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Immunohistochemical differentiation between lymphangiographically verified lymphatic vessels and blood vessels

Birthe Mørck Hultberg and Hans Svanholm
Department of Pathology, University Hospital of Odense, DK-5000 Odense, Denmark

Summary. In order to investigate the antigen profile in human lymphatic vessels when compared with blood vessels, postmortem retrograde lymphangiography was done via the thoracic duct on six patients. Formalin fixed, paraffin embedded tissue was stained immunohistochemically for Factor VIII-Related antigen (F VIII R:Ag), with Ulex Europaeus 1 lectin (UEA-1) and for laminin. The results show that the endothelium of blood vessels and lymphatics at all levels of the lymphatic system react positively following staining for Factor VIII-R:Ag and with UEA-1 lectin. The staining for F VIII R:Ag was generally weaker in the endothelial cells lining lymphatic vessels. Staining for the basement membrane component laminin can be used to distinguish lymphatic capillaries and smaller lymphatic collecting vessels from blood vessels.

Key words: Lymphatics – Factor VIII related antigen – Ulex europaeus 1 lectin – Laminin – Immunohistochemistry

Introduction

In surgical pathology vascular invasion is an important prognostic factor in malignant disease. However, by light microscopy, it is difficult or impossible to distinguish between small venules and lymphatic capillaries, and between smaller veins and lymphatic collecting vessels. It is not yet known whether the prognosis of patients with tumour invasion in blood vessels differs from that of patients with tumour invasion in lymphatic vessels. There is disagreement in the literature as to whether endothelial cells lining lymphatic vessels react positively following immunohistochemical staining for F VIII R:Ag and with UEA-1 lectin (Lee et al. 1986; Little et al. 1986; Ordonez et al. 1987; Walker 1985). Futhermore, some studies have indicated that immunohistochemical staining for the basement membrane component laminin can be used to distinguish lymphatic capillaries from small venules as lymphatic capillaries lack a basement membrane (Barsky et al. 1983). The aim of this study was to clarify further the relationship of F VIII R:Ag, UEA-1 and laminin and the human endothelial cells lining smaller lymphatic collecting vessels, capillaries and sinusoids of lymph nodes defined by lymphangiography, to find out whether these markers could be used to separate lymphatics from blood vessels.

Offprint requests to: B. Mørck Hultberg, Department of Pathology, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark

Materials and methods

The study was done on 6 patients on whom autopsy was performed less than 24 h p.m. In each case the thoracic duct was isolated,- as described by Bartholini as early as 1652. Retrograde lymphangiography was done, using 4–5 ml of a mixture of Mixobar gelatine and touluidine blue heated to 70° C. After half an hour the mixture was set, and the aorta with the thoracic duct and surrounding fatty tissue containing lymph nodes was removed. The tissue was fixed in 10% phosphate buffered formalin overnight, and embedded in Paraplast. From the paraffin embedded tissue blocks, 5 micron sections were cut and stained immunohistochemically by the following methods, routinely used in this department.

For the demonstration of F VIII R:Ag, the indirect two-step immunoperoxidase (IP) technique was used. Deparaffinized sections were pretreated with 0,05% trypsin (Sigma type II no. T-8128, in 0,1% $CaCl_2$ ph 7, 8, 37° C) for 20 min. Rabbit anti-human F VIII R:Ag (DAKO, A-082) diluted 1:200 was used as primary antibody, incubated 30 min at room temperature. As the second layer peroxidase-conjugated swine anti-rabbit (DAKO, P-217) diluted 1:20, was used, incubated 30 min at room temperature. For examination with Ulex Europaeus-1 lectin, a three step IP method was used (Holthofer et al. 1982). Deparaffinized sections were incubated for 30 min at room tem-

perature with UEA-1 lectin (DAKO, X 921) diluted 1:200, followed by a 30 min incubation with rabbit anti-UEA-lectin (DAKO, B 279) diluted 1:50. As the third layer, sections were incubated for 30 min with peroxidase-conjugated swine anti-rabbit Ig (DAKO, P 217) diluted 1:50. For demonstration of laminin, the avidin-biotin technique was used (Hsu et al. 1981). Depariffinized sections were pretreated with 0,4% pepsin (Sigma no. P 7012) in 0,1% HCl for 2 h at 37° C. Sections were incubated over night at 4° C with a polyclonal rabbit antibody (Z810) diluted 1:1000. This antibody was raised against human laminin and kindly donated by Albrechtsen, University Department Pathology, Copenhagen. Sections were incubated for 30 min at room temp. with biotin (Vector lab.), followed by incubation with the avidin-biotin complex (Vector lab.) for 30 min.

