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BASIC MECHANISMS OF LYMPHOCYTE RECIRCULATION IN LEWIS RATS

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INTRODUCTION

Recirculating lymphocytes emigrate from the blood into lymph nodes by selectively crossing the walls of high endothelial venules (HEV) (1). This process depends upon the ability of lymphocytes to attach to endothelium via membrane receptors (2,3) and on the capacity of the lymphocytes to propel themselves across HEV. We studied the role of microfilaments and microtubules (4-10) in attachment and migration of recirculating lymphocytes by using the cytoskeletal probes cytochalasin-A (CA) and colchicine (Col). The results indicate that both CA and Col inhibit entry of lymphocytes into lymph nodes. However, CA interferes with locomotion more than it affects recognition, and Col blocks recognition without significantly affecting locomotion.

MATERIALS AND METHODS

The adherent cell index for rat lymphocytes was determined in isolated perfused mesenteric lymph nodes prepared for scanning (SEM) and transmission (TEM) electron microscopy by previously described techniques (2). Thoracic duct lymphocytes (TDL) were collected from Bollman fistulae (1) for 2 days, after which the rats were euthanized. TDL were washed and resuspended in media to equal 1 x 10^8 cells/ml. ^{3}H-Uridine (spec. activity, 21 Ci/mmol) was added to a final concentration of 10 μCi/ml and the culture was incubated at 37° for 1 hour. The cells were washed and resuspended, 4 x 10^8 labeled lymphocytes were injected IV. Labeled and unlabeled TDL were used fresh or after incubation in media containing:

a) cytochalasin-A (0.2 - 10.0 μg/ml),

b) colchicine (10^{-8} - 10^{-2} M),

c) lumicolchicine (10^{-4} M) prepared by the method of Wilson and Friedkin (7), or

d) TDL incubated at 50° for 20 minutes.

Each suspension was assessed for viability by trypan blue dye exclusion, spontaneous locomotion during 10-minute incubation at 37°, and examined by SEM and TEM. All lymphoid tissues, liver and lungs were excised at timed intervals, weighed and disrupted in a tissue homogenizer. Aliquots were counted in a Beckman LS-233 scintillation counter. Lymph nodes for autoradiography were fixed, embedded in methacrylate and sectioned at 1.0-μm thickness. Sections were dipped in Kodak NTB-2 liquid emulsion and exposed for 8 - 12 weeks at 4°. After developing and fixing, the slides were stained and the distribution of labeled lymphocytes was tabulated per 10 high-powered fields.

RESULTS

Characterization of TDL used in these studies. Untreated TDL, which were 98.6% viable by trypan blue dye exclusion, exhibited 15.6 ± 3.2 % (mean ± SD) spontaneous locomotion during 10 minutes of viewing by phase microscopy at 37°. Locomotion began when a lymphocyte placed its hyaline pseudopod onto the surface. This initiated a wave of cytoplasmic contraction which flowed from front to rear as the cell advanced. Each cell rounded up after every 3 - 9 minute cycle of movement. SEM of moving lymphocytes confirmed the presence of this avillous constriction ring (Fig. 1a). Stationary TDL were rounded and had numerous microvilli on their surfaces (Fig. 1b). In TEM studies, microvilli and cortical cytoplasm excluded ribosomes and other organelles, but did not appear to contain obvious microfilaments or microtubules. However, approximately 12 segments of 25-nm microtubules were seen within a 1.0-μm radius from the centrioles in cells where the centrioles could be located.

Cytochalasin-A (0.2 - 8.0 μg/ml) treated lymphocytes showed markedly reduced spontaneous locomotion. Some TDL exhibited asymmetric and distorted locomotion. Blebs were extruded and retracted along lateral surfaces of moving lymphocytes. Doses of CA above 4.0 μg/ml completely immobilized TDL without affecting viability (Tab. 1). The effects of CA on locomotion were not reversed by washing and re-incubation in fresh media. SEM showed loss of microvilli and zeiotic blebing (Fig. 1c). Doses above 2.0 μg/ml

