Virchows Archiv Pathol Anat (1988) 413:563–571

Virchows Archiv A
Pathological Anatomy and Histopathology

Immuno-architecture of human fetal lymphoid tissues

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Summary. Spleen, thymus and lymph node of human fetuses from the 12th to the 38rd week (spleen from 9 weeks) were investigated in an immunohistological study on B5-fixed paraffin embedded tissues, employing a panel of recently developed monoclonal antibodies, reactive with antigens resistant against fixation and paraffin embedment. The monoclonal antibodies included were MT1, MT2, MB1, MB2, MB3, LN1, LN2, LN3, LeuM1, Leu7, VIE-G4, together with polyclonal antibodies reactive with immunoglobulin heavy and light chains, and with lysozyme and S100-protein. The preservation of morphological detail together with immunoperoxidase staining of cellular subsets, allowed an accurate determination of the ontogenic development of the different cell types in situ, in relation to their micro-environment. The use of paraffin tissue reactive (monoclonal) antibodies gives an extra dimension to the study of fetal lymphoid tissues. This is of particular advantage in studies on very fragile tissues as in early embryonal and fetal ontogeny.

Key words: Fetal lymphoid tissue – Immunohistology – Monoclonal antibodies – Ontogeny – Paraffin embedded tissues

Introduction

The study of the ontogeny of cells and tissues of the human immune system contributes to a better comprehension of the development of human immunity and the contribution of the different lymphoid organs to this. Subsequently, this comprehension may lead to a better understanding of the pathogenesis of immunodeficiency syndromes. In addition to morphological studies of the different cell types of the immune system during development, and their micro-environment (Kelemen et al. 1973; Markgraf et al. 1982), it is now possible to give an accurate determination of cellular subtypes based on specific immunophenotypes, as a result of the availability of monoclonal antibodies (MAb), recognizing a great variety of differentiation and maturation antigens on hemopoietic cells. However, the use of most MAb is limited to fresh cell suspensions or frozen tissue sections. The main disadvantage of frozen tissue sections is the generally poor morphology, whereas in cell suspension studies no information is obtained with respect to the micro-environment of the different cell subsets.

Recently, MAb have been developed that react with antigens that can withstand fixation and paraffin embedding (Epstein et al. 1984; Marder et al. 1985; Poppema et al. 1987; Poppema and Visser 1987; Poppema and Hollema 1988). The use of such MAb in an immunohistochemical staining procedure in combination with appropriate fixatives (like B5) has the advantages of both optimal morphology and specific determination of cell subsets. This is particularly useful in situations where frozen or fresh tissue cannot be obtained easily, or with very fragile tissues. In the present study, we have employed a series of par-MAb in an immunoperoxidase staining procedure on B5-fixed paraffin-embedded fetal spleens, thymuses and lymph nodes.

Materials and methods

Tissues of seven embryos of 5, 5–6, 6, 7, 9, 10 and 11 weeks (fertilization age), and of ten fetuses, varying in age from 12 to

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Table 1. Paraffin-tissue reactive monoclonal antibodies

MAb		M.W.	Specificity	Remarks
LCA (CD45)	[a]	200 kD	Leukocyte common antigen	Not reactive with some centroblastic and lymphoblastic lymphomas
MT1	[b]	190 kD 110 kD 100 kD	T cells, myeloid cells, monocytes, erythroid cells	Not reactive with mature B cells
MT2	[b]	200 kD 190 kD	Mature T and B cells	Not reactive with germinal center cells and immature T cells
MB1	[b]	200 kD 110 kD 100 kD	B cells, 50% T cells	–
MB2	[b]	28 kD	B cells (cytoplasmic)	Also reactive with endothelial and epithelial cells
MB3	[b]	31 kD	MHC class II related (cytoplasmic)	–
LN1	[b]	45–85 kD	Germinal center cells	Also reactive with erythrocytes and epithelial cells
LN2	[b]	31 kD	MHC class II related (cytoplasmic)	–
LN3	[b]	28, 32 kD	MHC class II antigen (membrane)	–
LeuM1 (CD15)	[c]	–	Lacto-N-fucapentaose III	Reactive with granulocytes, Reed-Sternberg-cells, and several types of epithelial cells
Leu7	[c]	–	Large granular lymphocytes; natural killer cells	Reactive with certain neural tissues and epithelial cells
VIE-G4	[d]	41 kD	Glycophorin A	Reactive with cells of erythroid lineage

