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FLUID PRESSURES IN THE RABBIT POPLITEAL AFFERENT LYMPHATICS DURING PASSIVE TISSUE MOTION

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ABSTRACT

The mechanisms of pressure and flow generation in the initial lymphatic network remain undefined in many details. Active pump mechanisms by lymph smooth muscle contraction have been demonstrated in collecting lymph ducts while passive mechanisms by periodic lymph compression in noncontractile initial lymphatics have received relative little attention. The aim of this study was to examine lymph flow before and during periodic passive tissue deformation under different lymph outflow pressures. Lymph flow rate and lymph outflow pressure were measured in rabbit popliteal prenodal lymphatics during whole leg rotations. The hind legs were rotated in a sagittal plane at selected frequencies. During constant leg rotation, lymph flow rates reached steady levels which depend on lymph outflow pressure. When lymph outflow was occluded, intralymphatic pressures increased progressively to levels which depend on leg rotation frequency. Both lymph flow rate and pressure showed higher values with foot edema than in the absence of foot edema. These results suggest that periodic tissue deformation, lymph outflow pressure, and interstitial free fluid volume are important determinants of the lymph flow rate.

The microscopic sized initial lymphatics have been looked upon as a passive network where the difference in hydrostatic pressure and in colloid osmotic pressure contributes to the maintenance of microcirculatory homeostasis by draining water, plasma protein, hyaluronan and cells from the interstitium (1,2). The unidirectional movement of fluid in initial lymphatics is supported by two valvular systems (3). One operates at the level of endothelial microvalves, which allow for unidirectional flow from the interstitium to the initial lymphatics (4), and the other operates in form of the well recognized intralymphatic valves, that prevent lymph reflux inside the lymphatic ducts (5,6). The existence of these valvular systems facilitate lymph formation/transport irrespective whether lymphatics are moved by active or passive mechanisms (3,7).

Previous microscopic studies of the end-lymphatics in the dorsum of the foot of rabbits have documented the effect of passive periodic mechanical force on the propulsion of lymph fluid into larger collecting lymphatics (8). Without external force applied to the leg, almost no lymph flow can be observed. Once periodic external force is applied, lymph flow rate increases with frequency or the size of the area over which the force is applied (8). Compression of the initial lymphatics serves to obliterate the vessel lumen so as to force fluid in initial lymphatics proximally across the successive lymphangions. While there is good evidence to suggest that the contractile collecting lymphatics generate positive lymphatic pressures (5), there are few reports which

focus on lymphatic pressure during periodic external force application in a region of initial lymphatics where mostly passive pump mechanisms are at work.

Thus, the purpose of this study was to investigate the magnitude of pressure formation during operation of passive and active lymph pump mechanisms. Three experimental protocols were designed, (1) to investigate the lymph flow rate during a periodic external force for different pressures in the collecting lymphatics, (2) to investigate the relationship between the frequency of the external force and lymph pressure when lymph flow rate was zero, (3) to investigate the relationship between body position and lymph flow rate under the condition of constant lymph outflow pressure. We used the popliteal prenodal lymphatic cannulation in the rabbit (9). By using this type of rabbit preparation, the relationship between lymph pressure and lymph flow rate could be determined precisely because the volume and composition of the lymph fluid have not changed proximal to the lymph node and by the fact that the lymphatics drain lymph from a clearly circumscribed tissue region (10,11).

METHODS

Experimental Preparation

The studies were carried out on male New Zealand White Rabbits (2 ~ 3Kg, Simunec, Vista, CA) anesthetized with ketamine chloride (20 mg/kg, i.v.) and sodium pentobarbital (Abbott Lab, Chicago, IL, 20 mg/kg, i.v.). Intermittent boluses were injected as needed during the experiments dictated by a toe pinch test. The trachea was cannulated to ensure patent airways. A catheter was inserted into the right external jugular vein (PE 90 tubing, Clay Adams, Parsippany, NJ) for administration of anesthetics and measurement of central venous blood pressure. The catheters were filled with 10 U/ml heparin (Elkins-Sinn Inc., Cherry Hill, NJ) in saline (Baxter Healthcare Corporation, Deerfield, IL). Esophageal temperature was monitored and maintained at 38 to 39°C by means of a water heating pad.

