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Original Article

Reticular Fibroblasts in Peripheral Lymphoid Organs Identified by a Monoclonal Antibody[1]

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We have produced a panel of monoclonal antibodies directed against nonlymphoid cells in central and peripheral lymphoid organs. In this paper we present the reactivity of one of these antibodies, ER-TR7. This antibody detects reticular fibroblasts, which constitute the cellular framework of lymphoid and nonlymphoid organs and their products. In frozen sections of the spleen incubated with this antibody, the red pulp and white pulp are clearly delineated. Furthermore, the major white pulp compartments—the follicles and periarteriolar lymphoid sheath as well as the marginal zone—are recognized by their characteristic labeling patterns. In lymph nodes, the capsule, sinuses, follicles, paracortex, and medullary cords are clearly delineated. In the thymus and bone marrow no such specialized compartments were demonstrated. ER-TR7 reacts with an intracellular component of fibroblasts. Since ER-TR7 does not react with purified laminin, collagen types I-V, fibronectin, heparan sulfate proteoglycan, entactin, or nidogen, it detects a hitherto uncharacterized antigen. The possible role of the ER-TR7 positive reticular fibroblasts in the cellular organization of peripheral lymphoid organs will be discussed.
KEY WORDS: Monoclonal antibodies; Reticular fibroblasts; Spleen; Lymph node; Immunohistology; Mouse.

Introduction

Peripheral lymphoid organs, such as the spleen and lymph nodes, are highly compartmentalized. Within these organs T and B lymphocytes each have their own domains. In the spleen, B cells localize in follicles in the peripheral part of the white pulp (de Sousa, 1971; Gutman and Weissman, 1973; Nieuwenhuis and Ford, 1976) and in the marginal zone (MZ) (Kumararatne et al., 1981), which separates the white pulp from the red pulp. T cells, on the other hand, occupy the central area of the peri-arteriolar lymphoid sheath (PALS) (Mitchell, 1972; van Ewijk et al., 1974). In lymph nodes, B cells localize in the follicles in the outer cortex, whereas T cells occupy the paracortical area (Parrott et al., 1966; van Ewijk and van der Kwast, 1980). Medullary cords predominantly contain plasma cells which migrate during differentiation from the outer cortex into this region. T and B cells both enter the splenic white pulp via the MZ (Ford, 1969; Nieuwenhuis and Ford, 1976; Brelinska and Pilgrim, 1982). In the lymph nodes they enter through high endothelial post-capillary venules (HEV) located in the paracortex (Butcher et al., 1980). Upon entry, T and B cells segregate and migrate into their respective domains.

The factors that direct the migration and specific homing of B and T cells into their respective domains are still unknown. From light and electron microscopic studies it is known that both spleen and lymph nodes contain several nonlymphoid cell types, such as interdigitating cells (IDC), follicular dendritic cells (FDC), macrophages, and reticular fibroblasts (Veerman and van Ewijk, 1975; Humphrey and Grennan, 1982). Morphologic observations indicate that FDC and IDC possibly play a role in the homing of B and T cells, respectively (van Ewijk et al., 1974, Dijkstra and Döpp, 1983). Little is known about the function of reticular fibroblasts in the lymphoid microenvironments. These cells constitute a supportive cellular framework and they may also help direct in the migration and localization of lymphocytes (de Sousa, 1969; Barclay, 1981).

We recently produced a panel of monoclonal antibodies directed against nonlymphoid cells of the mouse thymus (van Vliet et al., 1984a). These antibodies provide a new approach for a detailed structural analysis of the nonlymphoid constituents of the thymus. In this paper we present an extensive study of the reactivity of one of these antibodies, ER-TR7 (ER-TR = Erasmus University Rotterdam—Thymic Reticulum), which also reacts with the stroma of peripheral lymphoid organs.

The purpose of this study is threefold: first, to analyze in detail the anatomy of peripheral versus central lymphoid organs using the monoclonal antibody ER-TR7, second, to describe the reactivity of ER-TR7 in a variety of other tissues, and third, to analyze the nature of the antigen detected by ER-TR7. The tissue distribution of the antigen expressed on stromal cells and detected by

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monoclonal antibody ER-TR7 was studied using the immunoperoxidase technique on frozen sections.

Materials and Methods

Mice. Male and female C3H/HeJ and (CBA × C57BL/6)F1 mice, aged 6–12 weeks, were used for this study. They were kept in our animal colony under routine laboratory conditions.

Monoclonal antibody. Details of the production of rat monoclonal antibodies directed against stromal cells of the mouse thymus have been published elsewhere (van Vliet et al., 1984a). We obtained seven hybrid cell lines that produce antibodies against various stromal cell types in the thymus. In this study we describe the reactivity of one of these antibodies: ER-TR7, an immunoglobulin G2a (IgG2a) antibody, which also reacts with antigens of the reticular framework of lymphoid organs of the mouse.

