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Differential Expression of Leukocyte Common Antigen in Human Fetal Lymphoid Organs

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To investigate the differential expression of various types of leukocyte common antigen (LCA) isoforms during development, we analyzed human fetal lymphoid organs, including the thymus, liver, spleen, and bone marrow from 14 weeks to 29 weeks of gestational age by immunohistochemical and flow cytometric methods. In fetal thymus, over 90% of thymocytes throughout the entire fetal life expressed CD45RO and CD45RB, while CD45RA was expressed only in less than 5% of thymocytes. This expression pattern of LCA isoforms was established by a gestational age of 14 weeks or earlier, and persisted throughout the fetal period. The tissue distribution was different from each isoform ; CD45RO-positive thymocytes were found in both the cortex and medulla at the 14th week with low intensity, but was localized in the cortex with increasing fetal age. CD45RB-positive thymocytes distributed mainly in the medulla from early gestational age. Among extrathymic lymphoid organs, a small portion of lymphoid cells expressing leukocyte common antigens appeared first in the liver at 10-12 weeks of gestational age and was followed by a small number in the spleen and bone marrow by 13-15 weeks. All lymphoid cells in these extrathymic lymphoid organs at this stage were $CD19^+$ B cells. The number of these $CD19^+$cells increased abruptly during the early period of mid-gestational age. The pattern of tissue distribution of each LCA isoform in the fetal liver and spleen correlated well with the patterns of quantitative analysis by flow cytometry. In summary we found that different LCA isoforms expressed in cell-type-specific pattern and showed different tissue distribution during the period of fetal development, and that LCA was the earliest antigen expressed by lymphocytes in the thymus and extrathymic lymphoid organs in our series.

Key Words : *Leukocyte common antigen, Fetal thymus, Spleen, Liver, Bone marrow, Immunohistochemical study, Flow cytometry.*

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INTRODUCTION

Leukocyte common antigen (LCA/T200/CD45) is a family of cell surface glycoproteins found in nucleated cells of the hematopoietic lineage (Coffman and Weissman 1981 ; Dalchau and Fabre,

1980 and 1981 ; Osmary et al., 1980). In human, at least four LCA isoforms differing in molecular weight have been identified : they are 180-, 190-, 204-, and 220 kilodalton(kD) isoforms(Coffman and Weissman 1981 ; Dalchau and Fabre, 1880 and 1981 ; Osmary et al., 1980). Structurally, LCA has a well-conserved cytoplasmic domain that is known to have tyrosine phosphatase activity (Charbonneau et al., 1988 ; Clark and Ledbetter, 1989) as well as an extracellular domain that is heterogeneous in peptide sequence(Lai et al., 1991) and carbohydrate composition (Pulido et al., 1988 ; Lai et al., 1991). Molecular studies have shown that alternative splicing of mRNA generates the heterogeneity of LCA (Streuli et al., 1987 ; Thomas 1989). The result is that LCA isoforms differ in the expression of the peptide domains encoded by the three alternatives exon 4, 5, and 6. At the fourth Leukocyte Antigen Workshop, it was decided to distinguish these isoforms as CD45RA, CD45RB and CD45R0 antibodies. Antibodies recognizing common determinants of LCA (anti-CD45 or pan-leukocyte) are reactive with all four LCA isoforms. Antibodies recognizing a determinant on exon 7 that can only be detected in the absence of expression of all three variable exons are designated CD45R0. The pattern of these isoforms is controlled in a cell specific fashion. The B lymphocytes use all three exons resulting in the largest form, 220 kD (Coffman 1981 ; Dalchau 1981). In thymocytes, splicing occurs between exon 3 and 7 and thymocytes express the lowest molecular weight form of 180 kD (Engleman et al., 1981).

Several studies have reported the tissue distribution of CD45R antigens (Pulido et al., 1988 ; Lai et al., 1991). However, little is known about the LCA expression in fetal lymphoid organs. In this study, we analyzed three types of LCA antibodies that are reactive with the protein determinant of LCA, using fetal thymus and extrathymic lymphoid organs of various gestational age.

MATERIALS AND METHODS

Fetal and neonatal tissues

Twenty human fetuses from 10—36 weeks of gestation (gestational week, g.w.) were obtained by interruption of pregnancy on non-medical grounds. Permission to use these specimens for the present investigation was given by their parents. The heel-to-toe measurement of Chi et al. (1992) was used to standardize gestational age(Table 1). Postnatal thymi of 2 days to 9 years of age were obtained at open heart surgery.