Lymph Cannulation and Measurements.

In the following description the term *initial* lymphatics refers to the non-muscular terminal segment of microlymphatics which consist of a single endothelial layer embedded in the tissue parenchyma and with irregular lumen cross-sections. In contrast, *collecting* lymphatics have in addition to the endothelial layer a media, which in the skin of the rabbit hind leg is limited to a single smooth muscle layer. The initial lymphatics drain into the collecting lymphatics and both segments have intralymphatic valves in this tissue. Collecting lymphatics drain into the lymph nodes and their lumen cross-section tends to be more circular.

Lymphatic cannulation was carried out as previously described (9). Lymph fluid was collected via cannulae inserted under a stereo microscope into a collecting lymphatic vessel (300 to 500 µm in diameter with a single lymphatic smooth muscle layer) in the lower left leg (PE 10, Clay Adams) proximal to its entry into the popliteal node *(Fig. 1A)*. In the preparatory phase of the study, recognition of the lymphatic vessel and pilot cannulations were aided by injection of an intravital stain (Evans blue, 0.5%) into the dorsal skin so that the lymphatic pathways became optically enhanced. During the experiments presented in this report, however, no contrast medium was injected. The lymph cannulations were carried out with about a 90% success rate.

Development of Foot Edema.

In order to increase the venous pressure in the left foot to 40 mmHg, a blood pressure cuff was placed around the thigh proximal to the cannulation site and continuously monitored. In this series of experiments, a small incision was made on the lateral side of the

