EVIDENCE FOR A BLOOD-THYMUS BARRIER USING ELECTRON-OPAQUE TRACERS*

By ELIO RAVIOLA and MORRIS J. KARNOVSKY

(From the Department of Anatomy and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

(Received for publication 17 April 1972)

The failure of thymic lymphocytes to produce antibodies against circulating antigen and the appearance of antibody-forming cells in the thymus when antigen is injected directly into the organ have given rise to the concept of a blood-thymus barrier (1). The interpretation of these experiments is no longer acceptable because thymic lymphocytes seem to be incapable of undergoing transformation into antibody-forming cells (2–5); furthermore, reports have appeared in the literature which seem to contradict the existence of a barrier in the adult animal (6–8). There is evidence, however, that the blood vessels of the normal, adult thymus are scarcely permeable to circulating macromolecules and particulate matter (9–18), and an explanation has to be sought for the paradox that anti-thymocyte sera do not attack their target cells in the intact animal (19–23).

In the present study the problem of the putative blood-thymus barrier has been approached by assessing the permeability of the thymic vessels in young adult mice with ultrastructural tracers of different molecular weights. Evidence is presented that lymphoid cells in the cortex, in contrast with those of the medulla, are protected from circulating macromolecules.

Materials and Methods

(*A*) *General.*—Male and female Swiss albino mice, 6–8 wk old, were used. The fine structure of the thymic vessels was studied after both immersion fixation and vascular perfusion. For immersion fixation a paraformaldehyde (2%)-glutaraldehyde (2.5%) mixture (24) in 0.08 M cacodylate buffer, pH 7.3, containing 0.4% $CaCl_2$, was used. Initial fixation was performed at room temperature (1 hr); specimens were then held at 0°–4°C (3 hr). Tissue blocks were subsequently washed for 2 hr at 0°–4°C with 0.1 M cacodylate buffer, pH 7.3, containing 0.4% $CaCl_2$; postfixed for 2 hr at 0°–4°C with 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.3, containing 0.4% $CaCl_2$; rapidly dehydrated with ethanol; and embedded in an Epon-Araldite mixture.

Perfusion was performed through the abdominal aorta; blood vessels were washed out with oxygenated Ringer's solution at 37°C, followed by a warm, very diluted aldehyde mixture (paraformaldehyde 1%, glutaraldehyde 1.25%, sucrose 1% in 0.1 M cacodylate buffer, pH

* Supported by a Milton Fund Award of the Harvard University and grant HE-09125 from the National Institutes of Health, U.S. Public Health Service.

7.3), and finally by a concentrated fixative fluid at room temperature (paraformaldehyde 4%, glutaraldehyde 5%, $CaCl_2$ 0.4% in 0.07 M cacodylate buffer, pH 7.3).

In a few mice care was taken to retain the blood in the vascular bed; under anesthesia the abdominal cavity was opened and the fixative fluid was injected through the diaphragm into the mediastinum. A few minutes later, the thoracic walls were cut avoiding blood extravasation and the thymic blood vessels clamped. The whole organ was then excised and immersed together with the clamps into the fixative fluid. After 5–10 min, the thymus was cut into smaller blocks and processed in the usual way. With this method, the thin caudal edge of the thymic lobes was adequately preserved.

In many instances specimens were stained en bloc for 2 hr at 0°–4°C with 2% uranyl acetate in 0.05 M maleate buffer, pH 6.0, (24). Thick sections were stained with toluidine blue (25), thin sections with lead citrate (26). Micrographs were taken with RCA3F (RCA Corp., New York), Siemens Elmiskop 1 (Siemens Corp., Iselin, N. J.), and AEI 6B (AEI Scientific Apparatus Ltd., Essex, England) microscopes.

(*B*) *Vascular Injections.*—In order to visualize the vascular tree of the thymus, either the ascending or the abdominal aorta was cannulated with polyethylene tubing under anesthesia; blood vessels were perfused with warm Ringer's solution and subsequently injected with warm India ink suspension (Pelikan Werke, Hanover, Germany; batch No. C11/1431a, 10 ml in 100 ml 5% gelatin, containing 2.5% potassium iodide) or melted Ilford L4 photographic emulsion (Ilford Ltd., England) (27). After fixation, the thymus was sectioned in a Smith and Farquhar tissue chopper; the sections of the specimens injected with emulsion were developed in Kodak D-19 developer (Eastman Kodak Co., Rochester, N. Y.).

(*C*) *Experiments with Enzymatic Tracers.*—

(*1*) *Horseradish peroxidase*: The tracer was slowly (1–2 min) injected intravenously either via the tail veins in unanesthetized animals or via the femoral vein or the inferior cava under anesthesia. 5 mg of Sigma type II peroxidase (Sigma Chemical Co., St. Louis, Mo.; RZ value 1.0–1.5) or 2.5 mg of purified peroxidase (Worthington Corporation, Freehold, N. J.; RZ value 2.9–3.0) dissolved in 0.5 ml of isotonic saline were injected. Control animals were injected with saline alone. At the time intervals of 2-5-10-15-20-30 min and 1-7 hr, immersion fixation was initiated with the aldehyde mixture specified in paragraph *A*. Extreme care was taken to avoid extravasation, otherwise the superficial cortex of the thymic lobes is permeated by the tracer contained in the blood plasma. The organ was trimmed in an orderly fashion, so that blocks could be properly oriented during cutting. After aldehyde fixation, specimens were washed overnight at 0°–4°C in 0.1 M cacodylate buffer, pH 7.3, containing 0.4% $CaCl_2$, and sectioned in a Smith and Farquhar tissue chopper. After washing for 30 min in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.6, sections were incubated for 15–30 min at room temperature in the medium for demonstration of peroxidase activity (28); washed with both Tris-HCl (30 min) and cacodylate (1 hr) buffers; and postfixed for 2 hr at 0°–4°C either with cacodylate-buffered osmium tetroxide or with 1% osmium tetroxide, 1.5% potassium ferrocyanide in distilled water (29).

(*2*) *Cytochrome c:* 25–40 mg of cytochrome *c* (Sigma Chemical Co.; type VI) in 0.5 ml of phosphate-buffered saline adjusted at pH 7.3 were slowly (1 min) injected into the inferior vena cava of anesthetized mice, after ligation of the renal arteries to prevent rapid loss of the tracer into the urine. 1 min after end of the injection, the thymus was fixed and processed as in the experiments with peroxidase. Chopper sections were incubated for demonstration of cytochrome *c* activity (30). The following steps were the same as in the peroxidase experiments.

(*3*) *Catalase:* 20 mg of twice-crystallized beef liver catalase (Sigma Chemical Co.; stock No. C100) were injected via the tail veins into unanesthetized mice. In order to obtain a higher concentration of catalase for injection, approximately one-half of the supernatant was removed from the stock solution before dissolving the enzyme by sonication at 37°C (31). 15 min after the injection, the thymus was fixed and processed as in the experiments with peroxi-

dase. The chopper sections were incubated for demonstration of catalase activity (32). The following steps were the same as in the peroxidase experiments.

(*4*) *Ferritin*: 50 mg of cadmium-free ferritin in 0.5 ml of phosphate-buffered saline, pH 7.2, were injected into the femoral vein of anesthetized mice. At time intervals of 1-2-24 hr after the injection, the thymus was fixed by immersion avoiding blood extravasation. The following procedures were the same specified in paragraph *A*.

(*D*) *Experiments with Lanthanum Tracer.*—The vascular tree was perfused through the abdominal aorta with a weak paraformaldehyde-glutaraldehyde solution (cf. paragraph *A*), followed by a lanthanum-aldehyde mixture (2% lanthanum nitrate in deionized water, adjusted to pH 7.8 with 0.1 N NaOH, mixed with an equal amount of 8% paraformaldehyde, 10% glutaraldehyde in 0.1 M cacodylate buffer, final pH 6.8); tissue blocks were then washed in 0.2 M cacodylate buffer and postfixed in 2% osmium tetroxide in 0.2 M cacodylate buffer, both mixed with equal parts of lanthanum solution (33). Finally, specimens were stained en bloc with uranyl acetate.

RESULTS

(*A*) *The Vascular Supply of the Thymus.*—Circulating macromolecules can cross vascular endothelium by vesicular transport (34) and bulk flow through open intercellular junctions (24), fenestrae (35), or discontinuities (36). Their progression across the remaining vascular tunics is only impeded by a coarse sieving effect of the basal lamina, which restricts the passage of very large particles (35). Farther movement of blood-borne macromolecules and their organ distribution are influenced by the structural interrelationships of the parenchymal elements which surround the blood vessels and the geometry of the vascular tree. Therefore, an analysis of the morphology and distribution of the blood vessels represents an indispensible, preliminary step for a study of the vascular permeability of the thymus to circulating ultrastructural tracers. So far, such an analysis has never been carried out in a systematic fashion, although various reports on the organization of the thymic blood vessels are available in the literature (37–39).

The thymus of the mouse consists of two lobes which do not show the lobulation typical of larger mammalian species; in fact, only in very few places is the surface indented by incomplete connective tissue septa carrying blood vessels, which penetrate the thymic parenchyma as far as the cortico-medullary boundary. The vascular supply of the thymic lobes is unusual because of the pattern of distribution of the vessels and their relationships to the parenchymal elements. The intralobar vessels are represented by arterioles, capillaries, and venules; a clear-cut distinction between these three kinds of vessels can be made only in electron micrographs and the following description of the vascular tree, as it appears in specimens injected with India ink or photographic emulsion, takes advantage of the information gained through the electron microscope. The arterioles run at the boundary between cortex and medulla and give off capillaries, which ascend into the cortex being joined to each other by collateral anastomoses (Fig. 1). At the periphery of the cortex, but still within the thymic parenchyma, the capillaries form a network of branching and anastomosing arcades and curve back toward the interior of the lobe. In their

recurrent course through the cortex, the capillaries join to form larger vessels, which can still be classified as capillaries on the basis of their fine structure. These vessels merge into postcapillary venules at the cortico-medullary boundary and in the medulla. In addition to the postcapillary venules and the capillaries descending from the cortex, the medulla is supplied by a small

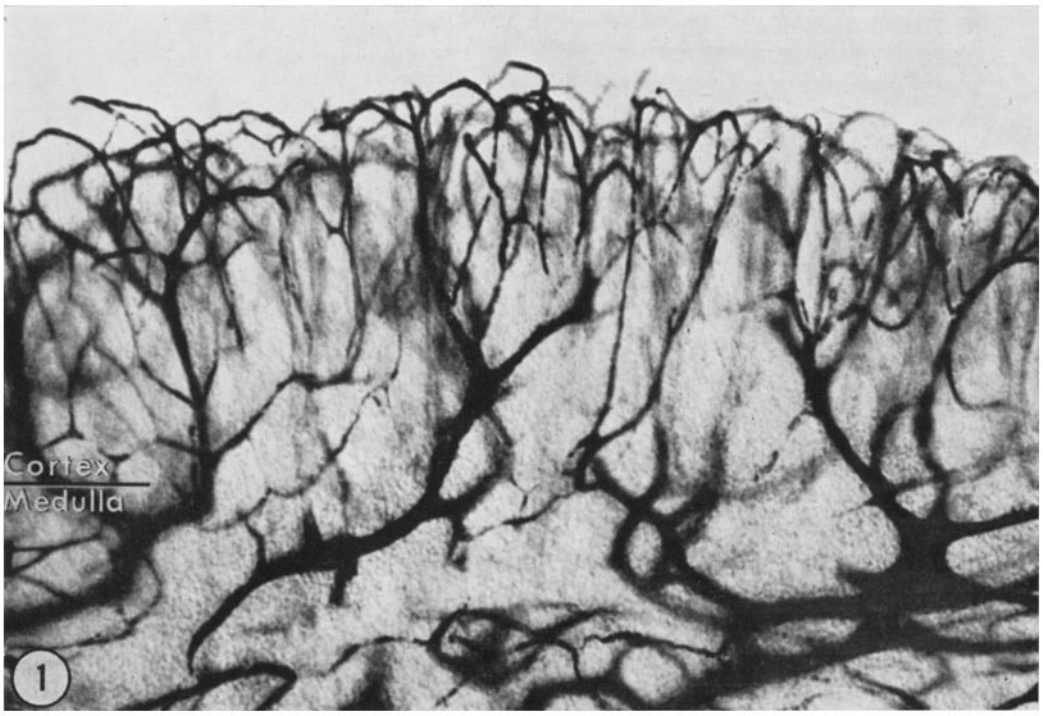


FIG. 1. Light micrograph of a 200 μ section of the mouse thymus after vascular injection with photographic emulsion. The capillaries, originating from arterioles at the cortico-medullary boundary, ascend into the *cortex* and form at the extreme periphery of the lobe a network of branching and anastomosing arcades. They afterwards curve back toward the interior of the lobe, run a recurrent course through the cortex, and finally merge into postcapillary venules deeply located in the *medulla*. $\times$ 200.

number of capillaries which originate directly from the arterioles at the cortico-medullary junction.

As an exception to this basic pattern, capillaries may cross the peripheral boundary of the cortex and course within the lobar connective tissue capsule. They may immediately reenter the cortex or follow one of the rare connective tissue septa which indent the surface of the lobes.

