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Vascular Permeability in the Thymus of the Mouse

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Summary. In this study, thymic blood vessels in adult mice were examined particularly in relation to the permeability of their walls. The pattern of distribution of the thymic vessels indicated that the cortex is supplied, for the most part, with capillaries, and the medulla with venules.

Cortical capillaries and medullary venules are usually encircled by epithelial reticular cells, but the investment is not completely continuous. Endothelial cells of cortical capillaries and medullary venules contain numerous micropinocytotic vesicles. In addition, larger vacuoles are also seen in the endothelial cells of the venules. After intravenous injection of ferritin, these micropinocytotic vesicles and vacuoles in the endothelial cells of the thymic vessels are labeled by ferritin. Ferritin particles also appear in the perivascular spaces of the thymic vessels. The vascular permeability in the thymus is different between cortical capillaries under the capsule, capillaries in the inner cortex and venules in the medulla. The injected ferritin in only limited amounts also invades the thymic parenchyma especially in the outermost cortex and the medulla. The permeability of ferritin across the walls of thymic blood vessels was compared with that in the splenic lymphatic tissue, mesenteric lymph node and pancreas. The results obtained were discussed with special reference to the vascular barrier mechanism in the thymus.

The existence of a blood barrier in the thymus has been postulated on the basis of the earlier findings that the thymus, unlike other lymphatic organs, does not show significant histologic changes associated with immune reactions to blood-borne antigens, whereas it responds to antigens injected directly into the organ (MARSHALL and WHITE, 1961). The barrier was previously thought to be ultrastructurally established by a continuous investment of epithelial reticular cells around the walls of the thymic blood vessels (CLARK, 1963; WEISS, 1963). However, it has been demonstrated by closer electron microscopy that the epithelial reticular cells are not completely continuous around the thymic blood vessels (ITO and HOSHINO, 1966). It should also be examined, however, whether or not blood-borne antigens actually permeate the vascular walls in the thymus, because failure of the thymic parenchyma to respond to antigens may account for the "non-permeable" vascular walls. Recently RAVIOLA and KARNOVSKY (1972) have assessed in detail, by electron microscopy, the permeability of the thymic vessels to ultrastructural tracers of different molecular weight including ferritin. In this study, we examined the permeability across the walls of thymic blood vessels by using ferritin as tracer. The choice of ferritin afforded, as shown in the present results, not only qualitative but also semiquantitative evaluation of the vascular permeability in electron microscopy.

Material and Methods

A total of 59 dd-mice of both sexes, two months old, were used. For examination of the pattern and distribution of the thymic vessels, 10 mice were injected intravenously with India ink. For each animal, 0.8 to 1.0 ml of India ink was injected slowly into a tail vein. Immediately after the injection 10% formol was injected into the thorax through the anterior thoracic wall. After 30 min the thymus was removed and immersed into 10% formol. After 24 hrs, the organ was usually cut into 0.5 to 1 mm thick slices with a razor blade. Then the slices or the organ in toto were immersed in glycerol for two or three days until they became transparent. They were examined with the light microscope to pursue the vascular trees. In 39 mice, 50 mg of horse ferritin in 0.25 ml of saline was injected into a tail vein. Ferritin for injection was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). For purification, a solution of 1 g of ferritin was dialyzed against 2 l of saline before use. Saline was changed four times during dialysis for 2 days. The dialysate was concentrated in vacuo. The ferritin solution thus obtained was diluted with saline to a final concentration of 20%. The mice were killed by chloroform at various time intervals after the injection (30 sec, 5, 10, 15, 30 and 60 min, 3, 6, 12 and 24 hrs). Ten untreated mice were used as controls. From each mouse, the thymus was removed rapidly, and pieces of the tissues were fixed in 5% formol in 0.05M-phosphate buffer (pH 7.5) containing 1.5% sucrose for 48 hrs at 4°C and then postfixed in 2% osmium tetroxide in the same buffer for 1.5 hrs. They were dehydrated in a series of graded ethanol, embedded in Epon and sectioned with glass knives on an LKB ultratome. Ultrathin sections unstained or stained with uranyl acetate alone or uranyl acetate and lead citrate were examined in a Hitachi electron microscope (HS-8). Semithick sections were stained with toluidin blue for light microscopy. Some of the specimens were also fixed in Zenker-formol-acetic acid for 6 hrs for light microscopy. They were embedded in paraffin, sectioned at 5 μ and stained with periodic acid-Schiff reagent (PAS)-hematoxylin-fast green. In addition, the mesenteric lymph node, spleen and pancreas were examined by electron microscopy in the same way as was the thymus.