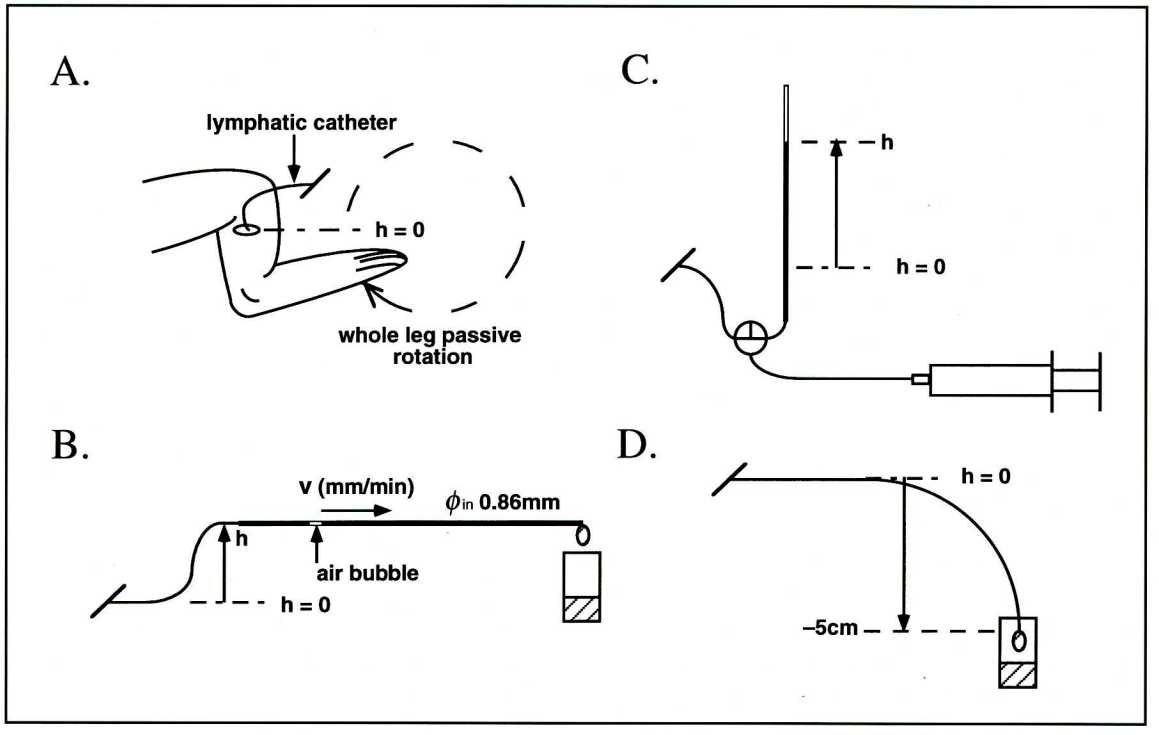

Fig. 1. Schematic diagram of the experimental setup. (A) Frontal view of rabbit left hind leg. Polyethylene tubing (lymphatic catheter) was inserted retrograde into a popliteal prenodal lymphatic. (B) Display of the method to adjust lymph outflow pressure and to measure the lymph flow rate by displacement of an interface in the cannula. (C) Display of the method to measure the intralymphatic pressure at zero outflow rate. (D) Shows the method to collect fluid via the lymphatic catheter at constant negative outflow pressure. See text for further details.

left lower leg. A polyethylene tube (PE 50, Clay Adams) was inserted into a small vein running parallel to the tendo calcaneus and connected to a pressure transducer. This increase in venous pressure led to a significant increase in skin wet/dry weight ratio of the rabbit foot (unpublished results).

Passive Whole Leg Rotation.

The left lower extremity was passively rotated in the sagittal plane with an electric motor *(Fig. 1A)*. The diameter of the circle of rotation was 8 cm. The rotation frequency was adjusted by means of a variable electrical resistance to the motor.

Experimental Protocols.

After lymph cannulation and a 15 min stabilizing period at 0.3 Hz passive, whole leg rotation was carried out in each experiment. We will outline here for each study protocol the complete time sequences of tissue motion since lymph flow rates depend on the past history of lymph pumping

Protocol #1: *Lymph outflow pressure - flow rate relationship.* Lymph flow rates were computed from the velocity of a small air bubble introduced into the outflow tubing and the lumen cross-sectional area of the tubing (PE 90 tube, 0.86 mm inner diameter; Clay Adams) *(Fig. 1B)*. The velocity of the air bubble was computed from its displacement in 5 minute intervals in a horizontal segment of the tubing without sedimentation. Since the pressure drop due to fluid movement in the cannula is negligible at the small lymphatic flow rates, the height (h, in cm units) of

the tube free end was considered to be equal to the lymph outflow pressure at the cannulation site (cmH_2O). The height of the lymph cannulation point was taken to be zero *(Fig. 1B)* and adjusted to be at the same height as the axis of the rotation.

After stabilization, the height (h) of the fluid filled free end of the lymph cannula was adjusted to an equivalent outflow pressure of 40 cmH_2O, 20 cmH_2O, 0 cmH_2O, -10 cmH_2O and then to -20 cmH_2O (n=4 rabbits). Lymph flow rates were recorded over a period of 30 min at each height.

A similar protocol was used after edema formation. In a pilot study, it was confirmed that the lymph flow rates reached steady values after 1.5 hrs at 40 mmHg venous pressure with 0.3 Hz whole leg rotation. Therefore, the leg veins were occluded with a cuff inflated to 40 mmHg for 90 min, after which period the same stepwise decrease of outflow pressure were carried out as described above (n=2 rabbits).

Protocol #2: *Leg rotation frequency-intralymphatic pressure relationship.* At the beginning of each experiment in this protocol, distilled water was inserted into a vertical tube (PE 160, Clay Adams) to a height of 50 cm by using a 10 ml syringe via a three way stopcock *(Fig. 1C)*. The value of 50 cm was selected since preliminary experiments had indicated that at 3 Hz hind leg rotation frequency each rabbit developed a lymphatic pressure of at least 50 cmH_2O. The cannulation point was taken as zero reference height. After stabilization, the 50 cm column delivering fluid into the lymph cannula was connected as soon as the leg rotation was stopped. The length of the lymphatic cannula (which are filled with lymph fluid) was sufficiently long to prevent reflow of the water column into the lymphatics. Then, the intralymphatic pressure, as reflected by the height of the water column, was observed over a period of 30 min. A similar protocol was repeated at leg rotation frequencies of 0.03 Hz, 0.3 Hz, and 3.0 Hz (n=6 rabbits). Each rabbit developed maximum pressure within 30 min at a rotation frequency of 3.0 Hz.

