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DIFFERENTIATION OF LYMPHATICS FROM BLOOD VESSELS IN THE BROAD LIGAMENT OF THE SWINE USING S-100 PROTEIN IMMUNOHISTOCHEMICAL LOCALIZATION IN THE VASCULAR ENDOTHELIUM

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ABSTRACT

We examined the immunohistochemical staining characteristics of S-100 protein in the vessels of the broad ligament of the swine uterus. The endothelial cells of arterial vessels, lymphatics and blood capillaries as well as nerve fiber bundles showed S-100 protein positivity. In contrast, the endothelial cells of veins did not react for the S-100 antiserum. Immunoreactivity for S-100 protein in the endothelial cells of lymphatics did not consistently demonstrate strong staining intensity. Accordingly, we filled lymphatics with colored gelatin before immunohistochemical staining to facilitate identification of lymphatics under light microscopy. Numerous arterioles and capillaries (of which the endothelial cells were immunopositive for S-100 protein) in the lymphatic walls, especially those in the paraovarian vascular plexus, support the existence of a microvascular arterio-arterial rete mirabile network.

Endocrinological studies suggest participation of lymph and blood vessels in the transfer of hormones between the uterus and the ovary within the broad ligament in various animals (1,2) including the swine (3-9). More investigation of morphology is needed, however, with the paucity of reports on the properties of lymphatic vessels in the broad ligament of the swine (10-12). From studies of other species it is known that the lymphatics of the reproductive tract vary in size and shape depending on the stage of the sexual cycle (13,14). In sheep, for example, lymphatics vary from large and filled with concentrated lymph during the luteal phase to narrow and barely detectable during the follicular phase (14).

Morphological studies on lymph vessels are severely hampered by difficulties in proper identification including distinction from blood vessels. Accordingly, we pursued immunohistochemical localization using S-100 protein in the vascular endothelium of conducting lymphatics and blood vessels in the broad ligament of the uterus in swine.

S-100 protein, so named for its solubility in saturated ammonium sulfate solution, was first isolated from the bovine brain (15). For a long time S-100 protein was regarded as specific for the nervous system (16). Later, biochemical and immunohistochemical studies revealed that S-100 protein was a dimer with subunits αα, αß and ßß, and that it was common among various cell types of non-neural tissues (17-19). Although the biological role of S-100 has not as yet been precisely recognized, its presence has been shown in the endothelial cells of lymphatics (20-23), arteries

(22-25) and capillaries of the continuous type (20,24) but not in the venous vessels (22,24). However, Amselgruber et al (20) who studied the cattle testis found immunoreactivity for S-100 protein in the endothelial cells of venous vessels and some immunoreactivity within the endothelium of arterioles, although the reaction was usually faint.

Because no other work describes localization of S-100 protein in the vasculature of the broad ligament of the swine, we examined the specificity of S-100 protein localization in various vessels of the mesometrium, mesovarium, and mesosalpinx and whether lymph vessels could be identified and differentiated from blood vessels.

MATERIALS AND METHODS

Ten mature pigs (about 1 year old, 90-120 kg body weight) from local industrial farms were investigated. The reproductive organs including the broad ligament of the uterus were removed immediately after slaughter. In five, the lymphatics were injected with carmine-gelatin mass (32-40°C) (26); interstitially into the ovary and subserously into the mesometrial margin of the uterine horn at 1-2 cm intervals. Both tissues with lymphatics filled with colored gelatin, and those not filled, were fixed in 10% buffered neutral formalin. Blocks of tissue (10x10 mm) cut out from various parts of the mesometrium, mesovarium and mesosalpinx were dehydrated and embedded in paraffin in conventional fashion. Paraffin sections were serially sectioned with a Reirchert-Jung 2040 microtome into 3-6 μm slices. Some slices were stained with hematoxylin and eosin (HE); others were immunohistochemically treated for localization with S-100 protein. Deparaffinized sections were submitted for immunoperoxidase technique—avidin-biotin peroxidase complex (ABC method—Vectastain® ABC kit Vector Laboratories). The antiserum used was a polyclonal rabbit anti-S-100 protein antibody obtained commercially (Sigma Chemical Co.). The sections were treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and then immersed in non-immune goat serum (diluted 1:10) and bovine serum albumin (diluted 1:100) to inhibit nonspecific binding of immunoglobulins. The sections were incubated with anti-S-100 protein serum (diluted 1:700) for 24 hours and then processed with the ABC method. Finally, the sections were treated with 3.3' diaminobenzidine. Some sections were counterstained with hematoxylin for better visualization of cell nuclei. All reactions were performed at room temperature. Normal goat serum and bovine serum albumin were used instead of the primary antibody as a control—the results were negative also using nerve fiber bundles. Observations and photographs were made with a light microscope (Nikon FXA, Japan).

