

## Immuno-electron-microscopic localization of types III pN-collagen and IV collagen, laminin and tenascin in developing and adult human spleen

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**Abstract.** The distribution of the extracellular matrix proteins types III pN-collagen and IV collagen, laminin and tenascin was investigated in fetal, infant, and adult human spleens by using immuno-electron microscopy. The presence of type III pN-collagen was assessed by using an antibody against the aminoterminal propeptide of type III procollagen. All the proteins other than type III pN-collagen were found in reticular fibers throughout development. In the white pulp of the fetus aged 16 gestational weeks, only an occasional type III pN-collagen-containing fibril was present, although type III pN-collagen was abundant in the reticular fibers of the red pulp. Conversely, in adults, most of the reticular fibers of the white pulp, but not of the red pulp, were immunoreactive for type III pN-collagen. Ring fibers, the basement membranes of venous sinuses, were well developed in both infant and adult spleens. The first signs of their formation could be seen as a discontinuous basement membrane, which was immunoreactive for type IV collagen, laminin, and tenascin in the fetus aged 20 gestational weeks. Intracytoplasmic immunoreactivity for all the proteins studied was visible in the mesenchymal cells of the fetus aged 16 gestational weeks and in the reticular cells of the older fetuses, which also showed labeling for type IV collagen and laminin in the endothelial cells. The results suggest that proteins of the extracellular matrix are produced by these stationary cells.

**Key words:** Spleen – Fetus – Development – Extracellular matrix – Immuno-electron microscopy – Transmission electron microscopy – Human

### Introduction

The characteristic structure of the human spleen begins to emerge on the 15th gestational week (GW), when the primitive red and white pulp become discernible (Ono

1930). These structures are composed of branched mesenchymal cells, which are arranged around the central arteries in the white pulp. In the red pulp, venous sinuses develop from lacunae in the reticular network and come into contact with the main venous system. Lymphoid colonization of the white pulp begins around the 18th GW (Vellguth et al. 1985), but germinal centers are only seen after birth (Barzanji and Emery 1978).

The spleen contains a rich meshwork of reticular fibers that are present early in the fetal red pulp and that, in adult red pulp, make up the honeycomb structure of Billroth's cords. Part of this meshwork, the ring fibers, consists of basement membrane (BM)-like structures arranged in the walls of the venous sinuses like hoops around a barrel. They mature relatively late and are clearly discernible after the 36th GW. In fetal white pulp, there is a distinct increase in the amount of reticular fibers at the time of lymphoid colonization (Liakka et al. 1991). In adult white pulp, the fiber network is less dense and mainly restricted to the periarterial lymphatic sheath and the marginal zone between the white and red pulp (Apaja-Sarkkinen et al. 1986).

The proteins of the extracellular matrix (ECM) are involved in cell adhesion, elongation, polarization, migration, and differentiation, and play an important role in organogenesis during embryonal development (von der Mark et al. 1992). The cells adhere to the ECM by means of receptors, among which integrins constitute the most important group. These, on the other hand, connect the ECM with the intracellular cytoskeleton and are thus able to function as signal transducers between the cell and the ECM (Burridge and Fath 1989; Hynes 1992). The glycoproteins laminin (Leivo et al. 1980; Dziadek and Timpl 1985) and tenascin (Chiquet-Ehrismann et al. 1986) appear early in embryonal development and play an important role in differentiation and cell migration. Apart from laminin, type IV collagen is the major component of basement membranes, in which it makes up complex three-dimensional networks to which other components attach (Weber 1992; Yurchenco and Ruben 1987).

Type III collagen, which is one of the fibril-forming collagens, is present in the interstitial connective tissue of many organs, being especially abundant in the skin and in blood vessel walls. Because of its ability to become rapidly cross-linked, it is thought to play a role in development and wound healing, where newly formed collagen is produced at a rapid rate (Burgeson and Nimni 1992). The cells synthesize a precursor molecule known as type III procollagen, which is secreted into the extracellular space. During extracellular processing, the carboxyterminal propeptides of the procollagen molecules are cleaved off by specific proteases. Some of the procollagen molecules also lose their aminoterminal propeptides, whereas in some of them, the propeptide remains uncleaved, i.e., type III pN-collagen. In these forms, the molecules are organized into small-diameter fibrils (Burgeson and Nimni 1992). The aminoterminal propeptide of type III procollagen has been shown to be present on the surface of mature type III collagen fibrils (Fleischmajer 1986; Karttunen et al. 1989). Type III pN-collagen seems to form co-polymers with type I collagen, and may play a role in the assembly of collagen molecules and the regulation of fibril diameter (Fleischmajer et al. 1990; Romanic et al. 1991).

According to our previous immunohistochemical studies (Liakka and Autio-Harmainen 1992; Liakka et al. 1991), splenic reticular fibers contain types III pN-collagen and IV collagen, and the glycoproteins laminin, fibronectin, tenascin, and vitronectin. On the basis of their protein composition, the ring fibers appear to be modified BMs that differ from the regular BMs in that they contain not only type IV collagen and laminin, but also tenascin and, at least in adult spleen, vitronectin (Liakka and Autio-Harmainen 1992; Liakka et al. 1991).

There are only a few studies available on the structural maturation of the human spleen. This investigation has been undertaken to elucidate the ultrastructure of the developing and mature human spleen and to extend our previous immunohistochemical light-microscopic findings on the distribution of types III pN-collagen and IV collagen, laminin, and tenascin by using immuno-electron microscopy.

## Materials and methods

### Tissue samples

The material comprised seven human spleens. They were from four fetuses (16–24 GW) obtained from spontaneous and legal abortions, from one infant who died of a congenital heart defect (1 month), and from two adults. The adult spleens were surgical splenectomy specimens. With the exception of hyaline arteriosclerosis in one adult spleen and slight congestion in the infant spleen, the spleens were both macroscopically and microscopically normal. The patient with gastric lymphoma had not received any radiation or chemotherapy before the operation. A detailed description of the material is given in Table 1. The ages of the fetuses were calculated from the crown-rump and foot lengths and from the mother's last menstruation. The indicated gestational weeks refer to the menstrual age. Because of a paucity of material, no infant spleens were included in the study for tenascin.

