the BM we found, and in the liver we deduced from the combined data an age-related decrease in the proportion of TdT$^+$ cells within the lymphoid population, as reported by Bodger *et al.* (1983). In BM they remained an almost constant proportion of nucleated cells, due to the increase in the relative number of lymphocytes. In the liver the fraction of lymphoid cells remained fairly constant during the fetal age period investigated (Asma *et al.*, 1983). The decrease in TdT$^+$ cells in the liver seems absolute. These data are similar to our previous observations on large pre-B cells in fetal liver and BM (Asma *et al.*, 1984), and confirm our earlier postulation that the role of fetal liver in the generation of precursor lymphoid cells is of limited duration, as reported by Kamps and Cooper (1982). Most of the TdT$^+$ cells in liver and BM bound to the OKT10, VIL-A1 and VIB-C5 MoAbs. This was also the case for the 'cμ^+' cells, most of which equally displayed the BA-2 and OKI1 determinants. The latter two MoAbs reacted to fewer TdT$^+$ cells, especially in the BM, where an

age-related decrease in the number of reactive TdT^+ cells was observed. Like Bonati *et al.* (1984) we found no TdT^+ cells in liver and BM which reacted with T cell markers like OKT6 and WT-1 (unpublished observations). It seems therefore unlikely that the TdT^+, Ia^- and/or BA-2^- cells are precursors of the T lineage. In rodents, TdT^+ precursors of the T cell lineage are found in BM (Silverstone *et al.*, 1976) but this has not been demonstrated in man. That many of the TdT^+ cells in human fetal liver and BM are probably precursors of the B lineage is indicated by the corresponding phenotype of TdT^+ cells and '$c\mu^+$' cells in these organs (Table 3) and by the following findings: except for the OKT10 MoAb, and to a lesser extent the BA-2 MoAb, the MoAbs used in this study did, in our hands, not react with thymocytes or $T3^+$ splenocytes (data not shown); the reactivity with BA-2, VIL-A1 and VIB-C5 is almost exclusively confined to the TdT^+ and '$c\mu^+$' cells in liver and BM; in fetal liver and BM, some cells contained both TdT and μ, defining them as pre-B cells. The latter was also found in postnatal BM by Janossy *et al.* (1979)

In human fetal thymuses of 12 and 14 weeks gestational age we found few TdT^+ cells as did Janossy et al. (1980). The relative numbers of these cells increased with age to about 30% of thymocytes. Bodger *et al.* (1983), however, found no TdT^+ cells in human fetal thymus before the week 21 of gestation, perhaps due to differences in technique. TdT was not detected in fetal thymocytes when preparations were kept overnight at room temperature, in contrast to BM and liver cells. Moreover, especially in the younger fetuses, the fluorescence of TdT^+ thymocytes was extremely weak.

TdT^+ thymocytes were different from TdT^+ cells in liver and BM. Most belong to the T cell lineage, as evidenced by their reactivity with the OKT6 MoAb. They did virtually not react with the VIL-A1 and VIB-C5 MoAbs. Similar differences have been reported between TdT^+ cells in postnatal BM and thymus (Janossy *et al.*, 1979). We therefore postulate that in man two different TdT^+ cell populations may exist: one in fetal liver and BM giving rise to cells of the B lineage and the other in the thymus belonging to the T lineage.

In liver and BM the '$c\mu^+$' population consists of pre-B and B cells. Despite the changing proportions of pre-B and B cells in the '$c\mu^+$' cells during gestation (e.g. the B cell fraction increases from $< 10\%$ at 8-weeks to $> 80\%$ at 20-weeks in the liver and from about 20% at 12-weeks to $> 40\%$ at 20-weeks in fetal BM (Asma *et al.*, 1984)), the percentage of '$c\mu^+$' cells which reacted with the various MoAbs remained the same during the fetal age period investigated. In view of the relatively high, i.e. $> 70\%$, proportion of '$c\mu^+$' cells staining with OKT10, VIL-A1 and BA-2 MoAbs, markers for immature lymphoid cells, this indicates that many sIg^+ B cells have the same immature features as the $sIg^-/c\mu^+$ pre-B cells in these organs.

In the spleen also many sIg^+ B cells reacted with the OKT10, VIL-A1 and BA-2 MoAbs. The number of sIg^+ B cells in the spleen which displayed VIL-A1 and BA-2 antigens decreased with progressing gestational age. B cells in the fetal spleen are therefore also more immature than those in blood and BM after birth, which are $CALLA^-$ and BA-2^- (Greaves *et al.*, 1980; Kersey *et al.*, 1981; Foon, Schroff & Gale, 1982). The decrease of VIL-$A1^+$/BA-2^+ B cells seen in the spleen of older fetuses was not observed in liver and BM, indicating that B cells in the latter organs are at all ages more immature than those in the spleen. Taken together with the finding of immature $T3^+$ cells in the fetal spleen (Asma *et al.*, 1983) this organ must be considered as a milieu for further maturation of lymphoid cells already expressing sIg or T3, during human fetal life.

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