For quantitative assessment of the vascular permeability, the number of ferritin particles in an area 0.25 μ^2 of the perivascular spaces was counted on electron micrographs enlarged at 36,000 $\times$. For each case a mean was determined from values obtained in 4 areas selected randomly.

Results

1. Distribution of the blood vessels in the thymus

Main blood vessels to the thymus enter and leave the medulla at the lateral margin of the lobes (Fig. 1a). In the medulla the arteries branch into arterioles, which then give off anastomosing capillaries into the cortex (Fig. 1b, 2). In the peripheral region under the capsule, the cortical capillaries run a recurrent course and return toward the inner cortex. Thus, they form branching and anastomosing loops in the outermost region of the organ. The cortical capillaries finally merge into postcapillary venules at the cortico-medullary boundary and in the medulla. In the medulla

Fig. 1. Distribution of blood vessels in the thymus injected with India ink. **a.** Main blood vessels enter and leave the two thymic lobes at the lateral margin. $\times 70$ **b.** Thick section of the thymus. The blood vessels show an anastomosing network in the cortex (C), and the cortical capillaries merge into venules in the medulla (M). $\times 180$

Fig. 2. The vascular pattern in the thymus. A superimposed illustration of microscopic images at different focuses in a thick section. Cortical capillaries reveal recurrent loops in the subcapsular region.

only small numbers of capillaries are derived from arterioles at the cortico-medullary boundary.

2. Thymic blood vessels in control mice

Capillaries in the cortex have attenuated endothelial cells which contain numerous vesicles, 600 to 1,000 Å in diameter. These vesicles are often seen to open on both the luminal and tissue fronts of the cells. The two adjoining endothelial cells are connected with tight junctions. The capillaries just under and within the thymic capsule sometimes have an extremely attenuated and fenestrated endothelium.

Venules in the medulla have wider lumina, and the endothelial cells contain small vesicles as those in the cortical capillaries. In addition, larger vacuoles, 1,000 to 4,000 Å in diameter, are also seen within the cytoplasm of the endothelial cells of the venules, and they are occasionally seen to open on the luminal surface. These vacuoles are often fused with each other or with smaller vesicles. Multivesicular bodies often appear in the endothelium of the venules. The walls of the medullary venules are sometimes infiltrated with migrating lymphocytes. The walls of the capillaries and venules usually appear to be encircled by epithelial reticular cells, but they occasionally abut directly on lymphocytes or macrophages. Arterioles at the cortico-medullary boundary and in the medulla have high endothelial cells which are surrounded with elastic fibers and smooth muscle cells.

3. Vascular permeability of thymic vessels in ferritin-injected mice

As early as 30 sec after the injection, ferritin particles are seen in high concentration within the lumen of all the blood vessels in the thymus (Fig. 3a). In the cortical and subcapsular capillaries, a few micropinocytotic vesicles close to the luminal surface of the endothelial cells are labeled by ferritin 30 sec after the injection. At 10 to 15 min after the injection, the vesicles containing ferritin are increased in number. Ferritin is also present within the vesicles opening on the tissue front of the endothelial cells. At time intervals after 15 min, the vesicles in the capillary endothelium do not appear to be increased in either number or concentration of tracer. Not all the vesicles are labeled by ferritin even when the vesicles show the highest concentration of the tracer.