Negative controls for the staining for F VIII R:Ag included omitting the primary antibody and substitution by normal peroxidase-conjungated swine anti-rabbit serum (DAKO P-127). Negative controls for the staining with UEA-1 lectin included omitting the lectin, the anti-lectin and substitution by normal swine anti-rabbit serum (DAKO P-127).

Control for the staining for laminin included omitting of the primary antibody, biotin and the complex.

All sections were developed with 0,04% 3-amino-9-ethylcarbazol and 0,01% H_2O_2, diluted in 0,05% M acetate buffer, pH 5,0. The sections were counterstained with Mayers haematoxylin and mounted with Aquamount (Bie & Berentsen Ltd, Gurr).

In addition to the material described, sections from 9 lymphatic collecting vessels removed from 8 patients who underwent lymphangiography as a staging procedure for their malignant diseases were examined for F VIII R:Ag and with UEA-1 and antibody to laminin. This part of the material has been used in a prior publication (Svanholm et al. 1984).

The staining was interpreted as being positive when a granular, brownish intracytoplasmic reaction was found in the endothelial cells following staining for F VIII R:Ag. Following staining with UEA-1 lectin a brownish staining reaction was found in the cytoplasm and along the cell membrane and a linear, brownish subendothelial staining reaction was seen following staining for laminin.

In all sections smooth muscle cells and peripheral nerves served as positive built-in controls of the staining for laminin, whereas blood vessels served as positive built-in controls of the staining for F VIII R:Ag and UEA-1.

We defined lymphatic capillaries as vessels containing luminal granular contrast but without surrounding smooth muscle cells. Lymphatic collecting vessels contained luminal contrast and were surrounded by smooth muscle cells.

Results

At the macroscopic, roentgenological (Fig. 1) and light microscopic level (Fig. 2), it was possible to identify lymphatic vessels and lymph nodes containing contrast material. In spite of tissue processing, contrast material was found in the lumen of the thoracic duct, lymphatic collecting vessels, lymphatic capillaries and sinusoids of lymph nodes. Microscopically the contrast material was granular and greyish. At light microscopic level the morphological similarity of lymphatic vessels with venous vessels was striking. The visually recorded results of the immunohistochemical stainings are summarized in Table 1.

F VIII R:Ag was found in the cytoplasm of endothellial cells of all blood vessels and of endothelial cells lining lymphatic collecting vessels (Fig. 3) lymphatic capillaries and sinusoids of lymph nodes (Fig. 4). However, the intensity of staining was weaker and more focal in lymphatic vessels when compared with blood vessels.