**Table 1.** Pertinent clinical data on the patients from whom tissue was sampled

Case	Age	Disease
Fetus	16 w	Fetal hydrops, hygroma of the neck
Fetus	20 w	Down's syndrome
Fetus	23 w	Abruption placentae
Fetus	24 w	Congenital heart defect
Infant	1 month	Congenital heart defect
Adult	25 years	Reflux esophagitis, fundoplication
Adult	72 years	Gastric lymphoma, centroblastic

### Antibodies

The polyclonal antibodies to the 7S domain of type IV collagen, the fragment P1 of laminin, and the aminoterminal propeptide of type III procollagen (PIIINP) were a generous gift from Dr. Leila Risteli and Dr. Juha Risteli, Collagen Research Unit, Department of Medical Biochemistry, University of Oulu, Oulu, Finland. The 7S domain of type IV collagen was purified from human kidney (Risteli et al. 1980), the fragment P1 of laminin from human placenta (Risteli and Timpl 1981), and the PIIINP from ascitic fluid (Niemelä et al. 1985). The antibodies were raised in rabbits and purified by cross-absorption with other ECM antigens immobilized on sepharose 4B columns. The final absorption was made with the protein used in the immunization. The specific antibodies absorbed onto the column were eluted, concentrated and used in the immunohistochemical stainings after verifying their specificity by radioimmunoassay.

The mouse monoclonal antibody (Mab) 143DB7 against tenascin has been characterized earlier (Tiitta et al. 1992). This antibody detects tenascin in formaldehyde-fixed tissue.

### Cryoimmuno-electron microscopy

Samples of 1 mm<sup>3</sup> from the splenic tissue were fixed with 8% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. They were cryoprotected by immersion in 2.3 M sucrose, and were frozen in liquid nitrogen. Thin cryosections of 90 nm were cut with a Reichert Ultracut FC4-E ultramicrotome.

The procedure for immunolabeling was a modification of the method described by Griffiths et al. (1984) and Sormunen et al. (1993). The sections were first incubated in 10% fetal calf serum (FCS) with 0.02 M glycine in phosphate-buffered saline (PBS-glycine) for 10 min. They were then incubated with the polyclonal antibodies to the 7S domain of type IV collagen, the PIIINP, or the fragment P1 of laminin diluted optimally with 5% FCS in PBS-glycine for 45 min and washed in PBS-glycine (5 changes in 15 min). Subsequently, the sections were incubated with protein A-gold complex diluted 1:20 in 5% FCS in PBS-glycine for 20 minutes. The protein A-gold complex, with a gold particle of 10 nm, was prepared as described by Slot and Geuze (1985). The sections were washed in PBS-glycine (6 changes in 25 min) and post-fixed in 2.5% glutaraldehyde for 2 min, followed by brief washes in PBS-glycine and distilled water (5 changes in 10 min). Finally, the sections were embedded in 2% methylcellulose with 0.3% uranyl acetate on ice for 10 min, dried, and examined in a Philips 410 LS transmission electron microscope with an acceleration voltage of 60 kV.

When Mab 143DB7 to tenascin was used, the sections were exposed, after incubation with the antibody, to rabbit anti-mouse IgG (Dakopatts, Denmark) diluted 1:200 with 5% FCS in PBS-glycine for 20 minutes and washed in PBS-glycine (6 changes in 15 min) prior to exposure to the protein A-gold complex. In the control sections, the antibody was omitted and replaced with PBS or chromatographically purified rabbit IgG or mouse IgG (Zymed Laboratories, San Francisco, Calif., USA).

### *Transmission electron microscopy*

Specimens of 1 mm<sup>3</sup> were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h. They were then washed in PBS and post-fixed in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer, pH 7.4, for 1 h. After dehydration in acetone, the specimens were embedded in Epon LX 112. Thin sections of 80 nm were cut with a Reichert Ultracut E-ultramicrotome. Finally, the sections were stained with uranyl acetate and lead citrate. Alternatively, to increase the contrast of the ECM, the sections were first treated with 0.01% tannic acid at 60° C for 3 min and then stained with uranyl acetate and lead citrate (Dingemans et al. 1990).

### *Analysis of structures*

The general histology of the specimens was assessed, from 1-μm-thick sections stained with toluidine blue, by light microscopy, and from ultrathin sections by transmission electron microscopy. In each age group, the findings were then correlated with the immunoelectron microscopic images of 90-nm-thick sections of the corresponding spleens. In this way, it was possible to analyze the different compartments of the developing and adult spleens. Special consideration was given to the BMs of the venous sinuses and the reticular fibers of the red and white pulp, and to the blood vessels in the different age groups. The capsules and trabeculae, if present, and the reticular cells and the endothelial cells of venous sinuses were also examined.

A semiquantitative scale was used to estimate the immunoreaction of the protein A-gold labeling, from + for weak labeling to +++ for abundant labeling. In the sections, the background labeling and the labeling in the nuclei served as internal controls, in addition to the control sections, where the antibody was omitted and replaced with PBS-glycine or nonspecific IgG.

## **Results**

### *Transmission electron microscopy*

In the fetal spleen of 16 GW, electron-microscopic features typical of endothelial and reticular cells were observed in some cells, but the cell population mostly consisted of mesenchymal cells. These made up a meshwork, in which lacunae filled with blood cells were seen. ECM material lay close to the plasma membrane of the mesenchymal cells, and consisted of variable amounts of fibril-forming collagen embedded in an amorphous electron-dense matrix. The more mature venous sinuses were lined by flat endothelial cells, which were often seen to build „bridges“ over the sinuses. On the cordal side of these cells, there were narrow stretches of ECM containing amorphous substance, but no continuous BM could be detected (Fig. 1). Immature white pulp areas enveloped the central arteries. They were composed of mesenchymal cells and thin fibers with an amorphous composition and only a few collagenous fibrils.

In the older fetuses (20–24 GW), reticular and endothelial cells were identifiable (Fig. 2), although some immature cell forms were also seen. Junctional complexes were detectable both between the reticular cells and between the endothelial cells. The ECM consisted of fibers that were thicker and that contained more amorphous material than those seen in the 16 GW fetus. The walls of the sinuses showed a discontinuous BM (Fig. 2). The

white pulp was readily recognizable because of its high lymphocyte content.

In the infant and adult spleens, a fully developed, elaborate network of reticular fibers of Billroth's cords and ring fibers around the venous sinuses was observed (Fig. 3). The ring fibers consisted of electron-dense amorphous material with a few occasional central fibrils of collagen. The ring fibers were connected to the reticular fibers of Billroth's cords, which were composed of fibril-forming collagen embedded in an electron-dense amorphous matrix. They were located in apposition to the long cytoplasmic processes of the reticular cells. The cytoplasm of the endothelial cells of the venous sinuses possessed abundant micropinocytotic vesicles and bundles of microfilaments at the abluminal side of the cell (Fig. 3). In the white pulp, reticular cells and reticular fibers were seen among the lymphocytes. Bundles of collagenous fibrils were embedded in an amorphous matrix between myofibroblasts and elastic material in the capsule.