Small fresh tissue blocks of fetal thymus, liver, and spleen were sliced 2-3 mm thick and quickly frozen with cold isopentane and stored in liquid nitrogen at −70℃

Preparation of cell suspension

Thymus cell suspensions were prepared by gentle teasing on a fine stainless steel mesh, passed through nylon mesh to remove cell debris, and washed twice with RPMI 1640 medium.

The fetal liver and spleen were minced in RPMI 1640 with 10% of fetal bovine serum (FBS), separated into a mononuclear fraction by Ficoll-Hypaque centrifugation (Sigmadiagnostics, St Louis, density : 1.077 g/ml) and interface cells were washed twice in RPM I1640 with 10% FBS.

The fetal bone marrow cells were collected by flushing both femoral shafts with RPMI 1640. Col-

Table 1. Age distribution of tested fetal lymphoid organs used in this study

Age(Week)	No. of Lymphoid Organs(No. of Morphologic Examination)			
	Thymus	Liver	Spleen	Bone Marrow
10-12	0	1	1(1)	0
13-14	1(1)	1(1)	1(1)	1
15-16	4(2)	3(1)	1(1)	4
17-20	5(3)	3(2)	4(2)	3
21-24	9(4)	6(3)	7(3)	4
25-28	0	0	0	0
29-32	1(1)	1(1)	1(1)	1
33-36	0	1	1	1
Total	20(11)	16(8)	16(9)	14

lected cells were filtered through a nylon filter to remove cell debris. Mononuclear cell fraction was also prepared by Ficoll-Hypaque centrifugation. Peripheral blood mononuclear cells were isolated from healthy adults by Ficoll-Hypaque centrifugation for the control study.

Antibodies

FITC-conjugated CD45RA(L48) (Coffman abd Weissman 1981) and UCHL-1(Norton et al,. 1986; Akbar et al., 1989) were purchased from Becton Dickinson (Mountain View, CA) and DAKO. Unconjugated CD45RB(PD7/26) (Warnke et al., 1983; Kurtin and Pinkus, 1985) was purchased from Dakopatt. FITC-conjugated rabbit anti-mouse IgG (DAKO) was used as a secondary antibody. For double immunofluorescence staining, phycoerythrin-conjugated CD3 (DAKO) and CD19 (DAKO) were used. For triple staining, peridinin chlorophyl protein-(Per-CP)-conjugated CD3 (DAKO) were used.

Immunohistochemical study

Four micrometer snap frozen sections, after immediate fixation with cold acetone, were stained with PD7/26 and UCHL-1 by immunoperoxidase method. L48 was stained by direct immunofluorescence method. The same sections were stained by hematoxylin-eosin.

Immunofluorescence assay

Fresh cell suspensions containing up to 1×10^6 cells (100 ul) were mixed with 10 ul of FITC-conjugated UCHL-1 or L48 and incubated in the dark at 4℃ for 10 minutes. Then they were washed with PBS and 2% bovine serum albumin. For the indirect staining, cells were stained with 4 ml FITC-conjugated rabbit anti-mouse immunoglobulin after incubation of PD7/26. Stained cells were washed and analyzed on a fluorescence-activated cell sorter (FACScan, BecktonDickinson, PaloAlto, CA). Fluorochrome-conjugated isotype-matched control antibodies were used in all immunofluorescence assays to establish the specificity of antibody binding. Fixation was done by mixing the sedimented cells with 1 ml 1% paraformaldehyde in 0.15 M NaCl. After staining procedure cells were stored at 4℃ in the dark and analyzed.

RESULTS

Tissue Distribution stained by Immunoperoxidase method

The results of the distribution studies with three types of CD45R reagents are demonstrated in Figure 1, 2, and 3. A detailed analysis of the staining results in thymus, liver, and spleen is given below.