MAb = Monoclonal Antibody; MW = Molecular Weight (in kiloDalton); MHC = Major histocompatibility complex.
[a]: Dakopatts, Glostrup, Denmark; [b]: Biotest, Dreieich, West-Germany; [c]: Becton-Dickinson, Mountain View (CA), USA; [d]: courtesy of Dr. W. Knapp, Vienna, Austria

38 weeks (=14–40 weeks menstrual age) were studied[1]. In all fetal cases spleen and thymus tissue was available, and in five cases (18, 20, 26, 27, and 36 weeks) also mesenteric lymph nodes. Embryo's were fixed and embedded in total. Age (fertilization age) was carefully determined by crown-rump (CR) length and the use of multiple precise morphological variables (O'Rahilly 1979; Moore 1982; England 1983). Tissues were obtained from intact embryos from medically approved abortions and from fetal autopsies. Cause of death in these cases was immaturity, hyaline membranes, or lung hypoplasia; no further pathology was found.

Tissues were fixed in B5 for 3 h, subsequently rinsed in alcohol for several h and embedded in paraffin. The B5-fixative was prepared as described previously (Jacobsen and Jacobsen 1984; Poppema et al. 1987): 90 ml aqua dest, 6 g mercuric chloride, 2.074 g sodium acetate ($CH_3COONa \cdot 3H_2O$), 10 ml 37% formaldehyde solution, pH 5.7. A series of paraffin-reactive monoclonal antibodies (Liszka et al. 1983; Warnke et al. 1983; Epstein et al. 1984; Marder et al. 1985; Poppema et al. 1987; Poppema and Visser 1987; Poppema and Hollema 1988) summarized in Table 1, were employed in an immunoperoxidase staining procedure on 2 µm paraffin tissue sections, using standard methods (Poppema et al. 1987). In short: sections were deparaffinized, dehydrated, and desublimated, and pretreated for 30 min with 0.3% methanol-H_2O_2 to block endogenous peroxidase reactivity. After a 30 min first step incubation with 100 µl of the respective MAb, sections were incubated for 15 min with 50 µl peroxidase conjugated rabbit anti-mouse immunoglobulin antibodies. Each step was followed by a 5 min wash. Diaminobenzidin tetrahydrochloride (DAB), together with H_2O_2, was used as a substrate, giving a brown reaction product. Sections were counterstained with Mayer's haemalum. Additionally, polyclonal antisera, reactive with lysozyme, S-100 protein, Factor VIII related antigen, immunoglobulin heavy chains gamma, alpha, and mu, and kappa and lambda light chains were used (anti-immunoglobulin antibodies after pronase-pretreatment of the sections). For conventional light-microscopy tissue sections were stained with haematoxylin/eosin and Giemsa.

[1] Informed consent was obtained and the study was approved by the local Medical Ethical Committee

Results

In all tissues, specific, reproducible staining patterns were seen (Table 2). Sections incubated with PBS or non-specific antibodies as a first step showed no non-specific- or background staining.

Staining for immunoglobulin heavy and light chains showed, in addition to surface-IgM staining of B cells and cytoplasmic plasma cell staining,

