23 *Szabó, Gy., Gy. Molnár, Zs. Magyar:* Transport of macromolecules and red blood corpuscles from the renal tissue (hung.). Kisérl. Orvostud. 23 (1971), 188-196
24 *Wasserman, K., H.S. Mayerson:* Dynamics of lymph and plasma protein exchange. Cardiologia 21 (1952), 296-307
25 *Whipple, G.H., S.C. Madden:* Hemoglobin, plasma protein and cell protein – their interchange and construction in emergencies. Medicine 23 (1944), 215-224

Dr. G. Szabó, National Institute of Traumatology, Mezó Imre ut 17, Budapest VIII, Hungary

Lymphology 6 (1973) 79-90

Ultrastructural Evidence for the Lymph Node-Venous Transport of Carbon Particles*

R.F. Dunn

Department of Surgery, Division of Head and Neck Surgery (Otolaryngology), UCLA School of Medicine, Los Angeles, California 90024

Summary

The primary response of the lymph node sinus endothelial cells to increased intrasinusoidal pressure appears to be an opening of overlap junctions between adjacent endothelial cells. When colloidal carbon particles are injected via an afferent lymphatic, the tracer is found within the patent overlap junctions, the sinus endothelial cells, and the interstitial tissue that separates lymphatic sinuses and nearby capillaries. Carbon particles generally are confined to capillary lumens when injected via the communicating vein that joins the lymph node and the adjacent internal jugular vein. The long-term removal of carbon particles appears to be via an endocytic process, primarily by sinus endothelial cells and sinus macrophages with secondary uptake by fixed parenchymal macrophages. Minimal uptake of the tracer by capillary endothelial cells precludes the capillaries as a major site of removal of tracers from lymph node sinuses.

Introduction

Experiments suggest that radioactive tracers and bacteria are transferred from lymph nodes directly into the circulatory system in response to increased intranodal pressures that occur when these tracers are injected into either the laryngeal submucosa or a lymph node afferent channel (1, 2, 3). Increase in recovery of the radioactive tracer coincides with the increased pressures of injection and subsides upon cessation of injection (1, 2). Since colloidal carbon particles have been utilized successfully as electron

*This work was supported by Public Health Service Research Grant No. CA-10923 from the National Cancer Institute, Training Grant No. NS-5295 from the Neurological Diseases and Stroke Institute, and the California Institute for Cancer Research.

dense tracers to establish the permeability of lymphatic capillaries (4-9), the present study with colloidal carbon particles was undertaken to delineate the morphological pathways involved in the direct lymph node-venous transfer of material (1, 2).

Methods and Materials

Either the right or left medial retropharyngeal lymph node was utilized from each of 15 asymptomatic adult dogs. Two methods of injection were used: 1) via cannulation of one afferent lymphatic channel as previously detailed (1); and 2) via the communicating vein between the lymph node and adjacent internal jugular vein. Pressures of injection were maintained digitally as previously described (1). The injection solution was prepared by adding 5% (V:V) of shellac-free India ink (Pelikan Spezialtusche C 11/1431a) to a 0.1 M monobasic and dibasic sodium phosphate buffer at pH 7.3 and an osmolarity of 240 mOs measured cryostatically. After a 2 cc injection of colloidal carbon solution, the lymph nodes were freed, sliced transversely at approximately 4 mm thickness, and placed immediately into 2.5% glutaraldehyde. The same phosphate buffer as in the injection solution was utilized both for the fixatives and buffer rinses. After completion of the initial fixation, the tissue was trimmed to 2-3 mm cubes in buffer and post-fixed in cold 2% osmium tetroxide in buffer for 3 hours. After a second buffer rinse, the tissue was dehydrated in a graded series of ethanol and embedded in Epon-812. Tissue sections were cut with either an LKB III or a Porter-Blum MT2 Microtome with glass knives. Image contrast was enhanced by section staining in a saturated aqueous solution of uranyl acetate (45 minutes at 60°C) and with lead citrate (10).

Three lymph nodes were perfused individually with buffer only and fixed by immersion as above or perfused with 2.5% glutaraldehyde in phosphate buffer followed by immersion fixation.

Electron micrographs were taken with a Siemens Elmiskop IA at primary magnifications of x2,000-26,000. Further magnifications were completed photographically for the final figures.

