Lymphology 23 (1990) 23-33

IMMUNE CELLS IN PERIPHERAL LYMPH AND SKIN OF PATIENTS WITH OBSTRUCTIVE LYMPHEDEMA

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ABSTRACT

Lymph stasis in the extremities caused by interruption of lymphatics or insufficient lymph propulsion is often complicated by recurrent skin infections. To shed further light on this subject, we studied the phenotypical and functional characteristics of cells in peripheral lymph and skin of patients with obstructive lymphedema. Compared with controls, patients with secondary lymphedema displayed a high concentration of lymphocytes and erythrocytes in peripheral lymph, sometimes increased numbers of B cells, increased density of Langerhans cells in the epidermis and occasionally in the skin papillary layer, strong expression of class II antigens on skin endothelial cells and mononuclear infiltration around blood vessels, and margination of granulocytes in skin blood vessels. Reactivity of lymph cells to mitogens was augmented. Taken together these findings indicate that ongoing chronic inflammatory processes persist in skin with lymph stasis, and, moreover, with impaired lymphocyte and Langerhans cell trafficking from skin to regional lymph nodes and inefficient clearance of foreign antigens, these lymphedematous limbs become susceptible to infection.

Obstructive lymphedema developing after removal of axillary or inguinal lymph nodes, nodal irradiation or chronic skin infection is often complicated by recurrent cellulitis (1). These episodes are usually diagnosed as lymphangitis when the inflammatory changes appear as "red streaks" along main collecting lymphatic trunks or as erysipelas when the inflammation encompasses a wide area of skin. There is also a latent form of skin infection which is characterized by tenderness and a slightly elevated surface temperature. These recurring infections have been attributed to local immunodeficiency, a theory supported by the development of lymphangiosarcoma, a virulent vascular neoplasm. This tumor arises in a lymphedematous limb (2) and its occurrence suggests a relationship between lymph stagnation and decreased tissue immunity. In tropical countries, bacterial and fungal infections of the skin commonly complicate filarial lymphedema, although the pathogenesis remains obscure. Three mechanisms to explain the tendency of lymphedematous limbs to infection seem plausible: a) the transport towards regional nodes of microorganisms which normally invade the skin (e.g., in the sole during walking) is retarded; b) lymphocyte and Langerhans cell trafficking which normally occurs from blood capillaries into the interstitium, and then into initial lymphatics and regional lymph nodes is impeded, and, accordingly cannot clear

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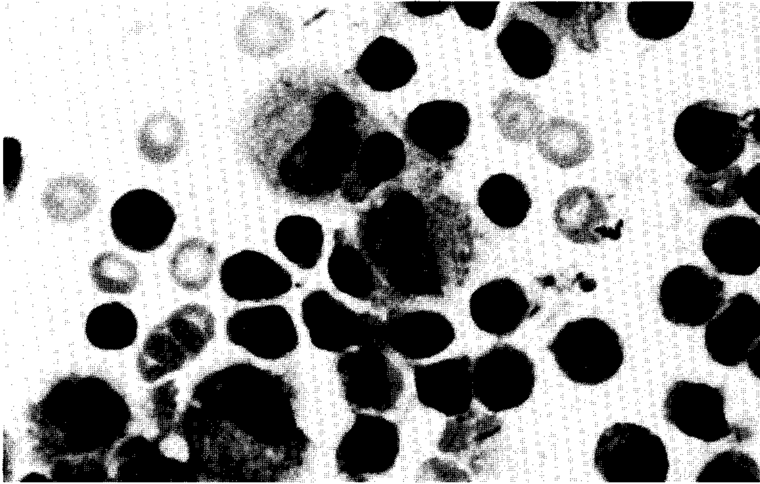

Fig. 1. Lymph smear in a patient with obstructive lymphedema stained with May-Grunwald-Giemsa method (x400). Note preponderance of lymphocytes with indented nuclei (seen in lymph but not blood lymphocytes), large macrophage-like cells with foamy appearance and veils (Langerhans cells). Some lymphocytes are in clusters with Langerhans cells. This phenomenon is partly due to centrifugation, but in fresh samples of lymph around 20% of Langerhans cells are seen with attached lymphocytes (Langerhans cell-lymphocyte clusters). The histological appearance of stagnant lymph is similar to normal lymph.

foreign antigens effectively; c) functional deficiency of individual lymphocytes from stagnation in the interstitial space, altered biochemical tissue environment and premature senescence. Thus far, only one report suggests that stagnant lymphocytes in peripheral lymph lose their cytotoxic properties (3), but no clinical studies on this issue has as yet been carried out.

This study, accordingly, addresses the question--what are the changes that occur in immune cells in stagnant lymph and skin of patients with obstructive lymphedema?