### *Immuno-electron microscopy*

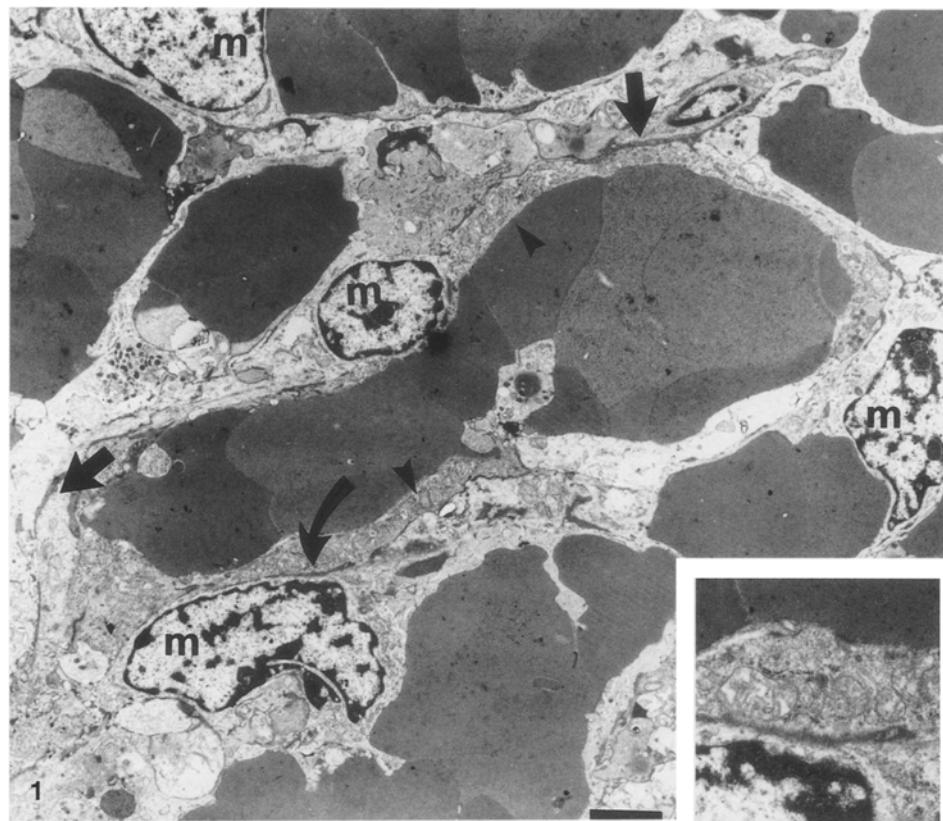
The immunoelectron microscopic findings in the splenic ECM are presented in Table 2.

**Type IV collagen.** In fetal, infant and adult spleens, moderate to intense labeling located in the amorphous electron-dense matrix of reticular fibers was seen with the anti-type IV collagen antibody (Fig. 5a, b). The first signs of ring fiber formation were observed in the walls of the venous sinuses in the fetus aged 20 GW; a discontinuous BM consisting of amorphous, electron-dense material showed moderate labeling (Fig. 5c). In the further developed infant and adult spleens, the ring fibers were well-formed and intensely labeled (Fig. 5d). Little labeling occurred in control sections, where the antibody was omitted and replaced with rabbit IgG (Fig. 4). The ECM of vascular walls showed moderate to intense immunoreactivity with the type IV collagen antibody in all the age groups.

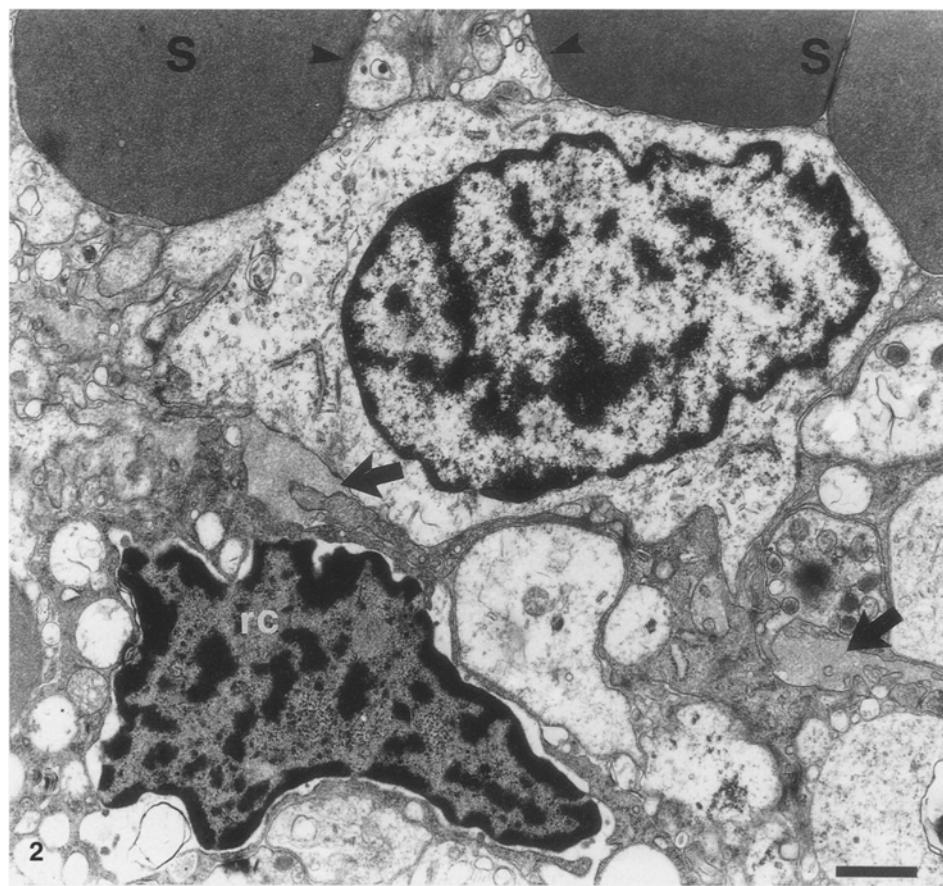
Intracytoplasmic labeling for type IV collagen was seen in the mesenchymal cells of the fetus aged 16 GW (Fig. 6a). It was also present in the endothelial and reticular cells of the older fetuses, the infant, and the adults (Fig. 6b, c).

One specimen from an adult spleen also contained material from the splenic capsule. Moderate labeling was seen in the amorphous matrix of the capsule and between the fibrils of collagen bundles. There was also weak labeling in the cytoplasm of the myofibroblasts of the capsule.