A similar protocol was used after edema formation with a cuff (n=4 rabbits). In this series of experiments, if a rabbit did not show maximum pressure at a rotation frequency of 3.0 Hz within 30 min, the observations were continued until the lymph outflow pressure reached a maximum value.

Protocol #3: *Body position and lymph flow rate.* In this series of experiments, lymph fluid was collected into 1 ml syringes. The lymph catheter tip was positioned inside the syringe about 5 cm below the cannulation site *(Fig. 1D)*. The volume of the collected lymph fluid was measured by using a micro-syringe. Lymph flow rate was computed from the collected lymph volume and the time required for the collection. A preliminary study served to confirm that evaporation of lymph fluid during the collection time was negligible over the period required for this study.

The effect of body position was tested in four different time sequences since it may be accompanied by significant fluid shifts. (a) After a 15 min stabilization with the rabbit in the face up position, the leg was rested, and then rotated at 0.3 Hz for 1 hr. At the end of this period, the animal's body was turned (around its long axis) into a face down position and the leg rotation was repeated at 0.3 Hz (n=1 rabbit). In each body position, the lymph flow rates were measured over a period of 1 hr. Next, the period of rest and leg rotation were reversed. (b) After 15 min stabilization in the face up position, the leg was rotated at 0.3 Hz, followed by rest without rotation. Then the rabbit was placed face down and the leg was rotated again at 0.3 Hz, followed by rest (n=1 rabbit). (c) After 15 min stabilization in face down position, the leg rotation was stopped, followed by rotation at 0.3 Hz. Then the body was reversed to the face up position and the leg rotation was stopped, followed again by rotation at 0.3 Hz (n=1 rabbit). (d) After 15