Conjugate. Rabbit anti-rat immunoglobulin coupled to horseradish peroxidase (RαRa-Ig-HRP) (Dako, Copenhagen, Denmark) was used. To prevent nonspecific binding of the conjugate, it was deaggregated by centrifugation in a Beckman Airfuge at 10^5 *g*. The conjugate was optimally diluted in PBS containing 0.5% bovine serum albumin (BSA) and 1% normal mouse serum (NMS).

Preparation and incubation of frozen sections. Frozen sections were prepared and incubated with monoclonal antisera and photography was performed as described elsewhere (van Ewijk et al., 1981).

Mouse fibroblast cell lines. Mouse fibroblast cell line 129 was initiated in our laboratory as a primary culture of strain 129 skin fibroblasts. A9 is a mouse L-cell derivative (Littlefield, 1964).

Preparation and incubation of fibroblast cell lines. Cells were isolated with a rubber policeman, fixed, and embedded in agar, and frozen sections were cut as reported before (van Vliet et al., 1984b). Sections were then incubated with ER-TR7 as described above.

Reticulin stain. Frozen tissue sections were stained for reticulin with routine silver impregnation according to Gomörri (1952).

Further characterization of the antigen. The possible antigenic relationship of the matrix component detected by ER-TR7 with laminin, types I–V collagens, nidogen, entactin, fibronectin, and heparan-sulfate-rich basement membrane proteoglycan was tested in the following ways.

Ouchterlony immunodiffusion. Double radial immunodiffusion in 1% agarose was performed in Immuno-Tek II OT agarose plates (Behringwerke, Marburg, West Germany). ER-TR7 antibody was put into the central well and the connective tissue antigens were put in the peripheral wells. The proteins were allowed to diffuse overnight at room temperature in a moist chamber.

Immunoelectrophoresis. Immunoelectrophoresis in 1% agarose was performed in electrophoresis base and agar gel plates (Hyland Laboratories, Costa Mesa, California). Laminin, fibronectin, or entactin was placed in the well and electrophoresed for 45 min at 30 mA in a barbital buffer system, pH 8.6. Either antibodies to these proteins or ER-TR7 were then placed in the trough. The proteins were allowed to diffuse overnight at room temperature in a moist chamber.

Radioimmunoassay. Interstitial and basement membrane connective tissue antigens were iodinated with ^{125}I by the chloramine-T method (McConahey and Dixon, 1966). Radioimmunoassay (RIA) was performed as described (Rohde et al., 1976), using purified antibodies to laminin, entactin, types I–IV collagens, fibronectin, heparan sulfate proteoglycan, or ER-TR7 antibody.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed as described previously (Voller et al., 1976). Serial dilutions of purified rabbit antibody to the connective tissue macromolecules were applied to microtiter wells (Cooke Laboratory Products Division, Alexandria, Virginia) coated with fibronectin, laminin, or the other biochemically characterized tested antigens.

Immunofluorescence. In order to determine whether the ER-TR7 antibody bound to the same antigenic moieties as did the antibodies to the matrix macromolecules, blocking studies were performed using mouse skin sections as substrate according to a previously described protocol (Yaoita et al., 1978). For example, purified antibody to laminin was conjugated with fluorescein isothiocyanate (Goldman, 1968). Unlabeled ER-TR7 antibody was reacted for 3 min. Unbound reagents were washed way, and fluoresceinated antibody to laminin was applied for another 30 min. The sections were then extensively washed and mounted. Conversely, in other studies, unlabeled rabbit antibody to matrix proteins was first applied to tissue sections for 30 min, unbound antibody was washed away, and ER-TR7 antibody was applied for another 30 min. After extensive washings, bound rabbit and rat antibodies were detected using fluorescein-conjugated antibody to rabbit or rat immunoglobulin. If the first antibody "blocked" binding of the second antibody, this would indicate either that they were binding to the same antigenic moieties or that there was steric hindrance by the first antibody. The specificity of the ER-TR7 antibody was also assessed by measuring its binding to agarose beads coated with connective tissue proteins, as described by Yaoita et al. (1978). Finally, in other studies ER-TR7 antibody was preincubated overnight at 4°C with 100 μg of each tested antigen in a test tube before immunofluorescence in order to block binding of antibody to the tissue section.

Immunoblotting. The reactivity of ER-TR7 with laminin, fibronectin, entactin, nidogen, and types I–V collagens was tested by immunoperoxidase staining of their polypeptides, after electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets as described by Towbin et al. (1979).

