Interstitial fluid, plasma protein, colloid, and leukocyte uptake into initial lymphatics

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Interstitial fluid, plasma protein, colloid, and leukocyte uptake into initial lymphatics. J. Appl. Physiol. 81(5): 2060–2067, 1996.—Lymphatics serve to remove from the interstitium a range of materials, including plasma proteins, colloid materials, and cells. Lymph flow rates can be enhanced by periodic tissue compression or venous pressure elevation, but little is known to what degree enhancement of lymph flow affects material transport. The objective was to examine the uptake of plasma proteins, a colloidal perfluoron emulsion (LA-11063, mean particle diameter = 0.34 µm), and leukocytes into lymphatics. Prenodal collecting lymphatics in the lower hindlimb of rabbits were cannulated with and without foot massage and after elevation of venous pressure (40 mmHg). The average lymph flow rates were elevated ~22-fold by the skin massage but only about threefold by venous pressure elevation. Lymph-to-plasma protein concentration ratio remained unchanged by the massage but decreased significantly after venous pressure elevation. Lymph colloid concentration and leukocyte counts were elevated on average 47 and 8.5 times, respectively, by foot massage, but both decreased after venous pressure elevation. These results suggest that skin movement by massage and elevation of the venous pressure lead to opposite lymph transport kinetics of protein, colloids, and cells. Massage is more effective to enhance material transport out of the interstitium into the initial lymphatics.

rabbit skin; skin massage; lymph formation; collecting lymphatics; perfluorocarbon; lymph fluid

THE LYMPHATIC SYSTEM carries tissue fluid, proteins, and leukocytes from the interstitial space to the intravascular compartment and takes up exogenous particles from peripheral tissue spaces. For example, chylomicra absorbed in the intestinal wall enter the intestinal lymphatics (4) and tattoo dye, carbon particles, or colloidal gold from subcutaneous tissue are carried in lymphatics and deposited in lymph nodes (18, 26, 28). Even larger liposomes are trapped in regional lymph nodes after subcutaneous injection (8).

An understanding of material and cell uptake by the lymphatics is important for analysis of immunological control and diagnosis as well as treatment of lymph node abnormalities (25, 30). However, little is known about the uptake kinetics of the initial lymphatics from the interstitial space. In this report, we study lymphatic transport from the interstitium of the skin on the rabbit hind leg.

In the skin, the arterial pulse pressure serves as a basic lymph transport mechanism (16). The level of lymph flow generated by the arterial pulse pressure can be enhanced by either tissue massage or by elevation of the venous pressure (2, 11). However, there are currently few studies that provide a quantitative picture of lymph colloidal or cellular constituents after enhancement of lymph flow by either massage or after venous pressure elevation (17). In skeletal muscle, elevation of the lymph flow rate by venous pressure elevation is accompanied by reduced lymph protein concentrations (6). However, there are no reports about lymph transport after controlled tissue massage.

Accordingly, the present study was designed to examine the protein transport, transport of a colloidal suspension (LA-11063, a brominated fluorochemical suspension of known particle dimensions), and transport of leukocytes in prenodal lymph vessels in the skin with and without massage and venous pressure elevation. A comparison is made under similar circumstances in a region of the skin where the microanatomy of the lymphatic network has previously been delineated (9, 11, 12).

METHODS

Experimental preparation. All animal procedures were previously reviewed and approved by the University of California, San Diego, Animal Subjects Committee. Studies were carried out on male New Zealand White rabbits (2.0–3.0 kg) anesthetized with pentobarbital sodium (30 mg/kg iv). Intermittent boluses of pentobarbital sodium were injected as needed during the experiment. The trachea was cannulated to ensure a patent airway. A catheter was inserted into the right external jugular vein for administration of drugs and into the carotid artery for systemic pressure measurement and blood collections. The catheters were filled with 10 U/ml heparin (original solution of heparin = 1,000 U/ml saline (Baxter Healthcare, Deerfield, IL). Oral temperature was monitored and maintained at 38–39°C by using a heating pad.

Lymph fluid was collected by cannulating one of the lymphatics (300–500 µm in diameter) in the lower left leg before it enters the popliteal node, as described previously (10). Briefly, cannulations were made under a stereomicroscope. The free tip of the lymph drainage cannula was positioned 5 cm below the cannulation site to create a constant negative hydrostatic pressure. To promote lymph flow, the toe portion of the foot was rotated passively in a sagittal plane with an electric motor on a circle with a diameter of 6 cm. The rotational velocity was constant at a rate of 0.1 Hz.