Thymus. As early as 14 g.w., the formation of the thymic lobular architecture was already similar to that of the infant thymus (Fig. 1). At 14 g.w., the majority of fetal thymocytes were UCHL-1(CD45R0) and PD7/26(CD45RB) positive, indicating the presence of either CD45R0 or CD45RB on the cell surface. There was no detectable difference in the intensity of staining between medullary and cortical thymocytes. However, compared to postnatal thymus, the intensity of staining was generally weaker. CD45RA-positive thymocytes as identified by L48 were not detected. Differences in staining intensity of both antibodies between the cortex and medulla became progressively prominent in the succeeding weeks and established at 21 g.w. Unlike the CD45R0 antibody that stained strongly in thymic cortex, the CD45RB antibody stained cells in the cortex were less strong than those in the medulla. L48 (CD45RA) was not reactive to the fetal thymocytes. This finding indicates that during the midgestational period, differences in LCA composition in the thymocyte surface are established.

Spleen. At 12 g.w., the spleen (1 mm in diameter) consisted of immature mesenchymal cells and no observable hematopoietic cells. Lymphocytes seldom appeared at 14 g.w. and were stained by PD7/26 (CD45RB). Small accumulations of PD7/26-positive lymphocytes around arterioles were recognized at 15 g.w. (Fig. 2). A few L48 (CD45RA)-positive cells were also found in the red pulps. In succeeding weeks, PD7/26 was restricted in lymphocytes forming the primary follicles which were formed at 22 g.w.. In white pulps, PD7/26 reactivity was diffusely positive with no zonal difference. In contrast, UCHL-1(CD45R0) reactivity was found at 17 g.w., and was scattered throughout the spleen without recognizable localization until late gestational age. The fetal white pulps composed of periarteriolar lymphatic sheath(PLAS) and primary follicles were established at 26 g.w.. UCHL-1 and L48 reactivities persisted in red pulp areas until the late gestational period without localization to white pulps.