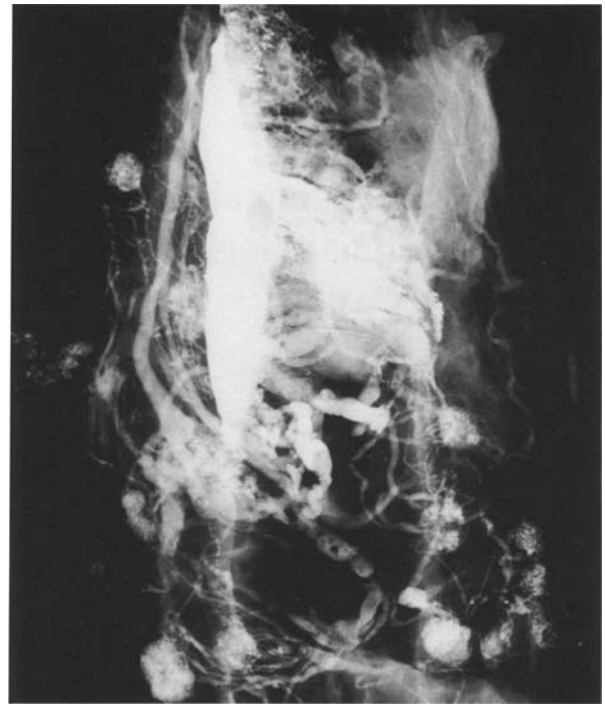


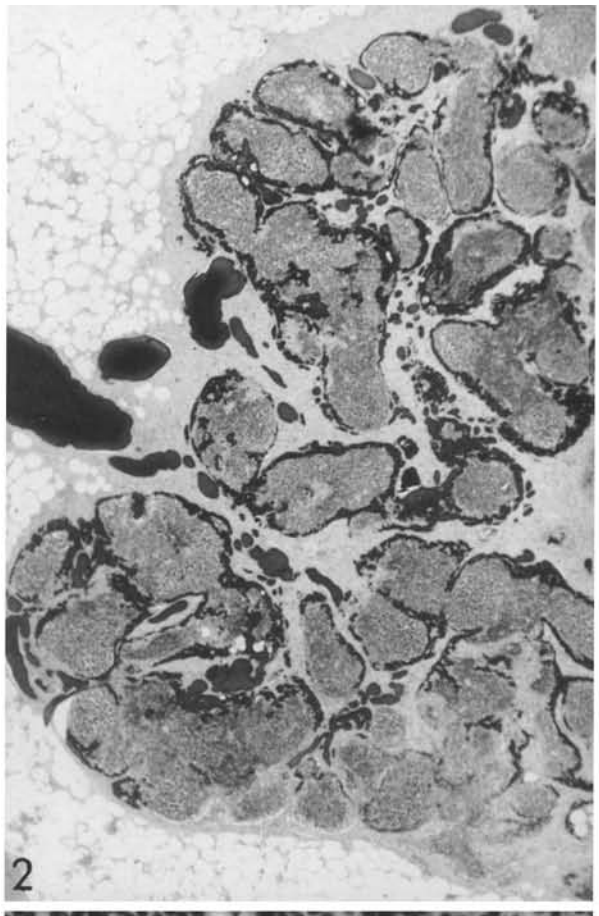
Fig. 1. Lymphangiography of the thoracic duct and adjacent lymph nodes filled with contrast. (× 1)

Fig. 2. A lymph node surrounded by fatty tissue. Dark stained contrast can be seen in the lymphatic collecting vessels, in the sinusoids of the lymph node surrounding the central lymphatic tissue, and in the lymphatic capillaries. (× 20). H & E

Fig. 3. Lymphatic collecting vessel stained for F VIII R:Ag, showing positiv reaction in the cytoplasm of the endothelial cells. The figure also shows strong staining of blood vessels. (× 345)

Fig. 4. Lymphatic capillaries and lymph node sinusoids filled with contrast. Positive reaction in the cytoplasm of the endothelial cells following staining for F VIII R:Ag (*Arrows*). (× 345)