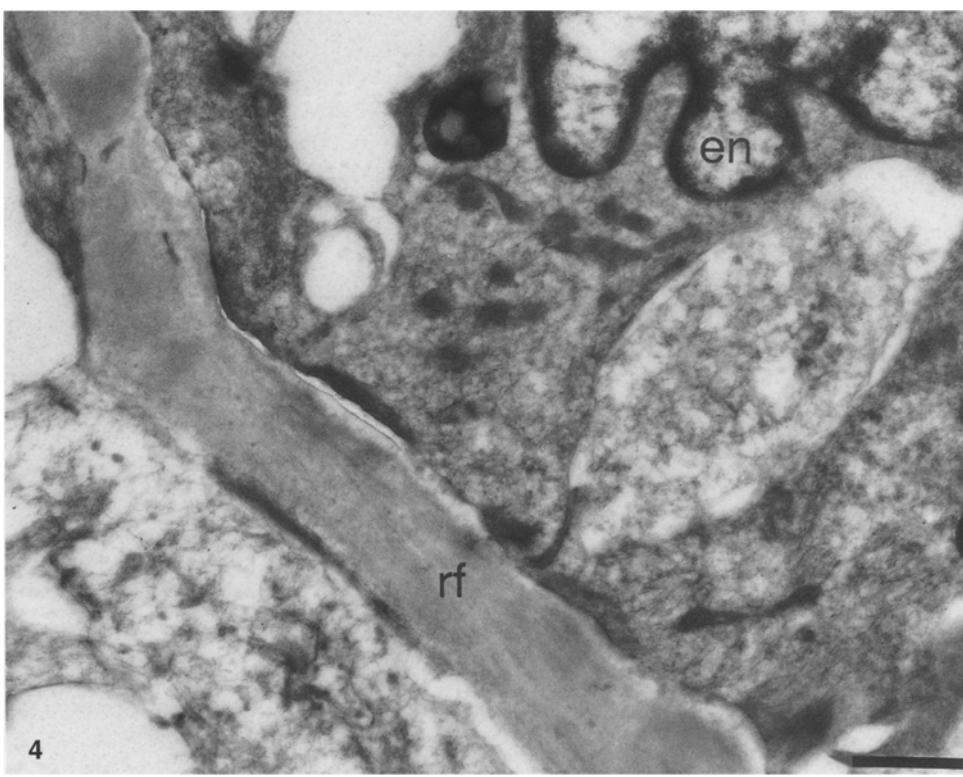
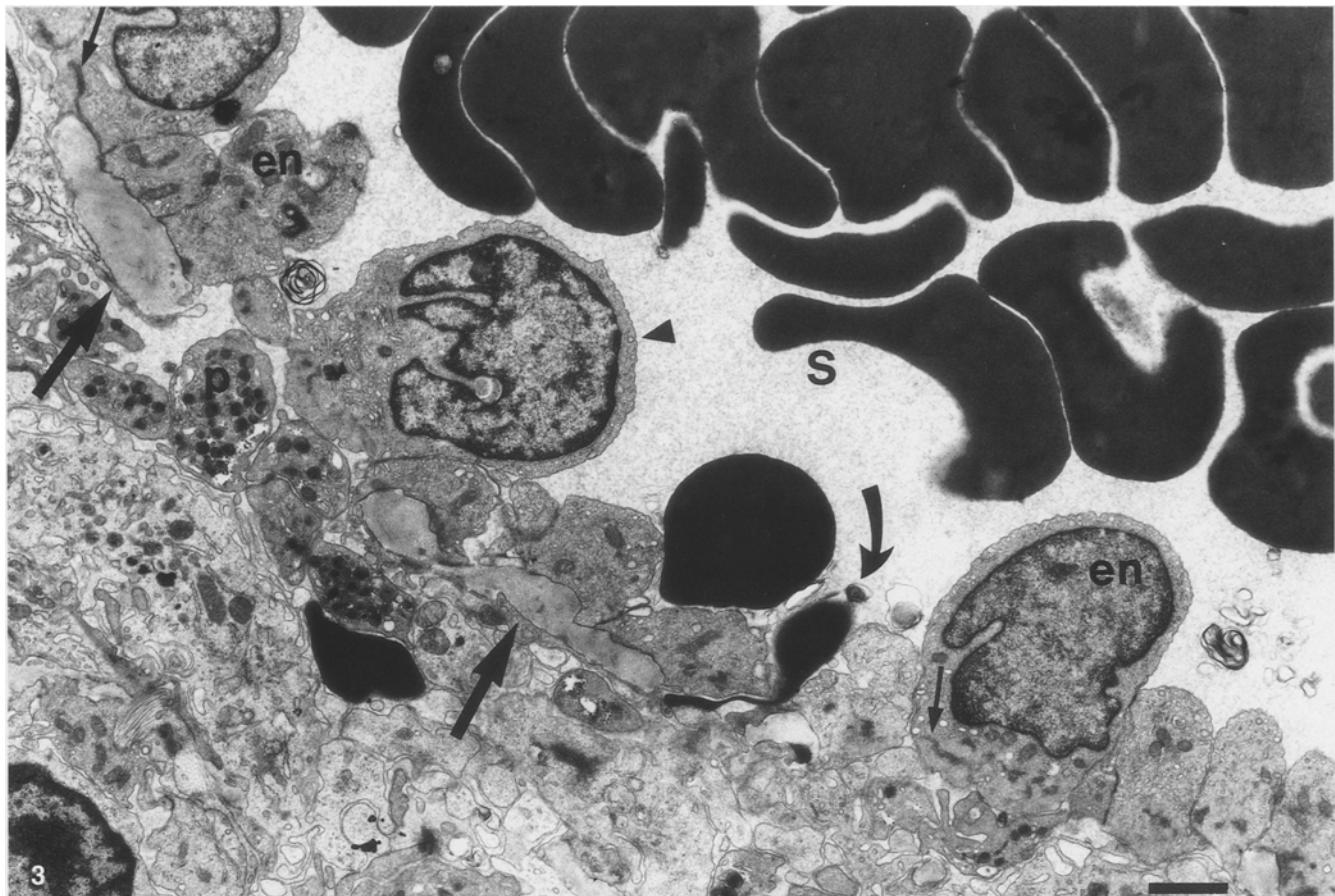
**PIIINP.** The reticular fibers of the red pulp of the 16 GW fetal spleen were intensely immunoreactive, the surface of the collagen fibrils being immunopositive (Fig. 7a, b). With increasing age, fewer immunoreactive fibers were seen, and the reticular fibers of the red pulp in adults showed only faint labeling. Conversely, in the white pulp of the 16 GW fetus, the ECM contained only a few labeled collagen fibrils, whereas in the adult white pulp,



**Fig. 1.** Transmission electron microscopy of the spleen of a fetus aged 16 GW. The red pulp is composed of immature mesenchymal cells (*m*) with abundant cytoplasm. Some large venous sinuses are lined by flattened endothelial cells (*arrowheads*). No continuous BM is seen, but thin stretches of ECM material (*arrows*) are present in the red pulp cords.  $\times 3200$ . Bar:  $3.0 \mu\text{m}$ . Inset: Higher magnification of the area indicated with a *curved arrow* representing the ECM between an endothelial cell and a mesenchymal cell.  $\times 10500$ . Bar:  $1.0 \mu\text{m}$