In the perivascular spaces, ferritin particles are first detected around the subcapsular capillaries at 10 min after the injection and around the cortical capillaries at 15 min. The permeability of ferritin across the capillary walls considerably varies in different sites of the thymic cortex (Fig. 3b-d). In general, ferritin particles are rarely seen in the perivascular spaces around the cortical capillaries, whereas they are often seen in relatively high concentrations around the subcapsular capillaries.

In the subcapsular region, the permeability of ferritin is almost the same in both fenestrated and non-fenestrated capillaries. Ferritin particles are distributed diffusely in the perivascular spaces, and they also accumulate within the basal lamina surrounding the endothelium. The findings indicate that if the basal lamina appears to retain ferritin particles temporarily, it would allow them eventually to pass out into the perivascular spaces.

Uptake of ferritin by pinocytotic vesicles in the endothelial cells occurs to a greater extent in the medullary venules than in the cortical capillaries. Ferritin particles are also entrapped by vacuoles in the endothelial cells of the medullary venules (Fig. 4a). At 30 sec after injection, vacuoles containing ferritin occur rarely on the luminal front of the cells. Then the number of the vesicles and vacuoles containing ferritin particles increases with increasing time. Ferritin is also present within multivesicular bodies in the medullary venules. At 30 min and later, the vacuoles and multivesicular bodies are enlarged in size, and ferritin is contained in higher concentration (Fig. 4b). The densely labeled multivesicular bodies measuring larger than 1μ in diameter are often seen at 24 hrs (Fig. 4c). In the spaces around the medullary venules, ferritin particles first appear at 15 min. The concentration

Fig. 3. Cortical capillaries. *En* endothelial cell, *PVS* perivascular space, *Ep* epithelial cell, *L* lymphocyte, *RBC* red blood cell. **a.** Cortical capillary at 30 sec after ferritin injection. Endothelial cell (*En*) contains numerous pinocytotic vesicles. Ferritin particles appear within the lumen, but are not present in the perivascular space (*PVS*). $\times 40,000$ **b.** Subcapsular capillary at 30 min after ferritin injection. Large amounts of ferritin particles appear in the perivascular space (*PVS*). $\times 40,000$ **c.** Capillary in the inner cortex at 60 min after injection. Small numbers of ferritin particles are visible in the perivascular space (*PVS*). The endothelial cell (*En*) abuts on a lymphocyte (*L*). **d.** Capillary in the inner cortex at 60 min after injection. Ferritin particles are rare in the perivascular space (*PVS*) around the capillary endothelium (*En*). $\times 40,000$

of ferritin in the perivascular spaces is variable, but it is generally higher than in the spaces around the cortical capillaries (Fig. 5). At 24 hrs, ferritin particles within both the vascular lumina and perivascular spaces are only small in amount.

Variable amounts of ferritin were also detectable in the intercellular spaces of the thymic parenchyma. In the outermost portion, particularly near the subcapsular capillaries, relatively large amounts of ferritin are seen in the intercellular spaces of the parenchyma (Fig. 6a). In the deep cortex, invasion of ferritin into the intercellular spaces of the parenchyma occurs only to a lesser extent. Some macrophages just around the cortical capillaries have membrane-bound bodies containing ferritin particles (Fig. 6b). Dense aggregations of ferritin are often seen within large round bodies of macrophages under and within the thymic capsule. In the medulla, ferritin permeates the intercellular spaces of the parenchyma across the walls of the venules, although it is variable in amount, and macrophages containing ferritin also appear (Fig. 6d).

Free ferritin particles are rarely found within the cytoplasm of lymphoid cells, epithelial reticular cells and endothelial cells of the vessels. Thymic lymphocytes

Fig. 4. Medullary venules. **a.** At 10 min after ferritin injection. Pinocytotic vesicles and vacuoles in the endothelium (*En*) are labeled on the luminal front. No ferritin particles are seen in the perivascular space (*PVS*). $\times 32,000$ **b.** At 30 min after injection. A multivesicular body in the endothelial cell is densely labeled with ferritin (arrow). $\times 35,000$ **c.** At 24 hrs after injection. A large membrane-bound body containing large amounts of ferritin particles is seen in the endothelial cell. $\times 35,000$

Fig. 5. a. Perivascular space around a medullary venule at 30 min after injection. The space contains numerous ferritin particles which are concentrated within the basal laminae (*BL*). *En* endothelial cell, *Ep* epithelial cell, *L* lymphocyte. $\times 40,000$ **b.** Ferritin particles are seen within a membrane-bound body (arrow) in the cytoplasm of a lymphocyte (*L*). *PVS* perivascular space, *EP* epithelial cell. $\times 40,000$

rarely have membrane-bound bodies containing ferritin particles (Fig. 5b).

