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Lymph Node Mapping in the Mouse

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Abstract

Accurate identification of lymph nodes in the mouse is critical for studies of tumor metastasis, and of regional immune responses following immunization. However, these small lymphatic organs are often difficult to identify in mice using standard dissection techniques, so that larger rats have been used to characterize rodent lymphatic drainage. We developed techniques injecting dye into the mouse footpad or tail, to label the lymphatic drainage of the hind leg and flank, pelvic viscera, prostate and mammary glands. While lymphatic drainage patterns were similar in mice and rats, the inguinal lymph nodes showed distinct differences in afferent and efferent drainage. These techniques allow accurate and rapid identification of lymph nodes and lymphatic drainage in normal as well as diseased mice.

Keywords

mouse; lymph node; lymphatic drainage; Evans Blue; lymphography

1. Introduction

The lymphatic system plays a crucial role in immune responses to foreign antigens and tumors, and in tumor metastasis in humans and rodent models (Saharinen, *et al* 2004). For example, foot-draining popliteal lymph nodes (LNs) were used to characterize the LN response to an acute inflammatory stimulus in the foot (Angeli, *et al* 2006). In addition, sentinel or tumor-draining lymph nodes (LNs) are used for the diagnosis of metastasis of a variety of cancers (Morton, *et al* 2003, Turner, *et al* 1997). Mice are widely used as the experimental model of choice for immunology and tumor biology experiments. However, the lymphatic drainage of different body regions has been extensively characterized only in rats (Hebel 1976, Tilney 1971), even though there could be species-specific differences in lymphatic drainage in mice and rats. While enlarged LNs can be readily identified in mice with acute inflammation or advanced cancer, the LNs of normal mice are small and difficult to distinguish from surrounding adipose and connective tissue. Historically, dyes such as colloidal India ink, or Evans Blue, a low molecular weight dye with high affinity for serum albumen, have been used to map lymphatic drainage in rodents. These dyes are of the correct diameter (less than 100 nm) to be preferentially drained via the lymphatics (Swartz 2001). Here we report the

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development of two dye injection methods using Evans Blue dye to identify commonly studied LNs, and to characterize lymphatic drainage in the mouse.

2. Materials and Methods

Male or female C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME) were used at 5 weeks to 1 year of age. Experimental methods involving animals were approved by the Fred Hutchinson Cancer Research Center Animal Care and Use Committee. Dye injections were performed with 1% or 5% Evans Blue dye (Sigma, St. Louis, MO) in 25 μ L Hank's Buffered Salt Solution, delivered using an 0.5 ml syringe with a 27 $\frac{1}{2}$ g needle (Becton-Dickinson, Franklin Lakes, NJ).

Mice were anesthetized with 2.5% isoflurane, and dye was injected subcutaneously into the second dorsal toe of the hindfoot, with the needle pointed in a rostral direction. Alternatively, the rear footpad was injected subcutaneously, with the needle pointed in a caudal direction. The lateral tail base was injected subcutaneously at 1 cm caudal to the rectum, and medial to the tail vein. The injection site should bleb lightly, before the dye is slowly taken up by lymphatic vessels. After 5 to 30 minutes of continuous anesthesia to allow the dye to travel through lymphatics, mice were euthanized with CO₂, and dissected to locate the lymphatic vessels and LNs of interest. The blue-labeled popliteal, inguinal, and axillary LNs were easily located after removal of the skin and fascia, however the iliac and renal LNs were not visible until the intestines were removed.

3. Results

3.1. Lymphatic drainage of the hind leg

We developed methods to identify the hind leg lymphatic drainage of mice, to facilitate our studies of lymph node responses to cancer. The popliteal LN drains the hind leg, as illustrated in Fig. 1A. Injection of 1% Evans Blue dye into the foot readily labeled this LN after subcutaneous foot injection, so that the LN was easily distinguished from the adipose and connective tissue of the popliteal fossa (Fig. 2A). Injection time was not critical, as euthanasia at any time after injection gave visible blue labeling of this node, whether the dorsal toe or the footpad were injected. In fact, dye uptake was detected in the popliteal LN even when the dye was injected immediately after euthanasia, indicating that there is some lymphatic drainage *post mortem*.