Table 2. Immunophenotype of fetal lymphoid tissues

Spleen	
– early lymphoid cells (12–14 wk)	all LCA1$^+$, MT2$^+$, MB1$^+$; a majority (60–80%) MB2$^+$, LN2/MB3$^+$, LN3$^+$, sIgM$^+$, and kappa$^+$ or lambda$^+$; 20–40% MT1$^+$
White pulp	
– follicles:	– Lymphoid cells: all LCA1$^+$, MT2$^+$, MB1$^+$, MB2$^+$, LN2/MB3$^+$, LN3$^+$, sIgM$^+$, and kappa$^+$ or lambda$^+$
– PALS:	– Lymphoid cells: all LCA$^+$, MT2$^+$, MT1$^+$; 20–50% MB1$^+$ – Scattered S-100$^+$, LN2/MB3$^+$, LN3$^+$, MT1$^+$ cells with dendritic morphology
– red pulp:	– Scattered IgM$^+$ (kappa/lambda$^+$) plasmacells – Cells with mature myeloid morphology: MT1$^+$, LeuM1$^+$ and lysozyme$^+$ – Cells with histiocyte morphology: MT1$^+$, lysozyme$^+$, additional weakly LN2/MB3$^+$ and LN3$^+$; up to 10% MB1$^+$, LeuM1$^+$
Lymph node	
– follicles:	– Lymphoid cells: all LCA1$^+$, MT2$^+$, MB1$^+$, MB2$^+$, LN2/MB3$^+$, LN3$^+$, sIgM$^+$, and kappa$^+$ or lambda$^+$
– interfollicular/medullary area	– Lymphoid cells: almost all* LCA$^+$, MT2$^+$, MT1$^+$; 20–50% MB1$^+$ – Scattered S-100$^+$, LN2/MB3$^+$, LN3$^+$, MT1$^+$ cells with dendritic morphology
– medullary/marginal sinuses	– Cells with mature myeloid morphology: MT1$^+$, LeuM1$^+$ and lysozyme$^+$ – Cells with histiocyte morphology: MT1$^+$, lysozyme$^+$, additional weakly LN2/MB3$^+$ and LN3$^+$; up to 10% MB1$^+$, LeuM1$^+$
Thymus	
– cortex:	– Thymocytes: all LCA$^+$, MT1$^+$; sporadically MB1$^+$ – Epithelial cells: LN3$^+$; perinuclear LN2/MB3$^+$; outer cortical rim Leu7$^+$
– medulla:	– Thymocytes*: all LCA$^+$, MT1$^+$; up to 50% MT2$^+$; 5–10% MB1$^+$ – Cells with dendritic morphology: S-100$^+$, LN2/MB3$^+$, LN3$^+$, MT1$^+$ – Epithelial cells: all strongly LN3$^+$; a minority MB2$^+$ – Hassal bodies: LN1$^+$, LN2/MB3$^+$, LN3$^+$, Leu7$^+$ – Cells with mature myeloid morphology: MT1$^+$, LeuM1$^+$ and lysozyme$^+$ – Cells with histiocyte morphology: MT1$^+$, lysozyme$^+$, additional weakly LN2/MB3$^+$ and LN3$^+$; up to 10% MB1$^+$, LeuM1$^+$

* Scattered cells with "B cell" (follicular cell) phenotype were present. PALS: periarteriolar lymphocyte sheath

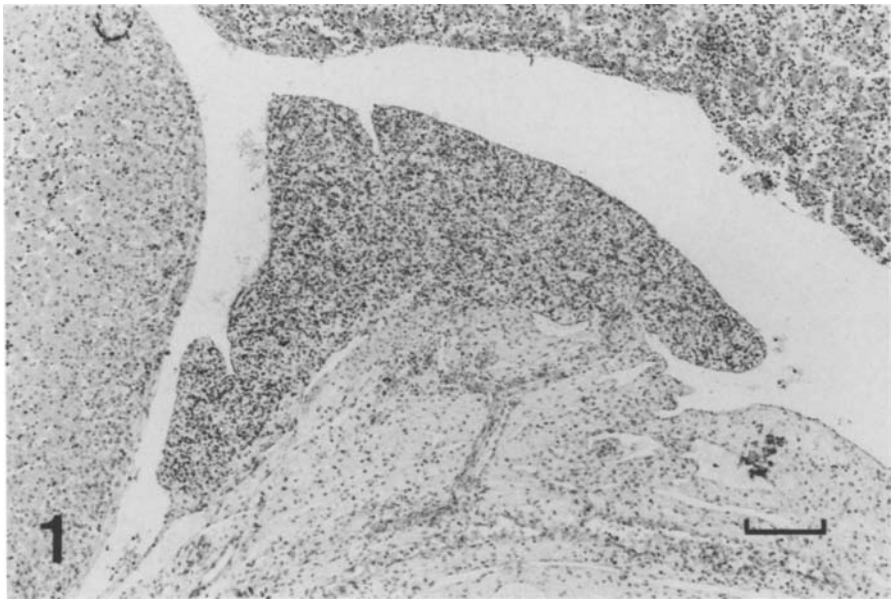

Fig. 1. 9 weeks fetal spleen. Except for some scattered erythroid cells no hemopoietic cells are present (hematoxylin/eosin, × 56; *bar* = 0.2 mm)