Fig. 5. Lymphatic collecting vessel following staining with UEA-1 showing strong positive reaction of the endothelial cells. (× 345)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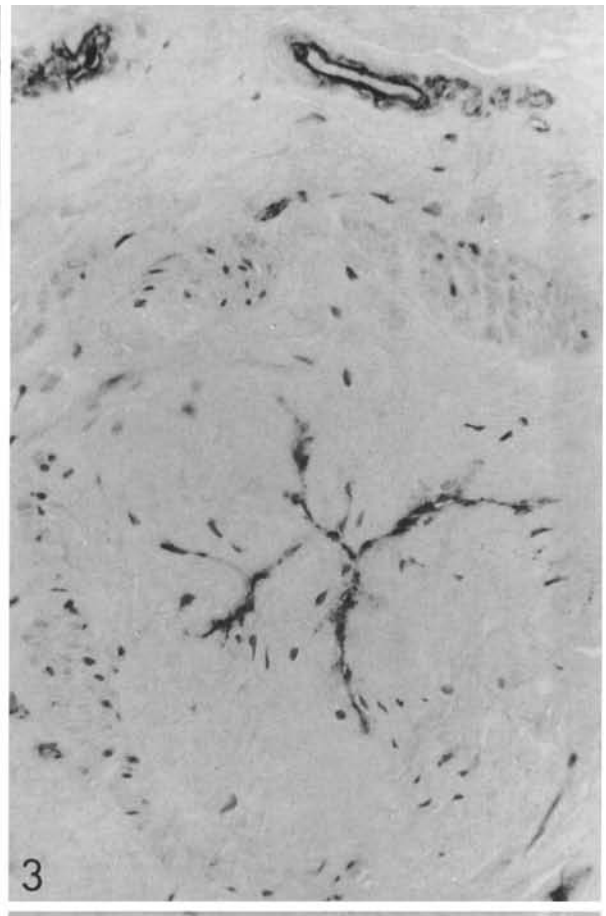
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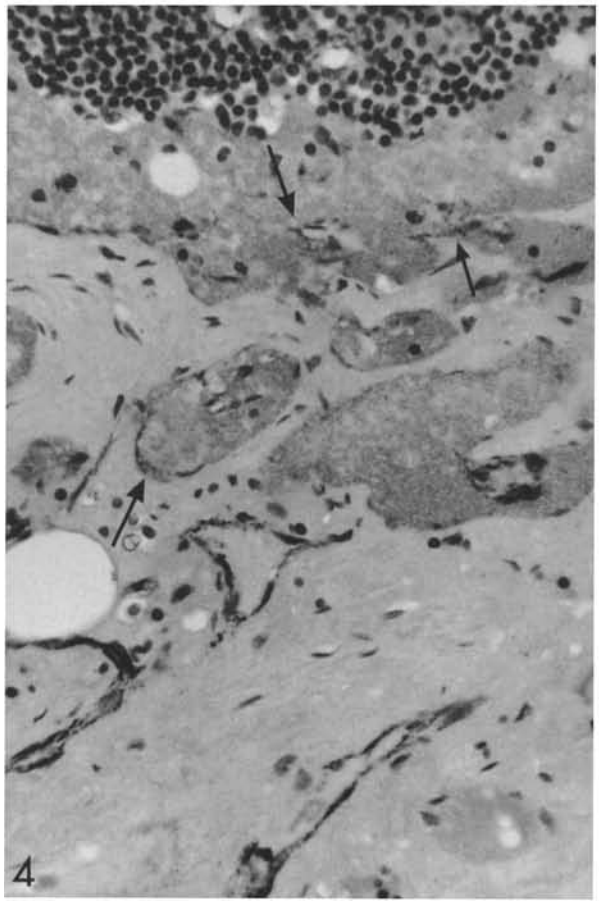
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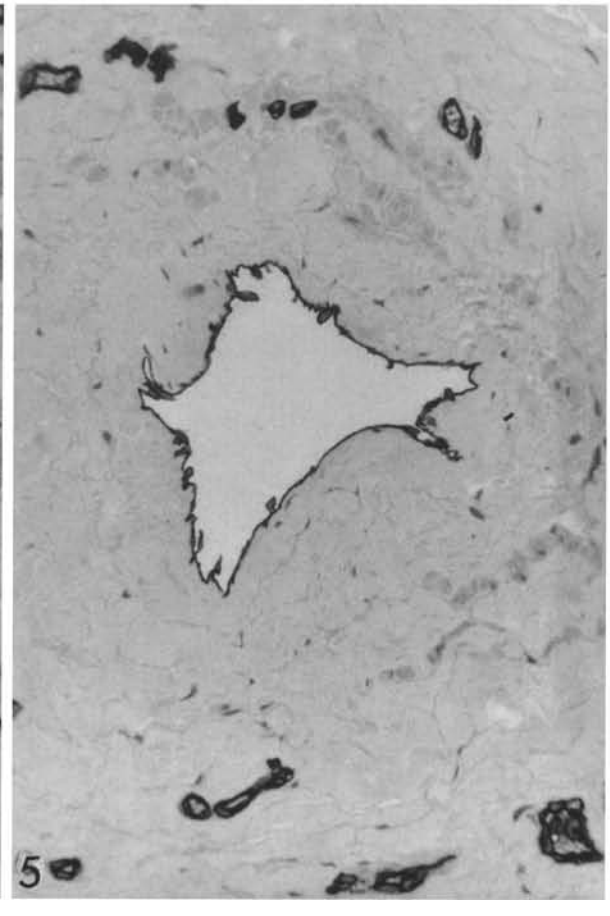