At the ultrastructural level the intralobar blood vessels appear to thread their way through a compact mass of thymic parenchymal cells, being enclosed

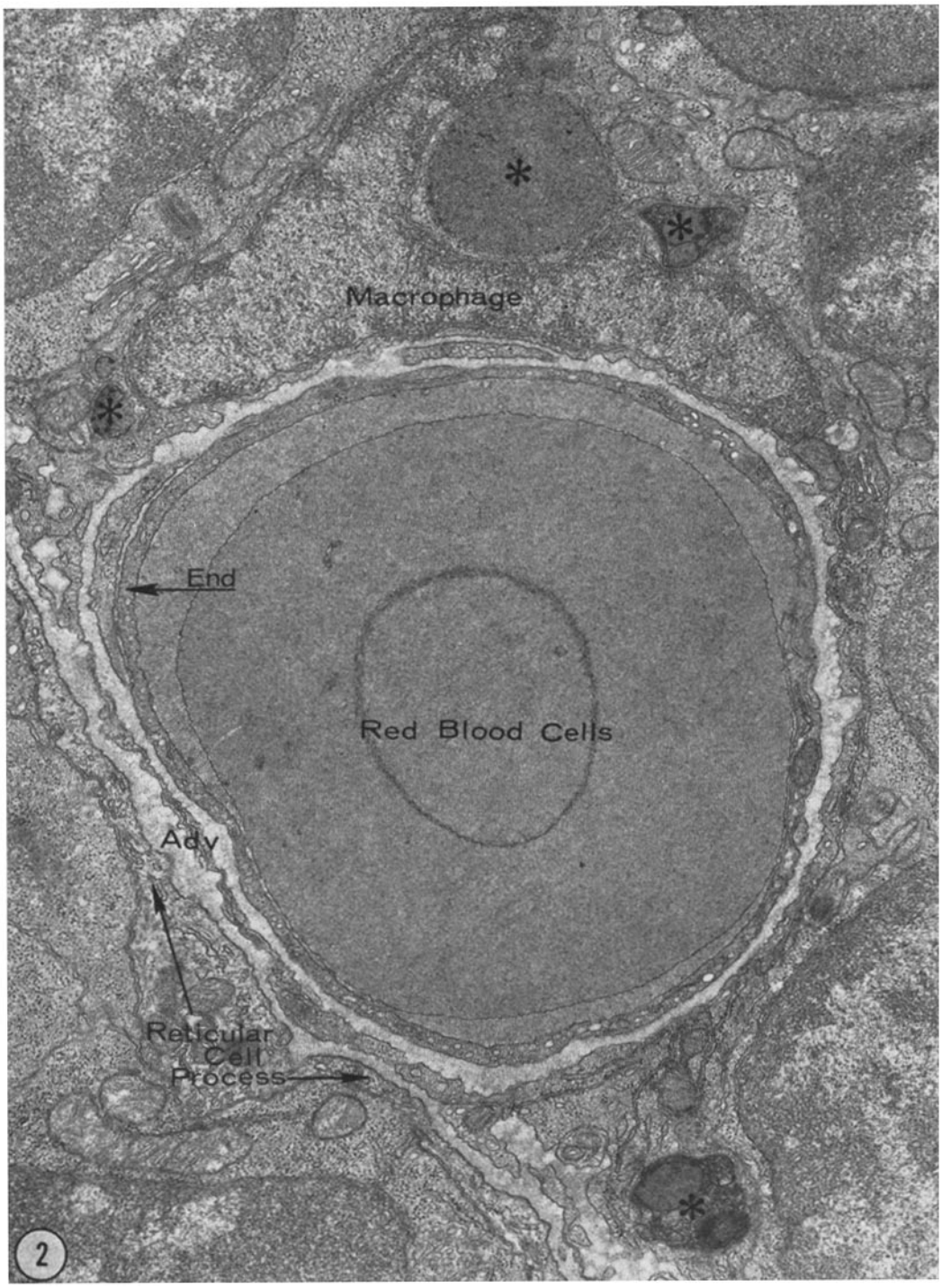


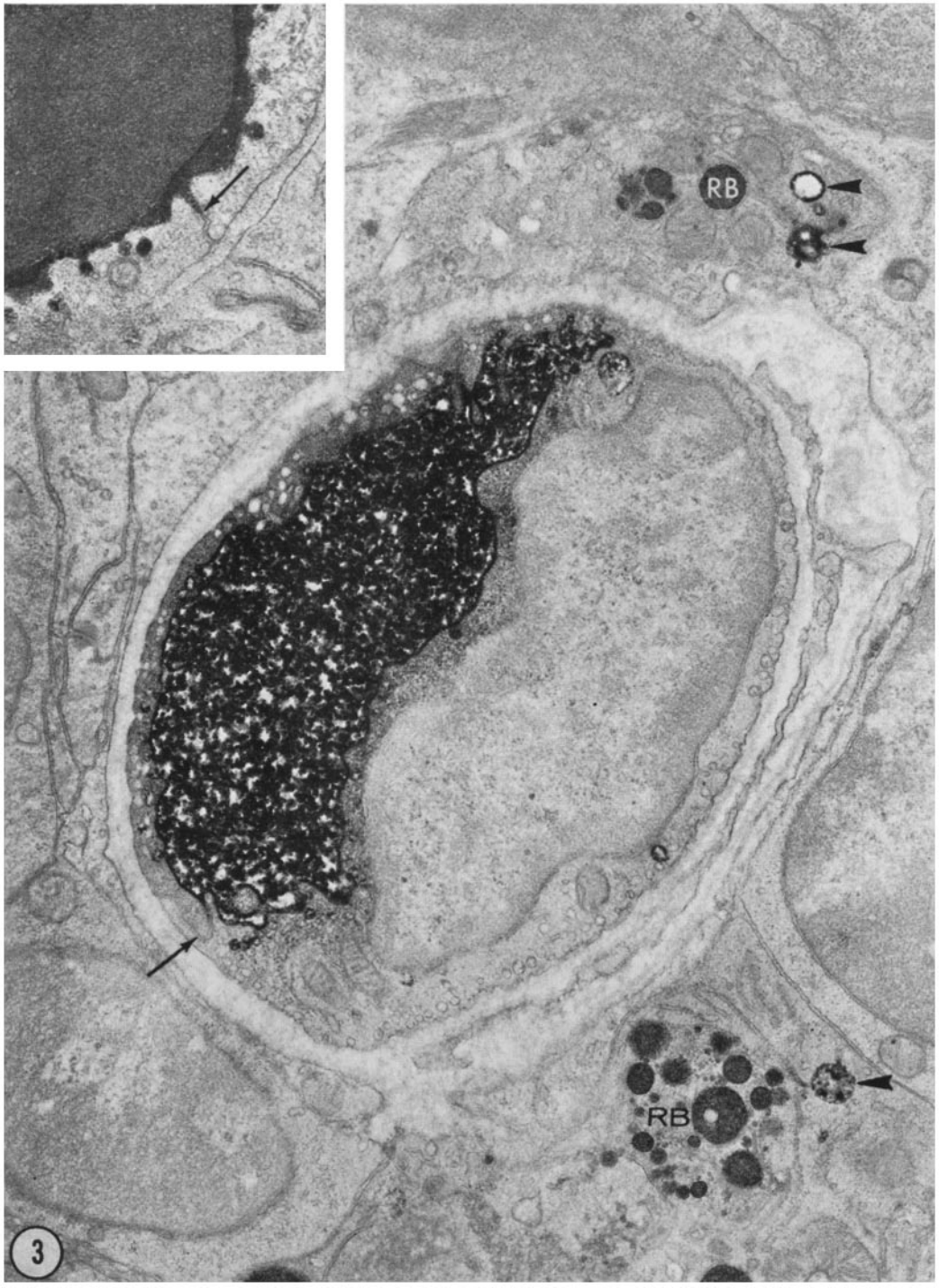
FIG. 2. Electron micrograph of a capillary of the cortex; in this specimen care has been taken to retain the blood in the vascular bed during fixation. The capillary lumen is wide open and contains both plasma and red blood cells; in the cytoplasm of the attenuated endothelial cells (*End*), plasmalemmal vesicles are few in numbers. The vessel is enclosed in a coaxial canal formed by the surrounding thymic parenchymal cells; a macrophage containing residual bodies (*asterisks*) encompasses most of the circumference of the vessel and sends processes into the vascular adventitia (*Adv*). Below and on the left, the parenchymal front bounding the vascular adventia is represented by a process of a reticular cell. × 27,000.

in a system of canals bounded by a continuous investment of reticular cell processes, macrophages, and lymphocytes (Fig. 2). Reticular cells encompass most of the circumference of the vessels, and they appear to be separated from the vascular adventitia by a boundary layer of amorphous material analogous to the basal lamina of epithelia. Also processes of the macrophages are consistently found adjoining the outermost layer of the vessel wall, whereas lymphocytes only occasionally abut on the vascular adventitia. In young adult mice, connective tissue fibers are limited to the adventitia of the vessels, with the exception of a few reticular fibers, which course through the medulla. These latter, too, are invested by processes of the reticular cells, with an intervening layer of material resembling a basal lamina.

All the vessels of the cortex are capillaries (Figs. 2 and 3). In specimens simply excised from the animal and trimmed in a drop of fixative fluid, their diameter varies from 3.5 to 7 μ. The capillary wall consists of endothelium, basal lamina, and a thin adventitial layer of collagen fibrils. The endothelial cells contain, besides the usual complement of organelles, a moderate number of micropinocytotic vesicles either budding from the luminal and basal surfaces or lying free in the cytoplasm. At high magnification, the intercellular spaces between adjoining endothelial cells appear to be sealed at one to three sites along their length by focal membrane fusions, which must represent the cross-sectional image of a simple but continuous zonula occludens (Fig. 15). In these regions a condensation of the cytoplasmic matrix is seen adhering to the inner leaflet of the endothelial cell membrane. Slender cell processes are commonly embedded in the adventitia; in places they are seen to be continuous either with the endothelial cells or with the reticular cells and macrophages encircling the vascular adventitia. The vascular injections show that the superficial cortex of the lobe is mainly irrigated by capillaries of small diameter, whereas the deep cortex contains vessels of varying size, the largest being the efferent limbs of the capillary loops. This peculiar arrangement of the small thymic vessels provides a unique opportunity to compare the fine structure of the different segments of the capillary bed, but the structural organization of the capillary wall does not seem to vary along the length of the vessel.

In a few capillaries at the extreme periphery of the cortex, the endothelium is penetrated by fenestrae. No more than one to three of these are found along the entire circumference of a cross-sectioned vessel. They are located in the region of the capillary wall where the endothelium is extremely attenuated. They have a diameter of about 400 A and appear to be closed by a diaphragm with a central thickening. Capillaries with a small number of endothelial fenestrae are more common within the thymic connective tissue capsule (Fig. 4), especially in association with heterogeneous clusters of connective tissue cells, such as macrophages, mast cells, adipose cells, and fibroblast-like elements. In the islands of pure adipose cells, fenestrated capillaries are very rare.

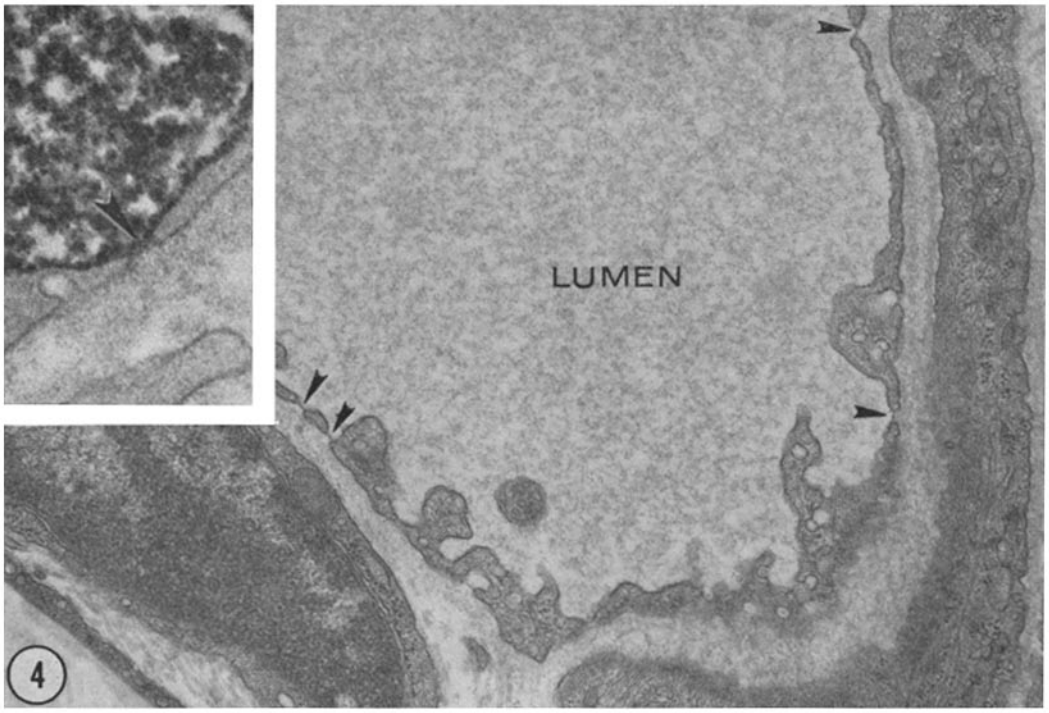
Arterioles 10–15 μ in diameter are found at the cortico-medullary boundary