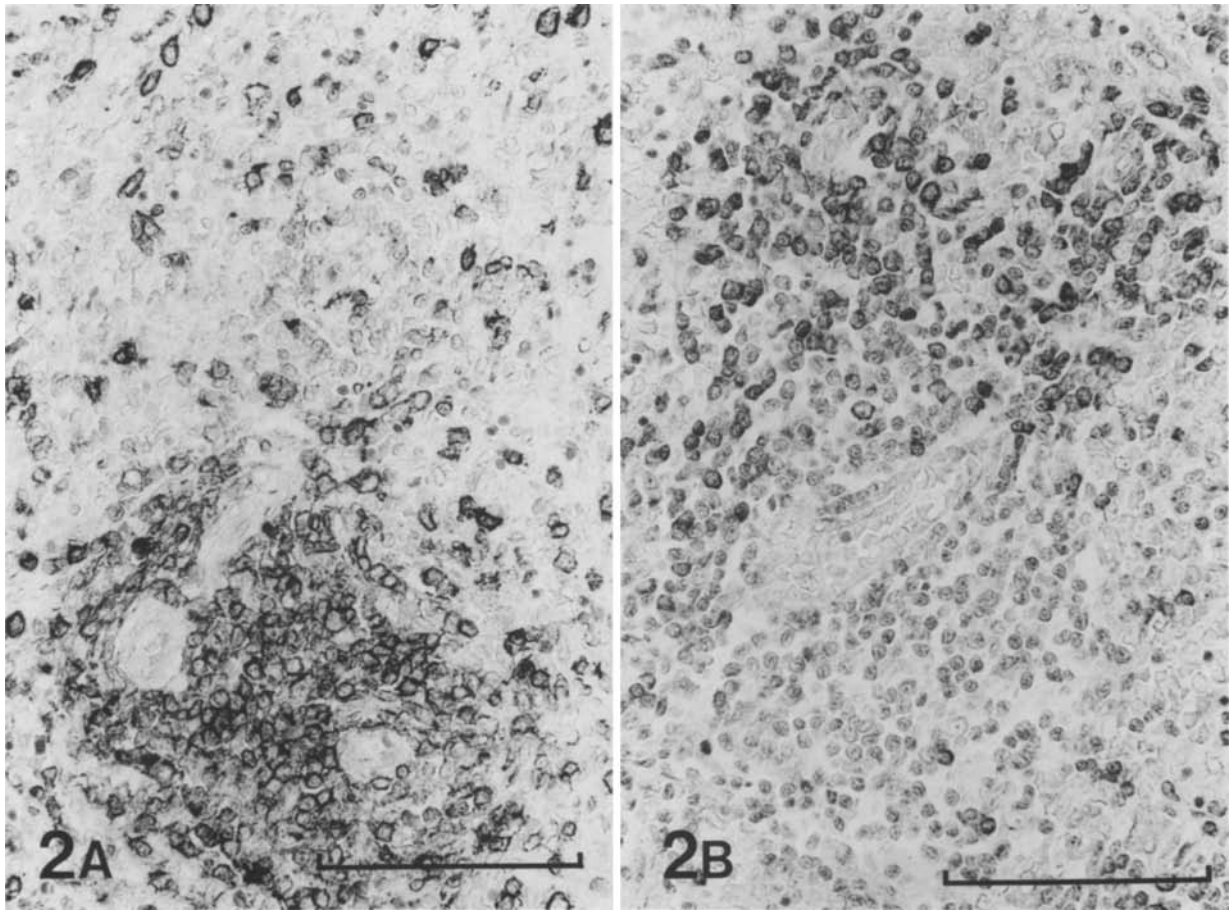

Fig. 2. Primitive white pulp in 20 weeks spleen stained for (**A**) MT1, and (**B**) MB2. The primitive periarteriolar lymphocyte sheath (PALS) is shown at the *bottom*, a primitive follicle at the *top* (immunoperoxidase, ×350; *bar* = 0.1 mm)