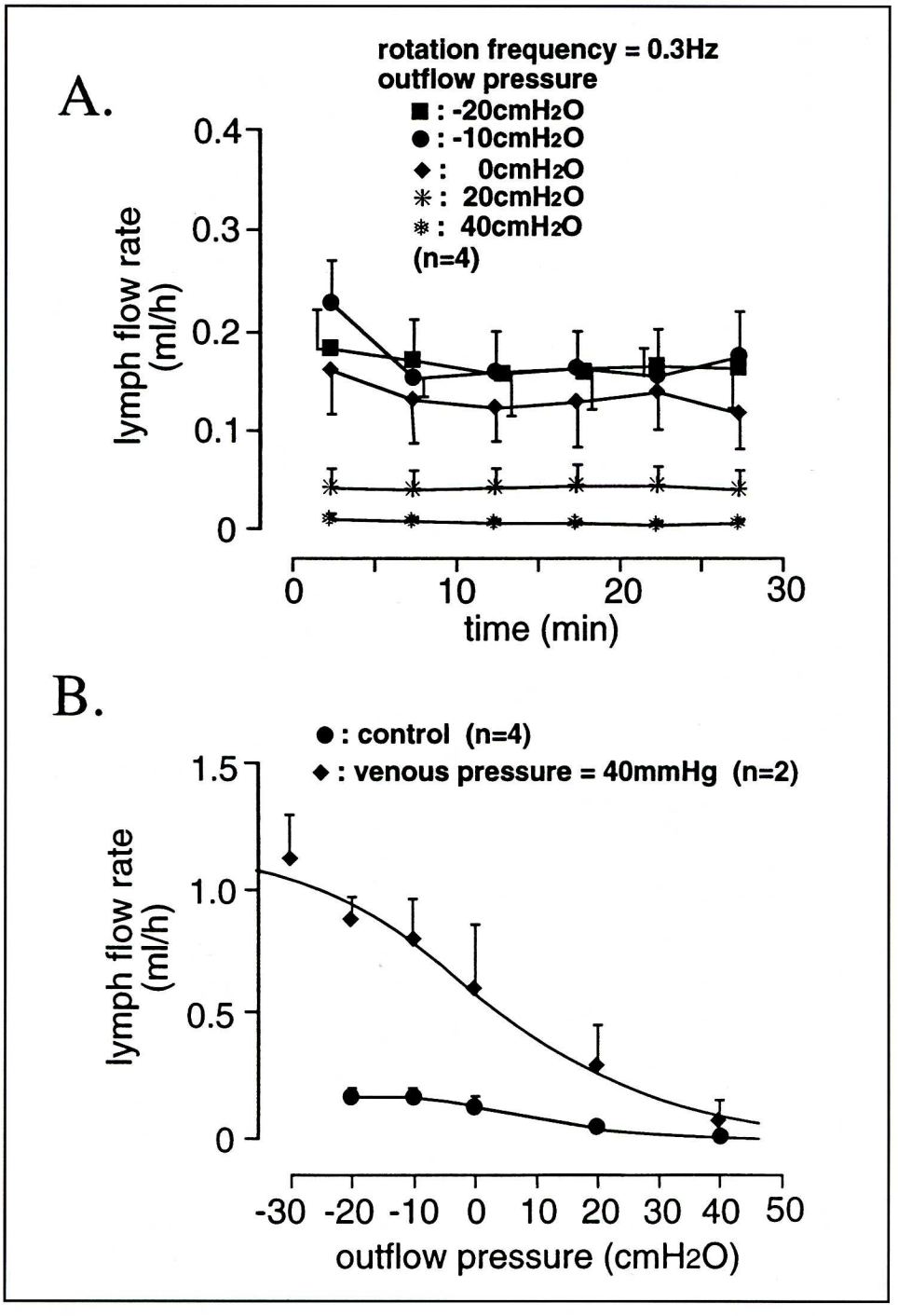

Fig. 2. Relationship between lymph outflow pressure and lymph flow rate in the rabbit popliteal prenodal lymphatics at 0.3 Hz hind leg rotation frequency. The mean lymph flow rate over a period of 10 to 15 min was used. (A) Time course of the lymph flow rate for different outflow pressures. (B) Lymph flow rate as a function of lymph outflow pressure without and with venous pressure elevation. n is the number of animals.

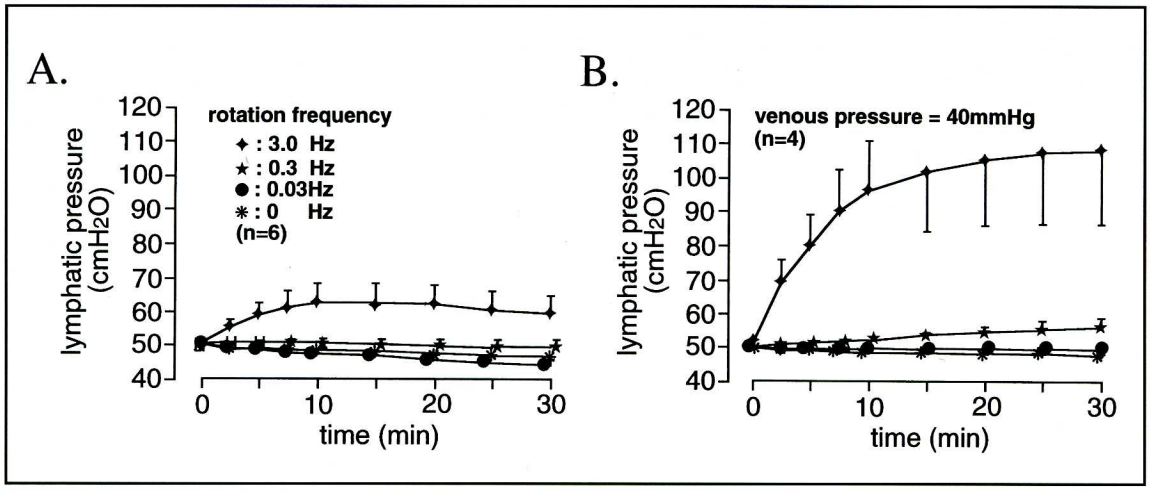

Fig. 3. Time course of intralymphatic pressure in the rabbit prenodal popliteal lymphatics during leg rotation at different frequencies with (A) and without (B) venous pressure elevation. At the beginning of these measurements, intralymphatic pressure was adjusted to 50 cmH_2O. n is the number of animals.

min stabilization in the face down position, the leg rotation was maintained at 0.3 Hz, followed by rest. Then the leg rotation was restarted at 0.3 Hz in the face up position, followed by rest (n=1 rabbit). The measurements during these four different time courses were similar and were combined.