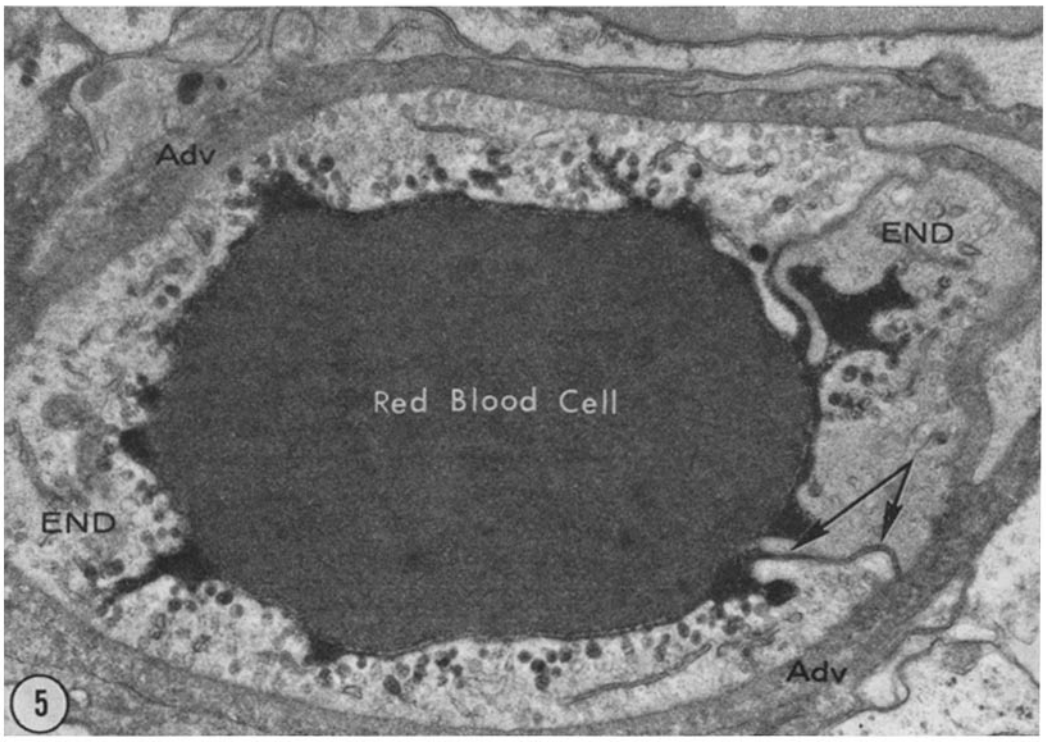

RB
RB
3

(Fig. 6); in cross section, their narrow lumen has a stellate shape, because of the highly irregular outline of the adluminal surface of the endothelium. This latter consists of cuboidal cells bulging into the lumen in the thicker perinuclear region and being deeply indented at the lateral boundaries of the cells. The luminal surface of the endothelial cells is consistently provided with marginal folds near the tortuous intercellular junctions; their basal surface follows the scalloped course of the elastica interna. The endothelial cells display the usual complement of cytoplasmic organelles. The Golgi complex is rather prominent and filaments about 75 A in diameter are especially numerous. Micropinocytotic vesicles budding inward from either surface of the endothelial cells occur with varying frequencies, but in some places they are extremely sparse. The elastica interna is thin and provided with wide fenestrations. A single layer of smooth muscle cells, in arterioles of smaller diameter a single muscle cell, encircles the tunica intima. The adventitia is very thin and consists of bundles of collagen fibrils interspersed with elastic fibers.

Postcapillary venules are defined here as large vessels located at the corticomedullary boundary and in the medulla, the walls of which are infiltrated with migrating lymphocytes (Figs. 7–12). Their diameter ranges from 10 to 50 μ; their walls consist of endothelium, basal lamina, and connective tissue adventitia. The endothelium is thick and, in collapsed vessels, displays an extraordinary diversity of form. The fine structure of the endothelial cells of the venules is similar to that of the capillary endothelium, but the Golgi apparatus is more developed. Micropinocytotic vesicles are highly variable in numbers from cell to cell, and in places are exceedingly scarce. Considerable difficulties were encountered in defining the morphology of the endothelial cell junctions in venules at high magnification, because the apposed membranes of adjoining cells are extremely irregular in contour and are so often oblique with respect to the plane of section, that the intercellular clefts cannot be followed throughout their course from the blood to the abluminal surface of the endothelium. Focal membrane fusions were commonly observed but along other clefts the membranes of adjoining cells were seen to converge to a distance of 40 A, but not

FIG. 3. Horseradish peroxidase, 5 min after i.v. injection. Intense staining is seen in the luminal plasma of a capillary of the cortex. The abluminal end of a cleft between endothelial cells is unstained (*arrow*); also most of the plasmalemmal vesicles situated close to or opening onto the tissue front of the endothelium are unstained. The endothelial basal lamina, the adventitia, and the intercellular spaces of the surrounding cortical parenchyma are free of peroxidase. Two macrophage processes adjoining the capillary adventitia contain phagocytic vacuoles stained with reaction product (*arrowheads*); the staining of the residual bodies (*RB*) is probably nonspecific and possibly due to lipid peroxides. Inset: cytochrome *c*, 1 min after i.v. injection. Part of a capillary of the cortex is seen, with a red blood cell filling most of the lumen. The reaction product is seemingly arrested at a waist of the cleft between two endothelial cells (*arrow*). The staining of the red blood cell is due to the peroxidase activity of hemoglobin. × 18,500; inset, × 26,500.


LUMEN
4


Adv
END
Red Blood Cell
END
Adv
5

fuse. Because of their tortuosity, however, one could not determine with any assurance that these clefts were patent throughout their length.

The endothelium rests upon a basal lamina, surrounded by bundles of collagen fibrils and occasional elastic fibers. Frequently, cell processes rich in cytoplasmic filaments are found underneath the endothelium; these may belong either to pericytes or to smooth muscle elements, but they are never seen forming a continuous investment around the entire circumference of the vessel (Figs. 8, 12). The thin adventitial layer is in turn encircled by a continuous front of thymic parenchymal elements, mainly processes of the reticular cells coated by their usual mucopolysaccharide boundary layer.

The venular walls are often infiltrated with large numbers of migrating lymphocytes (Figs. 7–9) and their architecture can be so remarkably distorted that the vessel is barely distinguished from the surrounding parenchyma. Lymphocytes are seen traversing the investment of reticular cell processes which encircles the venular walls. Others, lying aligned in concentric rows outside the endothelial basal lamina, dissect the adventitial connective tissue into multiple discontinuous layers. Lymphocytes are often found sandwiched between the endothelial cells and their basal lamina or penetrating the endothelium. Most commonly they seem to thread their way through the intercellular spaces, pushing aside adjoining endothelial cells, but the possibility cannot be excluded that they can also perforate the endothelial cells, as is reported to be the case for the postcapillary venules in lymph nodes (40). In a few instances, elongated lymphocytes were seen extending through the entire thickness of the endothelium, with a bulbous pseudopod projecting into the blood and the other pole lying in the adventitia (Fig. 8). The static morphological picture does not provide a clue as to whether these lymphocytes are leaving or entering the thymic parenchyma, but there is much circumstantial evidence suggesting that they are actually migrating into the bloodstream (cf. 41).

The morphology of the vascular wall is strikingly influenced by the procedure of specimen fixation. The endothelial cells especially display an extraordinary capacity for plastic adaptation to changes of the blood pressure.

If care is taken to retain the blood in the vascular bed by clamping the thymic

FIG. 4. The capillaries of the peripheral connective tissue capsule of the thymic lobes are in places provided with a small number of endothelial fenestrae (*arrowheads*), closed by a diaphragm. Inset: horseradish peroxidase, 5 min after i.v. injection. Very little enzyme is found in the basal lamina and adventitia opposite an endothelial fenestra of a capillary of the capsule (*arrowhead*). × 30,000; inset, × 61,000.

FIG. 5. Horseradish peroxidase, 5 min after i.v. injection. In a capillary at the corticomedullary boundary, a faint staining of both endothelial basal lamina and adventitia (*Adv*) is seen. The cleft between two endothelial cells (*END*) is stained throughout its length (*arrows*), but the concentration of the reaction product decreases abruptly at a short distance from the lumen. Notice that most of the plasmalemmal vesicles situated close to or opening onto the tissue front of the endothelium are very weakly stained in comparison with those adjacent to the lumen. × 24,000.

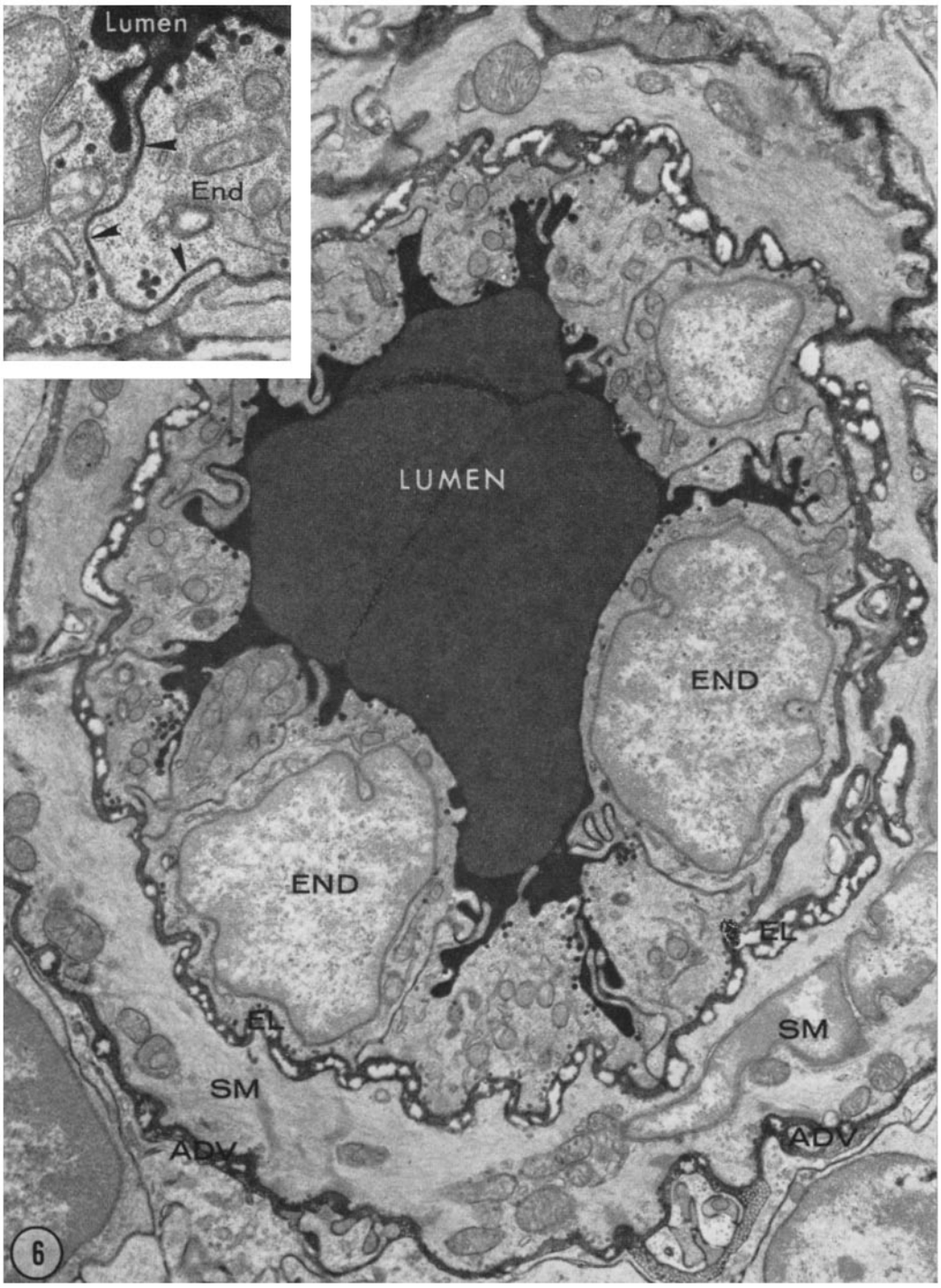


FIG. 6. Horseradish peroxidase, 5 min after i.v. injection. The walls of the arterioles at the cortico-medullary boundary consist of endothelium (*END*) with its basal lamina, elastica interna (*EL*), a single layer of smooth muscle cells (*SM*), and a thin adventitia (*ADV*). Reaction product is seen in the clefts of the endothelium, fenestrations of the elastica interna and adventitia; the lucid patches in the elastica interna are elastic fibers outlined in negative contrast by the reaction product. Inset: horseradish peroxidase, 5 min after i.v. injection. An endothelial cleft of an arteriole stains throughout its length with the same intensity as the blood plasma (*arrowheads*). *End*: endothelium. × 11,000; inset, × 19,000.