The splenic lymphatic tissue, mesenteric lymph node and pancreas in ferritin-injected mice were also examined for comparison of the extent of the vascular permeability in the thymus. In the splenic lymphatic tissue, antigens are known to be particularly concentrated in the marginal zone (Weiss, 1970). The blood vessels in the marginal zone are sinusoidal in nature, and their walls consist of two cell layers, endothelial cells and reticular cells, between which a basal lamina is present. The walls often show clefts or slits which are squeezed by migrating lymphocytes or macrophages. Soon after injection of ferritin large amounts of ferritin are found in the intercellular spaces of the parenchyma of the splenic lymphatic tissue. At 30 min after the injection and later, large amounts of ferritin particles are found in the perivascular and intercellular spaces in the marginal zone, where ferritin is also actively phagocytosed by macrophages and endothelial cells (Fig. 7). In the mesenteric lymph node, the endothelial cells of the capillaries are structurally similar to those of the thymic cortical capillaries, but the leakage of ferritin into the parenchyma is more prominent in the lymph node than in the thymic cortex. Large amounts of ferritin particles are contained particularly in macrophages and endothelial cells of the medullary lymphatic sinuses. In the pancreas, the capillary endothelium is fenestrated and considerably large amounts of ferritin particles are present in the perivascular spaces at 30 and 60 min after injection.

4. Quantitative assessment of the vascular permeability

The grades of permeability of the vascular walls to ferritin were assessed semi-quantitatively by counting the number of ferritin particles per unit area in the perivascular spaces of the above-mentioned tissues at 30 and 60 min after injection. The results obtained are given in Figure 8. As seen in this figure, considerable amounts of ferritin particles permeate across the walls of thymic vessels. Ferritin permeates the capillary walls in the subcapsular cortex more abundantly than those in the inner cortex. The venules in the medulla tend to be permeated by larger amounts of ferritin than the cortical capillaries. The permeability is higher in the lymph node and pancreas than in the thymus. Particularly in the marginal zone of the splenic lymphatic tissue very large amounts of tracer permeate through the walls

Fig. 6. Ferritin particles in the thymic parenchyma at 60 min after injection. **a.** Outermost cortex. Ferritin particles appear in the intercellular space (arrows), and they are engulfed by a vacuole (V) in the cytoplasm of a macrophage (M). L lymphocyte. $\times 38,000$ **b.** Uptake of ferritin particles by a macrophage (arrows) in the cortex. $\times 45,000$ **c.** Ferritin particles are seen in the intercellular space in the medulla (arrows). A macrophage (M) also contains ferritin particles. L lymphocyte. $\times 38,000$ **d.** Ferritin particles are visible within the membrane-bound bodies (arrows) of macrophage (M) abutting on the endothelium (En) of a medullary venule. $\times 50,000$

Fig. 7. Splenic lymphatic tissue at 60 min after ferritin injection. **a.** Marginal zone. Ferritin particles are phagocytosed by an endothelial cell (*En*) of the sinus. They are also densely distributed in the perivascular space (*PVS*). *RBC* red blood cell. $\times 38,000$ **b.** A macrophage showing active phagocytosis of ferritin. $\times 38,000$

Fig. 8. Numbers of ferritin particles in unit area ($0.25 \mu^2$) of the perivascular spaces of various tissues. *CC* cortical capillaries, *Subc C* subcapsular capillaries and *Mv* medullary venules in the thymus; *Panc* capillaries of the pancreas, *Lym N* capillaries in the mesenteric lymph node, *Spleen* sinuses in the marginal zone of the splenic lymphatic tissue. Open circles, at 30 min; solid circles, at 60 min after ferritin injection. Bars represent means and standard errors.