Formation of hind leg edema. To increase left hind leg venous pressure and generate a mild edema, a blood pressure cuff was placed on the upper left leg above the knee at a position that was proximal to the popliteal node and the cannulation site. The cuff was inflated to 40 mmHg. The venous pressure was continuously monitored in the lower left leg via a polyethylene cannula (PE-50 tubing) placed into a small vein parallel to the tendo calcaneus on the lateral side of the lower left hind leg (11).
Lymph and blood measurements. Lymph flow rates were measured by timed collection into 50-µl microsyringes. The leukocyte count in each lymph sample was determined with a hemocytometer. Osmotic pressure and protein concentration of plasma and lymph were measured with an osmometer with PM10 dialysis ultrafiltration membrane (10,000 mol wt cutoff; Amicon, Danvers, MA) (13, 31). This osmometer has a perforated stainless steel membrane support. Normal saline was used to fill the solvent compartment of the osmometer. About 0.1 ml of sample is required for each measurement, and equilibrium was obtained after 3 min. The osmometer membrane was washed three times with normal saline between readings. All measurements were performed at room temperature (22 ± 3°C).

The lymph-to-plasma protein concentration ratio was determined from simultaneously collected aliquots of plasma and lymph. Blood samples were collected from the arterial catheter into heparinized syringes (final heparin concentration = 10 U/ml) and spun for 10 min at 2,500 revolutions/min. The supernatant plasma was used in the osmometer measurements. Lymph samples were measured without any treatment. To construct a calibration curve, the osmotic pressure of bovine serum albumin (Sigma Chemical, St. Louis, MO) solution in saline was obtained (at 0.3, 1.0, 3.0, 10.0 g/100 ml). Protein concentration in each lymph sample was determined by means of this calibration curve. At the same protein concentrations, rabbit plasma and human albumin exhibited similar osmotic pressure (13, 31).

LA-11063 was used to investigate the colloidal particle uptake from interstitial space into the lymphatic system. LA-11063 is a brominated perfluorochemical emulsion (60% wt/vol perfluorobron, perfluorooctyl bromide, and 4% wt/vol egg yolk phospholipid; Alliance Pharmaceutical, San Diego, CA). The egg yolk phospholipid surfactant ensures a stable suspension in which each perfluorobron particle is covered by a phospholipid layer. Particle diameter of LA-11063 was 0.34 ± 0.24 (SD) µm (Alliance Pharmaceutical). To determine LA-11063 concentration, the number of particles of LA-11063 was counted under a microscope with ×100 oil-immersion objective (numerical aperture 1.25, E. Leitz, Wetzlar, Germany) in a hemocytometer under a glass coverslip. The number of particles in a 200 × 200-µm-square field were counted four times for each sample. Before injection of LA-11063, no particles or only a negligible number could be seen in the lymph sample. Lymph samples with foot massage were diluted 1:10–1:100 because the original lymph contained too many particles to be recognized individually. To construct a calibration curve, the counting procedure with the hemocytometer was repeated with dilutions of the original LA-11063 emulsion in 0.5% bovine serum albumin in saline (10⁻³, 10⁻⁴, 10⁻⁵ dilutions). The concentration of LA-11063 in each lymph sample was determined by using this calibration curve.

Local tissue massage. Gentle hand massage was performed on the rabbit's skin over the injection site of LA-11063. For this purpose, the forefinger and middle and third fingers were placed on the skin with slight pressure and rotated horizontally in a circular direction with diameter of ~3 cm at a frequency of ~3 Hz. Massage was applied for 15 min at the end of each experimental protocol. This procedure was carried out by a single operator and gave reproducible lymph flow rates (9).

Experimental protocol. Lymph was collected 1 h before and during the first, second, and third hours after LA-11063 injection (0.1 ml) into the dorsal skin of the rabbit's foot. After lymph sample collections, the foot was gently massaged for 15 min and then the lymph collection was repeated. The samples served to determine lymph flow rate, leukocyte count, LA-11063 concentration, colloid osmotic pressure, and protein concentration. After this procedure, Evans blue dye and an albumin solution were injected at the same location of the previous LA-11063 injection. This served to visually identify the drainage area feeding into the cannulated lymphatic vessel.

Statistics. All results are expressed as means ± SE. Two-tailed Student's t-test for paired or unpaired data or a one-way analysis of variance was used to test for significant differences between groups. Differences between groups were considered significant at P < 0.05.