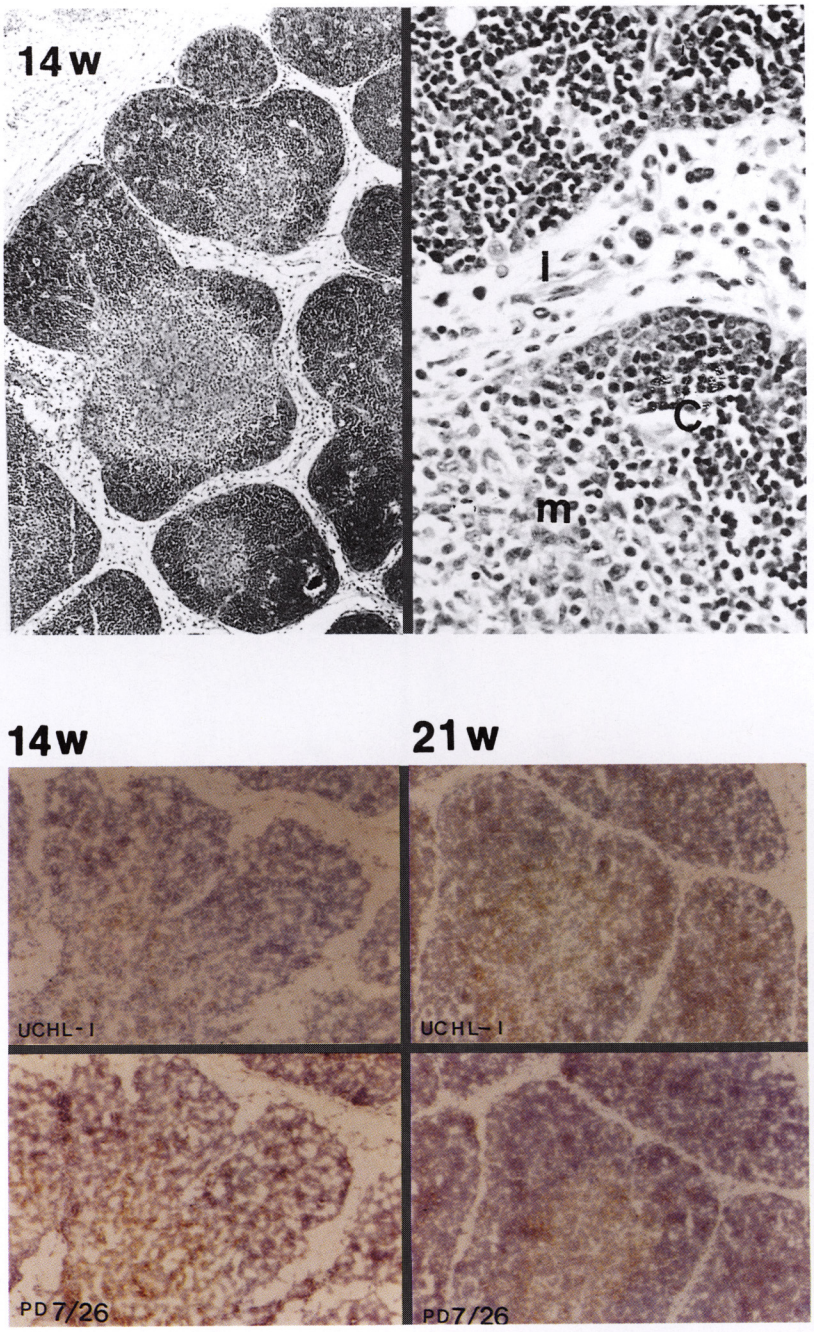

Fig. 1. The formation of thymic lobular architecture similar to that of the mature thymus is apparent already at 14 gestational weeks(H&E. X160). The lymphoid stem cells are scattered in broad interlobular septa infiltrated into the cortex or directly into the medulla(H&E.X800). M : medulla, C : cortex, I : interstitium Different tissue distribution of LCA isoforms in the fetal thymus at 14 and 21 weeks of gestation. The medulla is stained more intensely by PD7/26 and UCHL-1 at the early fetal stage. At the late stage, the distribution pattern of each isoform is similar to that of postnatal thymus, although the staining intensity is low(immunohistochemical staining,X330).

