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ENZYME TRIPLE STAINING FOR DIFFERENTIATION OF LYMPHATICS FROM VENOUS AND ARTERIAL CAPILLARIES

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ABSTRACT

5'-nucleotidase (5'-Nase)-dipeptidyl aminopeptidase IV (DAPase)-alkaline phosphatase (ALPase) triple staining was used to differentiate lymphatics from venous and arterial capillaries in a variety of mammalian tissue sections including human. This triple staining method facilitates specific identification under a light microscope of 5'-Nase activity in lymphatics, DAPase activity in venous capillaries and venules and ALPase activity in arterial capillaries and arterioles. This technique depicts initial lymphatics more clearly and extensively than other methods so far reported although some interspecies and tissue differences are obtained in each enzyme activity.

Previously we developed an enzyme-histochemical method with 5'-nucleotidase (5'-Nase)-alkaline phosphatase (ALPase) double staining to distinguish between lymphatics and blood capillaries on frozen sections (1-3), glycol methacrylate (JB-4) sections (4) and whole mount preparations (5) in a variety of tissues in laboratory animals and human. This enzyme staining using the heavy metal (lead) method could also be employed in conjunction with histochemical scanning and transmission electron microscopy (6,7). Although not all blood vessels react with ALPase staining, arterial blood vessels demonstrate predominantly high ALPase activity. These observations suggest that ALPase staining has variable but distinct degrees of heterogeneity for the enzyme activity of blood vessels. Most venous blood vessels have no ALPase activity and also little 5'-Nase activity. Thus, differential staining for venous blood vessels is needed to delineate lymphatics from blood vessels more reliably than 5'-Nase-ALPase double staining alone. Recently, Lojda (8) and Grim et al (9) have developed a histochemical procedure of dipeptidyl aminopeptidase IV (DAPase)-ALPase double staining, which makes it possible to distinguish between venous and arterial capillaries. In this context, this paper examines the differential triple (5'-Nase-DAPase-ALPase) staining for lymphatic, venous and arterial capillaries.

MATERIALS AND METHODS

Experimental animals

The following species, three or four animals in each, were used in these experiments: rats (Wistar), hamsters (Golden), mice (BALB/c), guinea pigs (Hartley), rabbits (domesticus), monkeys (Macaca fuscata), cats, dogs, cows, and pigs. After these animals were anesthetized with sodium pentobarbital according to the Guidelines for Animal Experimentation, Oita Medical University, several organs were excised: heart, tongue, stomach, intestine, spleen,