RESULTS

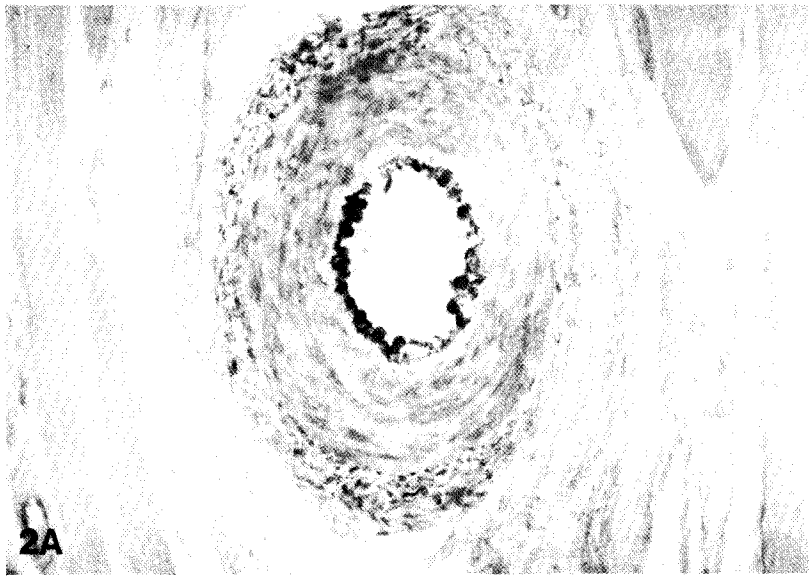
Immunoreactivity for S-100 protein in the broad ligament of the uterus of the swine was seen in the endothelium of both lymph and arterial vessels as well as nerve fiber bundles. The endothelium of venous vessels was immunonegative for S-100 protein *(Figs. 1A and B)*. Colored deposits of S-100 protein in the endothelial cells of lymphatics, visible also where lymphatics had a collapsed lumen *(Fig. 1B)*, permitted their positive identification. Additional counterstaining of slices with hematoxylin for better visualization of the cell nuclei corroborated a negative reaction to the presence of S-100 protein in the endothelium of veins and a positive reaction in the endothelium of lymph vessels *(Fig. 1B)*

A highly positive reaction for S-100 protein in the form of condensed brown precipitate was uniformly found in the endothelium of arteries in the broad ligament especially uterine and ovarian arteries and their large branches *(Figs. 1-3)*. There was a clear difference in the immunoreactivity of the endothelia of arteries and veins comparing adjacent slides containing the tissues of ovarian artery and branches of the utero-

Fig 1. Overview of various vessels of the mesometrium of the swine: positive reaction for S-100 protein in the endothelium of artery (A), arteriole (a), lymphatics (L) including those with narrow lumen (arrowheads) and nerve fiber bundles (N), and negative reaction in the endothelium of venous vessels (V). Condensation of reaction products around the cell nuclei counterstained with hematoxylin. 1A x250, 1B x500

ovarian vein, stained either with hematoxylin-eosin *(Fig. 3A)* or immunostained *(Fig. 3B)*. Venous endothelial cells did not demonstrate colored products of immunohistochemical reaction to S-100 protein whereas in the adventitia such reaction was seen only in tiny vessels (probably arterioles) and in nerve fiber bundles. In the artery, however, a positive reaction was evident in the endothelium and tiny adventitial nerves. Similar pheno-mena were observed in other parts of the swine broad ligament.