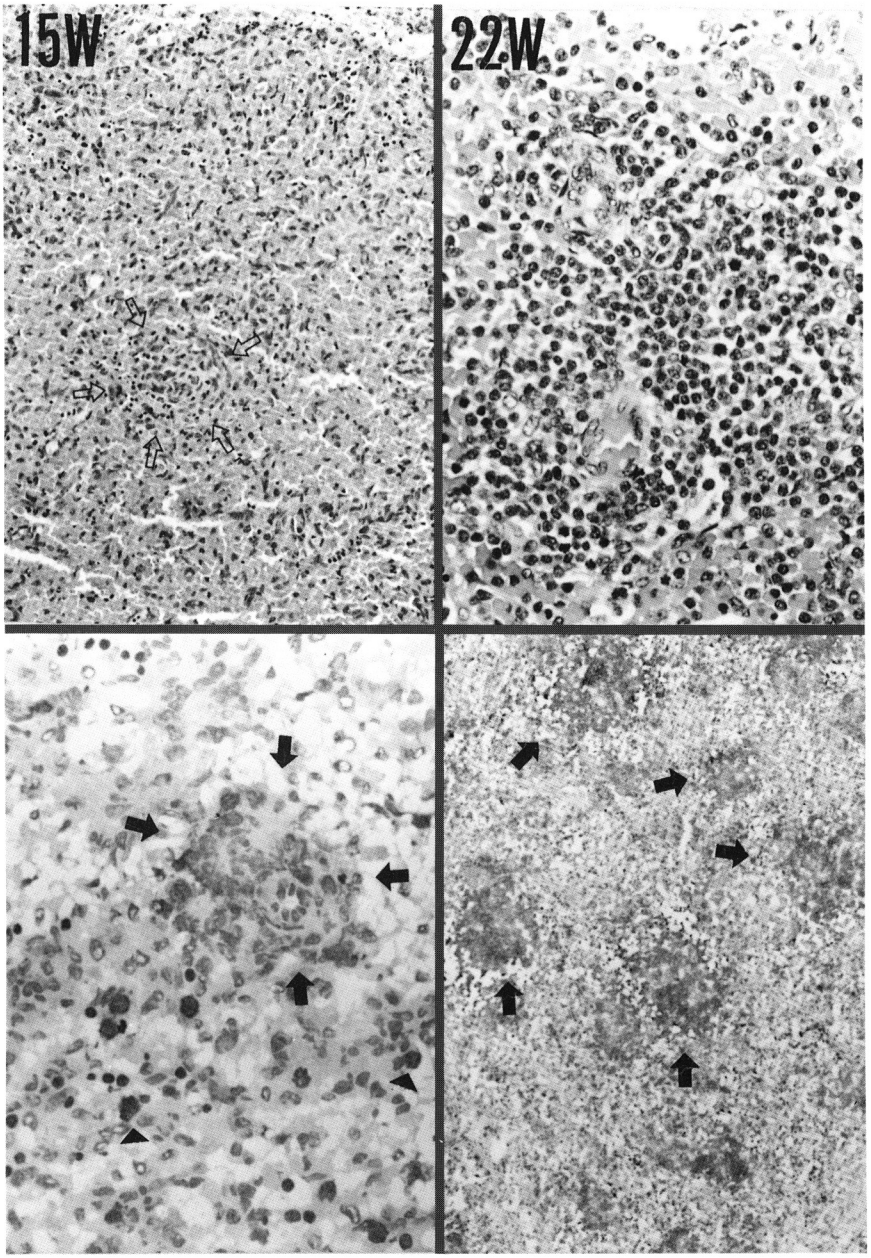

Fig. 2. Above : The formation of white pulps in the developing spleen. The periarteriolar lymphatic sheath(open arrow) appeared first by 15 weeks. Mature white pulp was formed by 22 weeks of gestation(H&E.×200 and×600). Below : Tissue distribution of PD7/26 in the fetal spleen at early(15 weeks) and late(22 weeks) gestational age. The periarteriolar and white pulp lymphoid cells express PD7/26(arrows). Positive cells are seldom found in red pulp(arrowhead) (immunohistochemical staining,×1000 and×160)

Liver. The hepatic hematopoiesis already appeared diffusely and extravascularly among the sheets of hepatocytes at 14 g.w. It was largely erythroid cells, although immature cells were rarely admixed. In the