Results

Untreated Tissue

Both irregular sinuses which predominate and the less frequent saccular sinuses of the canine medial retropharyngeal lymph nodes are lined with endothelial cells and occasional macrophages. The sinus endothelial cells contain a flattened, oblong nucleus surrounded by a thin rim of cytoplasm. The cytoplasm of these cells, extending radially from the nuclear area, becomes very attenuated and contains ribosomal particles, short profiles of rough-surfaced cytoplasmic membranes, mitochondria, occasional lysosomes, and an abundance of both smooth and rough-coated vesicles. The basement membrane at the abluminal surface of the sinus endothelial cells is incomplete. For example, in some sections the basement membrane is completely absent, in others it is interrupted, and in some it is present (Figs. 1-3). An irregular arrangement of collagen fibers or microfilaments is present in the abluminal connective tissue (Fig. 1).

Two regions of the sinuses are of particular interest in the present study, that of sinus

endothelial-endothelial cell apposition and that in which blood capillaries are in close proximity to the lymph node sinuses.

Sinus endothelial cells abut each other by the overlapping of processes from two adjacent cells. The plasma membranes in these overlap junctions generally are separated by a rather uniform distance of 130-160 Å. Although there may be considerable variation in the distance that one process extends over another, the usual pattern is a simple overlap without interdigitation. Areas of increased cytoplasmic density (*zonula adherens*) may or may not be present along the overlap junction near the luminal surface (Figs. 1-3).

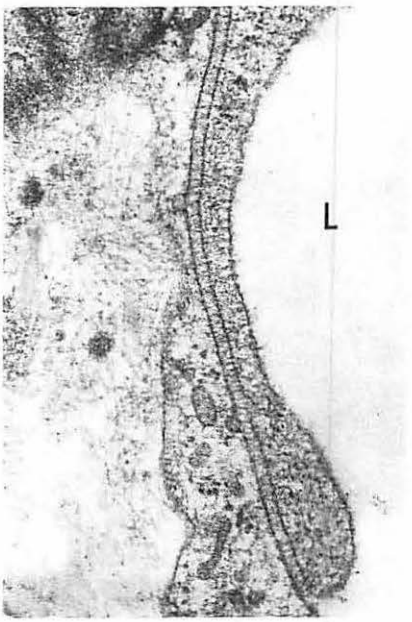

Fig. 1. A portion of an overlap junction shows the very regular separation of the plasma membranes. No basement membrane is present at the abluminal surface although numerous microfilaments are found in this region. The sinus lumen (L) is to the right. x 33,930.

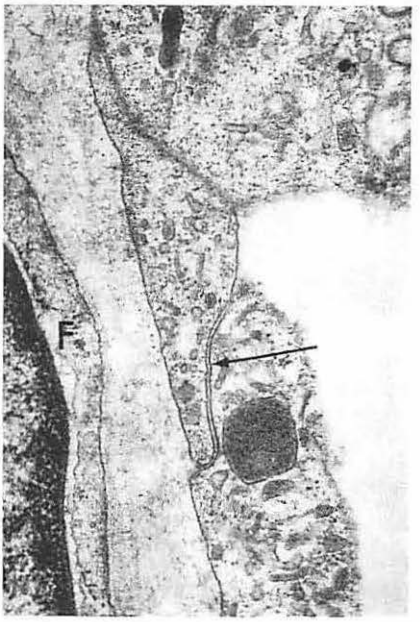

Fig. 2. A simple overlap junction is shown (arrow) which lacks a *zonula adherens*. The basement membrane is absent in this area; however, numerous filaments are present at the abluminal surface. A portion of a fibrocyte (F) is shown. x 19,800.

Blood capillaries often are located in close proximity to lymphatic sinuses, the two being separated only by a distance of 4 to 0.6 μ or less (Fig. 4). The capillary endothelial cells are similar in appearance to the sinus endothelial cells, however, the former is readily distinguished by a complete basement membrane that surrounds the abluminal surface of the capillary (Fig. 4). Two types of capillaries are present. The first is distinguished by the presence of numerous cytoplasmic vesicles in the capillary endothelial cells and an absence of endothelial fenestrations. This type appears to predominate. The second is characterized by very attenuated endothelial cells with fenestrations.

Regular crystalline bodies have been found in 6 of the 15 dogs investigated. These intracytoplasmic bodies are located in both sinus and capillary endothelial cells and con-

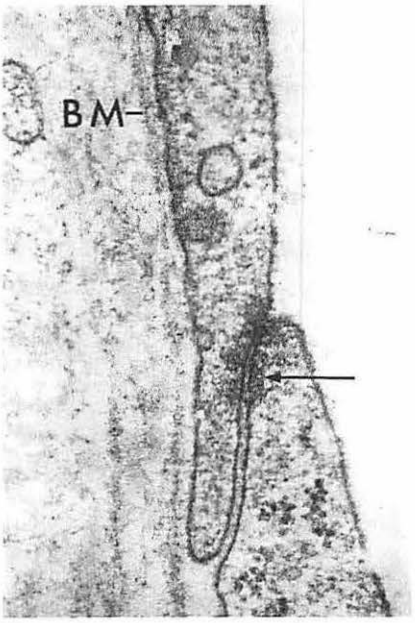

Fig. 3. This overlap junction includes a *zonula adherens* near the luminal surface (arrow). The *zonula* is characterized by an increased cytoplasmic density along the overlap junction. An interrupted basement membrane (BM) is located near the abluminal surface of the sinus endothelial cells. x 52,650.