**Fig. 2.** Transmission electron microscopy of the spleen of a fetus aged 20 GW. Islands of amorphous material (*arrows*) representing a discontinuous BM are seen in the wall of the venous sinus (*S*). The cordal side of the BM is covered by the cytoplasm of a reticular cell (*rc*). An endothelial cell bridges the sinusoid (*arrowheads*).  $\times 9680$ . Bar:  $1.0 \mu\text{m}$



**Fig. 3.** Transmission electron microscopy of the spleen of an adult. A venous sinus (*S*) with obliquely sectioned ring fibers (*arrows*) beneath the endothelial cells (*en*). Bundles of microfilaments (*small arrows*) and micropinocytotic vesicles (*arrowhead*) are seen in endothelial cells. Platelets (*p*) lie under the sinus wall and an erythrocyte „squeezes“ through an interendothelial slit (*curved arrow*).  $\times 5280$ . Bar: 2.0  $\mu\text{m}$

**Fig. 4.** Control section of an adult spleen; a wall of a venous sinus with endothelial cells (*en*) and a longitudinally cut ring fiber (*rf*). Immuno-electron microscopy. No labeling is detectable with rabbit IgG.  $\times 14200$ . Bar: 1.0  $\mu\text{m}$

**Table 2.** Occurrence of the ECM components in the fetal, infant, and adult spleens as assessed by immuno-electron microscopy<sup>a</sup>

	16 GW	20–24 GW	Infant	Adult
<i>Type IV collagen</i>				
Reticular fiber	++	++	+++	++
Ring fiber		++	+++	+++
Blood vessel	++	++	+++	++
<i>Type III pN-collagen</i>				
Reticular fiber	+++ <sup>b</sup>	++	+	+ <sup>c</sup>
Ring fiber	–	+	+	+
Blood vessel	++	++	+	+
<i>Laminin</i>				
Reticular fiber	++	++	++	++
Ring fiber		+	+	++
Blood vessel	++	++	++	++
<i>Tenascin</i>				
Reticular fibers	++	++	nd	++
Ring fibers		+	nd	+
Blood vessel	+	+	nd	+

<sup>a</sup> +, Weak; ++, moderate; +++, abundant labeling; –, no reaction; nd, not determined

<sup>b</sup> Reticular fibers in white pulp were negative

<sup>c</sup> Moderate labeling in the reticular fibers of the white pulp

the collagenous fibrils of the reticular fibers were distinctly immunoreactive (not shown). No labeling was seen in the electron-dense amorphous material of the ring fibers in the infant and adult spleens, although occasional labeled collagen fibrils were discernible within the ring fiber matrix. Collagenous fibrils in the outer part of the blood vessel walls were faintly to moderately labeled in all the spleens.

A cytoplasmic immunoreaction occurred in the mesenchymal/reticular cells of the fetal spleens, whereas only a faint reaction was present in the infant and adult spleens. The endothelial cells appeared negative. In the fetal, infant, and adult spleens, some trabecular myofibroblasts showed faint cytoplasmic labeling, whereas the surrounding fibrillary collagenous matrix was intensely immunopositive (Fig. 7c).

**Laminin.** The general distribution of laminin immunolabeling corresponded to that described for type IV collagen. A slight to moderate immunoreaction was seen in the reticular fibers in all the age groups. It was mainly localized around and between the collagenous fibrils (Fig. 7d). At 20–24 GW, faint labeling was seen in the amorphous electron-dense material in the walls of the venous sinuses. In the infant and adult spleens, enhanced labeling was present in the ring fibers (Fig. 7e, f). The ECM of the vascular walls showed moderate labeling in all the age groups.

Intracytoplasmic labeling for laminin was observed in the mesenchymal/reticular cells and endothelial cells of the fetal spleens. This labeling was faint in the infant and adult spleens. The amorphous substance in the trabeculae was moderately labeled and myofibroblasts showed intracytoplasmic immunoreactivity.

**Tenascin.** The amorphous electron-dense matrix of the reticular fibers was slightly to moderately immunoreactive in all the investigated spleens (Fig. 8a). The BMs of the venous sinuses of the 20–24 GW fetuses and the ring fibers of the adult spleens were also weakly labeled (Fig. 8b). The vascular walls showed immunoreactivity in their outer aspect, whereas the inner part was mostly negative.

An intracytoplasmic immunoreaction was seen in the mesenchymal/reticular cells of the fetuses. At the later stages of development, this reaction was less distinct. The endothelial cells appeared negative. In the fetuses aged 20–24 GW, the ECM of the trabeculae was moderately labeled, but this labeling was weak in the adults.