MATERIALS AND METHODS

Clinical population

Seventeen patients with obstructive lymphedema of the lower extremities were studied. Three patients had hyperplastic lymphedema for approximately 20 years complicated by foot skin inflammatory changes; others had inguinal lymph nodes removed or irradiated because of neoplasia; eleven had obstructive lymphedema probably from previous infections of the skin. Two patients from these last two groups had typical elephantiasis.

Cannulation of lymphatics, collecting of lymph and tissue fluid, skin biopsy

Of the 17 patients, cannulation of a lower leg lymphatic was successful in 3 of 4 with a visible lymphatic. In the others, including those with hyperplastic lymphedema, the dissected lymphatics were totally or partly obliterated. Of interest, the obliterative process also affected patients after inguinal or iliac lymphadenectomy. This phenomenon of lymphatic obliteration after a period of dilatation has been termed "die-back" (5). Following successful cannulation, lymph was collected continuously over a 3-day period. Skin edema fluid was obtained from 6 patients. Skin and subcutaneous tissue were punctured with an 18-gauge injection needle. The needle was kept *in situ* for the first 30 minutes until the dripping fluid became water-clear and then the edema fluid was collected for one hour. Edema

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Table 1

Phenotypes of Lymph Cells in 3 Patients with Obstructive Lymphedema as Compared with Normal Subjects

Cluster of Differentiation/Specificity	Monoclonal Antibody	Reactivity per 100 Cells: Patient 1		Patient 2		Patient 3		Controls	
		LLyc	LLgC	LLyc	LLgC	LLyc	LLgC	LLyc	LLgC
CD1	OKT6,DAKO T6	0	3	0	2	0	3.5	0	5.9±3.5
CD4	OKT4,DAKO T4	52	3	33.5	0.5	53	0	41.5±9.5	weakly 50%
CD8	OKT8,DAKO T8	38	0	21.5	0	19	0	18.4±6.2	0
CD19 (B cells)	DAKO CD19	2	0	34	0	17	0	2.0±1.0	0
HLA DR	OKIa1,DAKO HLA DR	0.2	7	32.5	2.5	15	3	3.0±5.2	9.2±5.6
CD35 (C3b receptor)	DAKO C3bR	4.0	1.5	ND	ND	ND	ND	1-2	all i.e. 6.0
CD25 (IL2 receptor)	DAKO IL2-R	1.5	0.5	0.6	0	0.5	0	6.2±2.2	0.25
Macrophage antigen	DAKO Mf	0	0.5	0	1.6	0	2.5	0	6.2±1.5
Dendritic cell	DAKO DRC	0	0	ND	ND	ND	ND	0	0
Epithelial cell membrane antigen	DAKO EMA	0	0	ND	ND	ND	ND	6.4±1.5	10.5±1.5

LLyc - lymph lymphocytes; LLgC - lymph Langerhans cells

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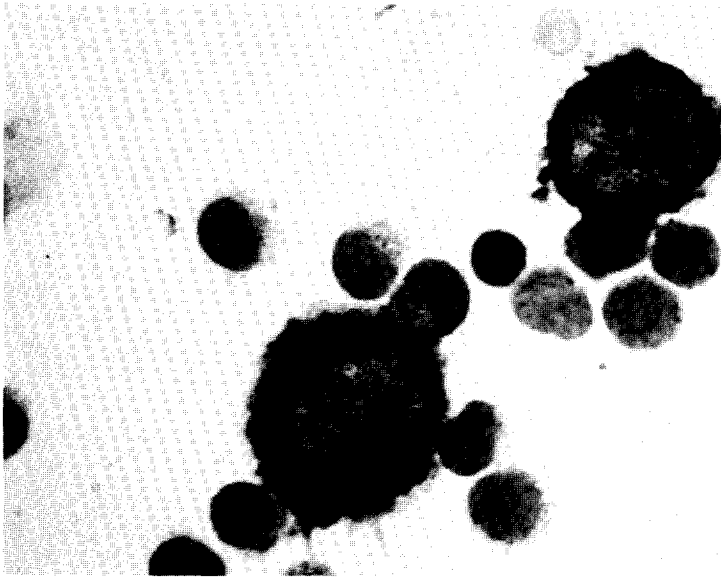

Fig. 2. Typical Langerhans cells of human afferent lymph. Note the large nucleus, abundant cytoplasm and veils, clusters with lymphocytes. This cell is $CD1^+$, weakly $CD4^+$, class II^+, $transferrin^+$, $C3bR^+$ (x400).

fluid visibly contaminated with blood was discarded. Skin and subcutaneous tissue specimens 2cm long and 1cm deep were taken from the anterior aspect of the calfabove the ankle joint.