Determining the paths of lymphatic vessels filled with carmine-gelatin mass *(Fig. 4A)* further confirmed localization of S-100 protein in the lymphatic endothelium *(Fig. 4B)*. Interstitial injection with carmine-gelatin mass did not consistently fill all the



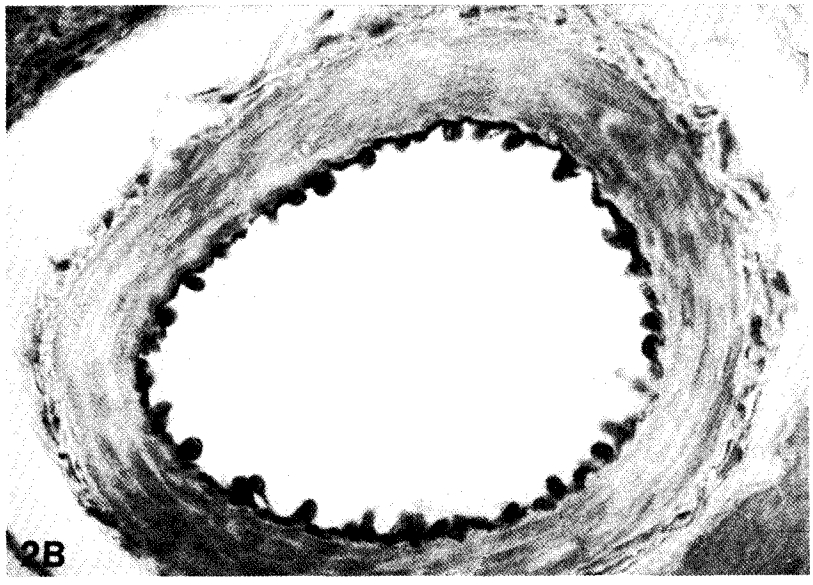


Fig 2. Cross-section of the uterine artery (2A) and ovarian artery (2B). 2A x250; 2B x500.

lymph vessels in a relevant area and consequently in identifying all the lymphatics. Here, however, the brown deposits of S-100 protein localized in the endothelial cells of the vessel served as the indicator for a lymphatic *(Fig. 4B)*. By these criteria, thin-walled, size-diversified lymphatic segments of various parts of the broad ligament of the swine were demonstrated *(Fig. 5A-C)*. Despite a delicate dash-like appearance for cross-section endothelium, a positive reaction for S-100 protein was clearly marked especially in slightly protruding nuclear areas of cells. Thus, the endothelium of lymphatics typically looked like a thin broken line which made it readily distinguishable from arterial and venous vessels *(see Figs. 5A-C and 6A-C)*. It should be emphasized, however, that the