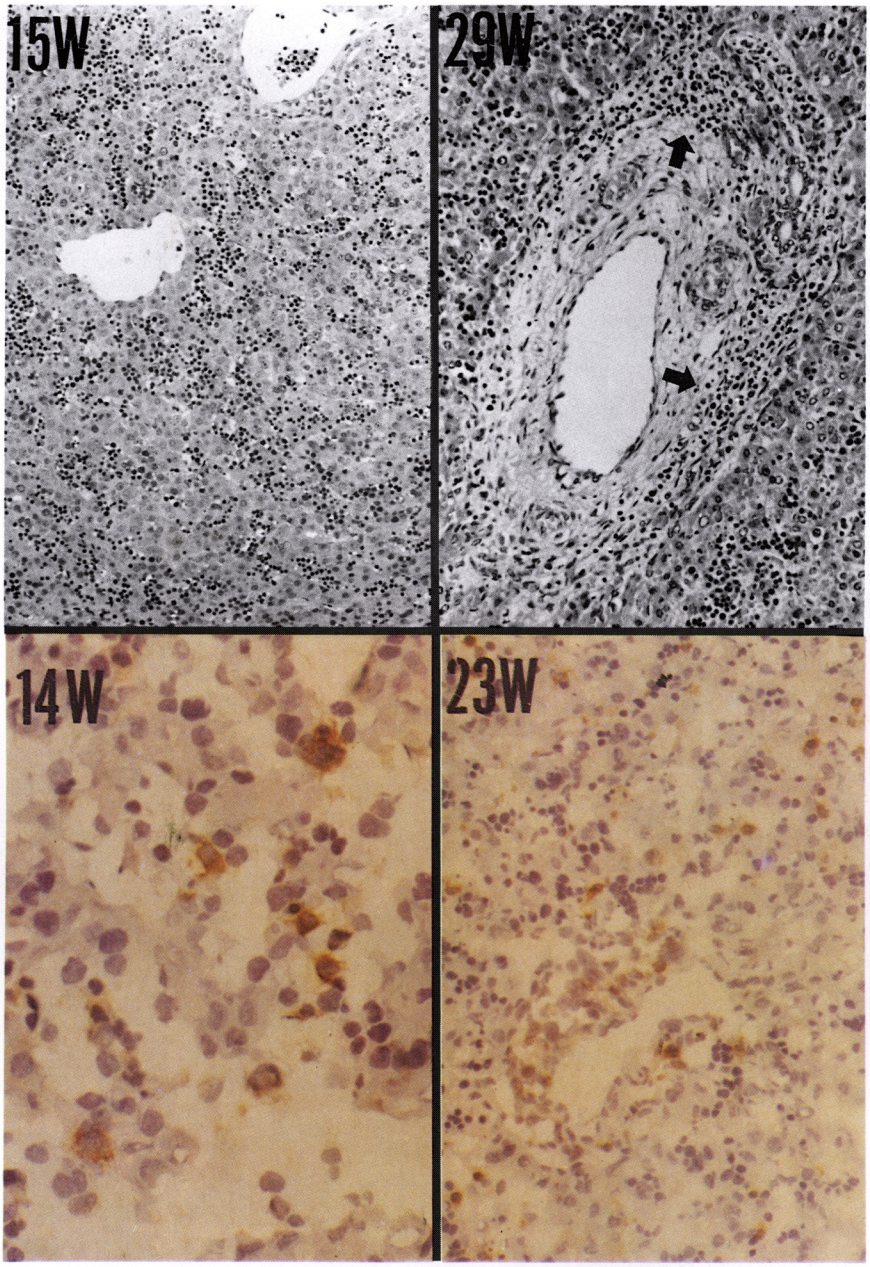

Fig. 3. The hepatic hematopoiesis is seen diffusely among the sheets of hepatocytes at 15 weeks of gestation(H&E.×300). Immature and mature hematopoietic cells(arrow) are heavily infiltrated in the portal spaces at 29 g.w(H&E.×400). PD7/26-positive cells are scattered throughout the hepatic parenchyma, mainly among sinusoidal hematopoietic cells at 14 weeks. At 23 g.w., the number and intensity of positive cells increased(immunohistochemical staining,×1000 and ×500).

connective tissue surrounding portal spaces, various types of hematopoietic cells were found. Among sinusoidal hematopoietic stem cells, PD7/26 (CD4-5RB)-positive cells were found at 14 g.w. and showed both cytoplasmic and nuclear membrane staining patterns (Fig. 3). Immature dendritic cells

were also positive for PD7/26 and were found among the hepatic cell cords. The staining intensity of the cells in the portal space was stronger than those in hepatic lobule. As the liver grows, the number of positive cells and the staining intensity of PD7/26 increased until 29 g.w. when the hematopoietic activity subsided. In contrast, UCHL-1-positive cells appeared first at 15 g.w. and were limited in the portal areas. Until the late g.w., UCHL-1 reactivity was not detected in the hepatic parenchyma. The L48-positive cells were not found in the fetal liver throughout the gestational period.

Differential expression analyzed by flow cytometry

Thymus. Over 90% of total thymocytes expressed UCHL-1 (CD45RO) and PD7/26 (CD45RB) while L48 (CD45RA) was expressed in less than 5% of thymocytes (Table 2). This expression pattern of LCA isoforms was established at 14 g.w.,which were the earliest samples examined and persisted throughout the fetal period.

Spleen. At 15-16 g.w. lymphoid cells of CD19-positive B cells and CD3-positive T cells expressed UCHL-1 (4.9%) and L48 (21.0%). Between 17-20 g.w., lymphoid cells expressing each isoform increased sharply. The majority of lymphoid cells expressed PD7/26 after mid-gestational age. The B lymphocytes and CD19-positive cells expressed mainly L48, and only a small population of them expressed UCHL-1 as well. In contrast, CD3-positive T cells, which were less than the B cell population, expressed UCHL-1 and PD7/26 although a small population was also positive for L48 (Table 3).

Liver. A few CD19-positive cells were found in the liver between 10-12 g.w.. Although these cells were not positive for LCA antibodies, a few cells were also positive for three isoforms of LCA antigens. In contrast, CD3-positive T cells first appeared at 13-14 g.w.. Lymphoid cells of fetal liver expressed three isoforms of LCA in a small population (less than 10%) until 21-24 g.w.. Most negative cells for all kinds of markers were normoblasts which were

Table 2. Mean percentage of fetal thymocytes positive for LCA isoforms

Age(weeks)	No. of cases	Positive cells(%) of LCA Isoforms		
		CD45RO (UCH L-1)	CD45RB (PD7/26)	CD45RA (L48)
15-16	4	93.5	91.0	5.9
17-18	3	96.6	97.3	6.9
19-20	2	93.4	98.3	4.2
21-22	6	95.0	97.2	2.0
23-24	3	92.2	97.2	1.8
29	1	92.9	98.7	2.5
Postnatal*	12	89.2	94.4	6.8

*: Postnatal thymi were 2 days to 9 years of age.