RESULTS

In both the control saline-injection group and the LA-11063 group, the lymph flow rates without massage remained constant in time throughout the experiment (Fig. 1). There was no significant difference between the saline and LA-11063 groups at any time point. Application of local skin massage led to a significant elevation of the lymph flow rates, on average from 0.10 ± 0.01 to 1.27 ± 0.11 (SE) ml/h in the saline-injection group (P < 0.01) and from 0.10 ± 0.02 to 1.61 ± 0.50 ml/h in the LA-11063-injection group (P < 0.01) (Fig. 1). The effect of massage on lymph flow rate was virtually instantaneous and could be maintained for several hours.

The lymph-to-plasma protein ratio remained below one and was unaffected by the saline or LA-11063 injections and had a tendency to reach, on average, slightly higher levels after local tissue massage (Fig. 2A, not significant). No significant changes were observed in plasma or lymph plasma oncotic pressure over a period of 3 h after saline/LA-11063 injection (Table 1). The lymph plasma protein clearance (lymph-to-plasma protein concentration ratio × lymph flow rate) was significantly elevated after massage, mostly due to the increased lymph flow rates (Fig. 2B). Massaging of the rabbit's skin at the injection site, however, increased the average LA-11063 concentration 47 times (Fig. 3A) and average lymph flow rates ~22 times (Fig. 3B).

Massage also increased lymph leukocyte count significantly in both the saline control and the LA-11063 groups (Fig. 4A). During the control period without massage, the lymph leukocyte counts were on the order of...
of 100 cells/mm³. After massage, the lymph leukocyte counts increased from 107 ± 620 to 1,414 ± 646 cells/mm³ in the saline-injection group and from 121 ± 631 to 1,264 ± 6278 cells/mm³ in the LA-11063-injection group (P = 0.01) (Fig. 5A). No significant differences were observed between the saline and the LA-11063 groups, with and without massage. As product of lymph leukocyte count and lymph flow rate, the average lymph leukocyte flux was elevated by massage some 183–194 times in the saline and LA-11063 group, respectively, compared with without massage (Fig. 5B).

In contrast to the situation during application of skin massage, which instantaneously raised lymph transport, a slower time-dependent increase in lymph flow rate was encountered after elevation of the venous pressure with the cuff (Fig. 5). Therefore, all measurements of lymph transport with venous pressure elevation by the cuff will be presented in form of a time course.

The elevated venous pressure increased lymph flow rate from 0.12 ± 0.03 ml/h in the control period to 0.35 ± 0.08 (0–1 h) and 0.56 ± 0.14 ml/h (1–2 h) in the saline-injection group and from 0.10 ± 0.03 to 0.40 ± 0.03 (0–1 h) and 0.58 ± 0.06 ml/h (1–2 h) in the LA-11063-injection group. The colloidosmotic pressure in lymph decreased after application of the cuff (Table 2). The lymph-to-plasma protein ratios decreased during pressure elevation (Fig. 6A), which led to only about a threefold increase in lymph plasma protein clearance (Fig. 6B). During swelling of the hind leg, the lymph LA-11063 concentration tends to fall (Fig. 7A), there-

### Table 1. Effect of local massage on colloid osmotic pressure of plasma and lymph

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Plasma Without massage</th>
<th>With massage</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>7</td>
<td>26.3 ± 0.8</td>
<td>11.6 ± 0.7</td>
</tr>
<tr>
<td>LA-11063</td>
<td>5</td>
<td>25.1 ± 1.3</td>
<td>11.4 ± 1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Values of colloidosmotic pressure are in cmH₂O. Saline or LA-11063 was injected subcutaneously into dorsal portion of rabbit's left hind paw 3 h before massage was applied over injection site.
fore resulting, despite the elevated flow rate, in only a mild increase in LA-11063 transport rate (Fig. 7B). The lymph leukocyte concentration also undergoes a decrease after swelling (Fig. 8A) without a significant shift in lymph leukocyte flux (Fig. 8B). There were no significant differences between the saline and LA-11063 groups.

**DISCUSSION**

These results suggest that tissue swelling by hind leg venous occlusion with a cuff and tissue movement by deformation of initial lymphatics lead to opposite lymphatic uptake kinetics of plasma proteins, colloidal particles, and leukocytes, despite the fact that both serve to enhance lymph flow rates. Repeated tissue deformation leads to instantaneous enhancement of the lymph flow rate (Fig. 1), in line with the frequency and tissue area that is subject to massage (11). The enhancement of lymph flow by tissue massage can be maintained over several hours, even after reduction of the central blood pressure by arrest of the heart (11). In contrast, enhancement of lymph flow after application of an inflated cuff requires time (Fig. 5) and is accompanied by reduced transport of materials with a wide range of dimensions, from plasma proteins to the size of leukocytes (Figs. 6–8).