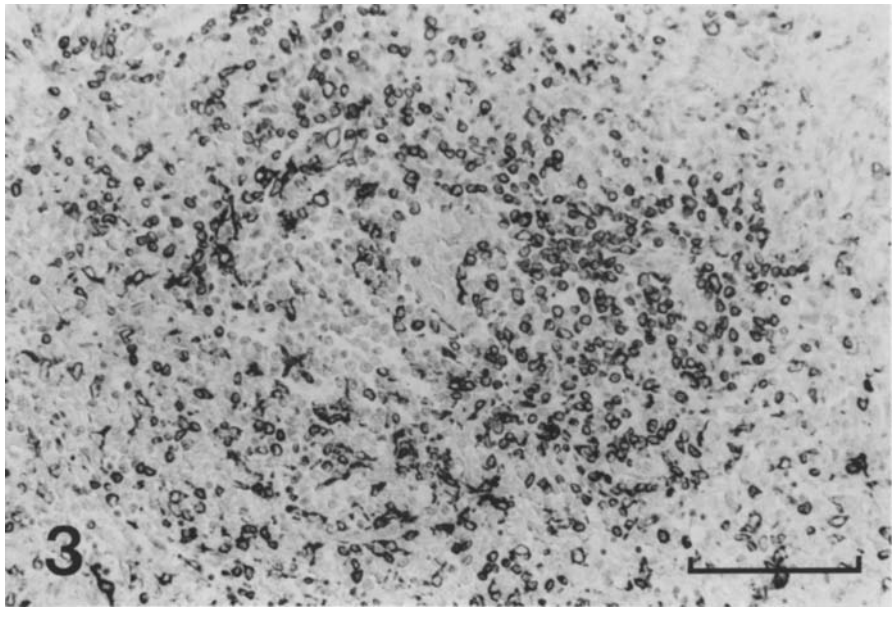

Fig. 3. LN2 staining of fetal splenic white pulp (20 weeks). Cytoplasmic B cell staining at the *right* and *top*, and negative T cells at the *left*, with strong staining interdigitating cells with dendritic morphology (immunoperoxidase, ×224; *bar* = 0.1 mm)