Table 3. Mean percentage of splenic lymphoid cells positive for LCA isoforms and T and B cell surface antigens

Age (weeks)	No. of cases	Positive cells(%) of LCA isoforms									$CD19^+$	$CD3^+$
		CD45RO (UCHL-1)			CD45RB (PD7/26)			CD45RA (L48)				
		Total	CD19	CD3	Total	CD19	CD3	Total	CD19	CD3		
13-14	1	0.0	0.0	0.0	—	—	—	0.0	0.0	0.0	0.0	0.0
15-16	1	4.9	0.7	0.8	—	—	—	21.0	2.8	1.5	2.6	2.9
17-20	3[a]	14.0	3.1	2.4	59.6	18.3	10.9	33.6	24.1	2.8	24.4	12.8
21-24	7[b]	12.3	8.4	7.8	88.3	—	—	50.8	38.2	6.1	42.6	23.0
25-32	1	7.5	—	—	87.4	—	—	64.7	—	—	—	—
33-36	1	5.8	—	—	—	—	—	35.9	12.7	13.7	16.1	31.0

a : Two out of three cases were examined by triple staining.
b : One out of seven cases was examined by triple staining.
— : not done.

Table 4. Mean percentage of hepatic lymphoid cells positive for LCA isoforms and T and B cell surface antigens

Age (weeks)	No. of cases	Positive cells(%) of LCA isoforms									$CD19^+$	$CD3^+$
		CD45RO(UCHL-1)			CD45RB(PD7/26)			CD45RA(L48)				
		Total	CD19	CD3	Total	CD19	CD3	Total	CD19	CD3		
10-12	1	0.4	0.0	0.0	0.5	0.0	0.0	0.2	0.0	0.0	0.9	0.0
13-14	1	2.7	0.8	0.0	0.1	0.0	0.0	1.6	0.8	0.0	2.6	0.4
15-16	3	3.4	1.1	0.2	0.3	0.3	0.1	3.1	1.6	0.0	3.0	1.6
17-20	3	7.5	0.7	0.3	4.3	0.8	0.2	5.1	2.8	0.0	4.2	0.5
21-24	6*	1.5	1.4	0.3	17.4	5.6	0.3	9.0	4.4	0.2	10.5	1.2
25-32	1	3.1	—	—	—	—	—	16.7	—	—	—	—
33-36	1	—	—	—	15.5	—	—	35.4	—	—	18.2	10.0

*: One(22 weeks g.w) out of six was examined by triple staining.
— : not done.

Table 5. Mean percentage of bone marrow lymphoid cells positive for LCA isoforms and T and B cell surface antigens

Age (weeks)	No. of cases	Positive cells(%) of LCA isoforms									$CD19^+$	$CD3^+$
		CD45RO (UCHL-1)			CD45RB (PD7/26)			CD45RA (L48)				
		Total	CD19	CD3	Total	CD19	CD3	Total	CD19	CD3		
13-14	1	0.2	0.0	0.0	0.3	—	—	0.0	0.0	0.0	0.0	0.0
15-16	4	11.5	1.1	0.2	5.9	4.4	0.1	14.4	10.8	0.2	23.9	2.0
17-20	3	10.0	0.7	0.0	30.9	8.6	0.4	17.9	7.7	0.1	33.9	0.4
21-24	4*	5.3	0.6	0.0	30.8	25.4	0.0	21.6	6.0	0.0	63.5	0.8
25-32	1	3.4	—	—	—	—	—	19.4	—	—	—	—
33-36	1	0.9	—	—	85.0	—	—	48.0	42.0	0.3	51.4	1.5

*: One(22 week g.w.) out of four was examined by triple staining
— : not done

confirmed by light microscopic examination of cell suspension (Table 4).

Bone Marrow. Between 13-14 g.w., a few lymphoid cells were positive for UCHL-1 and PD7/26. In contrast, CD3- and CD19-positive cells first appeared at 15 g.w.. Although CD19-positive B cells were observed in high percentage(28%) at 15 g.w., CD-3-positive T cells were seldom found throughout the fetal life. After 16 g.w., the LCA-positive lymphoid population increased abruptly. The UCHL-1-positive cells were less than 10% throughout fetal life although PD7/26 and L48-positive cells increased gradually up to 85% and 48%, respectively. The CD19-positive B lymphocytes expressed PD7/26 and L48 in a small population (Table 5).