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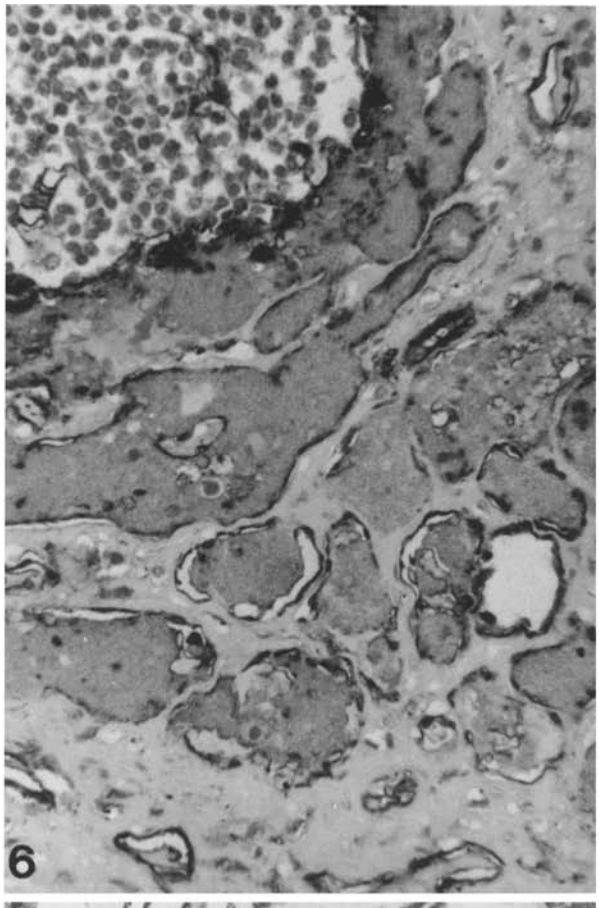
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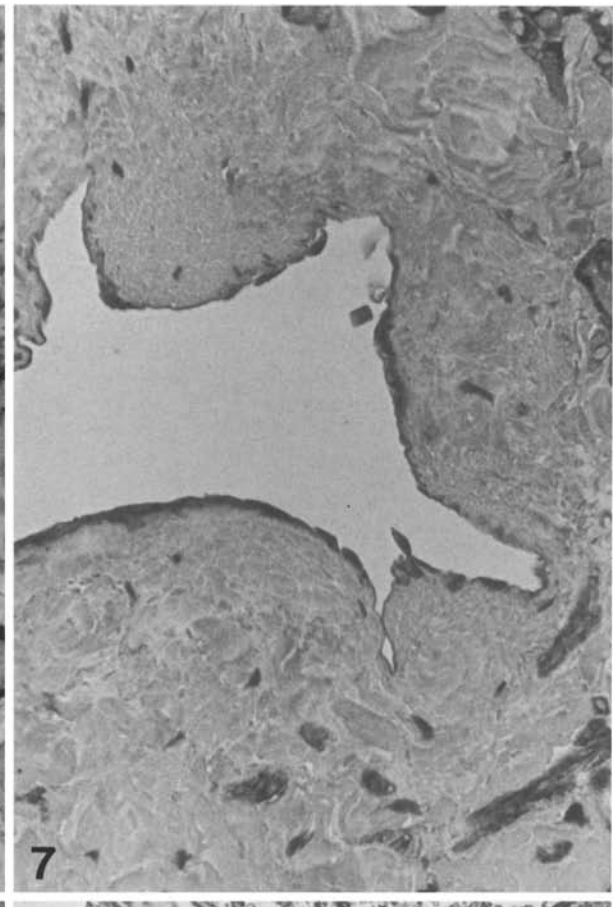
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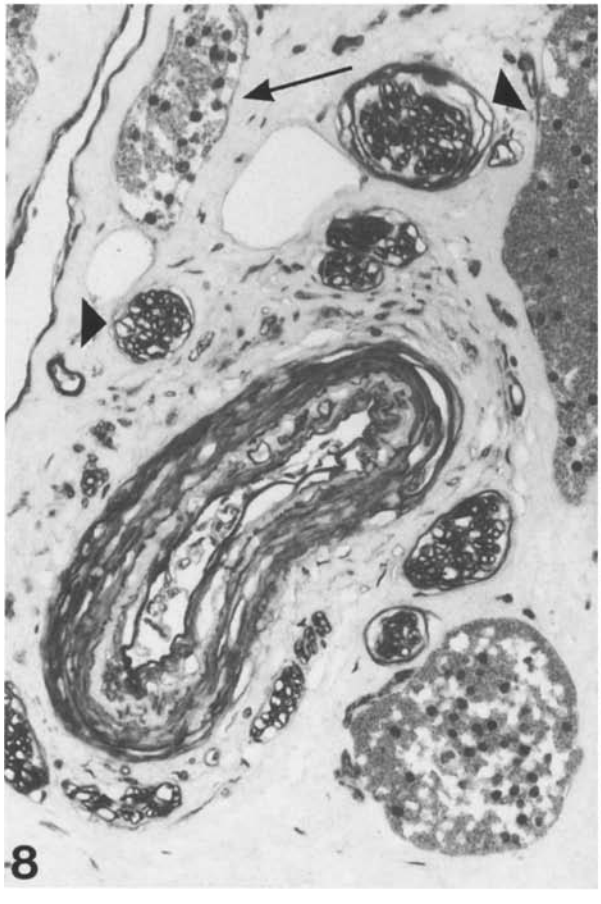
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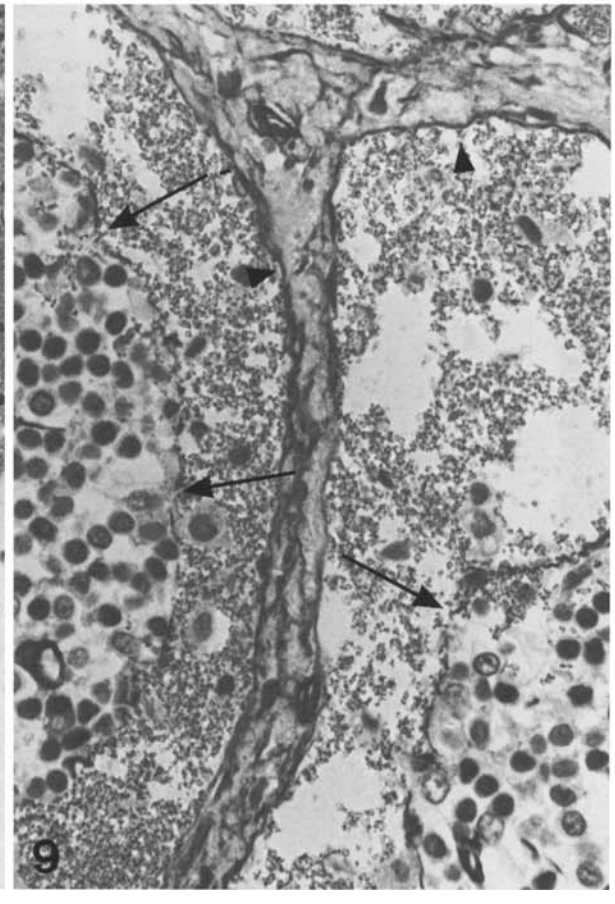
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Table 1. Summary of staining patterns