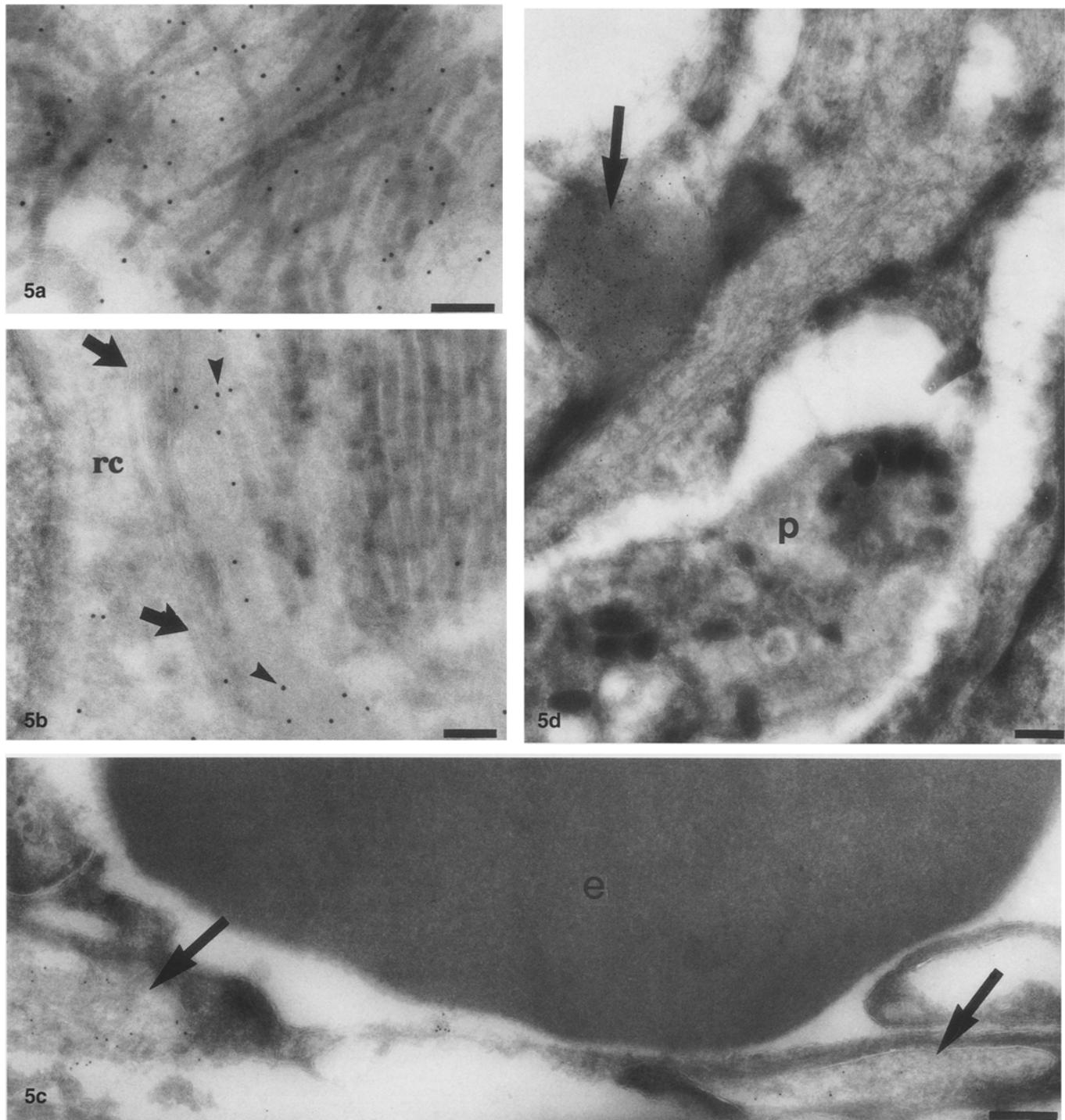
## Discussion

In this study, we have used immuno-electron and transmission electron microscopy to investigate the composition and fine structure of the ECM of developing and mature human spleens. In accordance with our previous immunohistochemical findings (Liakka and Autio-Harainen 1992; Liakka et al. 1991), this study demonstrates that type IV collagen, laminin and, to a lesser extent, tenascin are regular components of the reticular fibers of the red and white pulp of fetal and adult spleens.

This study confirms our previous suggestion that, even at an early stage of development, the ECM composition of the fetal spleen corresponds to that of the adult spleen. An important exception to this is type III pN-collagen, which is abundant in the fetal red pulp, but inconspicuous in the immature white pulp at 16 GW. Type III pN-collagen is however readily detectable in the white pulp at the age of 19 GW (Liakka et al. 1991), which is the age at which lymphocytes appear in larger amounts in the area. Contrary to our findings in fetal spleens, the reticular fibers of the red pulp in adult spleens only contain small amounts of type III pN-collagen, whereas those of the white pulp are rich in this collagen type. It seems therefore that, in the fetal spleen, type III collagen synthesis begins later in the white pulp than in the red pulp and that it continues in the mature white pulp more actively than in the red pulp.

The maturation of the structure of the reticular fiber meshwork in the fetal white pulp may be of importance for lymphoid colonization. The reticular fibers form a scaffold, to which interdigitating reticulum cells and dendritic reticulum cells, the stationary cells of the T-cell and B-cell regions, are attached. These cell types are present in the fetal white pulp prior to the appearance of T and B lymphocytes, and they have been suggested to have a role in the homing of lymphocytes (Timens 1991).

Type III pN-collagen containing fibrils are scarce in the adult red pulp. However, we have been able to identify bundles of cross-striated collagenous fibrils embedded in the amorphous electron-dense matrix of the reticular fibers of the adult red pulp. Some of these fibrils are reactive to the antibody against PIINP, but some collagenous fibrils are unreactive to our antibody. These fibrils may lack the aminoterminal propeptide or be composed of types I and V collagen, which are other types of



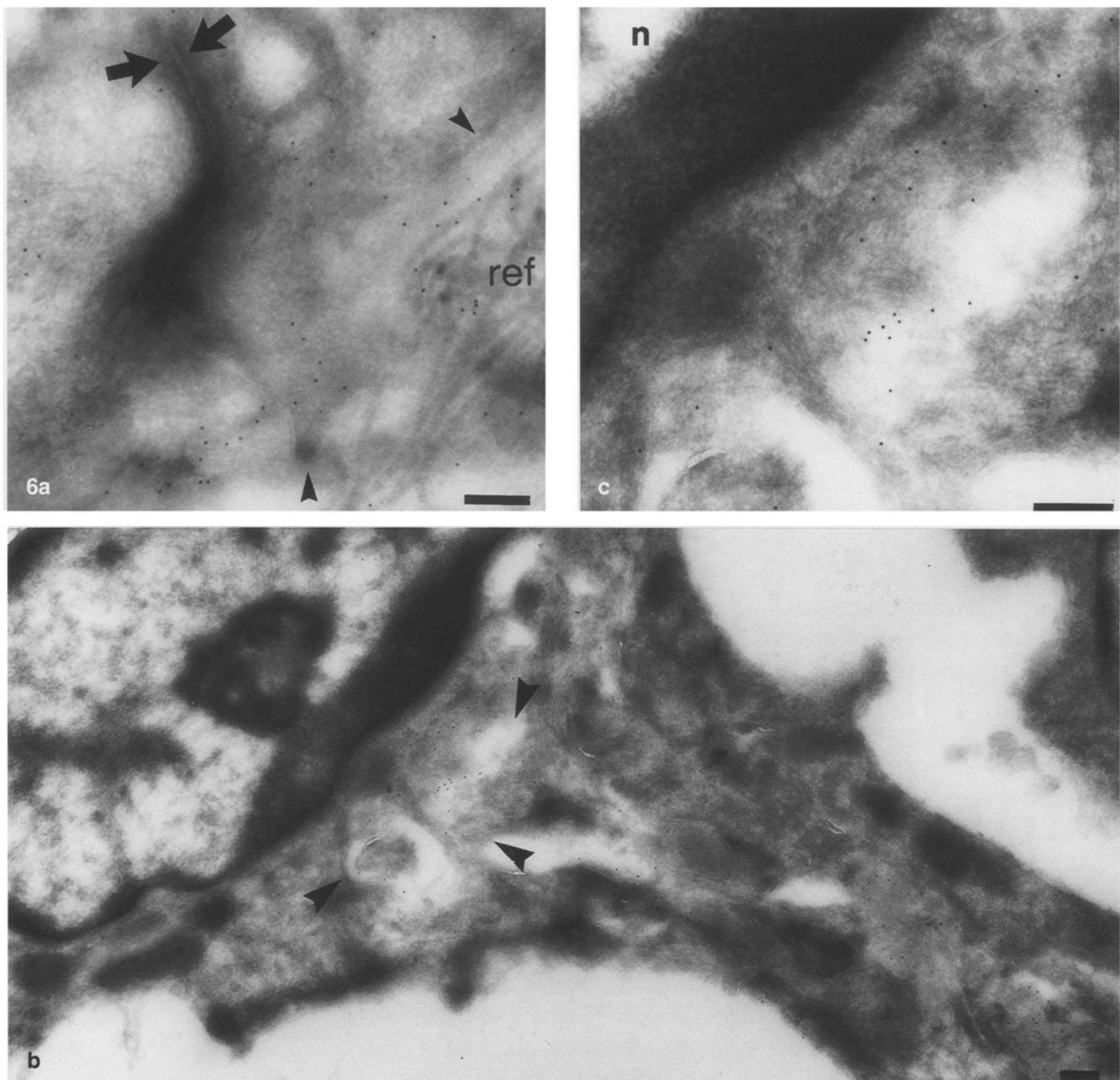
**Fig. 5.** Immuno-electron-microscopic localization of type IV collagen (a) in a reticular fiber of a fetus aged 16 GW, and (b) in the amorphous matrix of a reticular fiber (arrowheads) of an adult. A reticular cell (rc), the cell membrane of which is indicated with arrows, shows slight cytoplasmic labeling.  $\times 62000$ . a, b Bars: 150 nm. (c) Localization of type IV collagen is present in the wall of a