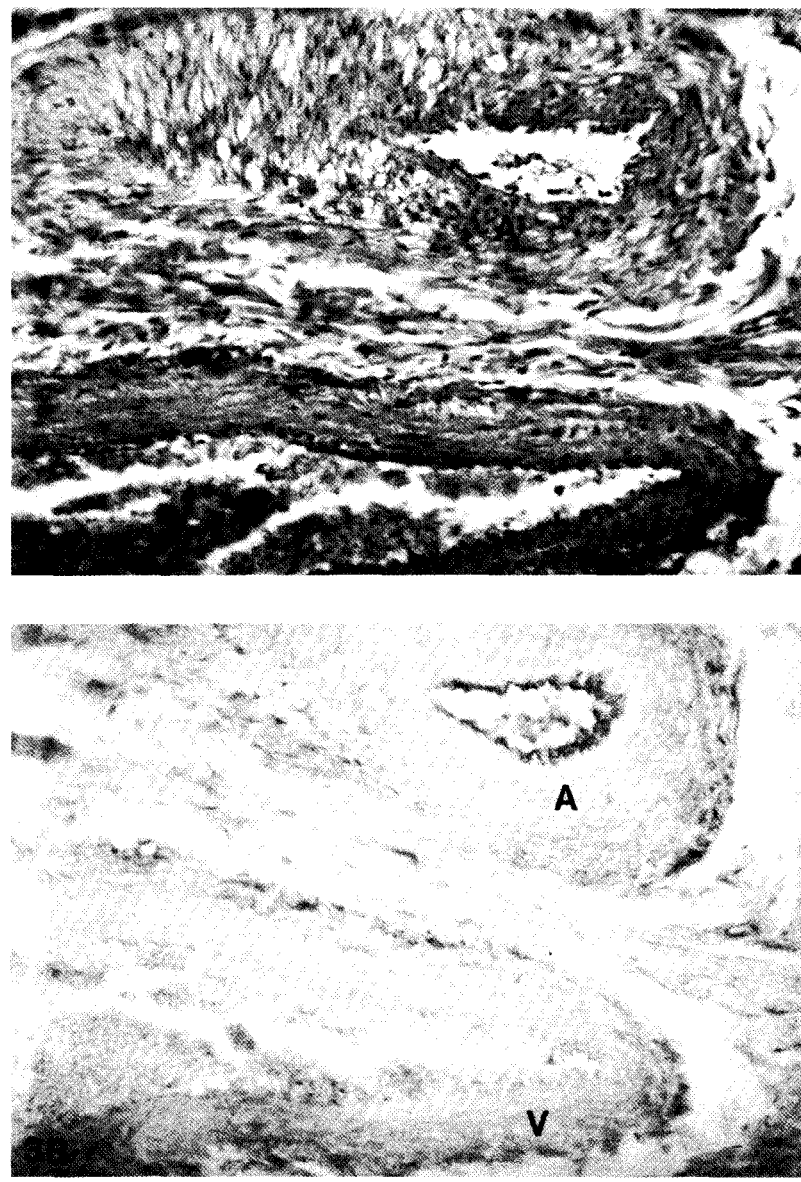


Fig 3. Sequential sections of the ovarian artery and branches of the utero-ovarian vein: (3A) - staining with hematoxylin-eosin; (3B) - immunohistochemical staining for S-100 protein positive only for arterial endothelium. 3A and 3B x250.

characteristic thin broken line of immunoreactivity was not due to counterstaining of the nuclei because the slices on *Figs. 5A-C and 6A-C* were not treated with hematoxylin.

Segments of lymphatics, though varied in size depending on the location within the ligament (e.g., smallest in the mesosalpinx—*Fig. 6A*, and largest in the mesovarium—*Fig. 6B*), invariably showed positive reactivity for S-100 protein not only in the endothelium but also in the endothelium covering intraluminal valves of the lymphatics *(Fig. 6B).*

DISCUSSION

Immunoreactivity of endothelial cells of lymph and arterial vessels of the broad ligament of the swine to S-100 protein verify