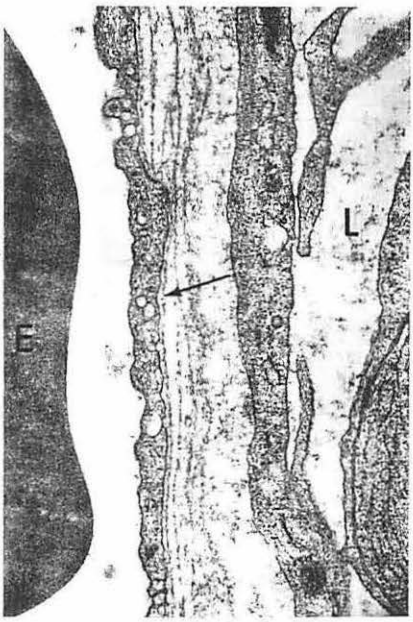

Fig. 4. The close proximity of a capillary with its included erythrocyte (E) to a lymphatic sinus (L) is shown. The sinus endothelial cell is attenuated, measuring 0.3-0.6 μ, and lacks a basement membrane. The basement membrane (arrow) of the capillary endothelium is present and complete. The capillary is separated from the sinus by a distance of 0.6 to 1 μ. x 13,050.

sist of rectilinearly arranged osmiophilic particles measuring 200-230 Å in diameter with the rows of particles intersecting at an angle of approximately 75° (Figs. 5A, B).

Perfusion with Fixative at Elevated Pressures

An immediate reaction to increased intrasinusoidal pressure appears to involve expansion of the overlap junctions of the sinus endothelial cells. Patent junctions, recognized by obvious gaps between adjacent sinus endothelial cells, are encountered with increased frequency when the fixative is perfused into the lymph node, via an afferent lymphatic channel, at pressures equivalent to those used with the carbon solution (Fig. 6). The separations range in size from a localized dilatation within an overlap junction to gaps of several micra.

Injection of Colloidal Carbon through an Afferent Channel

Colloidal carbon particles, when used, are observed within the sinuses, the expanded overlap junctions, sinus endothelial gaps, the sinus endothelial cells, and the tissue between the sinus and blood capillaries (Fig. 7). The tracer was found in the irregular sinuses and in well-defined saccular sinuses (Fig. 8).

Carbon particles have been observed in the interstitial spaces between the sinus and blood capillaries in experiments in which fixation was started immediately, 15, 30, 45

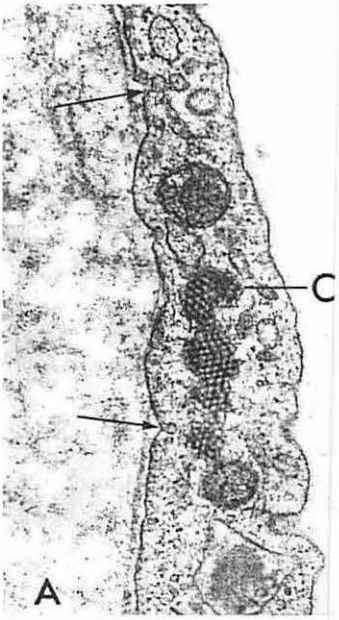

Fig. 5A. Crystalline bodies (C) are found in the cytoplasm of the sinus endothelial cells. Numerous coated invaginations are present along the abluminal surface (arrows). The abluminal basement membrane is interrupted. x 22,950.

Fig. 5B. A higher magnification of a crystalline body in the capillary endothelium. The regular rectilinear arrangement of the osmiophilic particles is well delineated. x 45,900.

and 60 minutes post-injection. At higher magnifications, carbon particles are seen in patent junctions between adjacent endothelial cells (Fig. 9). The distance of separation in these regions also showed considerable variation in size from moderate separations to endothelial interruptions of several micra.

Increased pinocytotic activity of the sinus endothelial cells was apparent from the higher frequency with which carbon-containing invaginations of the plasma membrane were observed, at both the luminal and abluminal surfaces (Figs. 10A, B). Increased number of intracellular vacuoles containing the tracer were found with increased time after injection (Fig. 10B).

In each of the timed experiments, carbon particles were observed in the interstitial tissue and at the capillary basement membrane. Carbon particles also were observed in intracytoplasmic vacuoles in the capillary endothelial cells (Fig. 11). Serial sections established that these vacuoles were contained entirely within the capillary endothelial cells.