TABLE 1: *In Vitro* Effects of Cytochalasin-A on Lymphocytes

Dose μg/ml	Phase microscopy of living cells % viable	% motile	Ultrastructure % loss of MV	% with blebs	Aggregated microfilaments
0	98.6	15.6	0	0	0
0.2	95.8	6.1	17.1	8.3	0
2.0	96.4	3.7	51.7	33.2	+
4.0	96.7	0.3	77.3	65.0	++
6.0	97.2	0	80.5	61.2	++++
8.0	94.8	0	89.7	65.1	++++

TABLE 2: *In Vitro* Effects of Colchicine on Lymphocytes

Dose M	Phase microscopy of living cells % viable	% motile	Ultrastructure % SEM changes	Microtubule No.
0	98.0	15.6	0	12.0
10^{-8}	95.3	14.9	0	9.9
10^{-6}	98.2	15.7	0	6.7
10^{-4}	94.6	14.7	0	1.3
10^{-2}	64.3	0	15	0.5

TABLE 3: Rheological Determination of Adherent Luminal Lymphocytes

	Perfusion conditions	ACI ± SE
A.	Normal rat nodes (no cells added)	
	Flushed with dextran/saline (DS)	0.235 ± 0.03
B.	Normal rat nodes (+ transfused TDL)	
	Flushed with DS 30 minutes after CA-TDL, IV	0.535 ± 0.09
	Flushed with DS 30 minutes after Col-TDL, IV	0.150 ± 0.01
C.	Nodes from rats given 1 μg/g Col, IP	
	(no cells added) Flushed with DS	0 ± 0

$$\text{Adherent cell index (ACI)} = \frac{\text{No. adherent lymphocytes}}{\text{No. endothelial cells}}$$

revealed partial separation of the microfilament mat from the plasmalemma, and formation of dense aggregates of 5 - 7 nm microfilaments by TEM. Fasces were usually seen crossing bases of zeiotic blebs (Fig. 1d).

Colchicine (10^{-8} - 10^{-2} M) treated lymphocytes did not show significant reduction of spontaneous locomotion until the dose reached toxic levels (Tab. 2). Hyaline pseudopods were extended but did not "stick" to the substratum, and lymphocytes with pseudopods did not appear to translocate. There were few gross changes in the surface appearance of colchicinized TDL by SEM. TEM studies revealed progressive reduction in the mean number of pericentriolar microtubules with increasing doses of colchicine. Lumicolchicine-treated TDL were indistinguishable from normal TDL in every respect.

Lymphocyte attachment. Approximately 80% of the lymphocytes found in the lumens of HEV retained their connections with the endothelial surfaces after all nonadherent blood elements were flushed away by 10% dextran/0.9% saline (DS) perfusion (Fig. 1e). The adherent cell index (Tab. 3) was 0.235 $\pm$ 0.03 in light microscopic and SEM preparations of perfusion-flushed lymph nodes. Normal rats which had received transfusion of CA-treated TDL 30 minutes prior to DS perfusion had significantly more adherent luminal lymphocytes than control nodes. Some of these adherent lymphocytes were smooth surfaced and resembled the *in vitro* preparations of CA-treated TDL shown previously (Fig. 1f). In rats transfused with colchicine-treated TDL, the ACI was not different from normal DS-perfused HEV. In contrast, no adherent lymphocytes were found on the luminal surfaces of lymph node HEV from rats which were injected IP with colchicine 1 hour previously (Fig. 1g). TEM examination of this endothelium revealed absence of cytoplasmic microtubules and there were fasces of 10-nm filaments coursing through the cytoplasm.