venous sinus of a fetus aged 20 GW. Labeling is seen in the discontinuous BM (arrows). An erythrocyte (e) lies in the sinus lumen.  $\times 36000$ . d Intense labeling for type IV collagen is present in a transversally sectioned ring fiber (arrow) of an adult. A platelet (p) is seen in the red pulp cord.  $\times 24000$ . c, d Bars: 300 nm

fibril-forming collagens. At least type I collagen is present in the reticular fibers of the spleen (van den Berg et al. 1993).

The presence of laminin and tenascin in the reticular fibers of the developing spleen is in agreement with the

suggestion that these molecules are important for embryonic development (Ekblom 1989; Chiquet 1992). Both laminin and tenascin show growth-promoting activities by being mitogenic for many cell types (Kubota et al. 1992; Panayotou et al. 1989; End et al. 1992). Tenascin



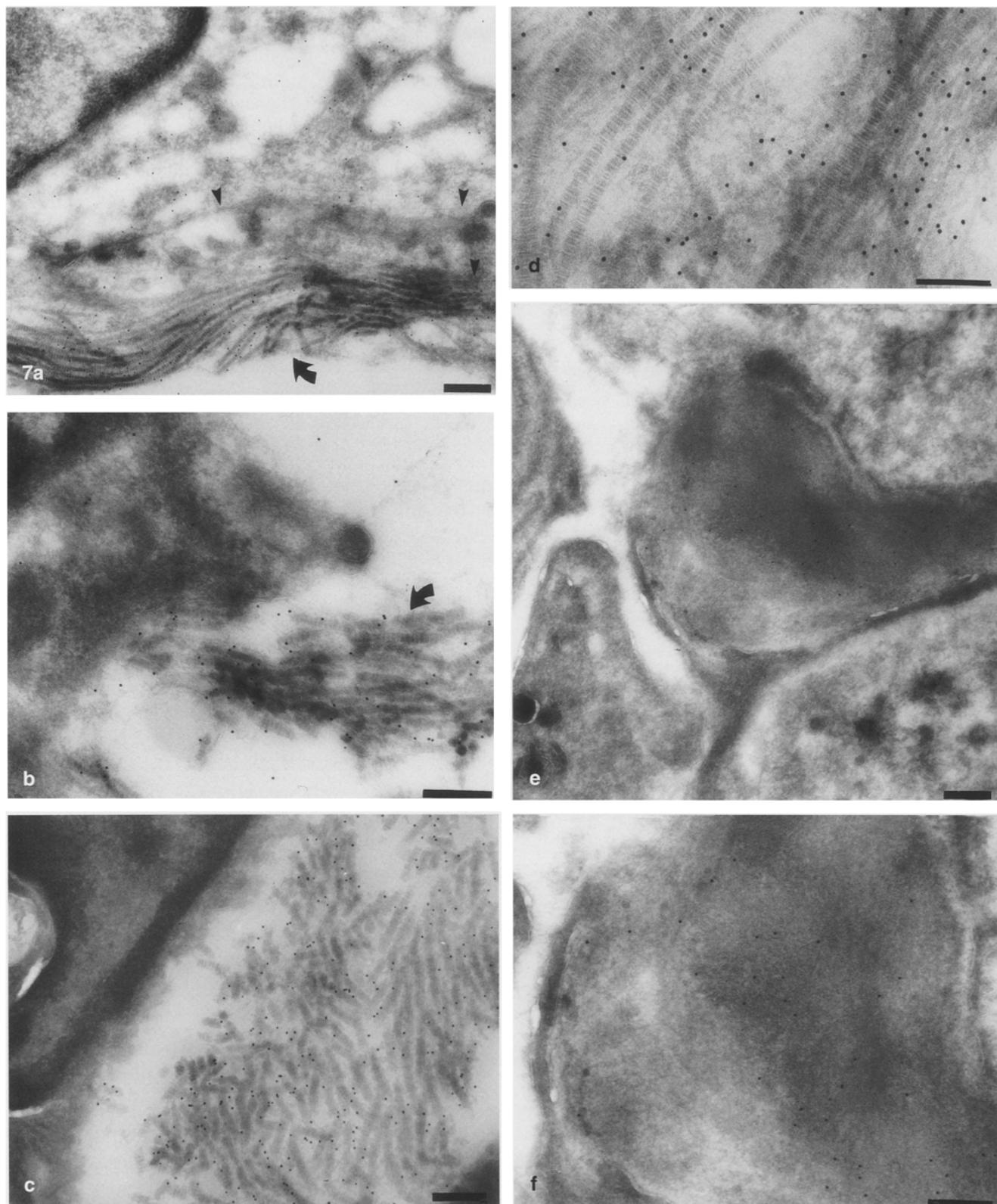
**Fig. 6.** **a** Intracytoplasmic labeling for type IV collagen in the mesenchymal cells of a fetus aged 16 GW. A junctional complex is indicated by the arrows. A reticular fiber (*ref*) is seen *lower right*. The cell membrane is indicated by arrowheads. **b**