	Factor VIII R:Ag	UEA-1	Laminin
Lymphatics:			
Small collecting	pos	pos	pos, focal
Capillaries	pos, perinuclear	pos	absent or weak pos. focal
Blood vessels:			
Venules	pos	pos	pos., strong, linear
Capillaries	pos	pos	pos., strong, linear

Following staining with UEA-1 lectin positive reaction was found in the cytoplasm and along the cell membranes of all endothelial cells lining both blood vessels, lymphatic vessels and sinusoids (Figs. 5 and 6). The staining was much more intense when compared with F VIII R:Ag.

Using antibody to laminin a subendothelial, continous, linear reaction was found lining blood vessels of all dimensions. A weaker, often patchy, subendothelial staining was seen in some smaller lymphatic collecting vessels (Fig. 7). Lymphatic capillaries generally stained negatively, but in some capillaries a focal and weak reaction were noticed (Fig. 8), especially located on the valves. In the sinusoids of the lymph nodes a continous, linear staining of varying intensity was found along the subcapsulary sinusoids, whereas a discontinous linear reaction was seen along the marginal sinusoids (Fig. 9).

No differences in staining pattern was observed between the material from autopsies and living patients, and between patients with benign and malignant diseases. All negative controls were without positive staining.

Discussion

This study demonstrates that it is possible to make a retrograde lymphangiography on autopsies and use immunohistochemical techniques.