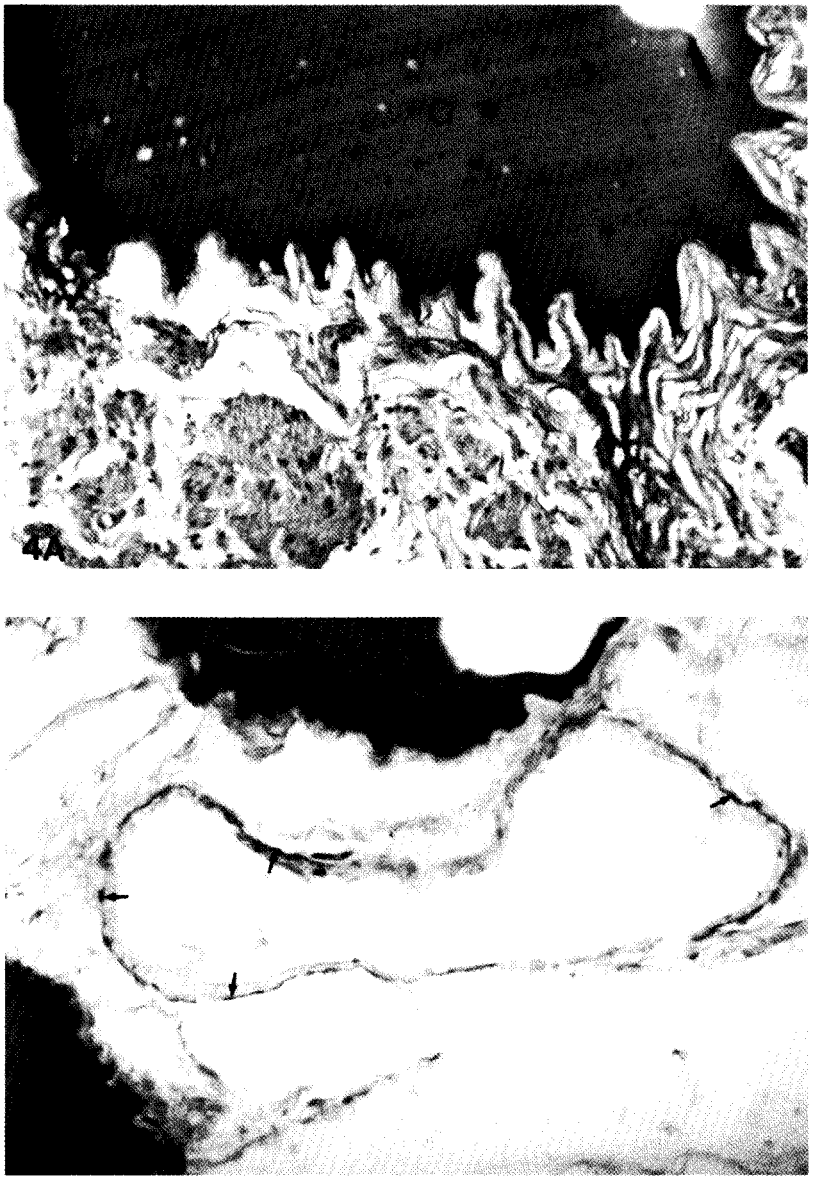
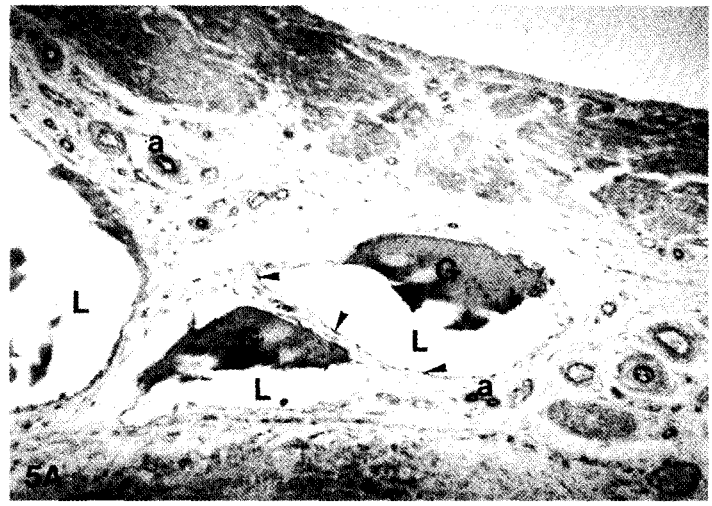
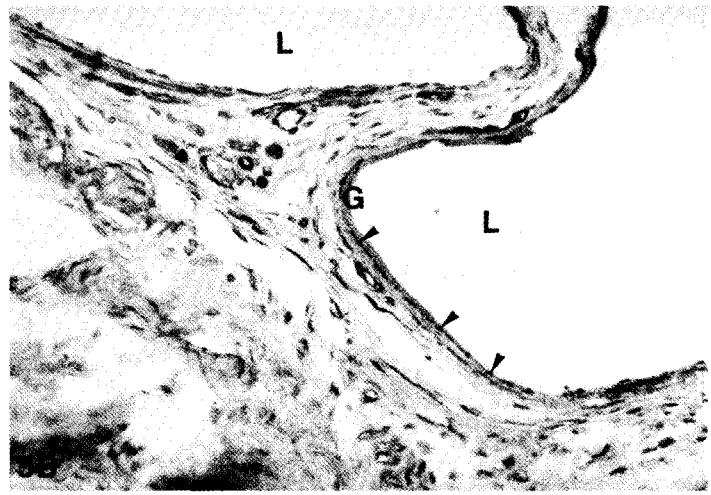