DISCUSSION

Our findings indicate that different LCA isoforms were expressed in cell-type-specific patterns and showed different tissue distributions during the fetal period. LCA was the earliest antigen expressed by the lymphocytes in the thymus and extrathymic lymphoid organs.

In thymus, in agreement with the view that LCA is one of the earliest antigens expressed in all cells of thymocytes with the exception of a small population were UCHL-1(CD45R0) and PD7/26(CD45RB) positive. By immunohistochemical staining, tissue distribution of these two antibodies was reciprocal and established by 21 g.w., ; UCHL-1 was stained in mainly cortical thymocytes, while PD7/26 was in the medulla. These results are similar to other reports in which UCHL-1 was stained in 90% of cortical thymocytes and in about 50% of medullary thymocytes after 20 g.w. (Norton et al., 1986). However, at early gestational age, tissue distribution was not distinct and showed low staining intensity in our series. These findings indicate that LCA seems to be expressed fairly early in T cell ontogeny, and that expression intensity increases progressively as the thymocytes mature. It has been suggested that

LCA on thymocytes plays a role in thymic education(Deans et al., 1989 ; Pilarski and Deans., 1989). In our study, about 5% of thymocytes were positive for L48(CD45RA) after 14 g.w. In another report(Serra et al., 1988), thymocytes included only 4-6% of CD45RA-positive cells, that were comprised mainly of single CD4-positive/CD8-positive, or CD3-positive medullary cells. We also tested another CD45R0 family, SHL-1 (Park et al., 1990) and L3B12 (Wood et al., 1984). They showed a tissue distribution in the thymus different from UCHL-1 (unpublished data). These findings indicate the diversity in the LCA expression pattern even in antibodies recognizing the same molecular weight of antigenic determinant.

Fetal liver earlier than 13 g.w. contained 70-80% erythroid cells, which diminished subsequently along with the increase in blastoid cells and lymphoid cells in reciprocal proportion (Namikawa et al., 1986). In our data, a very small population of lymphoid cells were already positive for UCHL-1 (0.4%), PD7/26 (0.5%), and CD45RA (0.2%) as early as 10-12 g.w, and this increased up to 3%, 15% and 35% as the fetal age progressed. The CD19-positive B cells and CD3-positive T cells were also found at 10-14 g.w. Although UCHL-1 was not expressed in peripheral B lymphocytes (Thomas 19-89), CD19-positive B lymphocytes in fetal liver expressed three isoforms of LCA after 13-14 g.w.. This finding showed the possibility that differential expression of LCA isoforms progressively changes as B lymphocyte maturation progresses because CD19 expressed in all B cell lines including pro-B cells. Our observations showed that portal spaces also provided hematopoietic areas as well as parenchymal sinusoid. Within the gated lymphoid poplulation by FACScan analysis, over 80% of gated cells were negative for all antibodies. These cells were confirmed as normoblasts by light microscopic examination. According to the previous reports (Shah et al.,), a small population of immature normoblasts is positive for pan-LCA antibody.

In the fetal spleen, the homing of T cells to the spleen occurred in fetus over 15 g.w. This finding was similar to a previous report (Namikawa et al., 1988) in which it occurs at 17 g.w.. Most lymphoid cells forming periarteriolar lymphatic sheath and white pulps were stained by PD7/26 in homogeneous pattern. Although L48 was expressed in over half of mononuclear cells by flow cytometry, we failed to find it in tissue.

In bone marrow, the expression pattern of LCA isoforms in the lymphoid population was established at 19 g.w. : It was a stronger expression of CD45RA and a weaker expression of CD45R0. We examined myeloid and monocyte subpopulations from several selected cases of 19 g.w. to 29 g.w.(unpublished data). In contrast to the lymphoid population, the expression pattern of LCA isoforms in these populations showed a reciprocal pattern. These results were very similar to those of adult peripheral blood (Norton et al., 1986).

Present study demonstrates the importance of studying lymphoid cells with both immunohistochemical and flowcytometric methods in order to understand the morphogenesis of each organ, in relation to the development of the immune system. With more types of LCA isoforms together with B and T cell markers, further study should focus on the functional role of LCA in fetal lymphoid cells.

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