Lymphocyte traffic studies. Immediately after transfusion of 4×10^8 untreated ^{3}H-labeled TDL, there was a rapid accumulation of label in the lung, liver and spleen, reflecting the blood flow to these organs (Fig.2). Traffic to the spleen was indicated by an initial period of high splenic labeling which then tapered off. In contrast, there was progressive "selective" accumulation of lymphocytes in lymph nodes which peaked 18 hours postinfusion. TDL treated with 4.0 µg/ml CA failed to accumulate in lymph nodes in appreciable numbers, and splenic uptake was also reduced. Liver uptake of CA-treated lymphocytes was not different from normal which attested to the viability of CA-treated TDL. Heat-killed lymphocytes were sequestered in the liver by Kupfer cells at the expense of all other tissues. Lymphocytes treated with 10^{-4} M colchicine showed depressed accumulation in lymph nodes, which was significantly reduced until 8 hours after infusion. When untreated

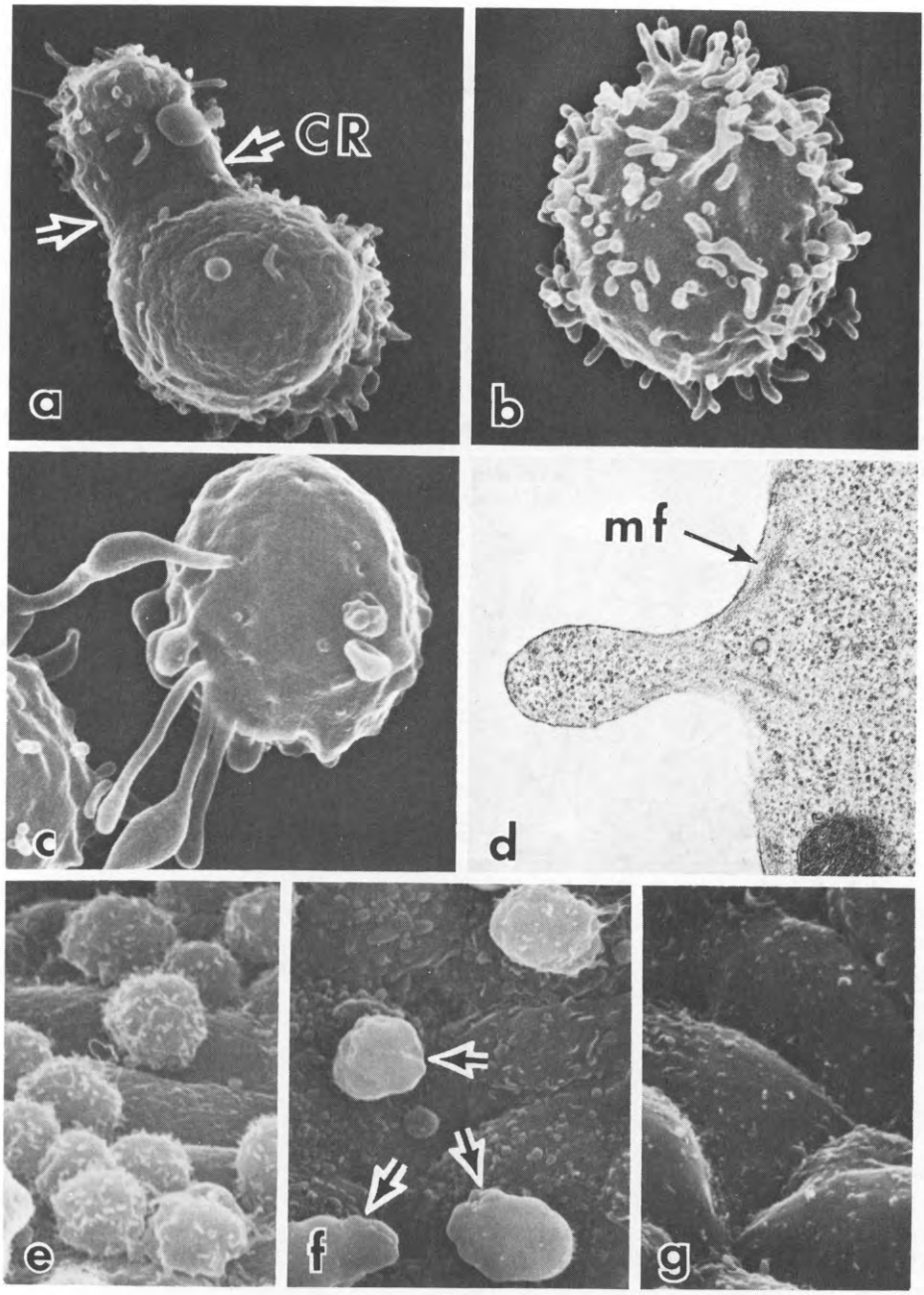

FIG. 1: SEM of untreated migrating (CR, contraction ring) (a) and sessile (b) TDL. SEM/TEM of CA TDL, showing blebs and microfilament aggregates (mf) (c and d). SEM of HEV lumina showing untreated (e), CA-treated (f) TDL (arrows). HEV from Col-treated rat (g).