With F VIII R:Ag our results show a weaker intensity and a more focal staining in lymphatic vessels at all levels when compared with blood vessels. This may be due to the sparse and attenuated cytoplasm of the endothelial cells of lymphatic vessels, combined with differences in staining patterns. Our results as regards F VIII R:Ag are in accordance with the results published by Burgdorf et al. (1981); Sehested and Hou-Jensen (1981); Svanholm et al. (1984); Lee et al. 1986), but are not in agreement with Mukai et al. (1980); Harach et al. (1983); Bettelheim et al. (1984); Beackstead et al. (1985); Howat and Variend (1985); Little et al. (1986) who found negative staining of lymphatic endothelium. Their results may be explained by different time of fixation or different fixatives since this has influence on the immunohistochemical technique (Sehested and Hou-Jensen 1981; Schmitt and Schmidt 1986).

Our results with UEA-1 lectin showed no differences between staining of blood vessels and lymphatics vessels and are in accordance with the results of Beackstead et al. (1985); Little et al. (1986); Lee et al. (1986); Ordonez et al. (1987). However they are not in accordance with the results of Walker (1985). This difference may be due to differences in fixation or staining techniques. At the ultrastructural level it has been found that lymphatic capillaries are not surrounded by a continous basement membrane like that of blood vessels but that it is discontuous and often absent (Leak 1976). Barsky et al. (1983) have demonstrated that in tissue from rats it is possible to distinguish lymphatic capillaries from blood capillaries and our study in Man partially confirms their experimental study. We found that it was possible to distinguish lymphatic capillaries from blood capillaries and blood venules using staining for laminin. Lymphatic capillaries reacted either completely negatively or showed only focal and very weak staining reaction, often located to the valves. Futhermore, we found a focal staining reaction for laminin in some of the lymphatic collecting vessels in which electron microscopy had not demonstrated a basement membrane (Svanholm et al.

Fig. 6. Lymphatic capillaries and lymph node sinusoids filled with contrast. Strong positive reaction following staining with UEA-1. (× 345)

Fig. 7. Lymphatic collecting vessel stained for laminin. Patchy positive subendothelal reaction. (× 550)

Fig. 8. Staining for laminin. Contrast-filled lymphatic capillaries showing absent or focal, weak subendothelial staining reaction (*Arrows*). Blood vessel surrounded by smooth muscle cells and peripheral nerves (*Arrowheads*) showing positive reaction. (× 345)

Fig. 9. Lymph node stained for laminin showing a continous, positive staining along the subcapsulary sinusoids (*Arrowheads*) and a discontinous reaction along the marginal sinusoids (*Arrows*). (× 550)

1984). This suggests that the basement membrane component laminin can be present in lymphatic capillaries and collecting vessels without a fully developed basement membrane being recognizable ultrastructurally. These findings are in accordance with the results of Bendelac et al. (1985) who also described divergence between electron microscopical and immunohistochemical findings. The staining pattern of the basement membrane surrounding the smooth muscle cells along the lymphatic collecting vessels was also of value in distinguishing these vessels from smaller venules. However, for practical light microscopic purposes it is possible by using anti – laminin to separate the strong, continous, linear subendothelial staining reaction of blood vessels from the focal and weak staining reaction of smaller lymphatics.

Our results following staining for laminin on lymph node sinusoids are in accordance with the results of Kartunen et al. (1986) who also found a continous basement membrane lining the subcapsulary sinusoids and a thin and discontinous basement membrane lining the marginal sinusoids.

We conclude that endothelial cells of lymphatic collecting vessels, lymphatic capillaries and sinusoids of lymph nodes all contain F VIII R:Ag. All react positively following staining with Ulex europaeus 1 lectin, the staining with Ulex Europaeus 1 lectin being more uniform and intense when compared with F VIII R:Ag. Neither F VIII R:Ag nor Ulex Europaeus 1 lectin seems to be able to differentiate between blood vessels and lymphatics. However this is the case for laminin which has a strong, linear, continous distribution in all blood vessels in contrast with lymphatic vessels which show absent or focal staining – depending on the type.

We recommend that staining with UEA-1 lectin is used to separate artificial slits from vessels and that staining for laminin is used to separate lymphatic capillaries from blood capillaries and vessels.

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