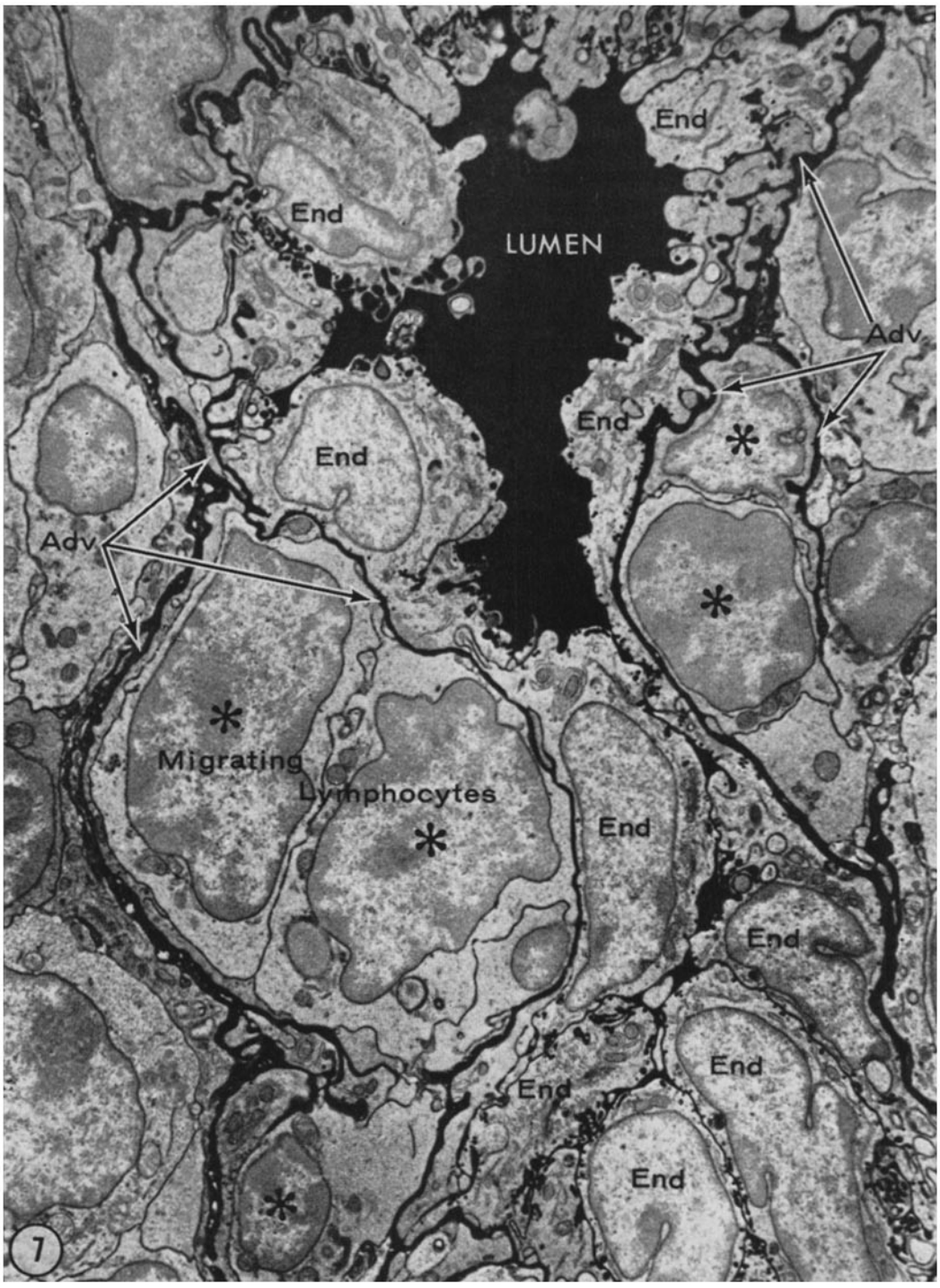


FIG. 7. Horseradish peroxidase, 5 min after i.v. injection. A postcapillary venule of the medulla is shown; notice its irregular endothelium (*End*) and the thin connective tissue adventitia (*Adv*), dissected by migrating lymphocytes (*asterisks*) into multiple, discontinuous layers. As a result of an impressive leakage of peroxidase, the endothelial basal lamina and the adventitia stain with the same intensity as the blood plasma. × 10,000.

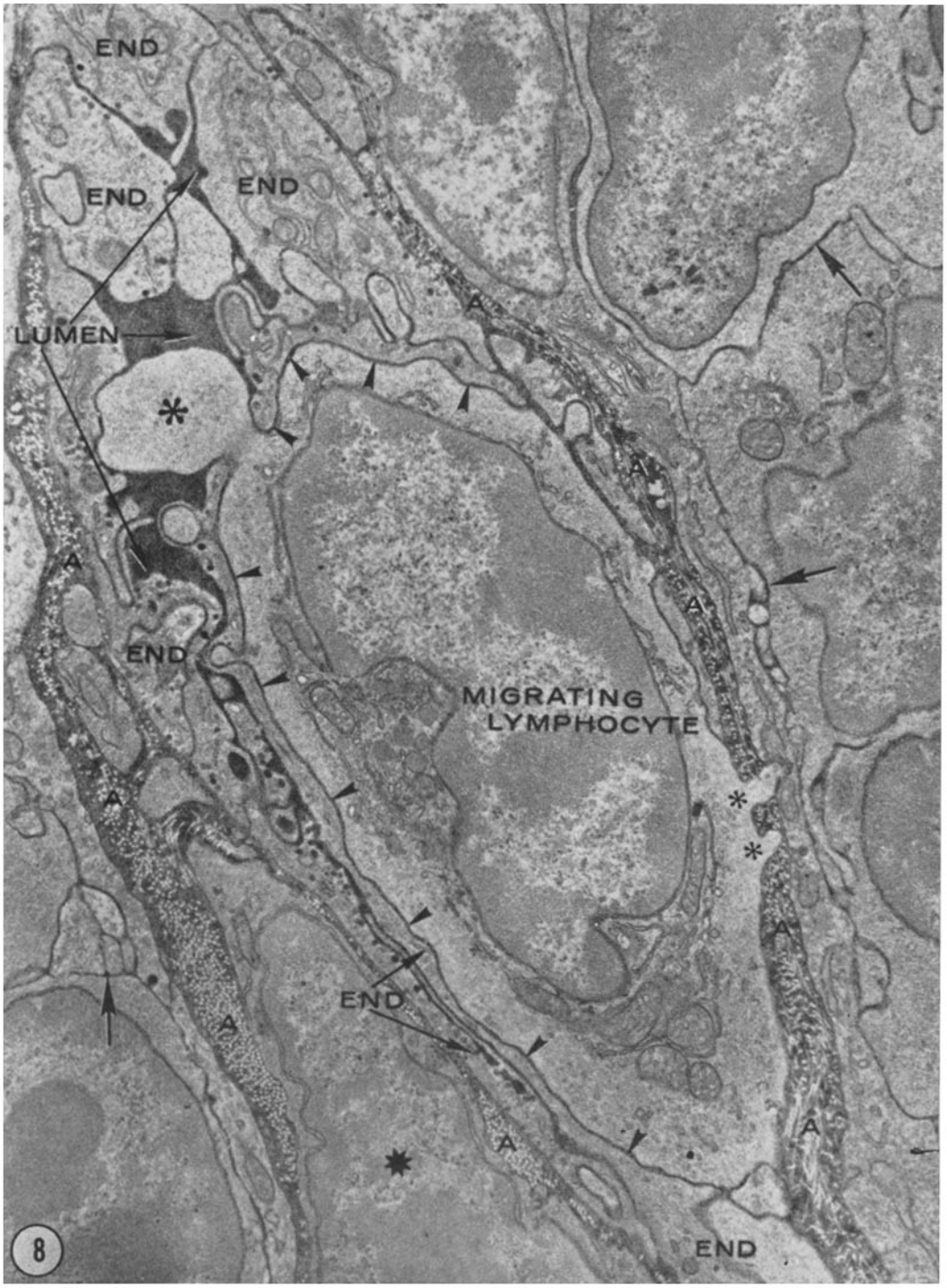


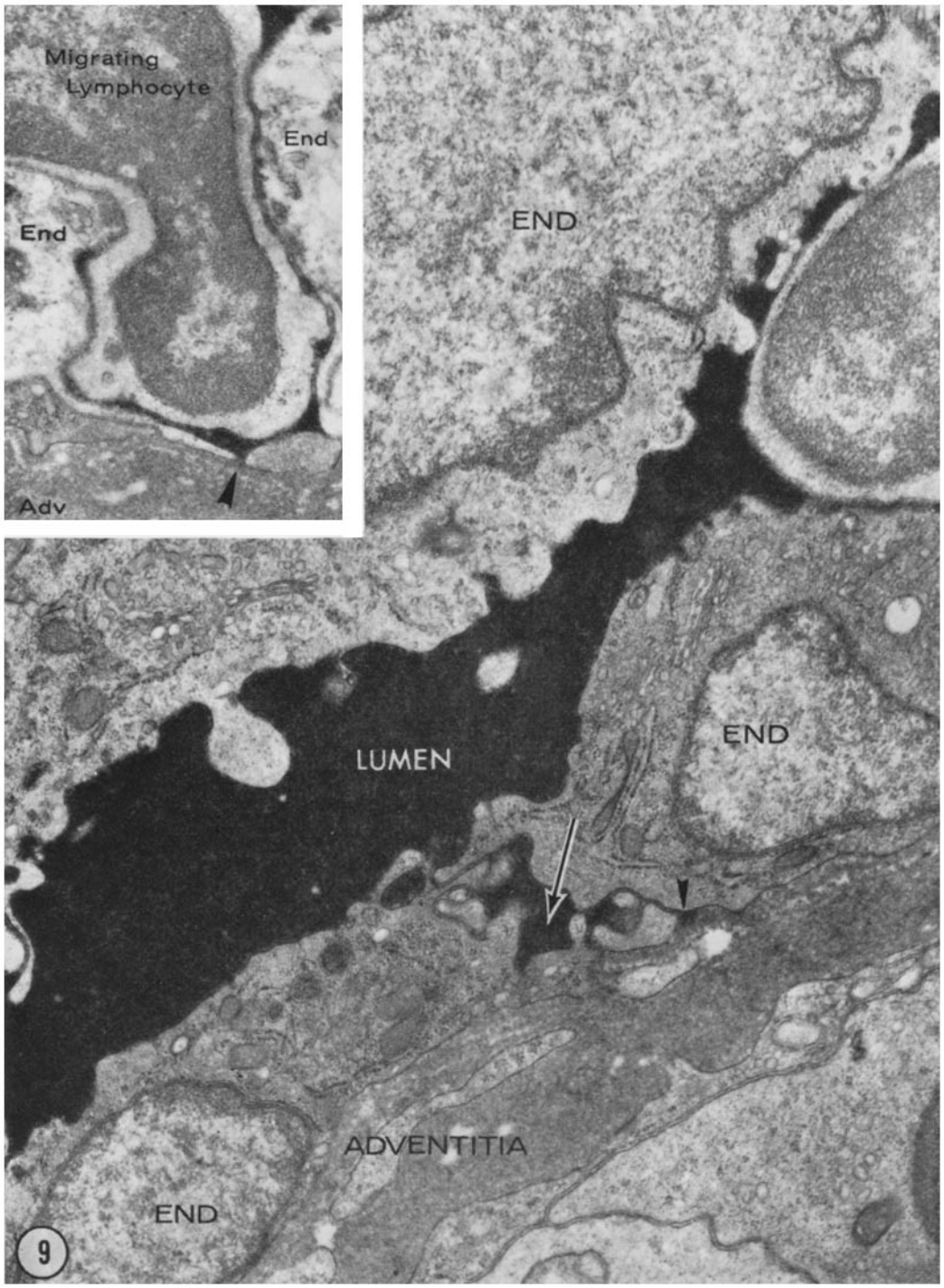
FIG. 8. Horseradish peroxidase, 5 min after i.v. injection. A lymphocyte is seen migrating through the endothelium (*END*) of a collapsed venule of the medulla. The migrating element has a pseudopod bathed in the blood plasma (*larger asterisk*) and two small processes (*smaller asterisks*) lying in the adventitia (*A*). The cleft between the lymphocyte and the adjacent endothelial cells is filled with reaction product (*arrowheads*). The collagen fibrils of the adventitia stand out in negative contrast against the intensely stained ground substance. Also the intercellular clefts of the surrounding medullary parenchyma contain peroxidase (*arrows*). A pericyte is embedded into the adventitia (*star*). × 12,500.

vessels before fixation, the shape and size of the intralobar vessels is much the same as in specimens fixed by vascular perfusion. Capillaries are perfectly cylindrical; they have an extremely attenuated endothelial lining and invariably contain red blood cells (Fig. 2). Therefore, the idea that only plasma skimmed from arterioles at the cortico-medullary boundary percolates through the capillary bed of the cortex (39) is no longer tenable. In venules the endothelium is thicker than in capillaries, but it does not occlude the lumen of the vessel nor does it show the myriad projections typical of collapsed venules.

In summary: (*a*) The thymic parenchyma mainly consists of tightly packed lymphocytes and reticular cells, with intervening narrow intercellular clefts, 100–200 A in thickness; blood vessels course through this compact cell mass, carrying a thin investment of adventitial connective tissue. (*b*) In the cortex, only capillaries are found; the arterioles are confined to the cortico-medullary boundary and the venules to the medulla. (*c*) Capillaries have tight endothelial junctions and a limited population of plasmalemmal vesicles; in the superficial cortex, however, and in the lobar connective tissue capsule, isolated capillaries display a small number of endothelial fenestrae. (*d*) Through the walls of the medullary venules large numbers of lymphocytes migrate from the thymic parenchyma into the bloodstream.

(*B*) *Experiments with Tracers.*—In the present analysis of the vascular permeability of the thymus, most of the tracers employed are enzymes, because their visualization by means of a histochemical reaction increases the sensitivity of the probe through the multiplying effect of the enzymatic activity; thus, the extent of the exchanges across the vascular walls can be roughly estimated. A wide spectrum of molecular sizes is tested with the purpose of sieving the dimensions of the vascular pores: horseradish peroxidase (a_e = 25–30 A, mol wt 40,000) and cytochrome *c* (a_e = 15 A, mol wt 12,000) penetrate both the small and the large pore systems (24, 30), whereas catalase (a_e = 52 A, mol wt 240,000) and ferritin (a_e = 61 A, mol wt 462,000) have the size required for a probe molecule of the large pore system (31, 34). Furthermore, the molecule of catalase is very close in both size and dimensions to IgG (42) and therefore could mimic the movement of an anti-lymphocyte serum through the vascular walls. Since both peroxidase (43) and ferritin (44) are immunogenic, their distribution within the thymus provides a reliable picture of the behavior of antigen. Finally, the highly electron-opaque colloidal lanthanum, suspended in the fixative fluid and perfused through the vascular tree, clearly outlines the clefts between the endothelial cells, thus allowing unequivocal identification of permeable intercellular junctions.