Fig. 9. Schema of the vascular permeability in various organs. The shaded arrows represent the amount of ferritin which permeates the endothelium (*End*) from the vascular lumen (*Vas Lumen*), appears in the perivascular space (*PVS*) and invades the parenchyma (*Parench*). *Spleen* sinus of the marginal zone in the splenic lymphatic tissue, *Lym NC* capillary of the mesenteric lymph node, *CC* cortical capillary, *SubcC* subcapsular capillary and *MV* medullary venule in the thymus. Endothelial cells of the sinus in the marginal zone of the splenic lymphatic tissue and medullary venule in the thymus phagocytose considerable amounts of ferritin.

through a vesicular pathway within the endothelial cells as in the case of other tissues such as muscle and intestinal capillaries (BRUNS and PALADE, 1968; CLEMENTI and PALADE, 1969).

In the brain, as is generally known, exists a physiological blood barrier. Here the vascular walls are generally invested by astrocytes which are thought to play an important role in the barrier mechanism (KUFFLER and NICHOLLS, 1966). In addition, the permeability of the endothelium itself appears to be related to the barrier mechanism, because the endothelial cells of the brain capillaries usually contain few vesicles and show little transport action of injected tracer substance by vesicular pathways (REESE and KARNOVSKY, 1967).

of sinusoids. The leakage of ferritin is presumably due to the presence of slits in the walls. The vascular permeability of the lymphatic tissues is schematically summarized in Figure 9.

Discussion

The vascular pattern of the thymus has been described in detail by previous authors (SMITH, et al., 1952; ITO and HOSHINO, 1966; RAVIOLA and KARNOVSKY, 1972). The present findings on the distribution of the thymic blood vessels are essentially in agreement with the earlier descriptions. As seen in the previous and present results, the thymic cortex is supplied mainly with capillaries and the medulla, with venules. From such an anatomical basis, it is reasonable that if blood-borne influences on the thymus are to be examined, the permeability of the vascular walls should primarily be considered in the cortical capillaries and medullary venules. As indicated by the present results, circulating ferritin particles can pass across the walls of both cortical capillaries and medullary venules, though their passage to the thymic parenchyma, particularly in the cortex, is far less in amount than in other lymphatic tissues and the pancreas.

As seen in the previous and present observations, endothelial cells in both the cortical and medullary vessels contained numerous micropinocytotic vesicles, which opened to both the blood and tissue fronts and were labeled with ferritin. Thus in the thymus, blood-borne substances are thought to be transported, though to a relatively lesser extent,

In the thymus, as mentioned above, the endothelial cells of the blood vessels contain numerous pinocytotic vesicles, and intravenously injected ferritin apparently is transported by the vesicles into the perivascular spaces across the basal lamina. As referred to in the introductory remark, a blood-thymus barrier was previously ascribed to continuous investment of epithelial reticular cells around the blood vessels (CLARK, 1963; WEISS, 1963), but closer inspection revealed that the epithelial investment around the cortical capillaries and medullary venules is not completely continuous but has frequent interruptions (ITO and HOSHINO, 1966). As seen in the present results, neither the layer of epithelial cells nor the basal lamina around the vascular walls can prevent the penetration of ferritin particles into the parenchyma, because the injected tracer is seen in the perivascular spaces and, to less extent, also within the parenchyma. It can be said therefore that the thymus lacks a vascular barrier in a strict sense. However, the thymic vessels generally appear to have, to some extent, a selective permeability to circulating tracers. The permeability is known to be relatively low as compared with that in other lymphatic tissues and the pancreas; the thymus clears little dye and protein from the blood (GREEN and BLOCH, 1963; KOSTOWIECKI, 1963; CLARK, 1964). Particularly in the peripheral cortex and the medulla, ferritin leaks into the parenchyma easier than in the inner cortex. In the cortex, as indicated by RAVIOLA and KARNOVSKY (1972), macrophages around the vessels phagocytise the permeated tracer actively and promptly. Nevertheless, very small amounts of the tracer permeate into the parenchyma. Even if thymic lymphocytes are thus exposed to permeated antigens, the thymus, as is generally accepted, would not respond to the antigens. This immunological unresponsiveness of the thymus requires an explanation other than the idea of a barrier mechanism.