Results

In the first part of this section we will describe the localization of cells expressing the antigen detected by ER-TR7 in peripheral lymphoid organs. In the second part we will describe the distribution patterns of ER-TR7 observed in central lymphoid organs such as thymus and bone marrow. To obtain further information on the nature of the antigen detected by ER-TR7, we studied the reactivity of ER-TR7 with various nonlymphoid tissues and with fibroblast cell lines, and we compared the reactivity pattern of ER-TR7 with a conventional reticulin stain. We also analyzed the possible antigenic relationship of the antigen detected by ER-TR7 with various purified connective tissue components.

Anatomical Distribution of ER-TR7 Positive Cells in Spleen and Lymph Node

Frozen sections incubated with ER-TR7 followed by RαRa-Ig-HRP and diaminobenzidine (DAB) clearly show the two major compartments in the spleen – the white pulp and the red pulp. Figure 1 shows that the white pulp is located around central arterioles which branch from the splenic artery. This area can be easily distinguished from the red pulp, which is characterized by a randomly distributed meshwork. Within the white pulp, three distinct areas can be delineated by their characteristic labeling

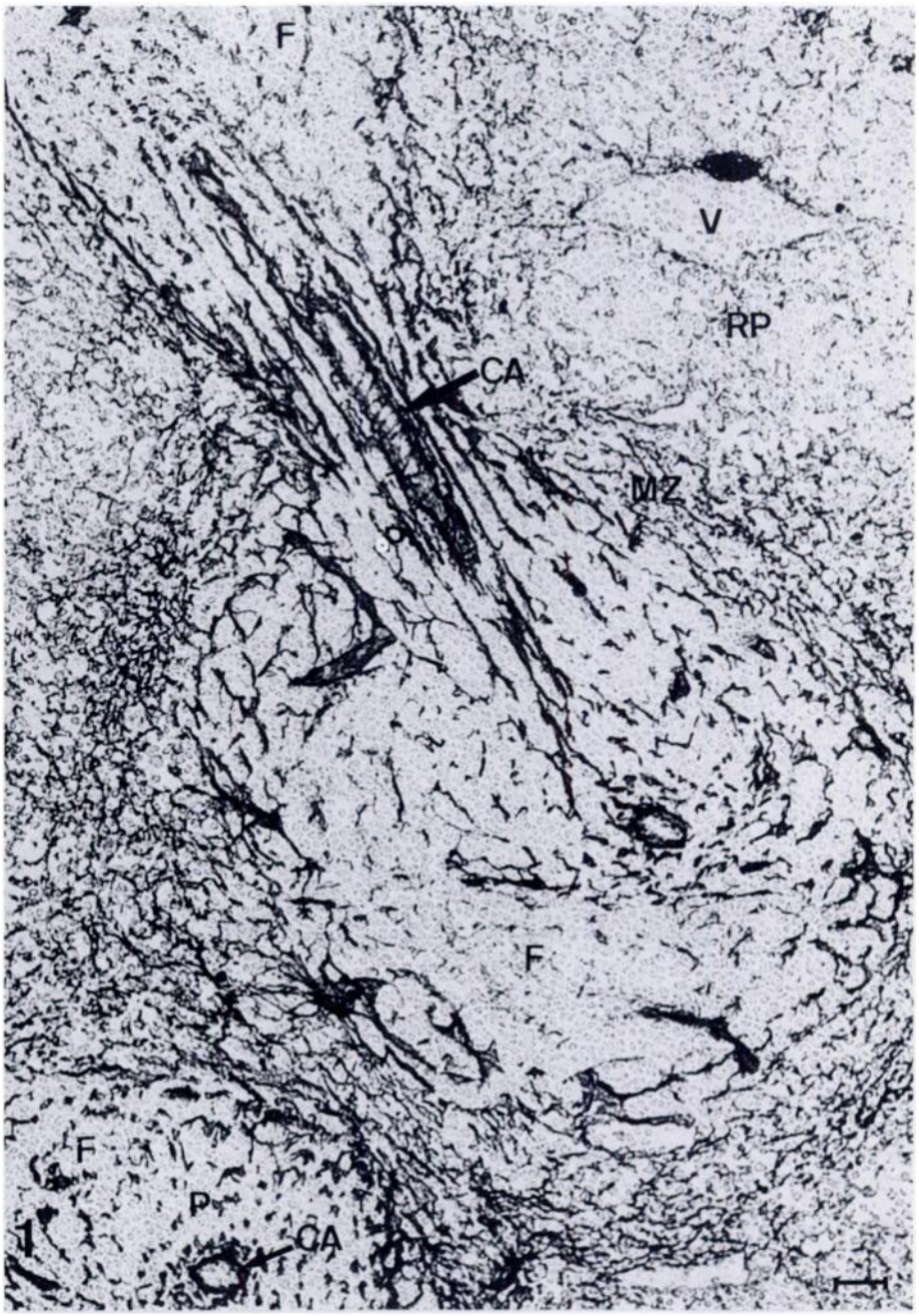

Figure 1. Immunoperoxidase staining pattern of frozen section of spleen. Section incubated with monoclonal antibody ER-TR7, followed by RαRa-Ig-HRP and DAB. Note both longitudinal and transverse sections of the PALS. CA, central arteriole; F, follicle; P, periarteriolar lymphoid sheath; MZ, marginal zone; RP, red pulp; V, venule. Original magnification × 280. Bar = 25 μm.