Injection of Colloidal Carbon through the Communicating Vein

When the tracer was injected retrograde through the communicating vein joining the medial retropharyngeal lymph node and the adjacent internal jugular vein, carbon particles were readily recognized in the lumens of blood capillaries (Figs. 12, 13A). Generally, the carbon was confined to the capillary lumen, with little evidence of exit via

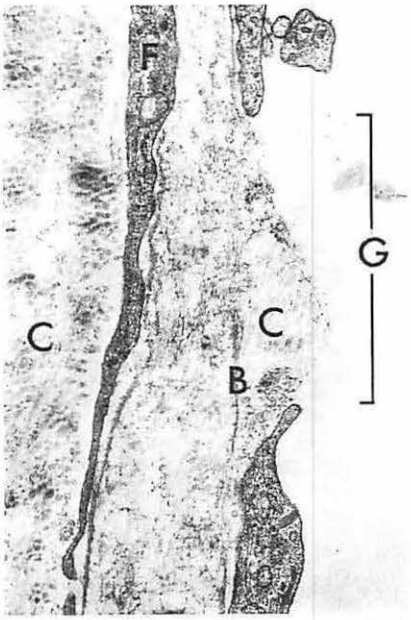

Fig. 6. When fixative alone is injected, obvious separation of overlap junctions (G) occurs. The basement membrane (B) is incomplete. Both collagen filaments (C) and irregular microfilaments are present. A portion of a fibrocyte (F) is also shown. x 13,050.

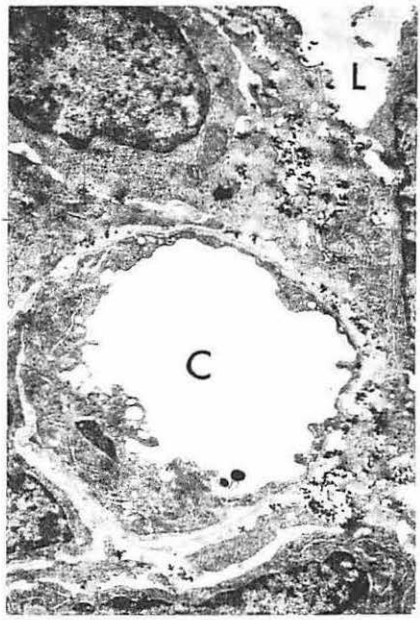

Fig. 7. A survey electron micrograph shows carbon particles in the sinus lumen (L), in the interstitial tissue, and around a nearby capillary (C). x 4,320.

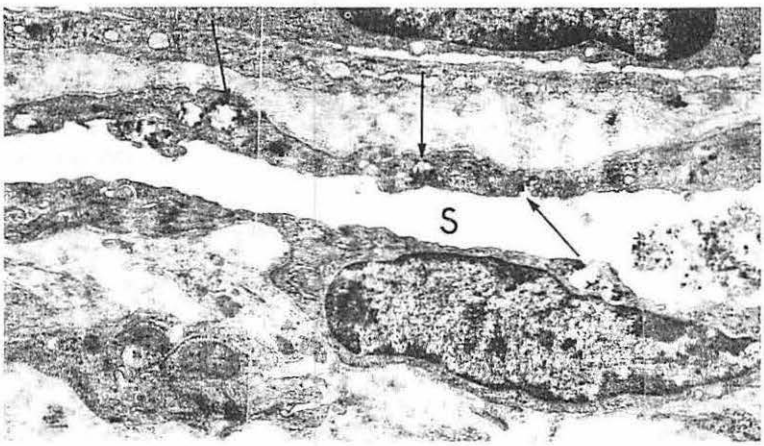

Fig. 8. Carbon particles are seen in the lumen of a saccular sinus (S) as well as in invaginating and intracytoplasmic vesicles (arrows). x 1,200.

intra- or intercytoplasmic transfer (Fig. 13B). Only rarely were carbon particles found in the interstitial spaces and never in the lymphatic sinuses.

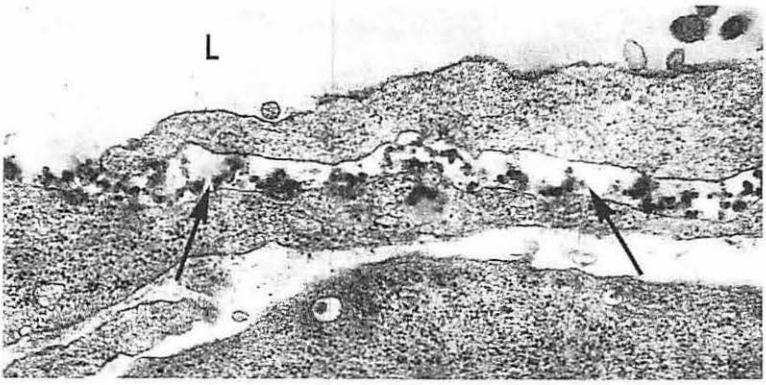

Fig. 9. Upon injection of the tracer, carbon particles are located in patent overlap junctions of the sinus endothelial cells (arrows), having entered from the sinus lumen (L). x 22,950.