The elevation of the lymph flow rates by raising venous pressure to 40 mmHg (Fig. 5) is close to the maximum effect that can be achieved in this preparation by cuff occlusion without significantly shifting vascular permeability (6). Lower pressure elevations lead to lower lymph flow enhancement, whereas elevation of the pressure >40 mmHg toward arterial pressure levels may lead to enhanced lymph flow rates. Lymph flow enhancement by tissue massage is significantly more effective and has not necessarily reached peak values because lymph flow rates are frequency dependent (11) and the current massage frequency of 3 Hz was selected only as a practical limit. Higher frequencies of massage may lead to further enhancement of lymph flow rates. Periodic tissue motion is also a more effective mechanism for removal of macromolecular and cellular materials from the interstitium.

**Table 2. Effect of local venous pressure elevation on colloid osmotic pressure of plasma and lymph**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Plasma (kPa)</th>
<th>Control 0–1 h (kPa)</th>
<th>1–2 h (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3</td>
<td>20.1 ± 1.7</td>
<td>8.2 ± 2.0</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>LA-11063</td>
<td>4</td>
<td>25.4 ± 0.6</td>
<td>10.9 ± 0.9</td>
<td>7.6 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Control, 2-h period before elevation of venous pressure (40 mmHg) at time 0. Saline or LA-11063 was injected subcutaneously into dorsal portion of rabbit’s left foot 24 h before VP elevation. *P < 0.01 compared with control value during control period (−2 to 0 h) before pressure elevation.
Inflation of a cuff over the upper hind leg is accompanied not only by venous and capillary pressure elevations in the lower hind leg but also by edema formation and possible reduction of blood flow in the hind leg. Interstitial and lymphatic edema formation serve to dilute interstitial material, which may, in part, be the reason for the dilution observed under these conditions. Thus inflation of the cuff may affect the entire chain of interstitial fluid transport and lymph formation, but it has less influence on the rhythmic expansion and compression of the initial lymphatics in this preparation.

The lymphatics in the skin of rabbit hindlimb are of the initial type, deeply embedded in connective tissue at the base of hair follicles and in the adventitia of subdermal arterioles (12). These initial lymphatics start as blind channels, form a meshlike network structure, and possess intraluminal valves. Initial lymphatics have a single attenuated endothelial lining and no smooth muscle (15). Their luminal cross section is irregular and may be collapsed. To pump lymph fluid, the initial lymphatics need to be cyclically compressed and expanded by either a pulse pressure, vasomotion, or external tissue compression in the form of massage. Initial lymphatic endothelial cells have discontinuous tight junctions and show an overlap between adjacent endothelial cells. Lymph and interstitial fluid communicate through gaps between two adjacent tight junctions of the endothelium. Also, flaps of initial lymphatic endothelial cells are attached by anchoring filaments to adjacent collagen fibers (3, 15). The characteristic overlap between adjacent lymphatic endothelial cells may provide a valvelike configuration (endothelial mi-
leukocyte count from 150,000 cells/mm³ in control period (0–1 h) and 50,000 cells/mm³ (1–2 h) in saline-injection group and from 213 ± 43 to 125 ± 32 (0–1 h) and 50 ± 0 cells/mm³ (1–2 h) in LA-11063-injection group. B: Lymph leukocyte flux (leukocyte count × lymph flux) in saline- and LA-11063-injection groups before and after VP elevation. Elevation of VP increased lymph leukocyte flux from 41,000 ± 11,000 cells/h (1–2 h) in saline-injection group (no significant differences) and increased it from 18,000 ± 1,000 to 53,000 ± 17,000 cells/h (0–1 h) and then decreased to 29,000 ± 3,000 cells/h (1–2 h) in LA-11063-injection group (not significant). Saline or LA-11063 was injected subcutaneously into dorsal portion of rabbit's left foot 24 h before VP elevation. *P < 0.01 compared with value during control period (−2 to 0 h).

Fig. 8. A: time course of lymph leukocyte count in saline (●; n = 2) and LA-11063-injection groups (●; n = 4) before and after VP elevation. Values are means ± SE. High VP decreased lymph leukocyte count from 150 ± 50 cells/mm³ in control period to 150 ± 50 (0–1 h) and 50 ± 0 cells/mm³ (1–2 h) in saline-injection group and from 213 ± 43 to 125 ± 32 (0–1 h) and 50 ± 0 cells/mm³ (1–2 h) in LA-11063-injection group. B: Lymph leukocyte flux (leukocyte count × lymph flux) in saline- and LA-11063-injection groups before and after VP elevation. Elevation of VP increased lymph leukocyte flux from 41,000 ± 11,000 cells/h (1–2 h) in saline-injection group (no significant differences) and increased it from 18,000 ± 1,000 to 53,000 ± 17,000 cells/h (0–1 h) and then decreased to 29,000 ± 3,000 cells/h (1–2 h) in LA-11063-injection group (not significant). Saline or LA-11063 was injected subcutaneously into dorsal portion of rabbit's left foot 24 h before VP elevation. *P < 0.01 compared with value during control period (−2 to 0 h).