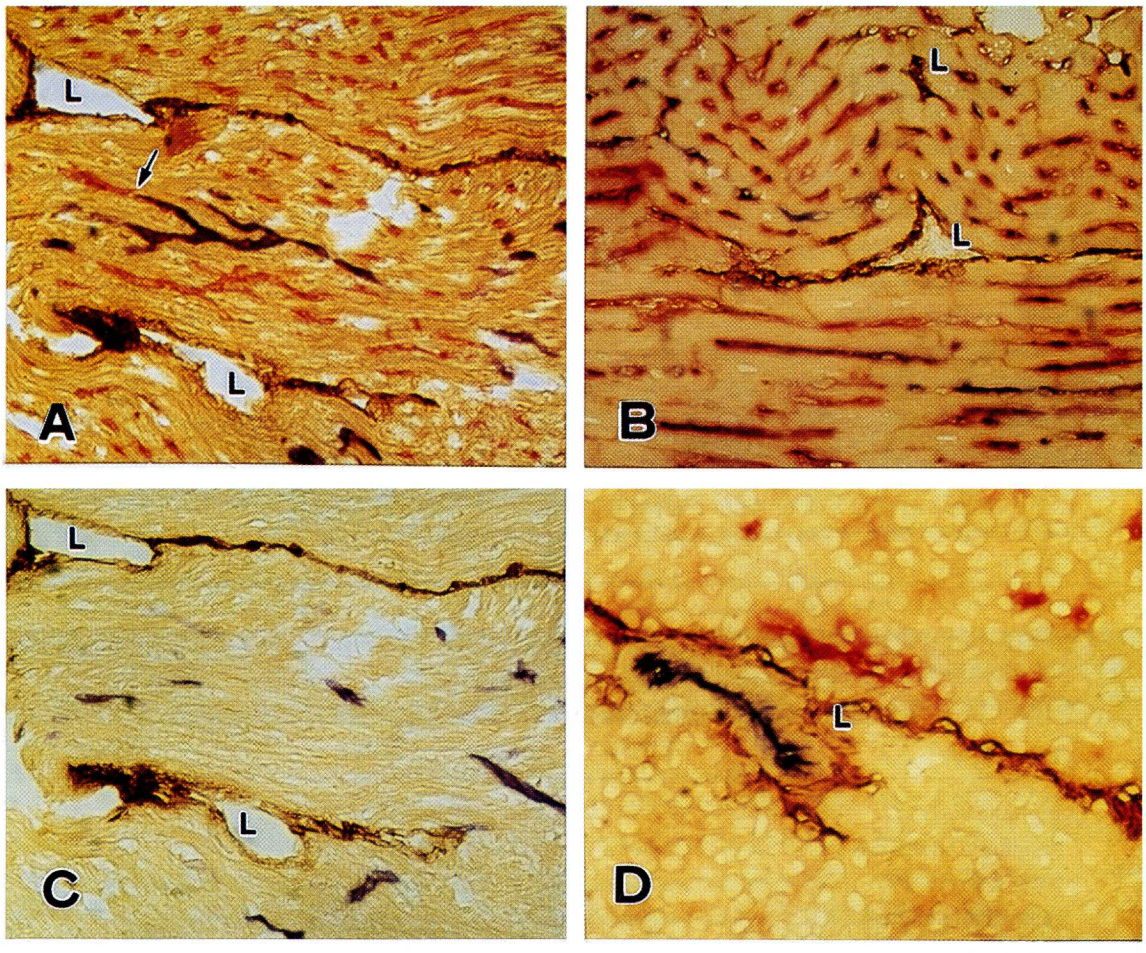

Fig. 1. Light micrographs of plastic (A,C) and cryostat (B,D) sections stained with enzyme triple (A,B,D) or double (C) staining for 5'-Nase-positive lymphatics (L, dark brown), DAPase-positive venous capillaries (red) and/or ALPase-positive arterial capillaries (blue). A: Monkey heart. An arrow shows the transitional segments of the arterial and venous capillaries. B: Rat heart. C: An adjacent section of A. D: Monkey pancreas. 260x.

lymph node, liver, pancreas, kidney, aorta, cisterna chyli and thoracic duct. Human subjects, skin and synovial membrane, were also collected at the time of operation in our University hospital.

Preparation of tissues for enzyme histochemistry

Some tissues were embedded in OCT compound (Miles, USA) and frozen at -80°C. The others were fixed by immersion with a chloroform-acetone mixture and embedded in the JB-4. Serial frozen or plastic sections, 7μm thick, were cut in a cryostat or with a steel blade on a microtome.

For 5'-Nase activity of lymphatics, the specimens were stained with the standard medium for the lead method of Wachstein and Meisel (10) as reported previously (3,4). The reaction medium contained 5'-adenosine monophosphate (Sigma) as substrate, lead nitrate (Taab) as capturing agent and L-tetramisole (Sigma) as an inhibitor of non-specific ALPase. For DAPase activity of venous blood vessels, the specimens were also

TABLE 1
5'-Nase, DAPase and ALPase Activity of Three Types of Vessel†

Enzyme Staining	Lymphatics	Venous Capillaries Venules	Arterial Capillaries Arterioles
5'-Nase-DAPase-ALPase	++*	++	++
DAPase-5'-Nase-ALPase	++	++	++
DAPase-ALPase-5'-Nase	++	++	++
5'-Nase-ALPase-DAPase	++	–	++
5'-Nase-ALPase	++	-/+	++
5'-Nase	++	±	/+
DAPase	±	++	–
ALPase	–	±	++

†tissue sections fixed in a chloroform-acetone fixative
*++: strongly positive, +: positive, ±: weakly positive, –: negative

TABLE 2
5'-Nase, DAPase and ALPase Activity of Various Vessel Types

Types of Vessel	5'-Nase	DAPase	ALPase
Lymphatic vessels			
lymphatic capillaries	++*	±	–
lymphatic sinus[1)]	–/+	–	–
central lymphatics[2)]	++	–	–
collecting lymphatics	+	±	–
cisterna chyli	+	±	–
thoracic duct	+	±	–
Blood vessels			
small arteries	±	–	+
arterioles	±	–	++
arterial capillaries	–	–/+	++
venous capillaries	±	++	–/+
splenic sinus	–	±	–
hepatic sinusoid	±	±	–
venules	±	++	–
high endothelial venules	–/+	–	–
small veins	–	±	–