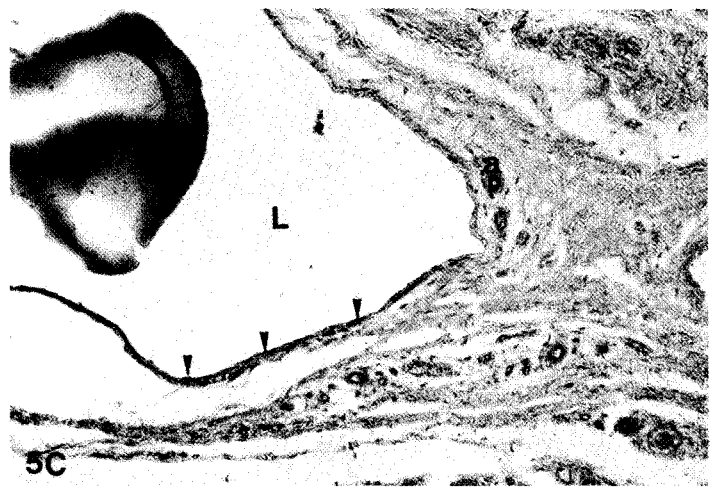
Fig 4. Fragment of a lymphatic of the mesometrium after filling with carmine-gelatin and H&E staining (4A) and lymph vessels of the mesovarium with carmine-gelatin in the lumen and positive reaction for S-100 protein (arrows) in the endothelium (4B). G - gelatin. 4A x125; 4B x250.

that this protein is not specific for the nervous system as previously assumed (18,19). On the other hand, our studies document that in the broad ligament as in other organs the endothelial cells of lymphatics (20-23) and arteries (22,24) are immunoreactive for S-100 protein whereas venous endothelium (22,24) is non-reactive. Our results are similar to those obtained in cattle ovarian vasculature (22) and other swine organs (24). Others report that immunoreactivity for S-100 protein is strongly positive in arterial endothelium cells (classified as subunit ß) but not in other cell components except for the adjacent nervous system innervating the walls of these vessels. This observation was confirmed by the changes in the products of reaction to S-100 in the endothelium of the arteries of the broad

Fig 5. Lymphatics from various regions of the broad ligament after application of double method of identification: segments of lymph vessels under the muscular layer of the mesometrium (5A), the lymphatic from the middle part of the paraovarian vascular plexus (5B), the lymphatic of the mesovarium (5C). L=lymphatic, a=arteri-ole, G=gelatin. Arrowheads point to a positive reaction to S-100 protein in endothelial cells of lymphatics. 5A and 5C x125; 5B x250.