The hindfoot of rats drains primarily through the popliteal LN to the iliac LN, with minor drainage to the inguinal LN (Hebel 1976, Tilney 1971). However, we found that this drainage pattern was different in mice, where the dye equally labeled the popliteal (Fig. 2A) and inguinal LNs (Fig. 2B). These findings indicate that in mice approximately half of the hind leg drainage enters the paired inguinal LNs, as illustrated in Fig. 1B. The inguinal LNs of rats drain to the axillary LNs (Tilney 1971). However, we only occasionally observed dye uptake in the axillary LNs, even when mice were anesthetized for 30 min (data not shown), indicating that lymph drainage from the foot on through the axillary LN is inefficient in mice.

The popliteal LN drains centrally to the iliac LNs in rats (Tilney 1971). We found that the iliac LNs also labelled with blue dye within 15 min after injection of mice (Fig. 2C). One or two iliac LNs are variably found on either side of the aortic bifurcation; the example shown features three iliac LNs labeled after injection of both hindfeet. In addition, an efferent lymphatic vessel draining the iliac LN was detected when more concentrated 5% Evans Blue dye was used for injection (Fig. 2C, arrowhead).

Lymph drains in a rostral direction from the iliac LNs to ultimately empty via the thoracic duct into the subclavian vein. We were interested to determine whether more central LNs can be detected after dye injection into the foot. Five percent Evans Blue dye was used for these injections, and the mice were held for 30 min before euthanasia, to increase labeling of these LNs. By this method, we were able to label the rostral renal LN (Fig. 1A), as shown in Fig. 2D (arrow). The renal LNs cannot be readily identified without using this dye labeling technique, as they are small and indistinct, even though they drain lymph from a large region including the hind limb, kidney, and pelvic viscera (Tilney 1971).

3.2. Lymphatic drainage of the lateral tail

The LNs draining the flanks and the mammary glands are of interest for our cancer studies. However, we were unable to obtain visible blue dye lymphatic drainage through these regions within 30 min after subcutaneous injection of the flank or back, suggesting that lymphatic drainage from these regions is less efficient than that of the limbs. The axilla of rats contains 4 axillary and 3 brachial LNs draining different regions of the forepaws, torso, and hindquarters (Tilney 1971), so that a labeling technique is required to unequivocally identify the LN draining the region of interest. We found that dye injection into the foot only sporadically labeled the caudal axillary LN (Fig. 1A), even at 30 min after injection of 5% Evans Blue. We therefore tested whether injection of the tail would more consistently label this drainage. In rats, the lateral tail and flanks drain to the paired inguinal LNs, and then up the “milk line” along the mammary glands, to the caudal axillary LN (Hebel 1976, Tilney 1971). The lateral tail also drains centrally through the iliac LNs (Fig. 1B).

In mice, we did find that subcutaneous injection of the lateral tail with 5% Evans Blue efficiently labeled this drainage basin within 30 minutes, as illustrated in Fig. 1B. The caudal axillary LN (arrow, Fig. 2F) and paired inguinal LNs (arrow, Fig. 2G) both strongly labeled blue by this injection technique. The lymphatic vessel connecting these LNs also labeled blue alongside the red superficial epigastric vein marking the milk line, shown at lower magnification (arrowhead, Fig. 2E), or at higher magnification (arrowhead, Fig. 2G). Thus lateral tail injection consistently labeled the lymphatic drainage from the inguinal LNs to the caudal axillary LN, for accurate distinction from adjacent LNs draining other regions.

4. Discussion

Murine lymph nodes are normally small and indistinct, which makes them difficult to reliably identify by gross dissection. The dye injection strategies we describe in this study allow confident identification of a number of major LNs in normal healthy mice, or in diseased mice. This study also characterized two major lymphatic drainage basins in the mouse (hindfoot and tail), which should be useful for local immune response or tumor metastasis studies.

The first dye injection approach of injecting the hindfoot reliably labels the popliteal, inguinal, and iliac LNs (Fig. 1A). With longer incubation times and the use of more concentrated 5% Evans Blue dye, the more central draining lymphatic vessels and renal LNs can be identified. Hence, this approach can be used to reliably identify the lymphatic vessels and lymph nodes draining the footpad, following injection of solid tumors for metastasis studies, or after immunization with particular antigens for immune response studies. In addition, because the iliac and renal LNs also drain the prostate, this dye injection method should also be useful for mouse prostate cancer studies.

The second dye injection approach using lateral tail base injection readily identified the inguinal and axillary LNs draining the mammary glands and flank, and the lymphatic vessel connecting them (Fig. 1B). The identification of these particular LNs and their drainage is of particular importance in studies of mammary tumors or of tumors implanted in the flank. A

major advantage of this dye mapping technique is the accurate identification of the caudal axillary LN draining the inguinal LNs, mammary glands, and torso, rather than the adjacent axillary and brachial LNs draining other body regions.