Statistics

All results are expressed as mean ± SE. Two tailed Student's t test for paired or unpaired data was used to test for significant differences between groups. Differences between groups were considered significant at $p<0.05$.

RESULTS

When lymph outflow pressure was shifted in steps from 40 cmH_2O to -20 cmH_2O at a constant leg rotation frequency of 0.3 Hz, a small transient increase in lymph flow rate was observed at each step after which lymph flow rate reached a constant value within about 10 min *(Fig. 2A)*. The initial transient may represent removal of 'extra' interstitial fluid, and the steady state values may be associated with microvascular fluid filtration. At positive outflow pressures the lymph flow rates were reduced compared to zero pressure. But at negative outflow pressure the lymph flow rates could not be elevated significantly. Lymph flow rates increased while outflow pressure was decreased and reached a plateau level at about -10 cmH_2O *(Fig. 2B; circles)*. After tissue edema formation by elevation of the venous pressure, the average lymph flow rates were elevated about 5 fold at almost all outflow pressure. Even at -20 cmH_2O pressure the lymph flow rate continued to increase *(Fig. 2B; diamonds)*.

During passive pumping by rotation of the hind leg, considerable pressures can be developed in the prenodal lymphatics *(Fig. 3)*. At 0 Hz and 0.03 Hz, intralymphatic pressure was continuously decreased from its initial level at 50 cmH_2O. At 0.3 Hz, after a small transient increase the pressure decreased gradually. At 3.0 Hz leg rotation frequency, the pressure reached a maximum after about 12 min and then decreased slightly *(Fig. 3A, Table 1)*. After edema formation, the lymphatic outflow pressure decreased

TABLE 1
Effect of Venous Pressure on Maximum Lymphatic Pressure

group	n	maximum pressure (cmH_2O)	time (min)
control	6	63.5 ± 6.1	11.7 ± 2.7
VP 40 mmHg	4	110.8 ± 20.9*	26.9 ± 7.7

Values are mean ± SE., n: animal number, time: required time to reach maximum value,
VP: venous pressure
* $p<0.05$ control group vs. VP 40 mmHg group

continuously from 50 cmH_2O at 0 Hz and 0.03 Hz, but was increased slightly at 0.3 Hz. At 3.0 Hz leg rotation in the edematous foot, the pressure rose dramatically and reached its maximum of approximately 110 mmHg at about 27 min *(Fig. 3B, Table 1)*. The maximum pressure in rabbits with edema was significantly greater than that in the rabbits without edema *(Table 1)*.

Lymph flow rates in the hind leg are affected by the body position *(Fig. 4)*. At both face up and face down positions, leg rotation significantly increased lymph flow rate. At a leg rotation frequency of 0.3 Hz, the average lymph flow rate in face down position was almost 3 times larger than that in the face up position *(Fig. 4)*.

DISCUSSION

This study serves to clarify the relationship between intralymphatic pressure and lymph flow rate during a controlled tissue deformation in prenodal lymphatics. Even without external compression of the skin, considerable pressures can be generated by the initial lymphatics, the magnitude of which depends on the frequency of passive tissue motion. Edema formation is associated with an increase in both intralymphatic pressure and lymph flow rate generated during passive tissue movement.

Tracer studies and morphological reconstruction of the lymphatics which drain into the lymphatic duct that was cannulated in the current study show the presence of non-contractile initial lymphatics. These initial lymphatics discharge into a few contractile collecting lymphatics (8). Both initial and collecting lymphatics have intralymphatic valves. Individual lymphangions act as a pump to set up unidirectional lymph flow, but the great majority of the microscopic lymphatics in the rabbit skin have no smooth muscle and are non-contractile. Only a single layer of smooth muscle cells are seen in the walls of the collecting lymphatics (8).