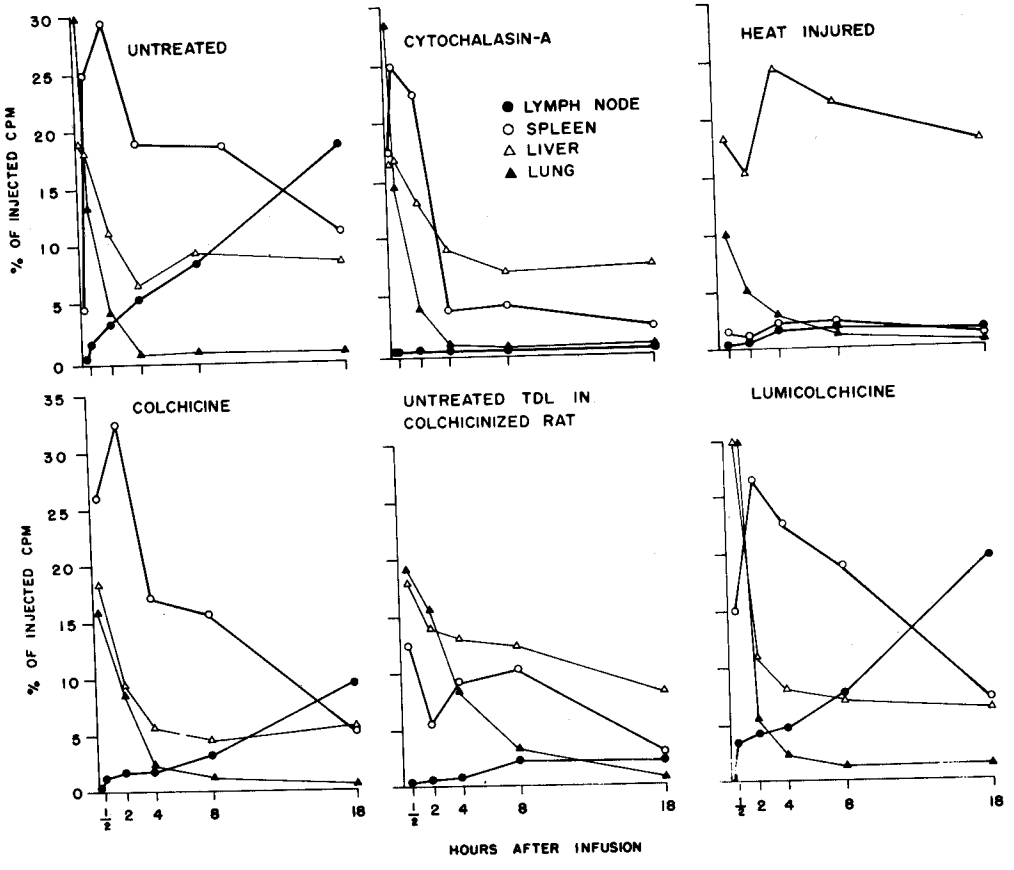

FIG. 2: Tissue radioactivity after IV infusion of TDL treated as shown.

TDL were infused into rats given IP colchicine at 1.0 μg/g body weight, both lymph node and splenic uptake was depressed. In contrast, 10^{-4} M lumicolchicine-treated TDL migrated normally when infused into untreated rats.