patterns. In a longitudinal section of the spleen, the PALS contains a network of fibers, concentrically arranged in sheaths, parallel to the central arteriole. The central arteriole is outlined by a brightly stained wall. Follicles at the periphery of the PALS are virtually unstained by the antibody, except for the outer boundary of the follicle with the MZ. The staining pattern in the MZ is a reticular meshwork, which is far more dense than in the red and white pulp. The marginal zone gradually merges into the reticulum of the cords in the red pulp. Red pulp sinuses clearly stand out as negative areas. Trabeculae and the splenic capsule are strongly positive.

In frozen sections of the lymph node, the cortex and the medulla can be delineated with ER-TR7 (Figure 2a). As in splenic sections, follicles located in the outer cortex are unstained, except for the outer boundary. The interfollicular areas of the outer cortex and the paracortex show a characteristic fine reticular staining pattern. The walls of HEV located in the paracortical area also react strongly with this antiserum. The capsule stains intensely with ER-TR7, whereas the subcapsular sinus is negative. In the medulla, strongly stained cords can be distinguished from negative sinuses. This staining pattern is specific since no such pattern was observed

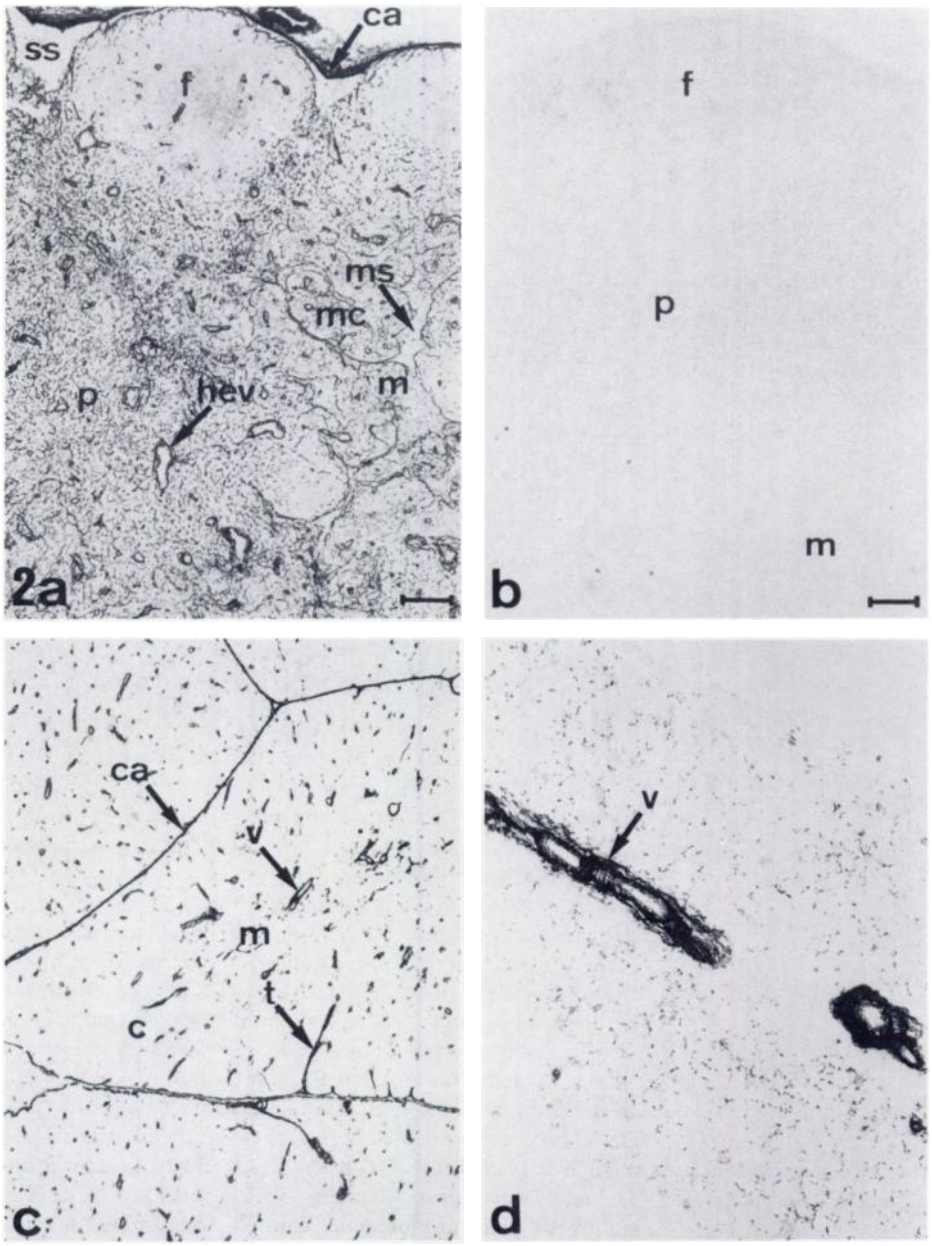

Figure 2. Immunoperoxidase staining pattern of (**A,B**) mesenteric lymph node, (**C**) thymus, and (**D**) bone marrow. **A,C,D,** incubation with ER-TR7; **B**, negative control. ca, capsule; c, cortex; f, follicle; hev, high endothelial venule; m, medulla; mc, medullary cord; ms, medullary sinus; p, paracortex; ss, subcapsular sinus; t, trabeculae; v, blood vessel. Original magnifications: **A,C** × 60 (bar = 115 μm); **B,D** × 140 (bar = 50 μm).

in negative control sections—sections incubated with RαRa-Ig-HRP and DAB only (Figure 2b).

Taken together, results of labeling of frozen sections of peripheral lymphoid organs with ER-TR7 demonstrate the various domains, to which T and B lymphocytes localize.

Anatomical Distribution of ER-TR7 Positive Cells in Thymus and Bone Marrow

To study a possible compartmentalization of thymus and bone marrow, we incubated frozen sections of these organs with ER-TR7.

Frozen thymus sections incubated with ER-TR7 show staining mainly of the capsule, blood vessels, and trabeculae (Figure 2c). The border between cortex and medulla is not clearly outlined, although these regions can be distinguished.

In frozen sections of bone marrow plugs labeled with ER-TR7 (Figure 2d), a slight reticular pattern can be noted, together with strong staining of the walls of blood vessels. However, in contrast with the spleen and lymph node, this labeling pattern does not demonstrate any compartmentalization within the bone marrow.

Anatomical Distribution of ER-TR7 Positive Cells in Nonlymphoid Organs