The initial lymphatics require an external force to compress and expand the lymphatic lumen, while active transport by spontaneous contraction of lymphatic smooth muscle is limited to the outflow portion of the lymphatic network close to the cannulation site. Active lymph transport has been demonstrated in larger lymphatics including the thoracic duct (5,12-16), all of which have a well developed smooth muscle layer (17). In the rabbit popliteal prenodal lymphatics almost no lymph flow can be observed when the leg motion ceases, indicating that passive transport mechanisms play a key role (8). Gravity (18), walking (19), exercise (20), massage (9), vibration (21), respiratory movement (22,23), intestinal villous move-

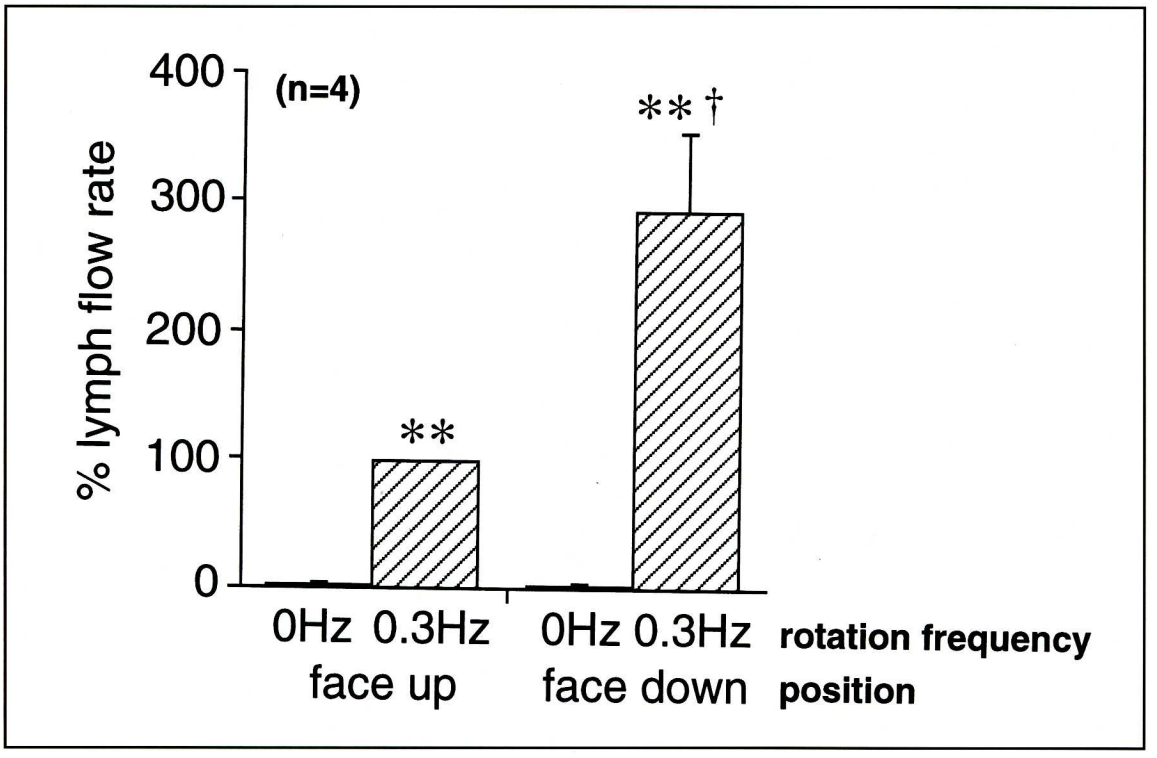

*Fig. 4. Effect of body position on lymph flow rate in the rabbit popliteal prenodal lymphatics. Values are expressed as the percentage of the value at 0.3 Hz in face up position (100% = 0.16 ± 0.08 ml/h). Left two columns are in face up position and right two columns are in face down position. The hind leg rotation frequency is 0 Hz (hatched column) and 0.3 Hz (striped column). n is the number of animals. ** p<0.01 vs. 0 Hz. † p<0.05 vs. face up position.*

ment (24), passive arthral movement (25,26), heart beats (27), arterial pulse (28), and vasomotion (29) have all been implicated as driving forces for the passive pump. Passive leg motion serves to enhance the lymph flow provided by pulse pressure or vasomotion in a fashion that depends on frequency of leg motion, and thus the frequency of rhythmic compression and expansion of the initial lymphatics (8,30). The current results show that the lymphatic pressures which can be developed in prelymphatic channels depend on the frequency of passive tissue movement.