(*1*) *Horseradish peroxidase:* A clear-cut picture of the pattern of distribution of peroxidase in the thymic lobes is first seen 5 min after the injection of the tracer. In the capillaries of the cortex (Fig. 3), the intense staining of the luminal plasma continues into the clefts between adjoining endothelial cells. However, the reaction product within the cleft abruptly stops at a variable


Migrating
Lymphocyte
End
End
Adv
END
LUMEN
END
ADVENTITIA
END
9

distance from the basal lamina where the apposed endothelial cell membranes approach and fuse. These sites are marked on the cytoplasmic aspect of the membranes by a condensation of the cytoplasmic matrix. Thus, the progression of the tracer toward the basal lamina of the capillary is blocked by one of the occluding junctions that seal the intercellular spaces of the endothelium. In the cytoplasm of the endothelial cells, the vesicles opening to the luminal surface contain dense reaction product. Numerous vesicles that are apparently free in the cytoplasm near the adluminal surface of the endothelium also contain peroxidase, but the possibility cannot be ruled out that these vesicles are continuous with the luminal plasma membrane at another level of sectioning. Most of the vesicles situated close to or opening onto the tissue front of the endothelial cells are unstained. In some capillaries, however, a few vesicles are seen discharging their content of peroxidase at the abluminal surface of the endothelium.

The endothelial basal lamina and the capillary adventitia, as well as the intercellular clefts of the surrounding parenchyma, are unstained. Both the reticular cells and occasional lymphocytes adjoining the capillary walls are free of peroxidase. On the other hand, in some places macrophage processes abutting on the vascular adventitia contain isolated cytoplasmic vacuoles 0.1–0.5 μ in diameter and a few small vesicles stained with reaction product. This is not endogenous enzyme, because in control specimens, incubated for peroxidase after injection of saline alone, thymic macrophages are unstained.

In the fenestrated capillaries located either at the extreme periphery of the cortex or in the lobar connective tissue capsule, the endothelial cell junctions prevent the tracer from escaping along the intercellular clefts of the endothelium. The periendothelial space is also free of reaction product, except in the vicinity of some of the fenestrae, where a weak staining of the basal lamina occurs; the tracer concentration, however, decreases abruptly from the luminal to the abluminal side of the fenestra (Fig. 4, inset). Whether a diaphragm is present or not in these fenestrae cannot be established, because the luminal aspect of the aperture is filled with dense reaction product. Within the connective tissue capsule and pericortical lobules of adipose tissue, macrophages contain peroxidase-reactive vacuoles and vesicles, in spite of the fact that interstitial spaces appear to be devoid of reaction product.

FIG. 9. Catalase, 15 min after i.v. injection. In a venule of the medulla, a dilated space (*arrow*) between two endothelial cells (*END*) is filled with catalase. Opposite the opening of this space onto the tissue front of the endothelium, a clear-cut concentration gradient of the reaction product is seen in the endothelial basal lamina (*arrowhead*). A lymphocyte is probably migrating through the endothelium at another level of sectioning, because enlargements of the endothelial clefts only occur where migrating elements cross the endothelium. The adventitia is weakly stained. Inset: in a medullary venule, the cleft between a migrating lymphocyte and the adjacent endothelial cells (*END*) is filled with catalase. Tracer is leaking from the cleft into the endothelial basal lamina and adventitia (*arrowhead*). *Adv*: adventitia. × 20,000; inset, × 17,500.

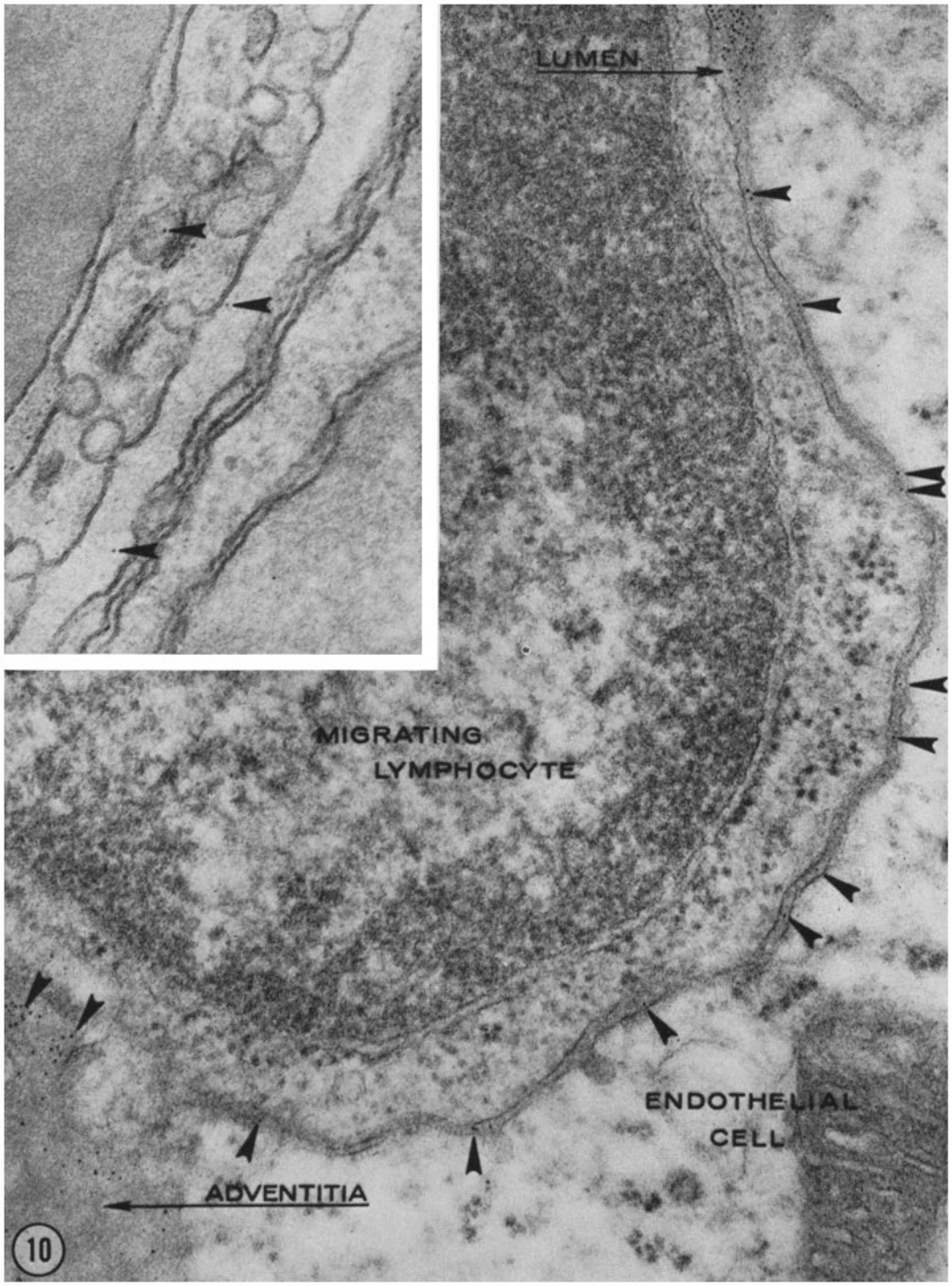


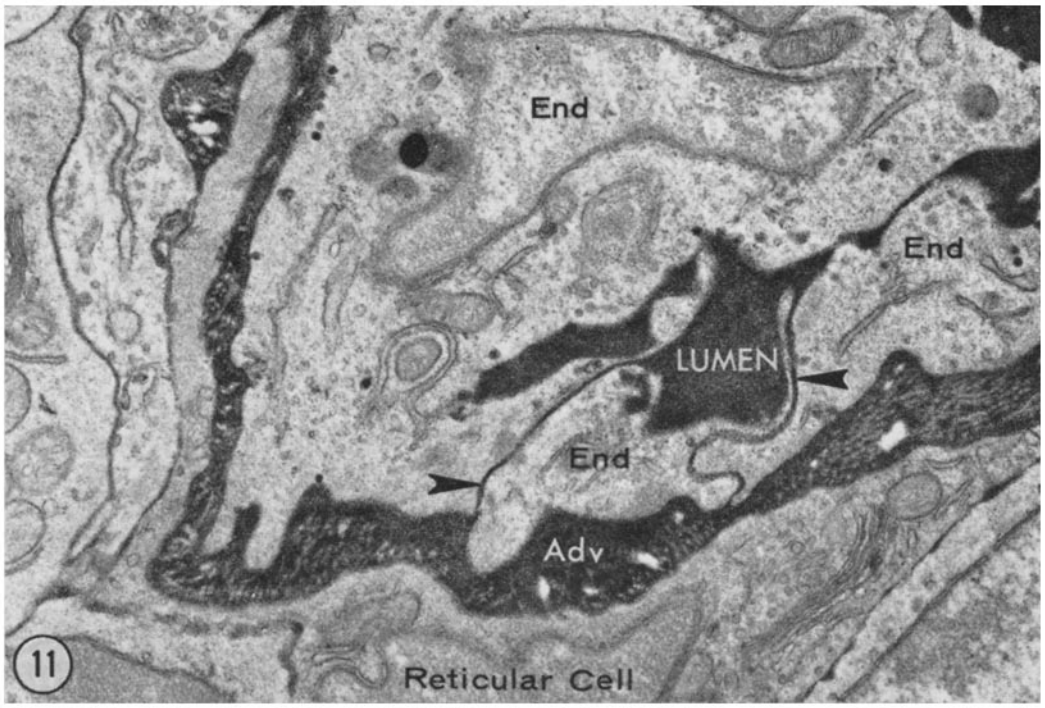
FIG. 10. Ferritin, 2 hr after i.v. injection. In the wall of a venule of the medulla, the cleft between a migrating lymphocyte and a neighboring endothelial cell is labeled with a row of ferritin molecules that extends from the lumen to the basal lamina (*arrowheads*). Opposite the opening of the cleft onto the tissue front of the endothelium, the concentration of the tracer in the basal lamina and adventitia is very high. Inset: 24 hr after the injection, very few ferritin molecules are seen scattered in the basal lamina and adventitia of the capillaries of the cortex (*arrowheads*). One particle is contained in a plasmalemmal vesicle of the endothelial cell. × 80,000; inset, × 100,000.

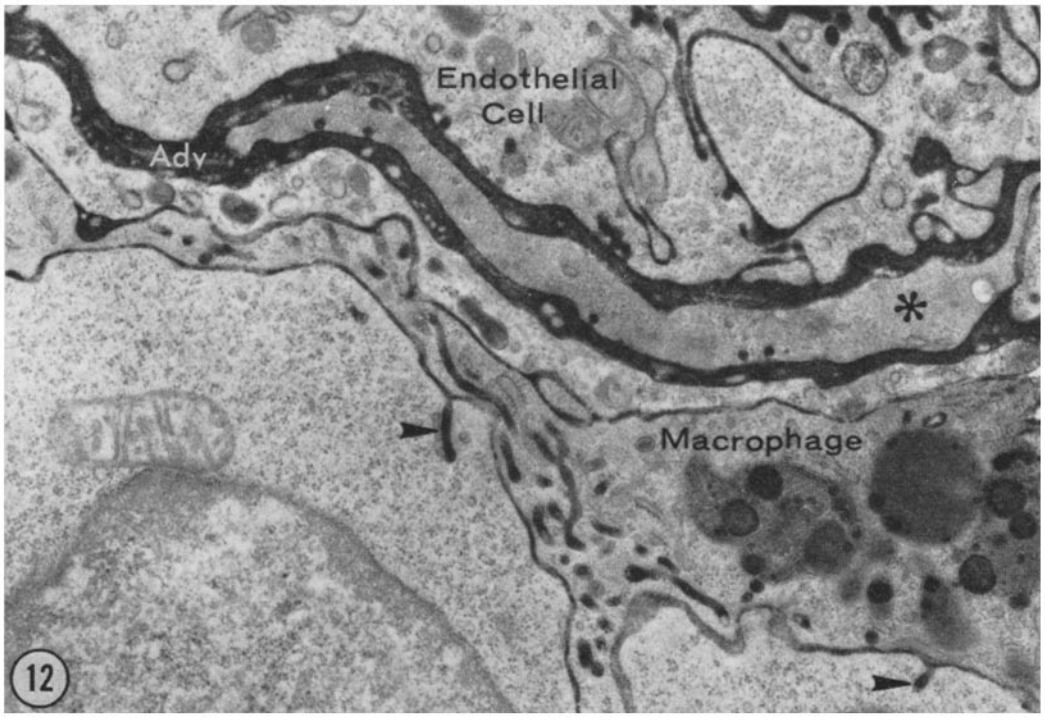
In the capillaries of the boundary region between cortex and medulla, a faint staining is detectable in the endothelial basal lamina and adventitia and it becomes more intense in capillaries situated more deeply in the lobe (Fig. 5). In the capillaries of the medulla, the staining of the periendothelial tunics reaches its greatest intensity, but it never equals the density of the reaction product in the vessel lumen. Here, the intercellular clefts of the endothelium are stained throughout their length, but the concentration of peroxidase falls off abruptly at a variable distance from the vessel lumen. This decrease in staining intensity consistently corresponds to a site of narrowing of the intercellular space, where a junctional specialization is presumably located.

At the cortico-medullary boundary, the arterioles show staining of the endothelial clefts, basal lamina, fenestrations of the elastica interna, and adventitia (Fig. 6). The concentration of the reaction product in the vascular wall varies in different arterioles, but it never equals that of the blood plasma. Along most of the clefts of the endothelium an abrupt diminution in tracer concentration is seen at a variable distance from the vessel lumen. Some of the clefts, however, are stained throughout their length with the same intensity as the blood plasma and a concentration gradient appears in the basal lamina opposite their abluminal end (Fig. 6, inset). Since the number of endothelial vesicles is low and very few heavily labeled vesicles seem to be successful in crossing the endothelium, the most plausible interpretation for the staining of the arteriolar walls is that peroxidase escapes from the lumen through a small number of patent clefts between endothelial cells and permeates the extracellular compartment of the vessel walls, including the abluminal portion of the endothelial clefts which are sealed by impermeable junctions.