Autoradiography. Autoradiography of lymphocyte entry into lymph nodes after IV infusion was quantitated. The data for untreated, CA-treated and Col-treated TDL are shown in Tab. 4. Untreated TDL were rapidly cleared from the vascular lumen by 30

TAB. 4: Intranodal distribution of labeled lymphocytes after IV infusion (effects of cytochalasin A and colchicine)

Time	Mean no. labeled lymphocytes/10 HPF								
	HEV lumen			HEV wall			Node cortex		
min.	TDL	CA	Col	TDA	CA	Col	TDL	CA	Col
3	10.1	27.6	1.8	15.8	7.6	0.3	1.0	0.5	0
30	1.8	27.1	3.6	27.8	36.1	4.1	28.8	19.3	1.4
60	0.4	18.3	4.2	12.1	21.5	6.4	150.8	15.6	11.7
240	2.2	14.5	0.8	12.0	19.1	0.9	302.8	57.8	10.0

minutes postinfusion. Migrating labeled cells were a constant proportion of interendothelial lymphocytes, and accumulated in the surrounding parenchyma in a nearly linear fashion. Lymphocytes treated with 4.0 μg/ml cytochalasin-A persisted in significant numbers along luminal surfaces of HEV. Increases in interendothelial cells were also seen, and a small percentage of the CA-treated TDL gained access to the lymph node cortex. Lymphocytes treated with 10^{-4} M colchicine showed reduced luminal accumulation at 3 minutes postinfusion, but failed to accumulate appreciably in the node parenchyma during the 4-hour study.

TEM of lymph nodes. In normal lymph nodes, some lymphocytes were seen which appeared to be in the process of migrating through the HEV at the time of fixation. These cells were structurally polarized and some had cytoplasmic constrictions with thickened microfilament mats indenting the nucleus on both sides (Fig. 3a). In lymph nodes from rats which received CA-treated TDL 30 minutes earlier, rare migrating lymphocytes were seen which had alterations consistent with CA effects. These cells were elongated, distorted and exhibited asymmetric distribution of microfilament bundles at sites of contact with the endothelium (Fig. 3b). In addition, smooth surfaced luminal lymphocytes in these nodes appeared to make contact only where aggregated microfilaments retained their connections with the membrane. No grossly abnormal lymphocytes were seen in TEM of lymph nodes from rats transfused with colchicinized TDL.

DISCUSSION

Long-lived lymphocytes recirculate continuously between the blood and lymph by entering lymphatic tissues through high endothelial venules (2,8). This phenomenon has received considerable attention (9) following Gowan's original demonstration (1).

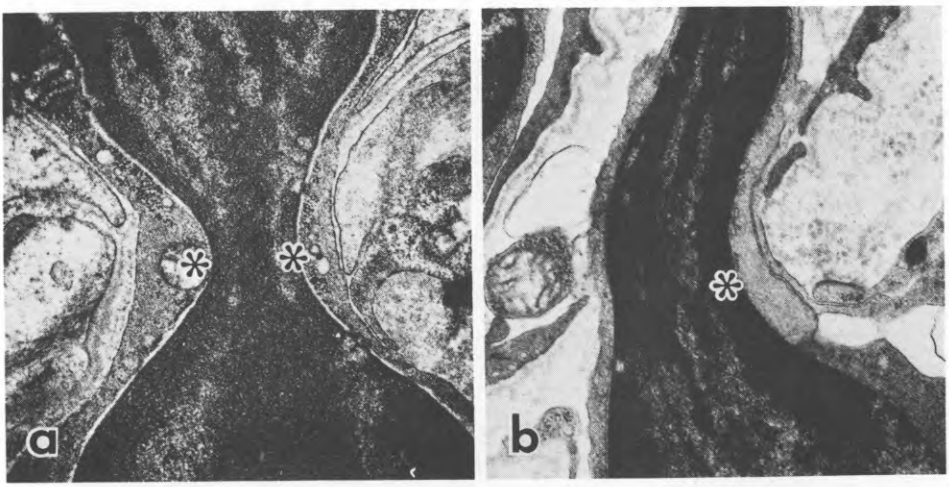

FIG. 3: Appearance of microfilament bundles (*) at contraction ring of untreated (a) and CA-treated (b) migrating lymphocytes.

However, relatively little is known about the molecular events associated with attachment and locomotion of lymphocytes in vivo.