The initial lymphatics drain into collecting lymphatics that can pump actively by using lymphatic smooth muscle (7, 32). At the cannulation point, the collecting lymphatics have valves and a single layer of loosely arranged smooth muscle cells surrounding the endothelium. Some smaller initial lymphatics are positioned in the adventitia of the collecting lymphatics. Lymph from specific areas in the dermis of the foot are relatively isolated and not significantly contaminated by connections from surrounding lymphatics (1). For this reason, lymph dilution from tissue adjacent to the injection site may not significantly affect the colloid concentration in the cannulated afferent lymphatic. This contention is supported by the observation that lymph from cannulated lymphatics becomes blue after injection of Evans blue albumin at the same sites as the LA-11063 colloid suspension or saline injections. Few, if any, other prenodal lymphatic channels away from the collection site contain Evans blue under these conditions.

Lymph protein flux was increased >10 times by massage (Fig. 2). This may be due, in part, to the fact that massage may serve to increase uptake of protein from the interstitium as well as leakage from blood vessels. Vibration of the canine hindlimb also increased lymph protein flux (19). In contrast, elevation of the venous pressure tends to reduce lymph protein concentration (6, 20) (Fig. 6A) so that, despite elevated lymph flow rates, the actual transport of proteins by the lymphatics in the skin is only slightly enhanced (Fig. 6B).

Many colloidal materials injected subcutaneously can be absorbed and transported by lymphatics, including colloidal gold (0.05–0.25 µm in diameter) (26), colloidal carbon (18), or fluorocarbon suspensions (10). The particles accumulate in regional lymph nodes. The transport is associated, however, with considerable dilution of the suspension. For example, the lymphatic colloidal concentration 1 h after subcutaneous injection was 0.05 mg/ml, which is much lower than that of the original density of 600 mg/ml. Local skin massage over the injection site served to increase the LA-11063 concentration in lymph by about two orders of magnitude, and LA-11063 flux increased by about three orders of magnitude (Fig. 3). Convective transport of LA-11063 through the large interendothelial gaps of initial lymphatics may increase with increased flow caused by massage. Clearance of radiolabeled colloids from pigskin can be increased by massage (17), in agreement with the present observations.

Colloidal particles may also be transported via an intracellular pathway after phagocytosis by tissue macrophages. The magnitude of intracellular transport after subcutaneous injection is smaller (3–4 orders of magnitude lower) than extracellular convective transport in the form of free particles. Several days after the injection, however, the extracellular transport becomes progressively less significant, and the intracellular removal of interstitial colloidal particle deposits becomes the dominating mode of removal by the lymphatics. The intracellular transport can also be enhanced by tissue massage (10).

LA-11063 emulsion is predominantly made up of perfluorobenzene-containing particles. Each particle is composed of perfluorobenzon and encapsulated by a phospholipid layer. Perfluorobenzon is radiopaque, has the ability to carry
interstitial space toward and into the initial prenodal well. Morphologically, the majority of leukocytes in trafficking from the vasculature may be enhanced as mechanical stimulation. The elevated leukocyte counts may have resided in the interstitium before leukocyte counts in the lymphatics, some of the leukocytes may have resided in the interstitium before venous pressure elevation (Fig. 8). This may be important for immunological control functions and intracellular transport by lymphocytes (9). The lymph leukocyte count without skin massage was, on average, 200 cells/μl in the prenodal lymphatics, which increased with massage to ~1,500 cells/μl. A previous study in the dog hindlimb suggests that after 2 min at 60 Hz mechanical vibration, the lymph leukocyte count increased from ~250 to 500 cells/μl (19). Because the mechanical massage leads to almost instantaneous increase in leukocyte counts in the lymphatics, some of the leukocytes may have resided in the interstitium before mechanical stimulation. The elevated leukocyte counts in the lymphatics can be maintained for several hours by massage, suggesting that eventually the leukocyte trafficking from the vasculature may be enhanced as well. Morphologically, the majority of leukocytes in prenodal lymphatics resemble blood lymphocytes. Their exact phenotype remains to be identified. Compared with leukocyte migration from blood vessel to interstitial, the mechanism of leukocyte transport in the interstitial space toward and into the initial prenodal lymphatics has not been well defined.

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