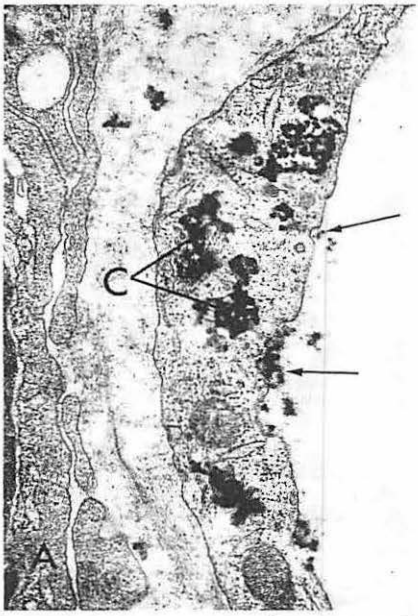

Fig. 10A. Intracytoplasmic accumulation of carbon particles (C) appears to increase with time after injection. Carbon particles are also seen in endocytotic vesicles at the luminal surface of the sinus endothelial cell (arrows). x 13,950.

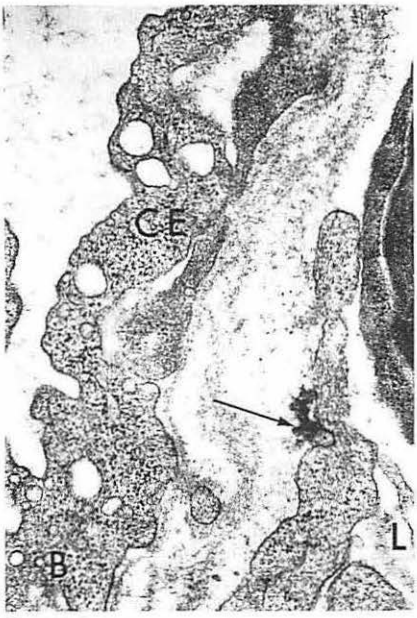

Fig. 10B. Carbon particles are also found in vesicles, apparently invaginating at the abluminal surface of the sinus endothelial cell (arrow). The capillary endothelium (CE) is very irregular and contains numerous clear membrane-bounded vesicles. Sinus lumen (L). x 18,900.

Discussion

The general morphology of the sinus endothelial cells shares many similarities to that of the lymphatic capillaries (4, 5, 11, 12). The attenuation of the sinus endothelial cells and the morphology of the cytoplasmic organelles appear quite similar to that described in the lymphatic capillary. The overlapping of adjacent sinus endothelial

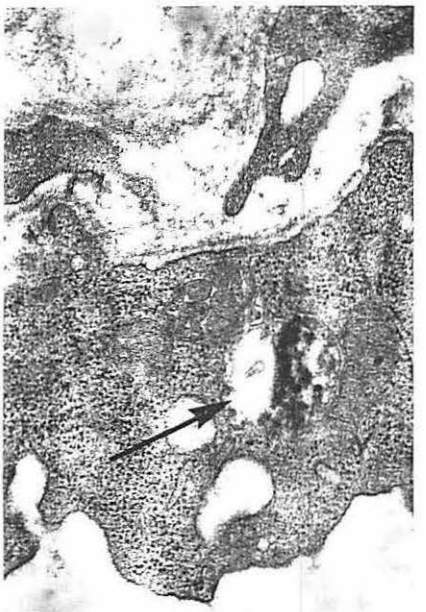

Fig. 11. Carbon particles were found in an intracytoplasmic vacuole in this capillary endothelium (arrow). Serial sections showed this vacuole to be entirely contained within the cytoplasm. x 19,800.