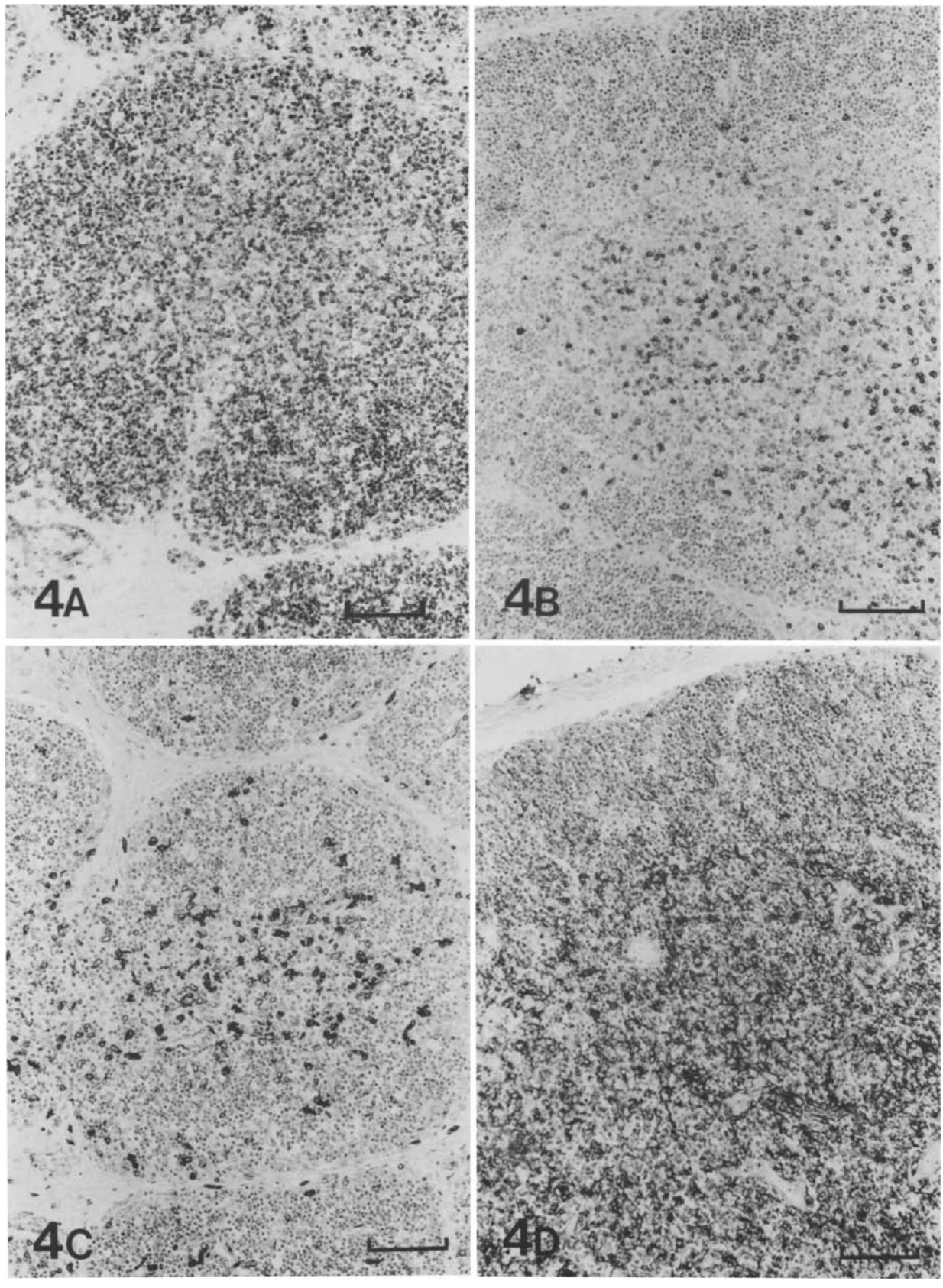

Fig. 4. Fetal thymus (**A**) (18 wk) MT1 staining of all thymocytes, and (**B**) (20 wk) MT2 staining of a minority of medullary thymocytes. (**C**) (20 wk) Staining of epithelial cells, interdigitating reticulum cells and few macrophages with LN2. Epithelial cells show staining, confined to the perinuclear space, in contrast with (**D**) (14 wk) showing branching epithelial cells expressing LN3 (all immunoperoxidase, $\times 140$; *bar* = 0.1 mm)

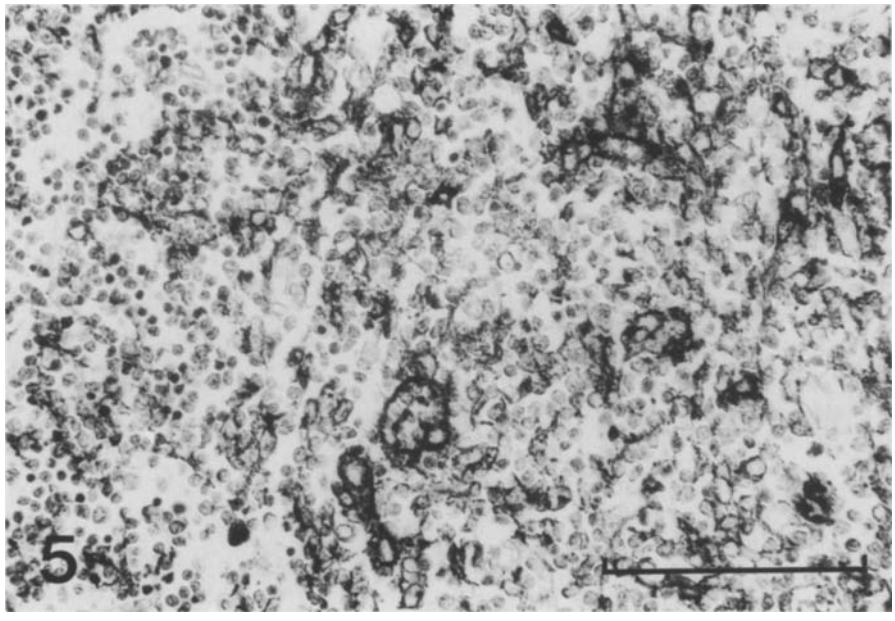

Fig. 5. 20 wk. fetal thymus: epithelial cells, positive for LN3, surrounding thymocytes in the thymic medulla (immunoperoxidase, ×350; *bar*=0.1 mm)

an irregular faint diffuse cytoplasmic staining in large cells with histiocyte/macrophage morphology and (in some spleens) of sinusoid lining cells. This was most probably due to passive uptake.

Monoclonal antibodies LN2 and MB3 gave identical staining results in all tissues, in agreement with previous reports that LN2 and MB3 recognize the same antigen (Poppema et al. 1987).

In most stages in our series, up to 21 weeks, several small clusters of erythropoiesis were seen in splenic red pulp as well as in the thymus. In all stages from 12 weeks mature erythroid cells were present in splenic sinusoids. All erythroid cells stained for VIE-G4, LN1, and MT1 (weakly in later stages).

In the earliest case we studied (5–6 weeks) no splenic "anlage" was present. In the 9 and 10 weeks embryo's a splenic "anlage" was discerned (Fig. 1), consisting entirely of loose mesenchymal tissue, with formation of primitive sinusoids. Staining for Factor VIII related antigen showed reactivity of small vessels, but no reaction in sinusoid lining cells. No haemopoiesis was seen; only occasionally was an erythroid cell observed.