The lymph flow rate could be raised as lymph outflow pressure was decreased *(Fig. 2B)*. The lymph flow rate increases as the pressure drop from the initial lymphatics to the cannulation point is lowered. But as the outflow pressure fell below 0 to -10 cmH_2O, the lymph flow rate reached a plateau because the lymphatics may have been collapsed, analogous to the "vascular waterfall effect" in veins. A similar mechanism has been demonstrated in canine lymphatics of the heart, liver, skeletal muscle, kidney, and small intestine (31). In contrast, in the rabbit hind leg with edema, the lymph flow rate increased about 5 fold compared with controls. The lymph flow rate continues to rise as the outflow pressure is lowered, even at -10 cmH_2O and -20 cmH_2O, suggesting edema may prevent the collapse of lymphatic channels at such pressure levels.

The pressure that is generated by lymphatics depends on how much the initial and collecting lymphatics are compressed, and therefore is a function of the stress in the tissue. The stress in the tissue depends on

initial (residual) stress in the skin, stress generated by muscle motion as well as the external force applied to the skin. The current analysis shows that the pressures generated by a normal connective tissue surrounding the lymphatics are already above about 50 cmH_2O even after the animal has been anesthetized, a condition which reduces muscle motion. Considerable higher pressures may be achieved if the hind leg and its lymphatics are compressed periodically from the outside. The outflow pressure required to stop lymph outflow depends on leg rotation frequency. With edema and enhancement of the fluid provided to the lymphatics by fluid filtration, the maximum pressure at 3 Hz was about 111 cmH_2O, which is similar to the arterial pressure and comparable to the highest fluid pressures in the body. Over the range of pressures tested in the current experiments, the maximum pressure was found to depend less on the mechanical strength of the valves, since even when the same lymphatics were tested, the maximum pressure created at each frequency of leg rotation was different. The maximum pressure required to induce reflux across a particular valve in the lymphatics is unknown, but it may not be necessary that the maximum pressure supported by one valve be as high as 111 cmH_2O. Inasmuch as the valves are arranged in series, each lymphangion can withstand a part of the elevated downstream pressure and each valve needs to support only fractions of the outflow pressure.

The maximum pressure that a valve can sustain to prevent reflux in bovine mesenteric lymphatics was reported to be about 69 H_2O (6) and about 48 cmH_2O in the canine thoracic duct (32). Intralymphatic pressures up to 40 cmH_2O without apparent valve failure have been recorded by micropipette technique in the rat mesenteric lymphatics (5). In the canine hind limb lymphatics, lymph pressure reached 18 mmHg and higher values when massaged in the region of the dorsal skin of the foot (33). Bovine mesenteric lymphatics can create lymph pressures of more than 20 cmH_2O by active contraction of the lymphatic smooth muscle cells (6) while pressures in human leg lymphatics reach more than 100 mmHg when the lymphatics are obstructed (34), values which are comparable to the current ones. The different values are reflections of the variable compression of the initial lymphatics by their surrounding tissue. Higher rotation frequencies or application of an external stress on the lymphatics may create even higher intralymphatic pressures.

Is there a need for the lymphatic system to create such high pressures? Only if there exist an outflow obstruction or when gravity needs to be overcome. In the intact animal, elevated intralymphatic pressures are expected in the presence of a lymph duct obstruction, but not during normal drainage of lymph fluid via the nodes into the central duct (5).

Body position is an important factor that influences local lymph flow rates. The normal position of the rabbit is in the face down position. When the rabbit position is changed from face up to face down, although the distance between right atrium and leg is not changed, lymph flow rate showed a 2 to 3 fold increase. The reason for this increase is unknown, but fluid shifts (35), neural (36), humoral (37) or local (38) control factors which modify capillary pressure or permeability may be involved in the phenomenon. In addition, a change in the compliance of the lymphatic wall may modify the characteristics of the passive transport mechanism (6).

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