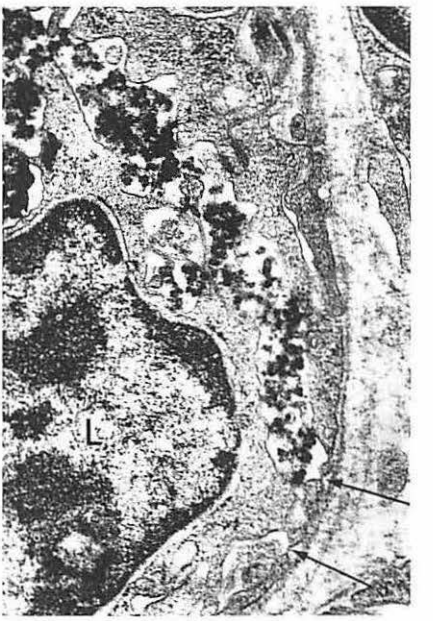

Fig. 12. When injected via the communicating vein, the carbon particles are generally confined to the capillary lumen. A circulating leukocyte (L) nearly fills the lumen of this capillary. Endothelial fenestrations are present (arrows). x 19,280.

cells is also similar to that of the lymphatic capillaries. However, the junctional complexes do appear slightly different. A *zonula adherens* is located near the luminal surface in the sinus endothelial cell junctions and is evidenced by cytoplasmic condensation. Neither *zonulae occludentes* nor *maculae adhaerentes* (13, 14) were found in the overlap junctions. The absence of these two junctional complexes, between sinus endothelial cells and lymphatic capillaries, indicates the potential for separation and the lack of effective impediment to the passage of tracers via this route (8). The appearance of patent junctions in response to increased sinus pressures tends to substantiate this postulation. The presence of carbon particles within the patent junctions appear to be a result of passive transportation of the tracer particles.

Whereas a basement membrane is lacking in the lymphatic capillaries (11, 12), an incomplete basement membrane is present at the abluminal surface of the sinus endothelial cells. Incomplete is the best term to describe the basement membrane because of its varied appearance, present or absent. For example, the absence underlying many patent and overlap junctions is evidence for interruptions of the sinus basement membrane. In such areas of patent junctions and basement membrane interruptions, no active transport of tracers is possible, and it is appropriate to assume a passive transport of the tracer in these regions. The plasticity of the overlap junctions could therefore serve as a mechanism to allow rapid exit of material from the sinuses in response to varying intrasinusoidal pressures.

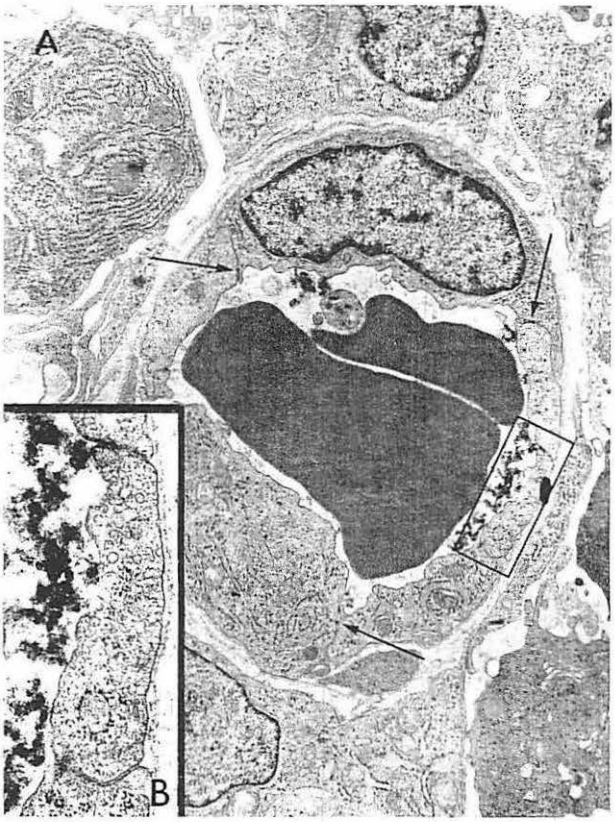

Fig. 13A. Carbon particles are also located within capillaries that exhibit no endothelial fenestrations. This capillary is comprised of processes from six endothelial cells with 1-3 *zonulae adherentes* along the cell junctions (arrows). x 5,130.
Fig. 13B. A higher magnification of the outlined segment in Figure 13A. No evidence was found of carbon particles exiting the capillary, although the tracer is in close proximity to the endothelial junctions. x 19,100.

Anchoring filaments characteristic of the lymphatic capillaries (11) do not appear at the abluminal surface of the sinus endothelial cell. In the lymph nodes, abluminal filaments rarely are seen attached to the basal surface of the sinus endothelial cells nor do they appear as well organized. The abluminal filaments found in the lymph nodes probably are randomly oriented connective tissue filaments that serve only to assist in parenchymal support. Observations that the filaments attach to the sinus endothelium most likely result from superposition effects within the thickness of the section.