In the present studies we have attempted to dissect the role of microfilaments and microtubules in lymphocyte recirculation using cytochalasin-A and colchicine. CA was used in these studies because it has been reported to cause prolonged disruption of microfilament function without altering hexose and nucleoside membrane transport (10). Its relative irreversibility and reduced metabolic effects made it useful for in vivo studies. Cytochalasins are thought to interfere with actin or myosin interactions with an integral membrane protein, possibly α-actinin; actin-binding protein or tropomyosin (10). Colchicine has well-documented effects on microtubule polymerization (6) and recent studies have shown that Col also affects the distribution of 10-nm filaments which apparently anchor to microtubules (10).

Cytochalasin-A clearly blocks lymphocyte locomotion without completely preventing lymphocyte homing. Despite the absence of microvilli, some of these lymphocytes retain the capacity to recognize and attach to HEV surfaces via segments of membrane which have intact networks of underlying microfilaments. CA-treated lymphocytes were not seen adhering via the membranes of zeiotic blebs. These bulbous structures are thought to result from herniation of endoplasm at sites where broken microfilament connections expose unsupported membrane. It is not known if adhesive receptors are present in

FIG. 4: Hypothetical mechanism of lymphocyte attachment and emigration in vivo.

zeiotic membranes; however, recent studies suggest that a major surface glycoprotein (fibronectin) is released into the media by treatment with cytochalasin (11). These observations indicate that the attachment receptor molecules, which are also trypsin- and EDTA-sensitive (2,3), may be linked to cytoplasmic actin-myosin filaments.

Colchicine has differential effects on attachment and locomotion, depending on the method of administration. IP injection of Col prior to transfusion with normal TDL blocks homing to spleen and lymph nodes, while Col-treated TDL showed normal motility in vitro but reduced homing into lymph nodes after IV infusion. This is in contradiction to in vitro studies by Woodruff et al. who suggested that homing receptors on lymphocytes were not affected by colchicine when treated cells were incubated on fixed frozen sections of HEV at 7° (3). We have ruled out any direct effects of colchicine binding to membranes through use of lumicolchicine, which has all the pharmacological characteristics of Col except the ability to alter microtubules. The reported role of microtubules in anchoring or stabilizing membrane receptors may help explain this effect of Col on homing. Microtubules apparently prevent ligand-induced receptor redistribution on lymphocytes and other cells; colchicine treatment releases these receptors from direct or indirect microtubule restraint, and capping occurs (6). If adhesive receptors on lymphocytes were released from localization on microvilli, this might lessen the likelihood of circulating lymphocytes adhering to HEV surfaces. Reciprocally, if complementary receptors on endothelial cells were free to diffuse in the membrane, lymphocytes might not "see" the correct receptor density or have a stabilized series

of membrane receptors against which the force of locomotion may be applied.

We propose a model of lymphocyte emigration using concepts of transmembrane cytoskeletal control of receptor movement (4-11) (Fig. 4). Normal lymphocytes attach to pits on HEV surfaces via receptors located on microvilli (2,12). Contact or some other stimulus, such as chemotactic gradient emanating from between endothelial cells (13) may signal locomotion. A wave of cytoskeletal contraction moves lymphocyte surface receptors toward the centriole. The force of receptor movement gains leverage against the resistance of microtubule and/or 10-nm filament stabilized complementary sites on lateral endothelial surfaces. This scheme is consistent with descriptions of lymphocyte locomotion in vitro (14); descriptions of the distorted asymmetric motion of CA-treated lymphocytes fit the contraction-wave concept, since deformation occurs toward residual connections of microfilaments with the membrane.

SUMMARY

Lymphocyte locomotion in vivo depends upon an intact network of subplasmalemmal contractile microfilaments which are linked through the membrane to surface receptors, and the distribution and stabilization of recognition receptors may be controlled by microtubules and/or 10-nm filaments in the cytoplasm. The differential effects of cytochalasin-A and colchicine on lymphocyte homing and locomotion have proven useful in dissecting the subcellular events underlying the process of lymphocyte recirculation.

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