Isolation and characterization of lymph cells

Cells were counted in a hemocytometer. Cytospin smears were performed with use of the SHANDON cytospin centrifuge. They were stained with May-Grunwald-Giemsa method or dried and frozen at -70°C for further processing and labeling with monoclonal antibodies (moabs). A portion of lymph cell samples was taken for culture studies.

Lymph and blood cell cultures

Cells from both sources were suspended in RPMI 1640 solution with 10% FCS and cultured at concentration of 10^5 cell/ml in Linbro 96-well flat-bottom culture plates for 72 hours at 37°C in the air plus 5% $C0_2$ gas mixture. H^3TdR was added to each well 20 hours before completing cultures. ^3H-thymidine incorporation was measured in a Beckman beta-scintillation counter.

Monoclonal antibody staining

A set of moabs as listed in *Table 1* was used. For labeling of lymph smears the alkaline-phosphatase-anti-immune phosphatase (APAAP) method was used as described in DAKOPATTS Manual. Frozen sections of skin were treated with moabs and peroxidase-anti-peroxidase (PAP) technique. Density of labeled cells was measured on skin cross-sections and expressed in numbers per linear mm.

RESULTS

Lymph and tissue fluid cell concentration

In three patients with obstructive lymphedema but patent collecting trunks, the lymphoid cell concentration in lymph was 1.03, 12.0, and 0.31 cells x 10^6/ml, respectively. These values contrasted sharply with the "low" normal values of 0.177±0.137 x 10^6/ml obtained from vol-

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Table 2
Responsiveness of Lymph and Blood Cells to PHA in a 72H Culture Samples of 3 Patients with Obstructive Lymphedema of Lower Limbs Mean Values of cpm of 6 Tests ± SD

Cells in Culture	90	18	4.5	1.8	Control
Lymph cells	33609±8822	32639±10662	10131±2890	2156±2747	709±611
p value	<0.05	<0.05	<0.05	<0.05	NS
Blood cells	12830±7806	9707±3470	3030±319	1035±569	569±70

Table 3
Responsiveness of Lymph and Blood Cells to ConA in a 72H Culture Samples of 3 Patients with Obstructive Lymphedema of Lower Limbs Mean Values of cpm of 6 Tests ± SD

Cells in Culture	5	2	1	0.5	Control
Lymph cells	31251±6730	33446±7472	30313±8473	23829±7480	709±611
p value	<0.05	<0.05	<0.05	<0.05	NS
Blood cells	5155±3686	3215±3168	2266±2348	1081±837	569±70

unteers (6). The erythrocyte concentration was high, namely 30.0, 4.0, and 3.13 x 10^6/ml, respectively. In normal subjects the erythrocyte concentration in afferent lymph remains at the level of 15% of all lymph cells (6). Tissue fluid samples were obtained from 9 patients. In three the samples were contaminated by lymph due to puncture of grossly enlarged dermal lymphatics. This occurrence was recognizable by sudden abundant fluid flow. These samples were discarded. The range of the level of tissue fluid lymphocytes was between 0 and 0.013 cells x 10^6/ml and erythrocytes between 0 and 31.5 cells x 10^6/ml. Collection of tissue fluid was always "threatened" by contamination with capillary blood; however, in none of the 6 investigated patients was the lymphocyte/erythrocyte ratio in tissue fluid comparable to that of blood. It was not possible to obtain control samples of tissue fluid from normal subjects in volumes sufficient for cellular analysis.

Morphological and phenotypical evaluation of lymph and tissue fluid cells

Smears of lymph stained with May-Grunwald-Giemsa method revealed lymphocytes with typically indented nuclei (note that this shape is seen in lymph but not blood smears), few mitotic figures, large macrophage-like cells morphologically resembling Langerhans cells, and erythrocytes *(Figs. 1,2)*. This picture was similar to that seen from peripheral lymph of normal subjects (7). In the smears of tissue fluid, some tissue macrophages could be seen among lymphocytes, but the density of cells was extremely low as compared with lymph. No granulocytes were detected.