We incubated frozen sections of a variety of nonlymphoid organs and tissues with ER-TR7 in order to study reactivity patterns in

these organs. The results are summarized in Table 1. A few examples are shown below to illustrate the specificity of ER-TR7.

In frozen sections of skin incubated with ER-TR7 (Figure 3a) the dermis was confluently labeled, whereas the epidermis was negative. Sections of the small intestine incubated with ER-TR7 show a similar confluent staining of the lamina propria (Figure 3b). No staining of the intestinal epithelium was observed. Similarly, in sections of the stomach a positive lamina propria and a negative epithelium were observed (not shown). The ovarian stroma was positive, whereas the follicles were negative (not shown). ER-TR7 stains the connective tissue that forms a supporting network between parenchymal cells in various organs, such as salivary gland, kidney, testis, liver, and pancreas. Cardiac and striated muscle also contain such an ER-TR7-positive connective tissue network. Examples of this staining pattern are in sections of salivary gland and kidney are shown in Figure 3c and d. In the salivary gland the epithelial cells of the acini and collecting ducts are negative, whereas the reticular connective tissue around the acini and ducts can be seen as thin lines (Figure 3c). A similar staining of connective tissue elements, but no staining of muscle fibers, was observed in striated muscle sections. In the testis the interstitial tissue between seminiferous tubuli is positive. Both glomeruli and interstitial connective tissue between kidney tubules are strongly labeled with ER-TR7 (Figure 3d). In the liver the lining of sinusoids are positive, whereas parenchymal cells are not labeled. ER-TR7 reacts with the extracellular matrix of cartilage and with the dermis in sections of ear (not shown). Blood vessels always showed a strongly positive wall.

In conclusion, in all the organs mentioned above, connective tissue compartments can be identified with the present monoclonal antibody.

Comparison of the Reactivity Pattern of ER-TR7 with a Conventional Reticulin Stain

Further study of the nature of the antigen detected by ER-TR7 involved conventional silver impregnation of spleen sections. This method is known to detect reticulin, which is defined as the connective tissue that stains with silver (Hay et al., 1978). Figures 4a and b show serial frozen spleen sections stained respectively with ER-TR7 or by conventional silver impregnation. Silver impregnation resulted in a reticular labeling pattern in the splenic *white* pulp that was in general similar to the staining pattern observed with ER-TR7, although less dense. However, no labeling of the ER-TR7-positive meshwork of the splenic *red* pulp and MZ was seen after silver impregnation. From this observation we tentatively conclude that ER-TR7 reacts with reticulin but also with other connective tissue components yet to be determined.