The most impressive leakage of peroxidase occurs through the walls of the venules in the medulla (Fig. 7) and corresponds to the regions where lymphocytes are crossing the endothelium. Where lymphocytes are found stretched out across the entire thickness of the endothelium, with processes in the adventitia and pseudopods bathed in the plasma, an uninterrupted cleft stained throughout with the same intensity as the blood plasma extends from the vessel lumen to the vascular adventitia (Fig. 8). Where lymphocytes are sandwiched between the endothelial cells and their basal lamina, the clefts intervening between the migrating elements and neighboring endothelial cells are commonly filled with reaction product. Their staining intensity, however, varies, in some places being equal to the blood plasma and in others matching the staining intensity of the basal lamina. These differences in tracer content probably depend upon whether or not the clefts are continuous with the vessel lumen at another level of sectioning. In a few places, also the clefts between adjoining endothelial cells in venules are outlined by reaction product throughout their length (Fig. 11).

Both the endothelial basal lamina and the vascular adventitia of the venules have a high content of peroxidase and in places stain almost as intensely as the


End
End
LUMEN
End
Adv
11
Reticular Cell


Endothelial
Cell
Adv
*
Macrophage
12

blood plasma. Where the adventitia is disorganized or delaminated by migrating lymphocytes, the tracer has rapidly permeated all connective tissue spaces continuous with the vessel wall (Fig. 7), beautifully outlining collagen and elastic fibers in negative contrast.

At the boundary between the cortex and medulla and in the medulla the intercellular spaces of the thymic parenchyma contain peroxidase for variable distances around the walls of capillaries, arterioles, and especially venules (Figs. 8, 11, 12). Thus, the perivascular envelope of reticular cell processes does not prevent the tracer from entering into the parenchyma. Especially rapid is the diffusion of peroxidase along the reticular fibers of the medulla which are stained with reaction product even in regions which are otherwise free of peroxidase.

Where the parenchyma is permeated by the tracer, reticular cells always appear devoid of reaction product, except for isolated vesicular invaginations of the plasmalemma (Fig. 11). Thus, the membrane-bounded vacuoles, a lysosomal equivalent, characteristically found in the cytoplasm of reticular cells (45) are not involved in an endocytotic process. A small number of lymphocytes contains a few cytoplasmic vesicles full of peroxidase and occasionally their surface shows staining in tubular or vesicular invaginations of the limiting membrane. Macrophages, on the other hand, display a large collection of intensely stained cytoplasmic vacuoles of varying diameter and their surface is provided with a multitude of tubular and vesicular invaginations filled with reaction product (Fig. 12).

The sequence of events after the injection of the tracer is the following: *2 min:* Intense staining is seen in the blood plasma. Throughout the cortex, reaction product is limited to the lumen of the capillaries. Only in macrophages of the capsule do isolated cytoplasmic vacuoles contain peroxidase. In the medulla, the endothelial basal lamina and adventitia of some of the venules and a few reticular fibers are outlined by reaction product. *10–15–20 min:* The staining intensity of the blood plasma remains high. In the superficial cortex both capillary outer tunics and parenchymal intercellular clefts are free of reaction product. The cytoplasm of perivascular macrophages, however, contains many

FIG. 11. Horseradish peroxidase, 5 min after i.v. injection. In a venule of the medulla two clefts between endothelial cells (*END*) stain throughout their length with the same intensity as the blood plasma (*arrowheads*). In the adventitia (*Adv*) the dense staining of the ground substance outlines in negative contrast collagen fibrils and a few elastic fibers. The perivascular investment of reticular cells does not prevent the tracer from entering the intercellular clefts of the surrounding medullary parenchyma; notice that the reticular cell is completely free of reaction product. × 17,000.

FIG. 12. Horseradish peroxidase, 5 min after i.v. injection. A macrophage located near a venule of the medulla contains many cytoplasmic tubules and vesicles filled with peroxidase. Also lymphocytes are occasionally provided with tubular or vesicular invaginations of the plasmalemma which are stained with reaction product (*arrowheads*). A process either belonging to a pericyte or to a smooth muscle cell is embedded in the adventitia (*Adv*) of the venule (*asterisk*). × 19,500.

more bodies staining for peroxidase. In the deep cortex the parenchymal intercellular clefts are free of peroxidase, but an increasing number of capillaries contains reaction product in their endothelial basal lamina and adventitia. At the cortico-medullary boundary and in the medulla, the walls of arterioles, capillaries, and venules are permeated by peroxidase. From the adventitia of the vessels the tracer has diffused throughout the intercellular clefts of the medullary parenchyma. *30 min:* The staining intensity of the blood plasma begins to fade. The pattern of tracer distribution in the thymic lobes has not changed significantly. *1 hr:* The blood concentration of the reaction product is very low. The staining in the cytoplasm of capsular and parenchymal macrophages reaches its peak. It appears quite clear that throughout the cortex the macrophages are stretched out along the capillaries. Only the walls of the medullary venules and neighboring reticular fibers are weakly stained with reaction product. *7 hr:* The blood plasma is free of peroxidase. Reaction product is confined to the cytoplasm of macrophages, but both number and staining intensity of the phagosomes have significantly decreased.

In summary: in the cortex, capillaries have endothelial junctions impermeable to peroxidase. A limited vesicular transport of tracer occurs through the capillary endothelium, but the vascular adventitia and the intercellular clefts of the surrounding cortical parenchyma are free of peroxidase. Tracer, however, is found in the digestive vacuoles of perivascular macrophages. Very little peroxidase leaks through the endothelium of fenestrated capillaries. In the medulla, large amounts of tracer escape from the walls of the venules where lymphocytes are migrating through the endothelium. The tracer permeates the intercellular clefts of the medulla and cortico-medullary boundary, but it never ascends into the intercellular clefts of the cortical parenchyma, even at long time intervals from the injection. Also some of the junctions of the arteriolar endothelium seem to be permeable to peroxidase.

(*2*) *Cytochrome c:* Only specimens fixed 1 min after the injection of the tracer were examined. At this time interval the distribution of cytochrome *c* throughout the thymus parallels that of peroxidase 15–30 min after the injection; in particular, the tracer does not leak along the clefts of the capillary endothelium and the intercellular spaces of the parenchyma in the superficial cortex are free of reaction product (Fig. 3, inset). A major difference, however, is seen in fenestrated capillaries: here, a large fraction of the fenestrae seems to be permeable to the tracer.

(*3*) *Catalase:* With the light microscope a faint staining is seen in the walls of a number of medullary venules 15 min after the injection of catalase. With the electron microscope, the capillaries of the cortex show staining in some of the endothelial clefts, but the reaction product does not extend to the basal lamina. Very few vesicles opening or very close to the blood front of the capillary endothelium are stained. The other tunics of the capillary wall and the surrounding parenchyma are free of catalase. A similar picture is seen in the arterioles at the cortico-medullary boundary.

In the walls of the venules of the medulla, leakage of tracer occurs where lymphocytes are stretched out across the entire thickness of the endothelium: the cleft between the migrating element and neighboring endothelial cells stains throughout its length; opposite its abluminal end a concentration gradient of tracer appears in the basal lamina (Fig. 9). The rest of the basal lamina and the adventitia are weakly stained. When lymphocytes are sandwiched between the endothelium and its basal lamina the intervening clefts are generally unstained.

(*4*) *Ferritin:* At time intervals of 1–2 hr after intravenous injection, ferritin is present in high concentration in the blood plasma, where it appears uniformly distributed. No ferritin is found along the clefts between endothelial cells in the capillaries of the cortex. Variable numbers of vesicles at or near the luminal surface of the endothelium contain a few ferritin molecules, but these represent a very small fraction of the whole vesicle population of the endothelial cells. Labeled vesicles, opening on the tissue front of the endothelium, are rare. Multivesicular bodies containing varying amounts of tracer are occasionally seen in the cytoplasm of the endothelial cells. Within the basal lamina and adventitia of the cortical capillaries, ferritin is present in low concentration. Most commonly, isolated particles or small groups of two to three are seen randomly scattered along the circumference of the vessel at considerable distances from each other. In places they are more numerous, but only exceptionally are they absent. The intercellular clefts of the cortical parenchyma are free of ferritin. In the macrophages which adjoin the vascular walls, rare phagocytic vacuoles contain a small number of ferritin particles and isolated molecules are occasionally found in infoldings of the plasma membrane.

In the medulla, ferritin is present in high concentration throughout the basal lamina and adventitia of some venules, and in the surrounding parenchyma reticular fibers are heavily labeled, even at considerable distances from the vessel walls. The leakage of ferritin from the bloodstream occurs where lymphocytes are migrating through the endothelium of venules. In a few instances, the cleft between the migrating cell and neighboring endothelial cells is found labeled with a row of ferritin molecules, extending from the lumen of the vessel to its basal lamina (Fig. 10); the concentration of the tracer is especially high in the adventitia opposite the opening of the cleft onto the tissue front of the endothelium.

The concentration of ferritin in the blood plasma is much lower 24 hr after the injection. In cortical capillaries very few endothelial vesicles are labeled and the number of molecules found in the periendothelial spaces is extremely small (Fig. 10, inset). The intercellular clefts of the cortical parenchyma are free of ferritin. The perivascular macrophages often contain a large collection of tracer particles in the cytoplasmic matrix, whereas phagocytic vacuoles and residual bodies are only occasionally labeled.

(*5*) *Lanthanum:* After perfusion with lanthanum-containing fixative, the capillaries of the cortex stain intensely in the adluminal end of the intercellular clefts of the endothelium. The tracer extends for varying distances along the

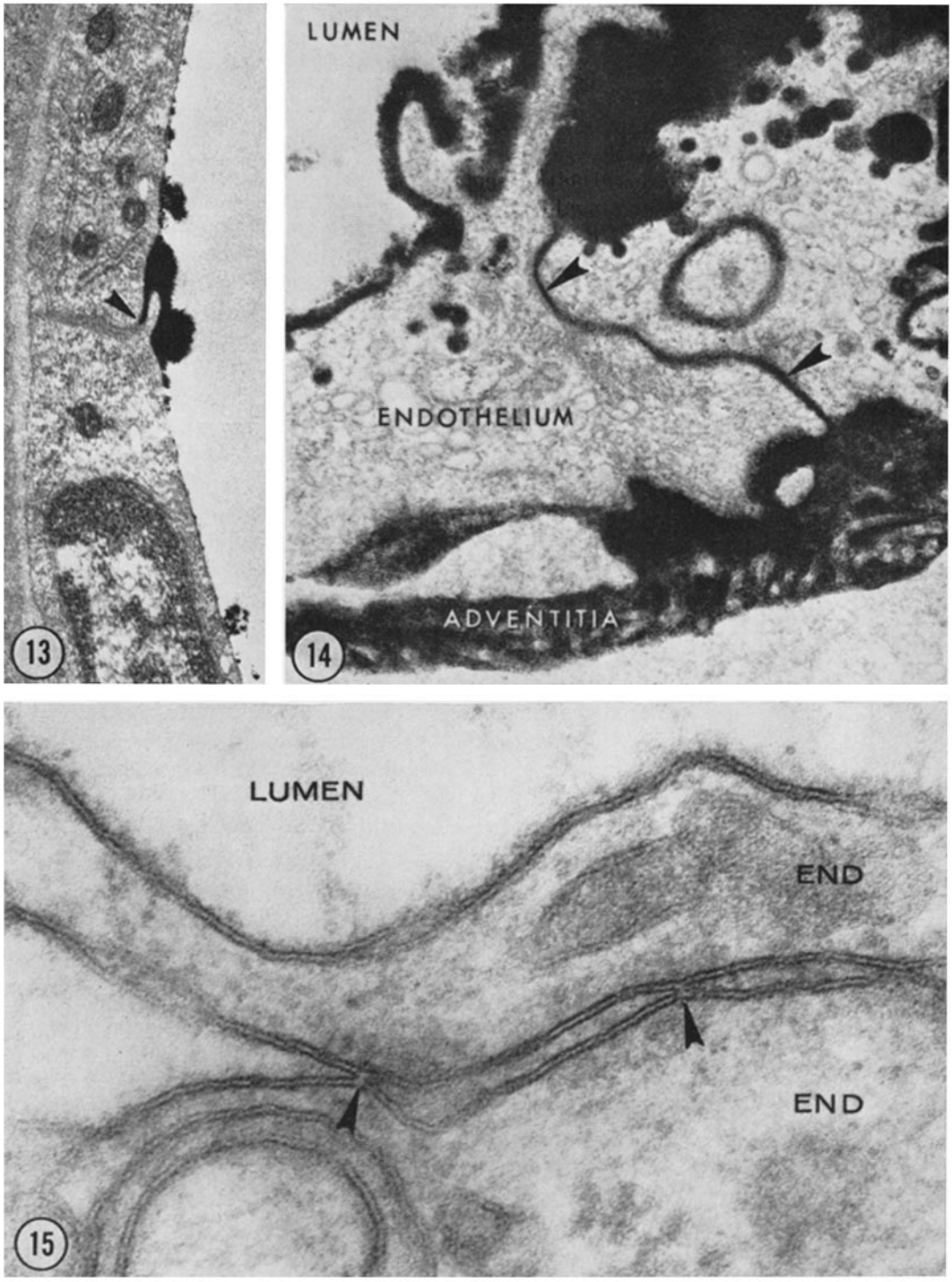


FIG. 13. Lanthanum perfusion. In the capillary of the cortex, only the luminal end of an intercellular cleft of the endothelium is filled with tracer (*arrowhead*). × 26,000.