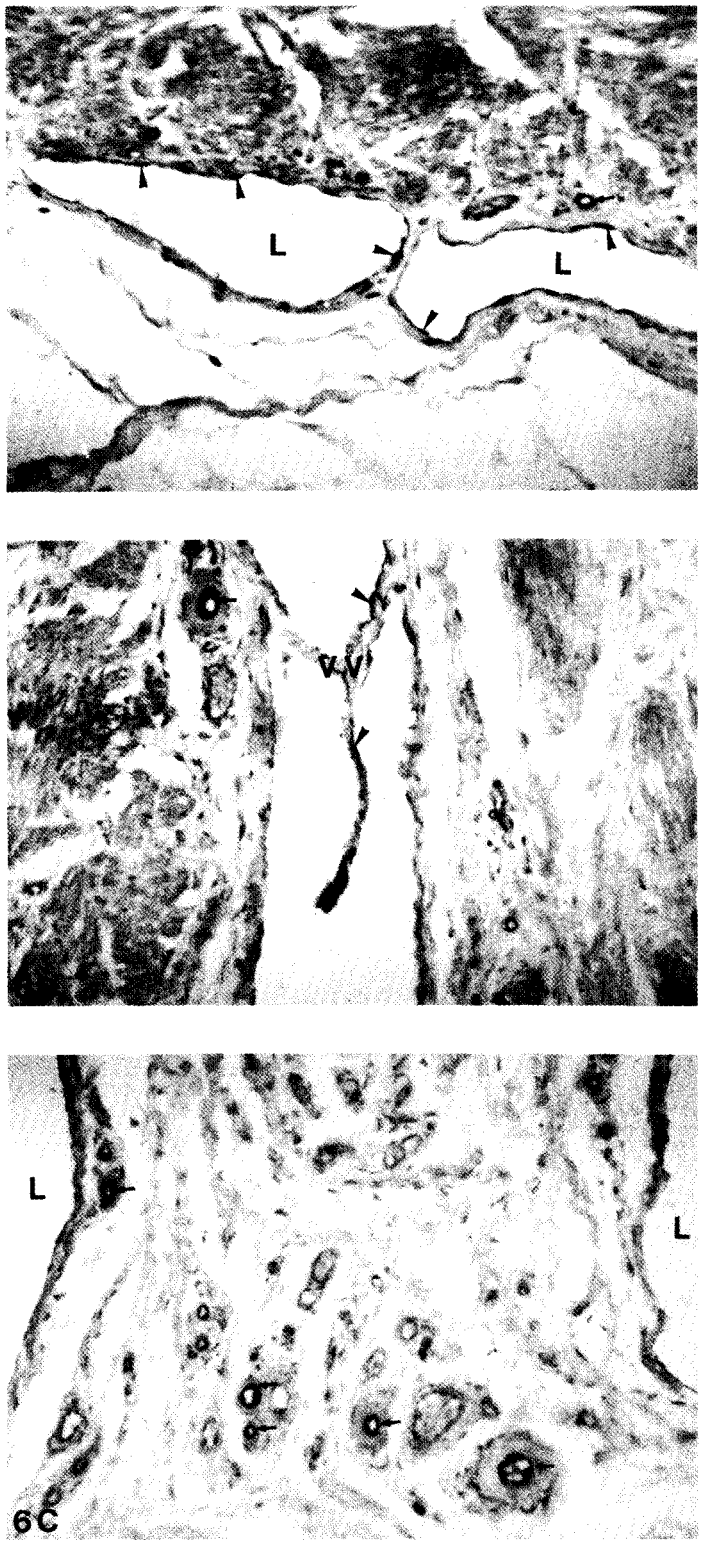


Fig 6. Various sized segments of lymphatics (L) of the mesosalpinx (6A), mesovarium (6B) and mesometrium (6C) of the swine. Clearly visible immunoreactivity in the endothelium cells (arrowheads) and in numerous arterioles (arrows). VV=lymphatic valve. 6A-6C x250.

ligament. Although there are no available reports on the localization of S-100 protein in the endothelium of arteries, we observed a highly positive condensation of the color reaction product primarily around the cell nuclei and also in the narrow parts of the endothelial cytoplasm.

Reaction for S-100 was consistently negative in the endothelium of variably sized venous vessels in various regions of the swine broad ligament. In veins of the tongue, stomach, small intestine, pancreas, kidney and lymph nodes of the swine and cow, Iwanaga et al (24) showed that the endothelial cells failed to react for S-100 antiserum specific for subunits α and ß with marginally visible immunoreactivity for subunit ß. They also found variable intensity of immunoreactivity for subunit ß of S-100 protein in the capillaries with continuous endothelium and negative immunoreactivity in fenestrated endothelium. Similar results for venous and capillary ovarian vessels were reported by Kamiya et al (22) who used polyclonal antibodies. On the other hand, Amselgruber et al (20) found S-100 protein in the endothelium of veins and capillaries of the continuous type in the testes of the cattle but absent or weak color reaction in the arterial endothelium. Some divergency in the localization of S-100 protein may occur among species, organ or endothelium function of blood vessels although separate reactions by polyclonal antibodies in relation to S-100 protein specific monoclonal antibodies (19) cannot be excluded.

Within the broad ligament of the swine, control preparations revealed negative reactivity not only in the endothelium but also in the nerve fiber bundles. Lack of immunoreactivity in the endothelium of venous vessels was typical as in other organs of the swine where veins revealed no S-100 protein even when monoclonal antibodies were used (24). As we observed, highly immunopositive reaction for S-100 protein was seen in the capillaries and other microvessels in the walls of or adjacent to lymphatics. The photomicrographs of these vessels suggest that they belong to the arterial system and furthermore support the existence of an arterio-arterial network on the lymph vessels of the paraovarian vascular plexus (10). This anatomic arrangement in turn supports the concept of a countercurrent transfer of materials (e.g., hormones) between the uterus and the ovary (6,7).

Localization of S-100 protein using color light micrographs and after extra tracing of the vessel path with carmine-gelatin substance favors positive immunoreactivity in the endothelium of lymphatics. Others (20-23) confirm that the endothelium cells of lymphatics contain S-100 protein. Although the role of the S-100 protein in endothelial cells of lymphatics has not as yet been determined, it may be involved in the mechanism of transcytosis of fatty acid as Haimoto et al (18) and Iwanaga et al (24) suggest for S-100 protein in arterial endothelium.

In summary, immunohistochemical testing with S-100 protein may be applied to other studies of the broad ligament of the uterus of the swine, renders it easier to identify lymphatics even those with a narrow or closed lumen, and aids in differentiating lymphatics from venules.

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