The active removal of the carbon particles from the lymph node sinuses appears to be a process of increased phagocytosis by the fixed sinusoidal macrophages and pinocytosis by the sinus endothelial cells with simultaneous passive transfer through patent sinus endothelial junctions. The first step in the active removal apparently consists of tracer particles adhering to the surface plasma membrane of the sinus endothelial cells, followed by successive invagination and eventual pinching off to form cytoplasmic vesicles-endocytosis. The cytoplasmic vesicles here are generally of the coated variety. Studies

by *Casley-Smith* (15, 16) concerning the dynamics of endocytosis suggest the uptake of particulate matter in response to a concentration gradient with either subsequent transfer across the endothelial lining or the possibility of intracytoplasmic coalescence to form larger tracer-ladened vacuoles. Similar conclusions resulted from observations on the response of the lymphatic capillary endothelium to intradermal injections of particulate tracers (8, 9).

Results from the present study tend to favor endocytotic uptake of carbon at both the luminal and abluminal surfaces and intracytoplasmic coalescence forming larger vacuoles that are retained in the sinus endothelial cells. This hypothesis is consistent with morphological evidence found in the timed experiments.

Uptake at the luminal surfaces would be an obvious response to a concentration gradient established by the intrasinusoidal presence of the tracer substance. Abluminal surface uptake of carbon passing through the patent junctions could also result from a locally established concentration gradient. Subsequent intracellular movement and coalescence implies a dynamic process. The primary differences between tissue taken at varying timed intervals appeared to be the frequency at which the large vacuoles occurred in the sinus endothelial cells. For example, at very short-term delay or immediate fixation, carbon was found in patent junctions, the interstitial space near blood capillaries, in close proximity to the sinus endothelial plasma membranes (both luminal and abluminal), and in small vesicles. With increased time, the large vacuoles appeared more numerous, and progressively less carbon particles were observed in the interstitial space surrounding the capillaries. Interpretation of these results would implicate the sinus endothelial cells and sinus macrophages as the primary cells responsible for removal of carbon, with parenchymal macrophages and capillary endothelial cells subserving a secondary and tertiary site of tracer removal. The latter would explain the infrequency (single observation) with which carbon was found in the capillary endothelial cells.

The passage of carbon from the lymph node to the adjacent internal jugular vein is easily demonstrated. For example, when colloidal carbon is injected through an afferent lymphatic to the medial retropharyngeal lymph node using moderate digital pressures of injection, the nodes are first observed to blacken. Shortly thereafter, a similar discoloration occurs in the communicating vein and then in the adjacent internal jugular vein. The retrograde competence of the valve-like structure observed in vessels that open into the saccular sinus (17) is suggested by free passage of carbon occurring via the "normal" route, impediment to passage via the retrograde route, and lack of carbon in any of the sinuses sampled in the retrograde experiments. The presence of saccular sinuses in the medial retropharyngeal lymph nodes has been established and is base upon a three dimensional analysis of their saccular configuration (17). The saccular sinus was found to be continuous morphologically with an irregular sinus. The presence of the carbon particles within the saccular sinus confirms the previous morphological observations and indicates free passage of material from the irregular sinuses to the saccular sinuses.

A regular lattice of osmiophilic particles has been reported in endothelial cells of canine retinal capillaries (18). The investigators suggested that these bodies might be either conjugated metal inclusions or perhaps virus particles but were not characteristic of retinal capillaries. The inclusion bodies found in the endothelial cells during the

present study are similar in crystalline array to reovirus inclusions found in monkey kidney cells (19) and mosaic virus inclusions found in plants (20).

Conclusions from the present experiments indicate that the sinus endothelial overlap junctions spread in response to increased intrasinusoidal pressure followed by passive distribution of carbon particles into the interstitial tissue due to a differential concentration gradient. Further, the sinusoidal macrophages and endothelial cells are responsible for removal of most of the intrasinusoidal carbon, whereas removal of carbon from the interstitial tissue is accomplished primarily by the tissue macrophages with only some removal by capillary endothelial cells. Finally, the competence of the valve-like structure previously described in the vessels communicating with the saccular sinuses is indicated by the failure of carbon to enter the sinuses when injected retrograde into the communicating vein.

The exit pathways of carbon as postulated here appear to be a secondary mechanism for removal of particulate matter from the sinuses. This seems reasonable since the endothelial uptake of carbon would be a terminal process that would preclude passage in quantities sufficient to account for the rapid discoloration of the adjacent internal jugular vein caudal to its junction with the communicating vein. The primary pathway for the rapid direct passage from the lymph node to the venous system therefore appears to be via a valved vessel that joins the communicating vein and the lymph node sinuses with the saccular sinuses acting as a mixing chamber.

Acknowledgement

The author wishes to express his appreciation to Mrs. Margot Brundage and Miss Hélène Lheridaud for their expert technical assistance in preparing the tissue sections.