FIG. 14. Lanthanum perfusion. In a venule of the medulla, an intercellular cleft of the endothelium is filled throughout its length with tracer (*arrowheads*). Also the endothelial basal lamina and the adventitia are permeated with lanthanum. × 53,000.

FIG. 15. Luminal portion of the cleft between two endothelial cells (*END*) of a capillary of the cortex; the intercellular space is sealed by two focal membrane fusions (*arrowheads*). × 240,000.

clefts, but it never reaches the basal lamina. Its leakage from the lumen is seemingly blocked at a constriction of the intercellular space, which probably corresponds to a tight junction between contiguous endothelial cells (Fig. 13).

In many venules of the medulla, an intense staining is seen along the clefts between migrating lymphocytes and adjoining endothelial cells; the underlying basal lamina and adventitia are also permeated by the tracer. However, the diffusion of lanthanum through the perivascular connective tissue spaces is rather limited, in comparison with peroxidase and ferritin; in general, only a short stretch of adventitia near the leak of the endothelium is stained, together with some of the neighboring intercellular spaces of the parenchyma. Most clefts between venular endothelial cells are only stained in their luminal portion; a few, however, are outlined by lanthanum throughout their length, and opposite their abluminal end the tracer permeates a limited region of the basal lamina and adventitia (Fig. 14). As filling of the abluminal portion of the cleft by lanthanum escaping from the lumen elsewhere is unlikely, these intercellular clefts may be permeable to the tracer throughout their length.

DISCUSSION

Although the thymus lacks the strict control of transendothelial exchanges which was demonstrated in the brain (46), several mechanisms concur in protecting the proliferating lymphocytes of the cortex from the influence of circulating antigen, so that the net effect is the same. On the contrary, blood-borne macromolecules have free access to the medulla. Making allowance for these restrictions, the concept of a blood-thymus barrier can be accepted, but limitedly to the cortex.

In contrast with the organs so far studied, the penetration of circulating ultrastructural tracers into the thymus and their distribution in the thymic parenchyma are not only governed by the permeability properties of the vascular walls, but also by the spatial distribution of the vessels within the lobe and the histological organization of the thymic parenchyma. In turn, the peculiar architecture of the thymus has provided a unique opportunity to analyze the differences in permeability of the various sections of the vascular tree. Arterioles have a small number of endothelial junctions which are permeable to peroxidase, but do not allow passage of tracers of higher molecular weight. The physiological significance of this finding is not known, nor is a correlation possible with the permeability properties of the arterial walls elsewhere in the body, because there is insufficient available information (47, 48). The capillaries of the thymus have endothelial junctions impermeable to all tracers injected including cytochrome *c* and colloidal lanthanum; in this respect they differ from the capillaries of skeletal muscle, myocardium (24), and testis (49). The endothelial cells, however, contain a moderate number of plasmalemmal vesicles and these transport across the endothelium limited amounts of all tracers injected, except catalase which is known to be a poor marker for micro-

pinocytotic vesicles (31). In postcapillary venules all tracers, including catalase and ferritin, escape from the bloodstream following along the clefts between migrating lymphocytes and endothelial cells. A similar finding has been reported in lymph nodes (50), but elsewhere in the body leakage of very large molecules or particulate matter through the vascular walls is an uncommon phenomenon in physiological conditions, occurring only through the walls of the sinusoids in the liver (36), bone marrow (51), spleen (52), and through the open fenestrae of the glomerular capillaries (35). A small number of endothelial clefts in venules may also be permeable, but their patency could not be established beyond doubt in high magnification studies nor could the possibility be ruled out that they fill throughout their length with tracer because a lymphocyte is migrating through the endothelium at another level of sectioning or that tracer which escaped elsewhere from the bloodstream has penetrated their abluminal end from the adventitia.

Despite the presence of permeable vessels, circulating tracers have access only to limited regions of the thymic parenchyma; in fact, the various segments of the vascular tree are spatially segregated within the lobe, in such a way that the cortex is exclusively supplied by capillaries with impermeable endothelial junctions while the medulla contains all the leaky vessels, namely postcapillary venules and arterioles. Therefore, in the cortex only the tracer than can be transported by plasmalemmal vesicles is released on the parenchymal front of the capillary endothelium, whereas there is massive extravasation of tracer through the venular walls in the medulla.

The extent of the vesicular transport through the walls of the cortical capillaries seems to be rather limited. Very few vesicles loaded with tracer appear to be successful in merging with the abluminal membrane of the endothelium. This is in accord with a theoretical model for vesicular transport proposed by one of us (53). The capillary adventitia always appears to be devoid of peroxidase and contains a very small number of ferritin molecules at short time intervals after the injection. Moreover, at increasing intervals of time after the introduction of ferritin into the bloodstream, this tracer does not accumulate in the periendothelial spaces as it does in muscle capillaries (34).

The question arises as to whether the tracer molecules released on the tissue front of the capillary endothelium come into contact with the lymphoid and reticular cells of the cortex. In considering this question one must take into account that another feature of the architecture of the thymic lobe, other than the arrangement of the blood vessels, influences the distribution of the tracers escaping from the bloodstream. That is, that the vessels are contained in coaxial canals bounded by a continuous investment of parenchymal cells, and the connective tissue compartment of the organ is almost exclusively represented by the vascular adventitia. Thus, the small amount of tracer released by the endothelium has free access to a very limited parenchymal surface, represented by the reticular cells, macrophages, and occasional lymphocytes that surround the

vascular adventitia. The tracer does not reach the rest of the cell population of the cortex, because it is promptly sequestrated by the perivascular macrophages stretched out in a continuous row along the cortical capillaries. With peroxidase, the surprising phenomenon is observed that at long time intervals after the injection, the perivascular macrophages are loaded with tracer, despite the fact that the latter is never seen in the vascular adventitia. Probably, the quantity of peroxidase released on the tissue front of the endothelium is diluted into the basal lamina and adventitia to such an extent that the concentration of the reaction product does not reach the threshold of visibility with the electron microscope. However, since most of the vascular front of the parenchyma is occupied by reticular cells which do not seem capable of phagocytosis, the tracer is channeled toward the processes of the macrophages which abut on the vascular adventitia and it accumulates in their digestive vacuoles. Thus, the burden of tracer in the perivascular macrophages of the cortex becomes a reliable indicator of the amount of vesicular transport which has taken place through the capillary walls.

In the medulla, the tracer escapes in large quantities from the walls of the venules, preferentially advances along the vascular adventitia, while it penetrates slowly the intercellular clefts of the surrounding medullary parenchyma. Its range of distribution is obviously dependent on its molecular weight, dose, and time interval from the injection. With peroxidase, at a dose of 5 mg, the invasion of the thymic parenchyma is maximal at 20–30 min from the injection; the tracer has permeated most of the intercellular clefts between lymphoid and reticular cells of both medulla and cortico-medullary boundary and following along the perivascular connective tissue spaces, it has reached the adventitia of the capillaries in the deep cortex where it appears confined to the vascular outer tunic. With longer time intervals from the injection, the concentration of the enzyme in the blood plasma declines, and the extravasated tracer begins to recede from the thymic parenchyma. Thus, the clefts between lymphoid and reticular cells of the cortex always remain free of peroxidase. The possibility exists that with higher doses and/or repeated injections, the tide of peroxidase rising from the medulla could invade the cortical parenchyma, but in our opinion such a finding would have very little biological significance, because the amount of tracer required to produce it would be disproportionately large. Tracers of higher molecular weight, such as ferritin and catalase, do not extend beyond the medullary parenchyma surrounding the leaky vessels.

The presence of fenestrated capillaries in the thymus was used as an argument against the existence of a barrier (54). They do not represent, however, a significant leak in the vascular tree, at least for macromolecules; few in number and mostly confined to the perilobar connective tissue capsule, they are only exceptionally seen in the outermost region of the cortex. Furthermore, their fenestrae, although readily penetrated by cytochrome *c*, are scarcely permeable to peroxidase. Their significance is unknown; a relationship to the presumed

endocrine function of the thymus (55) can be ruled out, as they are most commonly located outside the parenchyma. Since they are often surrounded in the capsule by heterogeneous collections of connective tissue cells, and they are very rare in mature fat lobules, it is tempting to suggest that they might be related with the development of adipose tissue which parallels thymic involution and that they disappear again after histogenesis of the fat lobules is completed.

It must be emphasized that in contrast with previous assumptions (11, 39), neither the perivascular investment of reticular cells nor the boundary layer formed by these cells at the periphery of the lobe prevent tracers from entering the thymic parenchyma; therefore, foreign materials, injected directly into the mediastinum can readily penetrate the thymic parenchyma (56), provided their quantity is large enough to saturate the numerous phagocytes contained in the connective tissue capsule of the thymic lobes.

The present study clearly demonstrates that in the cortex lymphocytes are protected from circulating macromolecules by a twofold mechanism: impermeability of the endothelial junctions and strategic location of the macrophages along the vessels. In most mammals, but not in the mouse, an additional mechanism is found: a concentration of macrophages at the cortico-medullary boundary (9, 57). Since both horseradish peroxidase and ferritin are immunogenic and the amounts of substance chosen for the present study by far exceed the doses required for effective immunization, there is no doubt that the blood-thymus barrier must be effective in preventing access of circulating antigen to cortical lymphocytes.

On the contrary, circulating macromolecules have free access to the lymphocytes of the medulla. The number of medullary lymphocytes which are exposed to blood-borne substances and the concentration of these latter in the medulla are clearly related to both the rate of bulk flow of macromolecules from the vascular bed and the rate of their removal from the parenchyma; in turn, the removal is probably a function of medullary macrophages, because there is no satisfactory evidence that lymphatic vessels are present in the thymic parenchyma. Therefore, a macromolecule which is not phagocytized by macrophages could have access to a very large proportion of the lymphoid population of the thymus.

While the access of circulating macromolecules to cortical lymphocytes is impeded, this may not necessarily be true for smaller substances. Small molecules, however, may enter the cortex by selective transport through the capillary endothelium and be restricted in their free movement across the vascular walls by the tight junctions which seal the endothelial clefts. This could explain why the distribution of a small solute such as tritiated thymidine is not uniform throughout the thymus after intravenous injection (58), and the pattern of cell labeling mimics the distribution of peroxidase. Steroid hormones, on the other hand, which selectively affect cortical lymphocytes (59) could freely cross the capillary endothelium in virtue of their lipid solubility.

On the basis of the results of the present study, it becomes clear why so many contradictory reports exist in the literature about the penetration of circulating tracers and macromolecules into the thymus. Previous light and electron microscope studies (1, 6, 7, 9–18) have shown that particulate matter, foreign proteins, and bacterial antigens either do not enter the thymus at all or they are found in small amounts inside the perivascular macrophages, especially in the medulla. In most of these studies, the existence of a blood-thymus barrier was claimed or denied on the simple basis of the presence or absence of foreign substances in the organ, but the possibility was overlooked that the barrier could be restricted to one region of the thymic parenchyma and that macrophages could be an integral part of the mechanism, which protects cortical lymphocytes from circulating tracers. On the other hand, the lack of a barrier in the medulla explains why studies on the whole organ or tissue homogenates were consistently able to detect the presence of injected radioactive proteins in the extravascular compartment of the thymus (8, 14, 19, 60, 61). This study, therefore, provides a satisfactory explanation for the phenomenon that the thymus is not affected by the injection of anti-lymphocyte sera, prepared by immunizing animals against suspensions of thymus cells (20–23).

The inadequacy of available information on the mechanisms responsible for the differentiation of the thymic lymphocytes does not allow a satisfactory correlation between the pattern of vascular permeability in the different regions of the thymic lobe and the developmental history of immunological competence in the lymphoid population of the thymus. There are, however, important coincidences which deserve to be mentioned. Very little doubt remains at present about the fact that thymic small lymphocytes originate from proliferation of larger precursors in the cortex, a proportion of them die *in situ,* while the survivors migrate to the medulla and finally leave the organ to enter the blood stream through the walls of the postcapillary venules (cf. 41). Among thymic lymphocytes a proportion seem to have graft-*versus*-host reactivity (62–65) or be capable of blastoid transformation subsequent to contact with phytohemagglutinin (66–68) or allogeneic cells (65, 69) and these seem to be located in the medulla (66, 70, 71); the suggestion has therefore been advanced that thymic lymphocytes become immunologically competent at the very moment of leaving the thymus (65, 72). If this proves to be true, then the presence of a barrier in the cortex and its absence in the medulla suggest that circulating substances might represent the trigger stimulus for thymic lymphocytes to complete maturation.

As a result of a growing body of experiments with antisera to antigenic markers on the thymic or thymus-derived lymphocytes (65, 72–74), it has been proposed that the differentiation of thymocytes to competent peripheral thymus-derived lymphocytes consists of an orderly succession of maturational steps, possibly reflected by the changes in the constellation of their surface antigens. These steps might well correlate with the different microenvironments the thymic lymphocytes experience on their way from the cortex to the bloodstream

and the pattern of vascular permeability in the thymus might therefore insure that inductive influences act upon thymic lymphocytes in an appropriate sequence.

SUMMARY

In order to verify the existence of a blood-thymus barrier to circulating macromolecules, the permeability of the vessels of the thymus was analyzed in young adult mice using electron opaque tracers of different molecular dimensions (horseradish peroxidase, cytochrome *c*, catalase, ferritin, colloidal lanthanum). Results show that although blood-borne macromolecules do penetrate the thymus, their parenchymal distribution is limited to the medulla of the lobe by several factors: (*a*) the differential permeability of the various segments of the vascular tree; (*b*) the spatial segregation of these segments within the lobe; (*c*) the strategic location of parenchymal macrophages along the vessels.