As is well known, thymic lymphocytes are actively produced in the cortex (for reviews see MILLER and OSOBA, 1967; EVERETT and TYLER, 1967; ELVES, 1972). Large and medium lymphocytes which are actively proliferating are most numerous in the peripheral cortex, and they are scattered throughout the remaining cortex. Such immature lymphocytes finally give rise to numerous small lymphocytes in the cortex. The latter migrate inwards and finally leave the organ from the medulla. There is evidence indicating that cortical and medullary small lymphocytes in the thymus are different in certain morphological and behavioral aspects (TROWELL, 1961; ITO and HOSHINO, 1962; KOSTOWIECKI and SHERMAN, 1963; SAINT-MARIE and LEBLOND, 1964; WEBER, 1966; ABE, SASAKI and ITO, 1972a). In previous papers we also observed by electron microscopy that thymic small lymphocytes are different in ultrastructural features between cortex and medulla (ABE and ITO, 1970b, 1972; ABE, SASAKI and ITO, 1973b). The morphological difference between cortical and medullary small lymphocytes is considered a reflection of their functional differentiation, because it is assumed that cortical small lymphocytes are immunologically incompetent and medullary small lymphocytes, competent. For these reasons, the immunological unresponsiveness of thymic lymphocytes to circulating antigens is probably due to functional immaturity of lymphocytes particularly in the cortex, as was suggested by WEISS (1972). If medullary small lymphocytes are immunologically competent, they may respond to antigens. Nevertheless, the immunological reaction of medullary lymphocytes cannot be observed morphologically either. This is probably because medullary small lymphocytes leave the thymus soon and they do not stay long enough to respond to

antigens within the medulla. Under certain experimental and pathological conditions, however, germinal centers have been observed in the medulla (MARSHALL and WHITE, 1961; MELLORS, 1965; KOTANI et al., 1967; GOLDSTEIN and MACKEY, 1969; ABE and ITO, 1970). This may suggest that the medulla is immunologically able to respond to antigens, although the mechanism of the formation of those germinal centers remains undetermined. From the above statement the lack of responsiveness of the thymus to circulating antigens is possibly because thymic lymphocytes are less responsive to antigens than lymphocytes in peripheral lymphatic tissues as suggested by WEISS (1963). Nevertheless, it is also a fact that the thymic vessels, that is, cortical capillaries and medullary venules, are relatively low in the permeability of the endothelial cells to circulating molecules. This peculiarity of the thymic vessels may protect the thymic parenchyma though to a limited extent. In this respect, the thymic vessels particularly in the cortex may be regarded as an incomplete barrier.

マウス胸腺血管の透過性

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成熟マウスを用いて、胸腺の血管を とくにその壁の透過性に関して検索した。胸腺に分布する血管は 皮質では主として毛細血管であり、髄質では主として細静脈である。皮質の毛細血管と髄質の細静脈は、一般に上皮性細網細胞で囲まれているが、この上皮性細胞は完全には連続性でない。皮質毛細血管と髄質細静脈の内皮細胞はともに多数の微飲小胞を含むが、細静脈にはやや大きな小胞もみられる。フェリチンを静脈内に注射すると、胸腺血管の内皮細胞におけるこれらの微飲小胞と小胞はフェリチンをとりこみ、フェリチンは血管周囲腔にも出現する。胸腺血管壁のフェリチンの透過性は、皮質被膜下と皮質内層の毛細血管、髄質の細静脈で異なる。注射したフェリチンは、とくに皮質外層や髄質で、胸腺実質内にも侵入する。さらに、胸腺血管のフェリチンの透過性を、脾臓リンパ組織・腸間膜リンパ節・脾臓の血管における透過性と比較し、とくに胸腺における血管の関門機構と関連して考察した。

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