Localization of type IV collagen in the cytoplasm of a reticular cell of an adult.  $\times 21300$ . The area indicated by arrowheads is seen at a higher magnification in **c**. **n** Nucleus.  $\times 50700$ . Bars: 250 nm

has been found to be concentrated at the epithelial-mesenchymal interface of the budding epithelia of many developing organs, such as the lung (Young et al. 1994), the kidney (Aufderheide et al. 1987), mammary glands, hair follicles, and teeth (Chiquet-Ehrismann et al. 1986), in a temporally restricted manner. The matrix-mediated effects of laminin on differentiation have also been explained by the differential expression of its chains, as has been shown for developing kidney (Klein et al. 1988), gut (Simo et al. 1991), skin (Olsen et al. 1989), and peripheral nerve (Jaakkola et al. 1993). Although we

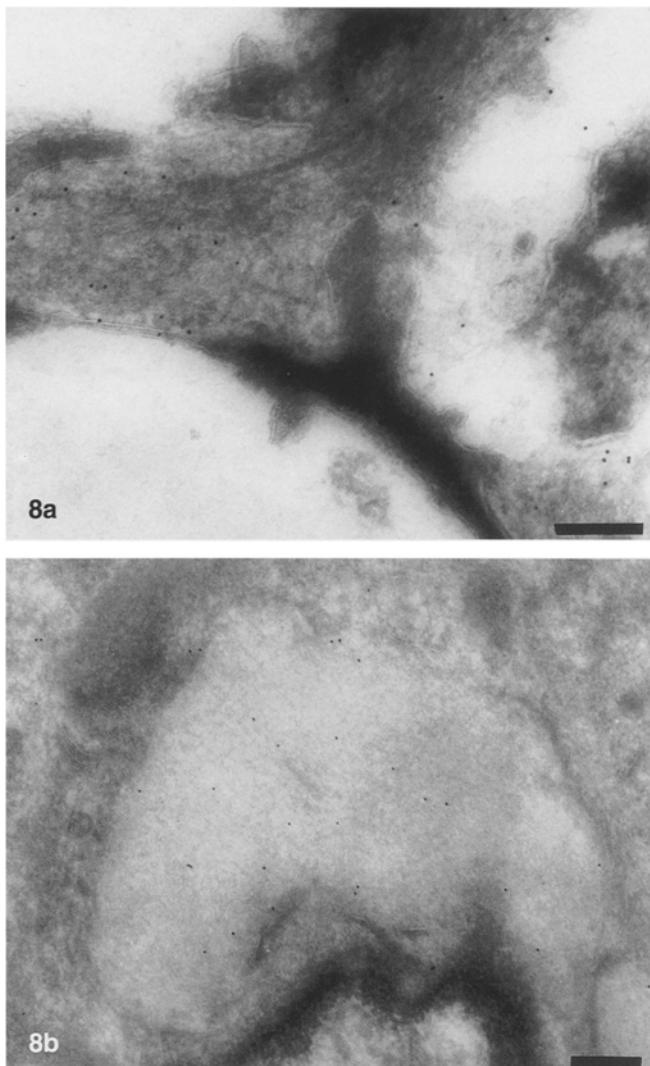
have used a polyclonal antibody against the fragment P1 of laminin, which apparently recognizes more than one laminin chain, it is not possible to say, on the basis of this study, whether there are temporal differences in the expression of the laminin chains during splenic development.

We have previously suggested that the primitive venous sinuses of the fetal spleen at the 16th GW are surrounded by a continuous BM (Liakka et al. 1991). No corresponding structures could however be demonstrated by electron microscopy. The fetal cell population is



**Fig. 7.** **a, b** Localization of type III pN-collagen in the reticular fiber (arrows) of the red pulp of a fetus aged 16 GW. Label for PIIIINP is seen on collagen fibrils and, in (a) in the cytoplasm of the mesenchymal cells. The plasma membranes of two adjacent cells are indicated by arrowheads. **a**  $\times 20800$ , **b**  $\times 42000$ . **c** Label for PIIIINP is seen on the collagen fibrils of a trabecula of a fetus

aged 23 GW.  $\times 27300$ . **a-c Bars:** 300 nm. **d** Laminin is mainly localized in the amorphous matrix of the reticular fiber of a fetus aged 24 GW.  $\times 62000$ . **Bar:** 200 nm. **e, f** Localization of laminin in the amorphous matrix of an obliquely sectioned ring fiber of an adult. **e**  $\times 22320$ , **f**  $\times 39060$ . **Bars:** 300 nm



**Fig. 8. a, b.** Immunoreactivity for tenascin is seen in the reticular fibers of a fetus aged 20 GW (a) and in an obliquely sectioned ring fiber of an adult (b). **a**  $\times 39060$ , **b**  $\times 28000$ . Bars: 300 nm

largely composed of mesenchymal cells with abundant cytoplasm, which, together with the ECM material, appear to form the linings of the immature venous sinuses. It is therefore likely that the impression of a continuous BM surrounding the venous sinuses, which is notable in the immunohistochemical preparations, comes from intracytoplasmic staining of the mesenchymal cells and the reactivity of the reticular fibers. At 20–24 GW, however, distinct but discontinuous BMs are visible around the venous sinuses by transmission electron microscopy. These structures correspond to the alternation of thick and thin stretches (dots) seen by light-microscopic immunohistochemistry (Liakka et al. 1991; Liakka and Autio-Harmainen 1992).

The ring fibers of the adult spleen consist of an electron-dense amorphous substance immunoreactive for type IV collagen and laminin and, to a minor extent, tenascin. With respect to laminin, our finding is contradictory to that of Drenckhahn and Wagner (1986) who, by using a light-microscopic indirect immunofluorescence

method, have found laminin immunoreactivity to be concentrated at the periphery of the ring fibers. In the present study, laminin immunoreactivity has been found to be evenly distributed throughout the ring fiber matrix. The reason for this discrepancy might be in the resolution power of the two techniques.

Within the ring fiber substance, occasional central collagenous fibrils label for PIIIINP. In their transmission electron microscopic work on the rat spleen, Saito et al. (1988) have also demonstrated collagenous fibrils in the ring fibers. These findings further support our previous notions that the splenic ring fibers seem to be specialized forms of BMs, with respect not only to their morphological arrangement, but also to their protein composition (Liakka and Autio-Harmainen 1992).

In this study, an intracytoplasmic immunosignal has been seen for both PIIIINP and type IV collagen and for laminin and tenascin in the mesenchymal/reticular cells of the fetal spleens. This suggests that the proteins may be synthesized by these cells. Further studies are however needed to confirm this. To exclude non-specific labeling, we have always used negative control sections. The nuclei and the background serve as an internal control for nonspecific labeling and the amount of labeling that they exhibit is low.

In conclusion, the present findings indicate that types III pN-collagen and IV collagen, laminin, and tenascin are components of reticular fibers during early splenic development. The composition of the developing splenic fiber meshwork may be important for the differentiation of various cell types, for lymphoid colonization, and in a mature spleen, for the anchoring of stationary and itinerant cells that fulfill the main functions of the spleen, i.e., phagocytosis and the immune response.

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