Reactivity of ER-TR7 with Fibroblast Cell Lines

To study the reactivity of ER-TR7 with fibroblasts, we incubated frozen sections of cell pellets of mouse fibroblast cell lines 129 and A9 with ER-TR7. ER-TR7 was shown to react with the cytoplasm of fibroblasts of each of these cell lines (Figure 4c). 80–90% of the cells are ER-TR7 positive. A negative control section is shown in Figure 4d.

Table 1. *Reactivity of ER-TR7 with various nonlymphoid organs of the mouse*

Organ	Structures with which ER-TR7 reacts[a]
Submandibular salivary gland	Interstitial CT[b] between acini
Stomach	Lamina propria, CT of muscularis, serosa
Small intestine	Lamina propria, CT of muscularis, serosa
Pancreas	Interstitial CT between acini
Liver	Lining of liver cords
Skin	Dermis
Ear	Extracellular matrix of cartilage, dermis
Striated muscle	Interstitial CT between muscle fibers
Cardiac muscle	Interstitial CT between muscle fibers
Tendon	Fibers
Ovary	Connective tissue stroma, tunica albuginea
Testis	Interstitial CT between seminiferous tubuli
Kidney	Glomeruli and interstitial CT between tubuli
Brain	Blood vessels, meninges

[a] In tissues tested ER-TR7 reacts with blood vessel walls and capsule.
[b] CT = connective tissue.

Characterization of the Antigen Detected by ER-TR7

The reactivity of ER-TR7 with purified laminin, fibronectin, types I–V collagens, heparan sulfate proteoglycan, entactin, and nidogen was investigated by the Ouchterlony technique, immunoelectrophoresis, RIA, ELISA, immunoelectroblotting and indirect immunofluorescence blocking and inhibition studies. ER-TR7 reacted with none of the tested matrix components in any of the test systems, whereas the control antisera specific for these matrix components detected them in mouse tissue sections. These results show that ER-TR7 does not detect a strict basement membrane component or any major collagen type or fibronectin. In addition, ER-TR7 reacts in immunofluorescence studies with interstitial stroma and matrix cartilage but not with the basement membrane matrix deposited by EHS sarcoma or L2 tumors, two transplantable murine and rat tumors that synthesize a matrix of basement membrane (Timpl et al., 1979; Wever et al., 1981). In culture, ER-TR7 reacts with murine fibroblasts but not with L2 cells, vascular endothelial cells, or glomerular epithelial cells, which suggests that the antigen detected by ER-TR7 is synthesized by mesenchymal cells rather than by epithelial or endothelial cells.

Discussion

In this study we analyze the anatomical distribution of the cellular framework of lymphoid and nonlymphoid organs, detected by monoclonal antibody ER-TR7 and the nature of the antigen detected by ER-TR7. Our results clearly demonstrate that ER-TR7 can be used to study the micro-anatomy of various organs. In summary, we have demonstrated that 1) ER-TR7 outlines the various compartments of peripheral lymphoid organs by characteristic labeling patterns; 2) no such compartments are found in central lymphoid organs; 3) ER-TR7 delineates various types of connective tissue compartments in nonlymphoid organs; and 4) the antigen