The cortex is exclusively supplied by capillaries, which have impermeable endothelial junctions. Although a small amount of tracer is transported by plasmalemmal vesicles through the capillary endothelium, this tracer is promptly sequestrated by macrophages stretched out in a continuous row along the cortical capillaries and it does not reach the intercellular clefts between cortical lymphocytes and reticular cells.

The medulla contains all the leaky vessels, namely postcapillary venules and arterioles. Across the walls of the venules, large quantities of all injected tracers escape through the clefts between migrating lymphocytes and endothelial cells; also the arterioles have a small number of endothelial junctions which are permeable to peroxidase, but do not allow passage of tracers of higher molecular weight. The tracers released by the leaky vessels penetrate the intercellular clefts of the medulla, but they never reach the cortical parenchyma, even at long time intervals after the injection.

Therefore, a blood-thymus barrier to circulating macromolecules does exist, but is limited to the cortex. Medullary lymphocytes are freely exposed to blood-borne substances.

We gratefully acknowledge the help of Mr. Ronald Takvorian during the initial phases of this work.

REFERENCES

1. Marshall, A. H. E., and R. G. White. 1961. The immunological reactivity of the thymus. *Br. J. Exp. Pathol.* **42**:379.
2. Kennedy, J. C., L. Siminovitch, J. E. Till, and E. A. McCulloch. 1965. A transplantation assay for mouse cells responsive to antigenic stimulation by sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* **120**:868.
3. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant, and E. V. Elliott. 1967. The failure of thymus-derived cells to produce antibody. *Transplantation.* **5**:222.
4. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev.* **47**:437.

5. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **59**:296.
6. Green, I., and K. Bloch. 1963. Uptake of particulate matter within the thymus of adult and new-born mice. *Nature (Lond.)*. **200**:1099.
7. Kostowiecki, M. 1967. Does the poor immunological reactivity of the thymus really depend on the vascular barrier of Marshall and White? *Z. Mikrosk.-Anat. Forsch.* **76**:320.
8. Kouvalainen, K., and D. Gitlin. 1967. Passage of antigen across the vascular barrier of the thymus. *Nature (Lond.)*. **214**:592.
9. Bargmann, W. 1943. Der Thymus. *In* W. v. Möllendorff's Handbuch der mikroskopischen Anatomie des Menschen. Springer-Verlag GmbH., Berlin. **6**(Pt. 4): 62.
10. Kaplan, M. H., A. H. Coons, and H. W. Deane. 1950. Localization of antigen in tissue cells. III. Cellular distribution of pneumococcal polysaccharides types II and III in the mouse. *J. Exp. Med.* **91**:15.
11. Clark, S. L., Jr. 1964. The penetration of proteins and colloidal materials into the thymus from the blood stream. *Thymus, Symp., Wistar Inst. Monogr.* **2**:9.
12. Bridges, J. B., and C. H. Calvert. 1966. Some observations on the blood-thymus barrier. *J. Anat.* **100**:934.
13. Irmscher, J., M. Müller, and F. Arnold. 1966. Untersuchungen zur Frage einer Blut-Thymusschranke bei neugeborenen und 4 Wochen alten Kaninchen. *Frankfurt. Z. Pathol.* **75**:252.
14. Nossal, G. J. V., and J. Mitchell. 1966. The thymus in relation to immunological tolerance. *Thymus: Exp. Clin. Stud., Ciba Found. Symp.* 105.
15. Jaroslow, B. N. 1967. Genesis of Hassall's corpuscles. *Nature (Lond.)*. **215**:408.
16. Lance, E. M. 1969. The mechanism of action of anti-lymphocyte serum. Studies of antibody eluate. *J. Exp. Med.* **130**:49.
17. Chapman, A. L., and W. J. Bopp. 1970. Electron microscopy of vascular barrier in thymus, tonsil, and lymph node of beagle pups. *Am. J. Vet. Res.* **31**:1255.
18. Blau, J. N. 1971. Histological changes and macrophage activity in the adult guinea-pig thymus. *Br. J. Exp. Pathol.* **52**:142.
19. Denman, A. M., and E. P. Frenkel. 1968. Mode of action of anti-lymphocyte globulin. I. The distribution of rabbit anti-lymphocyte globulin injected into rats and mice. *Immunology.* **14**:107.
20. Taub, R. N., and E. M. Lance. 1968. Histopathological effects in mice of heterologous antilymphocyte serum. *J. Exp. Med.* **128**:1281.
21. Tyler, R. W., N. B. Everett, and M. R. Schwarz. 1969. Effect of anti-lymphocytic serum on rat lymphocytes. *J. Immunol.* **102**:179.
22. Röpke, C. 1971. Cytological and histological events following treatment with anti-thymocytic globulin in mice. Studies on the increased decay in lymphatic tissue. *Acta Pathol. Microbiol. Scand. Sect. B: Microbiol. Immunol.* **79**:23.
23. Simpson, E., and S. L. Nehlsen. 1971. Prolonged administration of anti-thymocyte serum in mice. II. Histopathological investigation. *Clin. Exp. Immunol.* **9**:79.
24. Karnovsky, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:213.

25. Richardson, K. C., L. Jarett, and E. H. Finke. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35:**313.
26. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25:**407.
27. Olson, I. A., and D. E. Skuse. 1969. The use of liquid photographic emulsion for demonstrating blood vessels in histological sections. *J. Microsc. (Oxf.)* **89:**345.
28. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14:**291.
29. Karnovsky, M. J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. *In* Abstracts of the 11th Annual Meeting of American Society of Cell Biology, New Orleans. 146.
30. Karnovsky, M. J., and D. F. Rice. 1969. Exogenous cytochrome *c* as an ultrastructural tracer. *J. Histochem. Cytochem.* **17:**751.
31. Schneeberger, E. E., and M. J. Karnovsky. 1971. The influence of intravascular fluid volume on the permeability of newborn and adult mouse lungs to ultrastructural protein tracers. *J. Cell Biol.* **49:**319.
32. Venkatachalam, M. A., and H. D. Fahimi. 1969. The use of beef liver catalase as a protein tracer for electron microscopy. *J. Cell Biol.* **42:**480.
33. Revel, J. P., and M. J. Karnovsky. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33:**C7.
34. Bruns, R. R., and G. E. Palade. 1968. Studies on blood capillaries. II. Transport of ferritin molecules across the wall of muscle capillaries. *J. Cell Biol.* **37:**277.
35. Farquhar, M. G., S. L. Wissig, and G. E. Palade. 1961. Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* **113:**47.
36. Hampton, J. C. 1958. An electron microscope study of the hepatic uptake and excretion of submicroscopic particles injected into the blood stream and into the bile duct. *Acta Anat.* **32:**262.
37. Smith, C., E. Conant Thatcher, D. Ziegler Kraemer, and E. Sayer Holt. 1952. Studies on the thymus of the mammal. VI. The vascular pattern of the thymus of the mouse and its changes during aging. *J. Morphol.* **91:**199.
38. Clark, S. L., Jr. 1963. The thymus in mice of strain 129/J, studied with the electron microscope. *Am. J. Anat.* **112:**1.
39. Weiss, L. 1963. Electron microscopic observations on the vascular barrier in the cortex of the thymus of the mouse. *Anat. Rec.* **145:**413.
40. Marchesi, V. T., and J. L. Gowans. 1964. The migration of lymphocytes through the endothelium of venules in lymph nodes: an electron microscope study. *Proc. R. Soc. Lond. B Biol. Sci.* **159:**283.
41. Sainte-Marie, G., and F.-S. Peng. 1971. Emigration of thymocytes from the thymus: a review and study of the problem. *Rev. Can. Biol.* **30:**51.
42. Samejima, T., and J. T. Yang. 1963. Reconstitution of acid-denatured catalase. *J. Biol. Chem.* **238:**3256.
43. Leduc, E. H., S. Avrameas, and M. Bouteille. 1968. Ultrastructural localization of antibody in differentiating plasma cells. *J. Exp. Med.* **127:**109.

44. De Petris, S., G. Karlsbad, and B. Pernis. 1963. Localization of antibodies in plasma cells by electron microscopy. *J. Exp. Med.* **117**:849.
45. Raviola, E., and G. Raviola. 1962. Ulteriori osservazioni submicroscopiche sui rapporti fra lisosomi e cromolipoidi. *Monit. Zool. Ital.* **70**(Suppl.):83.
46. Reese, T. S., and M. J. Karnovsky. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**:207.
47. Florey, L., and B. L. Sheppard. 1970. The permeability of arterial endothelium to horseradish peroxidase. *Proc. R. Soc. Lond. B Biol. Sci.* **174**:435.
48. Walters, M. N. -I., J. M. Papadimitriou, T. A. Robertson, and J. M. Archer. 1970. Permeability of pancreatic endothelium to horseradish peroxidase. *J. Pathol.* **100**:31.
49. Fawcett, D. W., L. V. Leak, and P. M. Heidger, Jr. 1970. Electron microscopic observations on the structural components of the blood-testis barrier. *J. Reprod. Fertil.* **10**(Suppl.):105.
50. Mikata, A., and R. Niki. 1970. Permeability of postcapillary venules of the lymph node. An electron microscopic study. *Exp. Mol. Pathol.* **14**:289.
51. Zamboni, L., and D. C. Pease. 1961. The vascular bed of red bone marrow. *J. Ultrastruct. Res.* **5**:65.
52. Weiss, L. 1963. The structure of intermediate vascular pathways in the spleen of rabbits. *Am. J. Anat.* **113**:51.
53. Karnovsky, M. J., and S. M. Shea. 1970. Transcapillary transport by pinocytosis. *Microvasc. Res.* **2**:353.
54. Kramarsky, B., R. Siegler, and M. A. Rich. 1967. Presence of endothelial fenestrations in thymic capillaries of mice. *J. Cell Biol.* **35**:464.
55. Clark, S. L., Jr. 1968. Incorporation of sulfate by the mouse thymus: its relation to secretion by medullary epithelial cells and to thymic lymphopoiesis. *J. Exp. Med.* **128**:927.
56. Sainte-Marie, G. 1963. Antigen penetration into the thymus. *J. Immunol.* **91**:840.
57. Raviola, E., G. Raviola, and M. T. Tenconi. 1961. Lisosomi e cromolipoidi nei macrofagi del timo di ratto. *Riv. Istoch. Norm. Patol.* **7**:521.
58. Sainte-Marie, G., and B. Messier. 1970. Thymus and node labeling by intramediastinal injection of tritiated tracers and its bearing on present concepts of lymphocyte cytokinetics. *Scand. J. Haematol.* **7**:163.
59. Ishidate, M., and D. Metcalf. 1963. The pattern of lymphopoiesis in the mouse thymus after cortisone administration or adrenalectomy. *Aust. J. Exp. Biol. Med. Sci.* **41**:637.
60. Garvey, J. S., D. V. Eitzman, and R. T. Smith. 1960. The distribution of S^{35}-labeled bovine serum albumin in newborn and immunologically tolerant adult rabbits. *J. Exp. Med.* **112**:533.
61. Hinz, B., and M. M. Webber. 1965. Antithymic antibody localization in the mouse. *Nature (Lond.)*. **208**:797.
62. Billingham, R. E., V. Defendi, W. K. Silvers, and D. Steinmuller. 1962. Quantitative studies on the induction of tolerance of skin homografts and on runt disease in neonatal rats. *J. Natl. Cancer Inst.* **28**:365.
63. Cohen, M. W., G. J. Thorbecke, G. M. Hochwald, and E. B. Jacobson. 1963. Induction of graft-*versus*-host reaction in newborn mice by injection of newborn or adult homologous thymus cells. *Proc. Soc. Exp. Biol. Med.* **114**:242.

64. Cantor, H., M. A. Mandel, and R. Asofsky. 1970. Studies of thoracic duct lymphocytes of mice. II. A quantitative comparison of the capacity of thoracic duct lymphocytes and other lymphoid cells to induce graft-*versus*-host reactions. *J. Immunol.* **104**:409.

65. Colley, D. G., A. Y. Shih Wu, and B. H. Waksman. 1970. Cellular differentiation in the thymus. III. Surface properties of rat thymus and lymph node cells separated on density gradients. *J. Exp. Med.* **132**:1107.

66. Weber, W. T. 1966. Difference between medullary and cortical thymic lymphocytes of the pig in their response to phytohemagglutinin. *J. Cell. Physiol.* **68**:117.

67. Winkelstein, A., and C. G. Craddock. 1967. Comparative response of normal human thymus and lymph node cells to phytohemagglutinin in culture. *Blood.* **29**:594.

68. Claman, H. N., and F. H. Brunstetter. 1968. The response of cultured human thymus cells to phytohemagglutinin. *J. Immunol.* **100**:1127.

69. Schwarz, M. R. 1967. Transformation of rat small lymphocytes with allogeneic lymphoid cells. *Am. J. Anat.* **121**:559.

70. Warner, N. L. 1964. The immunological role of different lymphoid organs in the chicken. II. The immunological competence of thymic cell suspensions. *Aust. J. Exp. Biol. Med. Sci.* **42**:401.

71. Blomgren, H., and B. Andersson. 1969. Evidence for a small pool of immunocompetent cells in the mouse thymus. *Exp. Cell Res.* **57**:185.

72. Williams, R. M., A. D. Chanana, E. P. Cronkite, and B. H. Waksman. 1971. Antigenic markers on cells leaving calf thymus by way of the efferent lymph and venous blood. *J. Immunol.* **106**:1143.

73. Leckband, E., and E. A. Boyse. 1971. Immunocompetent cells among mouse thymocytes: a minor population. *Science (Wash. D.C.).* **172**:1258.

74. Raff, M. C. 1971. Evidence for subpopulation of mature lymphocytes within mouse thymus. *Nat. New Biol.* **229**:182.