[1)]lymph nodes, [2)]intestinal villi
*++: strongly positive, +: positive, ±: weakly positive, –: negative

TABLE 3
5'-Nase, DAPase and ALPase Activity in Various Mammals

Animals	5'-Nase (lymphatics)	DAPase (venous cap.)	ALPase (arterialcap.)
Monkey	++*	++	++
Mouse	++	±	++
Rat	++	++	++
Guinea pig	++	–/++	++
Hamster	++	++	++
Rabbit	±/+	±	+
Cat	±/–	ND	+
Dog	±/–	ND	±
Cow	++	++	++
Pig	++	++	++
Human	+/++	++	++

*++: strongly positive, +: positive, ±: weakly positive, –: negative
ND: not determined, cap: capillary

stained according to the azo-dye method (8) with a slight modification. Briefly, the reaction medium for DAPase contained 12-16 mg of Glycyl-L-Proline-4-Methoxy-ß-Naphthylamide (substrate: Bachern Feinchemukalien, A.G. Bubendorf, Switzerland) dissolved in 2 ml of NN'-dimethylformamide (Sigma) and 40 mg of o-Dianisidine tetrazotized (Fast blue BN, Sigma) dissolved in 40 ml of 0.1 M phosphate buffer (pH 7.3). The specimens were incubated in the reaction medium for 2 hrs at 4°C. For ALPase activity, the specimens were stained for 20 min to 1 hr at 4°C according to the azo-dye method of Burstone (11) with a slight modification (2) using naphthol AS-MX phosphate (disodium salt, Sigma) as substrate, along with fast blue BB (Sigma) as simultaneous coupler.

After incubation for 5'-Nase and/or DAPase activity, the specimens were further incubated in the reaction medium for ALPase activity as double or triple staining. The reaction medium was altered as control experiments for DAPase, being substrate free or containing inhibitor (E600) as described for 5'-Nase and ALPase (2,3).

RESULTS AND DISCUSSION

5'-Nase activity was consistently seen as a dark brown precipitate of the reaction product on the walls of lymphatics on the tissue sections of the experimental animals, whereas little or no activity was detected on the walls of the blood vessels. When the section was then incubated to demonstrate DAPase activity, walls of the blood vessels were visualized in red. The dark brown and red staining provided a clear distinction between lymphatics and venous capillary segments. On the same section stained further with ALPase staining (5'-Nase-DAPase-ALPase triple staining), ALPase-positive arterial capillaries (blue) were clearly differentiated from lymphatics and venous capillaries *(Figs. 1A,1B)*. The adjacent sections were incubated either for 5'-Nase,

DAPase or ALPase activity separately in order to facilitate coloring and demonstrate the activity of each enzyme. *Fig. 1C* shows the 5'-Nase-ALPase double staining section adjacent to that in *Fig. 1A*. For the combination staining of three enzymes, DAPase staining was performed first and followed immediately by ALPase, before or after 5'-Nase staining *(Table 1)*. Neither 5'-Nase and ALPase staining changed, regardless of whether or not the section had previously been stained with DAPase staining. However, when ALPase staining was followed by the DAPase procedure, that is, in the case of 5'-Nase ALPase-DAPase triple staining, DAPase activity was weak or absent in the venous capillaries and venules *(Table 1)*.

Table 2 shows enzyme-histochemical staining for 5'-Nase, DAPase and ALPase activity in several tissues. Some differences in the stainability of these three enzymes were noted in various types of vessels, but there were only few interspecies differences *(Table 3)*.

Based on these findings, certain staining characteristics emerge. Vascular DAPase-positivity is more prominent than ALPase-positivity *(Figs. 1A,1B,1D)*. In longitudinal section we were able to follow ALPase activity of the arterial endothelial wall from the smallest arteriole to a region of the capillary bed that stained exclusively for ALPase. Some blood capillaries reacted both for DAPase and ALPase with the reactivity of one enzyme usually more distinct than that of the other *(Fig. 1A, arrow)*. Such examples probably represent transitional segments of the capillary network. The longer the relative incubation time the longer the area of the capillary bed that demonstrated positive reactivity to both enzymes. Accordingly it is important to use a standardized incubation time for purposes of quantification. Nonetheless, the triple staining method as outlined is useful to differentiate lymphatic from venous and arterial capillaries on the same specimen in a variety of mammalian tissues. Moreover, the triple staining technique makes the vascular distinction of lymphatics from blood vessels clearer than any other method to date using light microscopy.

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