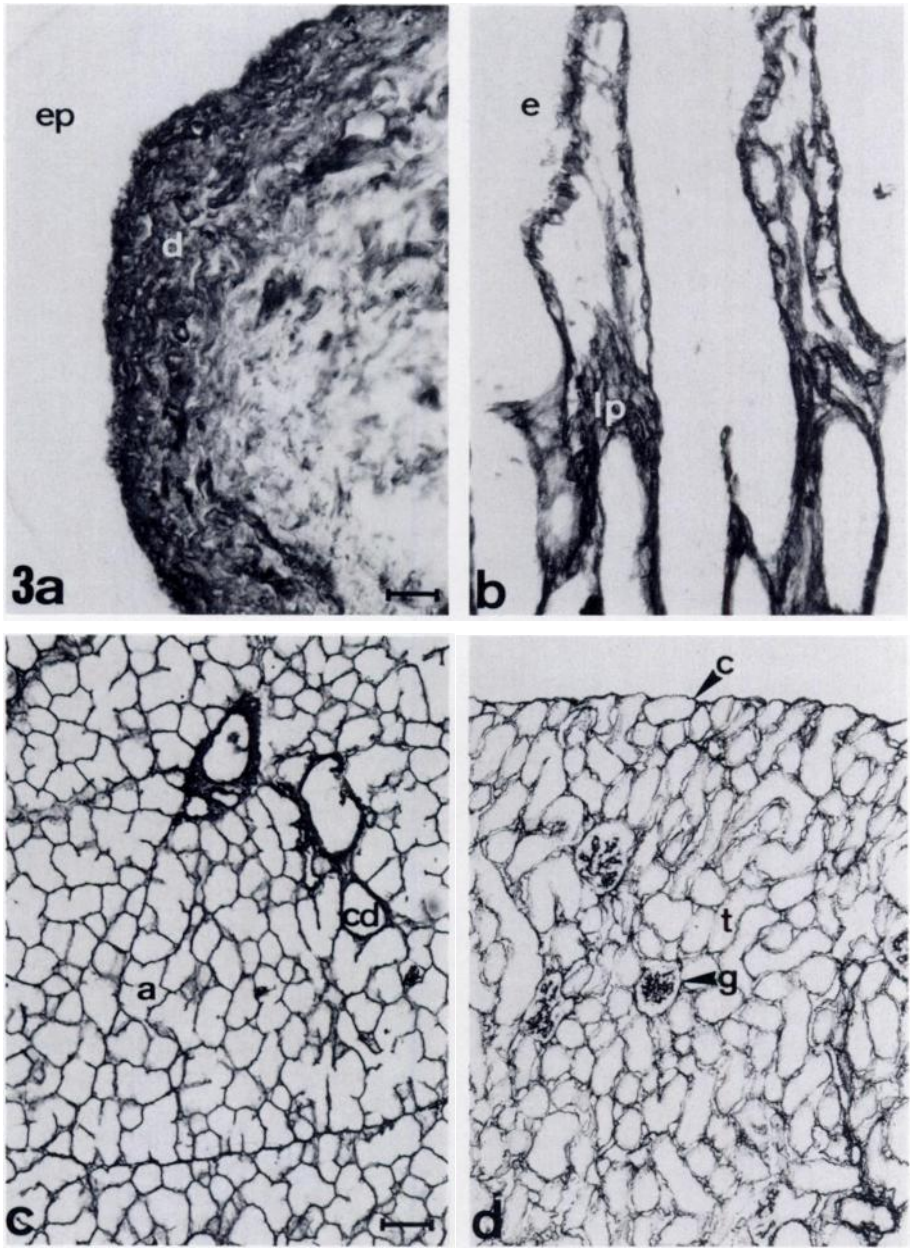

Figure 3. Immunoperoxidase staining pattern of (**A**) skin, (**B**) small intestine, (**C**) salivary gland, (**D**) kidney. a, acinus; c, capsule; cd, collecting duct; d, dermis; e, epithelium; ep, epidermis; g, glomerulus; lp, lamina propria; t, tubules. Original magnifications: **A,B** × 350 (bar = 20 μm); **C,D** × 140 (bar = 50 μm).

detected is not a basement membrane component, nor any major collagen type or fibronectin.

Our results show, furthermore, that ER-TR7 reacts with the basic cellular framework in peripheral lymphoid organs. This cellular framework consists of the reticular fibroblasts, described by Müller-Hermelink et al. (1974), Veerman and van Ewijk (1975), Villena et al. (1983), and their products. The intracellular reactivity of ER-TR7 with fibroblast cell lines and the confluent staining in the dermis of the skin and the lamina propria of the intestine shows that ER-TR7 not only detects intracellular components of fibroblasts, but also reacts with extracellular products. The spleen sections incubated with ER-TR7 and stained by conventional silver impregnation clearly demonstrate that the antigen detected by ER-TR7 has a tissue distribution wider than that of reticulin. The major difference between these two staining procedures is that reticular components in the marginal zone and in red plup are detected by ER-TR7 antibodies but not by silver impregnation. Although the tissue distribution suggests that this antibody detects a major component of the extracellular matrix such as collagen, the fact that ER-TR7 does not react with a variety of collagens, glycoproteins, or basement membrane proteoglycan with comparable tissue distributions demonstrates that ER-TR7 does not detect a strict basement membrane component or any major collagen type, or the biochemically characterized glycoprotein fibronectin.