The lymphatic drainage patterns we have characterized in mice are largely similar to those previously characterized in the rat, indicating that the available characterization of the rat lymphatic system (Tilney 1971) should be useful to guide the mapping of lymphatic vessels and draining lymph nodes in the mouse. However, we did find that there are some differences of note. In mice, the inguinal and popliteal LNs both drain lymph from the hind leg, while in rats most lymph drains via the popliteal LN. In addition, mouse inguinal LNs drain to the central LNs and to the axilla, while in rats the inguinal LNs preferentially drain to the axilla. Further dye mapping comparison of the mouse and rat will determine if there are additional differences in lymphatic drainage between these species.

Our mapping studies indicate that the caudal inguinal LN compartmentalizes lymphatic drainage from different regions, as has been observed in human LNs (Morton, *et al* 2003). Dye injection of the lateral tail readily labeled both the inguinal and axillary LNs, while injection of the foot only labeled the inguinal LN, with inconsistent minor flow to the axillary LN. This suggests that the tail base preferentially drains to one region of the inguinal LN, which sends efferent lymphatic vessels to the axillary LN, while the footpad drains to a distinct region of the inguinal LN and then to the midline.

This Evans Blue dye injection method provides an inexpensive and simple approach for mapping other lymphatic drainages of interest in the mouse. This technique could also be useful in immunodeficient mice where lymph node remnants are very small (Custer *et al.*, 1985). We have found that injected Evans Blue dye does not interfere with flow cytometry, immunofluorescence staining, or nucleic acid purification and analysis, making this a useful approach for rapid harvesting of the correct draining LNs from valuable experimental subjects. Fluorescent high molecular weight dextran (Ruddell, *et al* 2003) or near infrared quantum dots (Harrell, *et al* 2007) can also be useful to identify LNs by fluorescent imaging, although these molecules can potentially interfere with experimental assays involving fluorescent detection. These dye injection techniques should also be useful to characterize lymph flow in mice bearing lymphatic abnormalities, such as the abnormal drainage associated with lymphedema (Saharinen, *et al* 2004) or the increased lymph flow associated with tumors (Harrell, *et al* 2007, Ruddell, *et al* 2003).

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Abbreviations

LN	lymph node
AX	axillary LN
IL	iliac LN
IN	inguinal LN

PO

popliteal LN

RE

renal LN

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Figure 1. Lymphatic drainage in the mouse

Schematic of dye-labelled lymphatic vessels and draining LNs is shown in blue. Abbreviations used are: AX, axillary LN; IL, iliac LN; IN, inguinal LN; PO, popliteal LN; RE, renal LN. A). Hindfoot injection of Evans Blue dye labels the popliteal LN, which drains centrally to the iliac and renal LNs along the midline. The hindfoot also drains to the inguinal LN, and then to the midline. Drainage from the inguinal LN to the axillary LN is only occasionally observed, as indicated by the dotted line. B). Lateral tail base injection consistently labels the central iliac LN, and the inguinal and caudal axillary LNs.

Figure 2. Dye labeling of mouse lymph nodes and lymphatic vessels

A). The popliteal LN is labelled with 1% Evans Blue dye after hindfoot injection (arrow), and is easily distinguished from surrounding adipose and connective tissue in the popliteal fossa. B). Paired inguinal LNs are strongly labeled after hindfoot injection of 1% Evans Blue (arrow). C). Three iliac LNs (arrows) and an efferent lymphatic vessel (arrowhead) are identified after injection of both hindfeet with 5% Evans Blue. D). Left renal LN (arrow) dorsal to renal veins is labeled after hindfoot injection of 5% Evans Blue. E). Injection of 5% Evans Blue into the lateral tail base labels the inguinal LN (bottom arrow) draining to the caudal axillary LN (top arrow), and the connecting lymphatic vessel (arrowhead) running alongside the superficial epigastric vein on the milk line. F). Enlargement of a region from panel E demonstrating strong

labeling of the axillary LN (arrow) after lateral tail injection. G). Enlargement of a region from panel E showing dye labeling of the inguinal LN (arrow) and efferent lymphatic vessel (arrowhead) after lateral tail injection. In all panels, an asterisk points in the direction of the tail for orientation purposes.