In the 12–14 weeks cases scattered intermediate-sized lymphoid cells were present, partially in small clusters, without showing a particular configuration. From 18 weeks small demarcated clusters of intermediate-sized lymphoid cells were observed: the primitive white pulp (WP), which increased in size during maturation (Fig. 2). The cells were arranged in periarteriolar regions, with in the periphery formation of primitive follicular structures. No morphologically recognizable separate compartments (e.g. germinal centers; marginal zones) could be discerned.

In the periarteriolar area scattered individual cells were observed with dendritic morphology and irregular nuclei (Fig. 3). These cells were already present at 12 weeks, when only few scattered T cells were observed.

In the red pulp, cells with mature myeloid and with histiocyte morphology were irregularly scattered in varying amounts in all cases, increasing gradually during development.

Lymph node lymphoid cells were also of intermediate size. In the subcortical area few small lymphoid follicles could be recognized. In the medulla, and less abundantly also in interfollicular areas, several individual cells with dendritic morphology were present. Few myeloid cells and a considerable amount of histiocytes were seen in medullary and marginal sinuses, and in capsular fibrous tissue.

The studied thymuses all showed similar staining results, irrespective their stage of development (Table 2; Fig. 4). In the earliest thymus studied (14 weeks), a clear demarcation of cortex and medulla was observed. In the thymic medulla a considerable number of dendritic cells was present (Fig. 4c), similar to the appearance in splenic and lymph node T cell areas. Strongly $LN3^+$ thymic epithelial cells (Fig. 4d) were present most abundantly in the medulla; staining for LN1 and LN2/MB3 was weak or absent. The less conspicuous $LN3^+$ cortical epithelial cells were least pronounced in the outer cortex, with increasing pres-

ence towards the deep cortex and the medulla (Fig. 4d). An intimate contact was seen between thymocytes and epithelial cells, established by dendritic epithelium (mainly in the cortex), or by epithelium surrounding lymphoid cells (Fig. 5a). An abundant amount of histiocytes and few myeloid cells were present in the medulla, mainly in marginal areas at the corticomedullary junction, and in fibrous tissue of lobular septa and of the thymic capsule.

Discussion

The goal of this study was to delineate the architecture of developing fetal lymphoid tissues immunohistologically, with maximum preservation of histomorphological detail. This has been obtained by the use of recently developed monoclonal antibodies reactive with antigens in fixed, paraffin embedded tissues. The use of B5 fixative provides reliable immunohistological results, combined with good morphology (Epstein et al. 1984; Poppema et al. 1987).

In fetal spleen and lymph node, the T and B cell immunophenotype are similar to that observed in small resting lymphocytes in adult lymphoid tissues (Epstein et al. 1984; Poppema et al. 1987). T cells are mainly characterized by MT1, and B cells by MB2, LN2/MB3 and LN3. The staining patterns with these MAb, observed in T and B cell areas seem to be mutually exclusive. MB1 and MT2 show less specific patterns for T and B cells, but appear useful in combination with the other antibodies.

The splenic white pulp shows a gradual increase in organization from few small lymphoid cell clusters with "random" presence of intermediate-sized T and B cells, progressing to a loosely arranged primitive white pulp and an adult-like white pulp configuration. An increasing separation is observed of cells with T cell immunophenotype in the periarteriolar region and cells with B cell immunophenotype in peripheral primitive primary follicles. Except for the T and B cell areas, no separate white pulp cellular compartments are discerned by immunohistological staining, nor by morphology. Fetal lymph nodes show similar results.

The absence of germinal centres may be explained by an intrinsic inability of fetal B cells to proceed into a germinal centre reaction and/or inadequate T help function, or maybe by inadequate antigenic stimulation (Butler et al. 1987).

The absence of an immune response against certain thymus-independent antigens, especially polysaccharide antigens, in the human fetus and newborn may be explained at least partially by the absence of a marginal zone in fetal and newborn spleen, as this represents a cellular compartment supposed to be specifically related to this particular immune response (Timens et al. 1987).