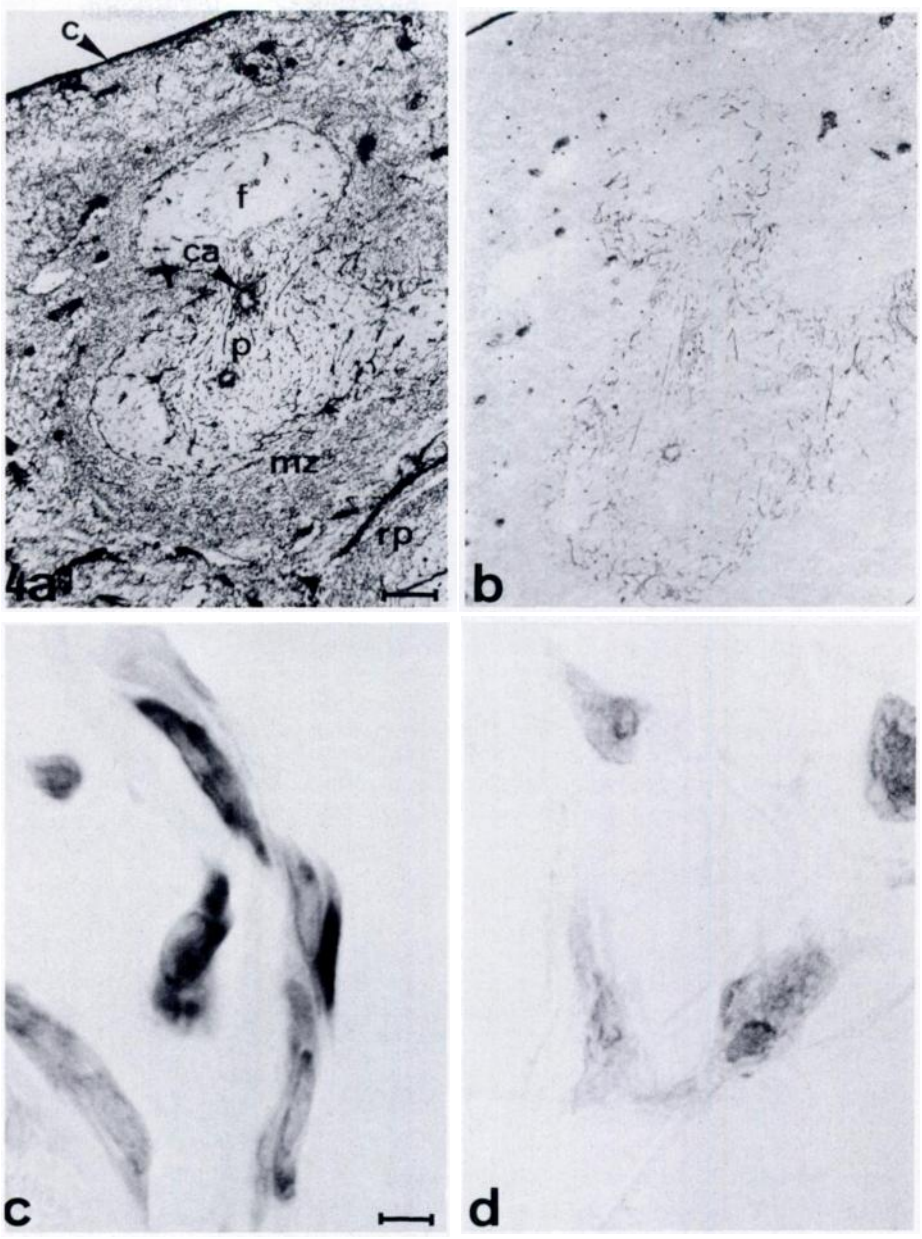

Figure 4. (**A**) Immunoperoxidase staining pattern of spleen with ER-TR7. (**B**) Silver impregnation pattern of spleen. (**C,D**) Immunoperoxidase staining pattern of frozen sections of a pellet of A9 cells, incubated with ER-TR7 (**C**) and a negative control section (**D**). c, capsule; ca, central arteriole; f, follicle; p, periarteriolar lymphoid sheath; mz, marginal zone; rp, red pulp; t, trabeculae. Original magnifications: **A,B** × 60 (bar = 115 μm); **C,D** × 875 (bar = 8 μm).

The tissue distribution of the antigen recognized by ER-TR7 is clearly distinct from those reported for all other biochemically characterized connective tissue macromolecules—the ER-TR7 antigen is a ubiquitous component of stromal (interstitital) matrix cartilage and of at least some basement membrane zones. ER-TR7 is particularly useful as a tool for distinguishing various lymphoid compartments in central and peripheral lymphoid organs. Thus, based on the distribution pattern of ER-TR7-positive fibroblasts, the various compartments in the spleen—red pulp, MZ, PALS, and follicles—and in lymph nodes—paracortex, follicles, and medulla—can be clearly distinguished. This antibody also shows that the thymus, a lympho-epithelial organ, shows only mesenchymal components in the medulla and in the capsule. As can be judged from the staining pattern, these fibroblasts are not only present as components in a general framework structure, but also create various microenvironments in the different lymphoid compartments. In the spleen this is most obvious in the marginal zone and in the central part of the white pulp (T zone). We speculate that this arrangement of fibroblasts participates in two major functions of the spleen—phagocytosis of blood-borne substances (e.g., erythrocytes, antigens) and initiation of the immune response. The dense meshwork in the marginal zone might then function as a filter, slowing the flow of blood into this particular area (see also Veerman and van Ewijk, 1975). By this mechanism,

mononuclear phagocytes associated with this reticular meshwork can optimally clear the in-flowing blood (van Vliet et al., 1985). The typical arrangement of fibroblasts in lymphoid organs might also guide migration of lymphocytes after their entry into the splenic white pulp or into the lymph node paracortex (de Sousa, 1969; Barclay, 1981), and promote the intercellular contact between lymphocytes and the cell types that regulate the ultimate homing of lymphocytes into their respective domains.

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