References

1 *Dunn, R.F., M.V. Burtz, P.H. Ward:* Studies on the lymph node-venous communications. I. The passage of radioactive serum albumen. Lymphol. 5 (1972), 15-26

2 *Dunn, R.F., M.V. Burtz, P.H. Ward:* Studies on the lymph node-venous communications. II. The passage of labeled exogenous erythrocytes. Lymphol. 5 (1972), 120-127

3 *Pressman, J.J., R.F. Dunn, M.V. Burtz:* Lymph node ultrastructure related to direct lymphaticovenous communication. Surg.Gyn. Obstet. 124 (1967), 963-973

4 *Leak, L.V., J.F. Burke:* Fine structure of the lymphatic capillary and the adjoining connective tissue area. Amer.J.Anat. 118 (1966), 785-810

5 *Leak, L.V.:* Lymphatic capillaries in tail fin of amphibian larva. J.Morph. 125 (1968), 419-446

6 *Leak, L.V., J.F. Burke:* Electron microscopic study of lymphatic capillaries in the removal of connective tissue fluids and particulate substances. Lymphol. 1 (1968), 39-52

7 *Leak, L.V., J.F. Burke:* Ultrastructure of lymphatic capillaries in the transport of colloidal particles. In: Electron Microscopy, Proc. Electron Micros. Soc. Amer., C.L. Arceneaux (ed.), Claitor's Pub.Div., Baton Rouge (1967), pp. 186-187

8 *Leak, L.V.:* Studies on the permeability of lymphatic capillaries. J.Cell Biol. 50 (1971), 300-323

9 *Leak, L.V.:* Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue – lymph interface. Microvasc.Res. 2 (1970), 361-391

10 *Reynolds, E.S.:* The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J.Cell Biol. 17 (1963), 208-212

11 *Leak, L.V., J.F. Burke:* Ultrastructural studies on the lymphatic anchoring filaments. J.Cell Biol. 36 (1968), 129-149

12 *Bullon, A., F. Huth:* Fine structure of lymphatics in the myocardium. Lymphol. 5 (1972), 42-48

13 *Farquhar, M.G., G.E. Palade:* Junctional complexes in various epithelia. J.Cell Biol. 17 (1963), 375-412

14 *Brightman, M.W., S.L. Palay:* The fine structure of ependyma in the brain of the rat. J.Cell Biol. 19 (1963), 415-439
15 *Casley-Smith, J.R.:* Endocytosis: The different energy requirements for the uptake of particles by small and large vesicles into peritoneal macrophages. J.Micros. 90 (1969), 251-269
16 *Casley-Smith, J.R.:* The dimensions and numbers of small vesicles in cells, endothelial and mesothelial and the significance of these for endothelial permeability. J.Micros. 90 (1969), 251-269
17 *Dunn, R.F., R.W. Strahan:* Studies on the lymph node-venous communications. III. The presence of saccular sinuses and their function. Lymphol. 5 (1972), 161-169
18 *Bloodworth, J.M., D.L. Molitor:* Crystalline bodies in retinal capillary endothelial cells. Investig.Ophthal. 4 (1965), 285-289
19 *Easterbrook, K.B., K.R. Rozee:* The ultrastructure of the reovirus inclusion: A freeze etching study. J.Ultrastruct.Res. 34 (1971), 303-315
20 *Weintraub, M., H.W.J. Ragetli:* Electron microscopy of the bean and cowpea strains of southern bean mosaic virus within leaf cells. J.Ultrastruct.Res. 32 (1970), 167-189

Dr. L.F. Dunn, Department of Surgery, Rehab. 31-19, UCLA School of Medicine, Los Angeles, California 90024

Lymphology 6 (1973) 90-97

The Influence of Prednisolone and Thymectomy on the Thoracic Duct Lymphocyte Population of the Rat*

P.M. Lundin, J.C. Schooley

The Institute of Pathology, University of Göteborg, Sweden, and
Donner Laboratory, Lawrence Radiation Laboratory, University of California, Berkeley, California

Summary

The number of mononuclear cells in the thoracic duct of 8-10 week-old rats were counted three and seventeen hours after a single dose of prednisolone and the concomitant involution of the lymphoid tissues was studied histologically.

Both in animals thymectomized at weaning age and in shamoperated controls the number of cells per volume and per hour were strongly depressed after three hours. At seventeen hours after injection the original level was reached again. The sensitivity of thoracic duct lymphocytes to prednisolone in vitro was tested, and cells from thymectomized animals were found to be about as sensitive as cells from shamoperated animals. The mechanism for the disappearance and rapid regeneration of the lymphocytes in the circulating pool is discussed.

Histologically no differences was observed between shamoperated and thymectomized animals in

*This investigation was supported by a grant from the Swedish Medical Research Council (B71-12X-592-06C)