Virtually all thymocytes are stained by MT1. As MT1 also is reactive with all T cells in peripheral lymphoid tissues (Poppema et al. 1987; this study), it can be considered as a pan-T (and not mature-B) cell reagent, although not lymphocyte lineage restricted (Table 1). MT2-staining of normal fetal and infant thymuses shows similar results (unpublished observations). As the expression of MT2 is observed on all fetal as well as adult mature non-activated peripheral T cells, the finding of a minority of medullary thymocytes positive for MT2, seems discrepant with studies on frozen tissue, where a majority is found to be of mature phenotype (Bhan et al. 1980; Janossy et al. 1981). With respect to this discrepancy, it seems probable that the population of mature medullary thymocytes is subdivided in $MT2^+$ "peripheral" type T cells and $MT2^-$ "pre-peripheral" type T cells. The presence of $MB1^+$ medullary thymocytes is consistent with the observed $MB1^+$ lymphocytes in peripheral T cell areas, and represents a mature T lymphocyte subset, comprising all $CD8^+$ cells and part of the $CD4^+$ subset (Poppema et al. 1987; Poppema and Visser 1987), identical to the population recognized by 2H4 (Akbar et al. 1988). This population of 2H4 (CD45R) positive T cells has been shown to include the inducer-suppressor T cell population (Morimoto et al. 1985), but also to represent a virginal T cell population, in contrast to $CD45R^-$ $UCHL1^+$ memory T cells (Akbar et al. 1988; Serra et al. 1988). It has been demonstrated in vitro, that 15–25% of medullary thymocytes express CD45R and that stimulation of thymocytes induces further CD45R expression (Serra et al. 1988). After leaving the thymus, approximately 50% of peripheral T cells express CD45R and loose the antigen upon antigenic stimulation in vitro (Serra et al. 1988).

Besides thymocytes, thymic cortex and medulla contain localized subsets of epithelial cells, which each play a role in thymic lymphopoiesis and T cell maturation and differentiation (Janossy et al. 1980; Weissman 1986). Three subsets are recognized with the MAb employed in this study. Leu7 reactive epithelial cells are restricted to the outer margin of the cortex (Chan et al. 1984; Kodama et al. 1986), in the area where proliferating blast cells are observed (Weissman 1986). Deep cortex epithelial cells and medullary epithelial cells show

strong reactivity with LN3, as is known from other HLA-DR reactive antibodies (Janossy et al. 1980; Bhan et al. 1980; Rouse et al. 1982; Weissman 1986). The LN3 expressing epithelial cells are located in the area where the acquisition of the CD3 antigen, related to the T cell receptor, is also observed (Bhan et al. 1980; Janossy et al. 1981). Thus it may be suggested that these epithelial cells may be involved in education and selection during T cell development in the thymus. Finally, a small number of MB2-reactive medullary epithelial cells is present.

The weak reactivity of LN2/MB3 with thymic epithelial cells seems to be in contrast with strong LN3 expression, as LN2/MB3 reacts with a major histocompatibility complex (MHC) class II related cytoplasmic antigen, which generally shows a similar distribution as LN3, with intensive staining (Epstein et al. 1984; Poppema et al. 1987; this study). However, the LN2/MB3 staining is confined to the perinuclear space of the cells and therefore does not show the extensive dendritic membrane network seen with LN3 and other MHC class II related antigens.

Other, non-epithelial, accessory cells are cells with dendritic morphology, mainly present in the medulla, which, in contrast with the epithelial cells, show strong expression of LN2/MB3 as well as LN3 (Figs. 4c, d), and in addition MT1, and S100-protein. Similar cells are seen in T cell areas in fetal spleen and lymph node. Immunophenotype and morphology are consistent with that of interdigitating dendritic cells (Janossy et al. 1986), and these cells were easily distinguished from epithelial dendritic cells. In general, IDC are thought to play a role in antigen presentation to T cells, and in "homing" of T cells (Janossy et al. 1980, 1986). An important role in fetal T cell homing is suggested by the presence of these cells in the earliest stages in fetal spleen and lymph node, when hardly T cells are present (Namikawa et al. 1986).

In conclusion, the use of paraffin tissue reactive monoclonal antibodies provides possibilities for preservation of morphological detail combined with immunohistological determination of cell subtypes and their micro-environment. The advantages are especially obvious in very fragile tissues, as in early ontogeny.

Acknowledgements. The authors thank their colleagues in the Department of Pathology for their co-operation, Mr. Hilbrand Wierenga for the preparation of the micrographs, and Mrs. Truus Bartels for secretarial assistance.

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Received June 3, 1988 / Accepted September 5, 1988