The quantitative evaluation of phenotypes of lymph cells is shown in *Table 1*. The percentage of helper (CD4) and suppressor (CD8) cells remained within normal limits for lymph (7), whereas that of Langerhans cells (CD1) was decreased. A relatively high number of Langerhans cells failed to stain with the OKT6 antibody. Of note, a high percentage of CD19-positive cells (i.e., B cells) was found in two patients, exceeding by factors of 8 and 17 the normal value. Labeling lymphocytes with anti-HLA DR antibody confirmed that a large number of B cells was present in these two patients. B cells are class-II positive and 32% and 15% of lymph lymphocytes bound the anti-HLA DR antibody. The IL2-receptor was detected on a smaller number of cells

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Table 4
Phenotypic Characteristics of Cells in Epidermis and Skin in Various Types of Lymphedema (Patients 1-11)

Monoclonal	Normal	Postinflammatory or Post Surgical						Hyperplasia			Elephantiasis	
Antibody		1	2	3	4	5	6	7	8	9	10	11
CD3	+	+	+	+	+	-	-	-	+	-	-	--
CD4	+	+	+	+	+	-	+	+	+	+	-	-
CD8	+	+	+	+	+	-	+	+	+	+	-	-
CD1												
epidermis	40/mm	60	48	72	60	100	40	64	56	40	28	32
cutis	+	+	+	+	+	+	-	+	-	-	-	-
CD19 (B)	-	-	-	-	-	-	-	-	-	-	-	-
Transferrin	-	-	-	-	-	-	-	-	-	-	-	-
HLA DR												
epidermis	20/mm	8	8	20	40	20	40	+	8	10	+	+
cutis	++	+++	+++	+++	+++	+++	+++	-	-	-	-	-
Macrophage	+	+	+	+	+	+	+	+	+	+	-	+
C3b	-	-	-	-	-	-	-	-	-	-	-	-
M4 (PMN)	-	-	-	-	-	-	-	-	-	-	-	-
IL2R	-	-	-	-	-	-	-	-	-	-	-	-

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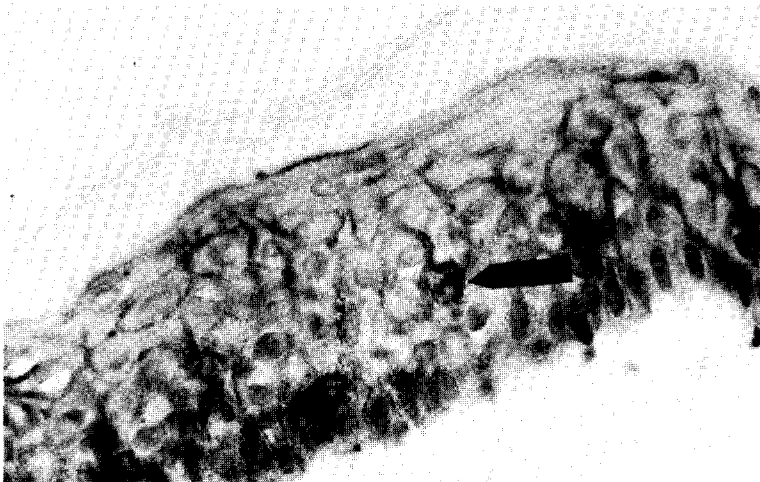

Fig. 3. Skin specimen of a patient with obstructive lymphedema. Frozen section stained with anti-CD1 monoclonal antibody and PAP technique. Note numerous Langerhans cells in the epidermis (marker). Normal epidermis contains less of these cells.

as compared with normal lymph. The same was found for the macrophage antigen labeled with DAKO-Mf antibody. No cell with epithelial cell membraneantigen was found in the population of stagnant compared with normal lymph. The number of cells obtained from tissue fluid was too few for phenotypical analysis.

Cell cultures

The responsiveness of cells in lymph to PHA and ConA was high compared to that of blood mononuclear cells *(Tables 2 and 3)*. Generally, the shape of curves of reactivity to increasing concentrations of mitogens was similar to that of normal lymph (8). However, there were differences concerning the level of responsiveness. At high concentrations of PHA, cells in lymph retained the tendency to respond vigorously, which was not the case in lymph from normal subjects (8). Also, the differences in responsiveness of lymph and blood cells to ConA were much higher in lymphedema than in controls (8).

Cluster formation

Formation of clusters of Langerhans cells and lymphocytes did not show the predilection of helper (CD4) cells to attach to large cells, as has been observed in normal lymph. The percentage of helper cells in clusters (66%) corresponded to the percentage (68%) of free-floating cells. This test was performed in only two patients.

Immunochemistry of skin

Histology of the skin showed typical changes of hyperkeratosis, abundant fibroblasts, dense bundles of collagen fibers, and multiple lymphatic "lakes" with borders not lined by cells. These "lakes" were empty of cells but did contain proteinaceous fluid. The same kit of moabs was used for labeling of skin as for lymph cells, but only some skin cell populations stained with the antibodies listed in *Table 1*; others remained negative *(Table 4)*. No mononuclear cells with microscopical appearance of lymphocytes, including those in perivascular infiltrates, were CD3 positive. Some scattered CD4 and CD8-positive cells were seen in the Malpighian

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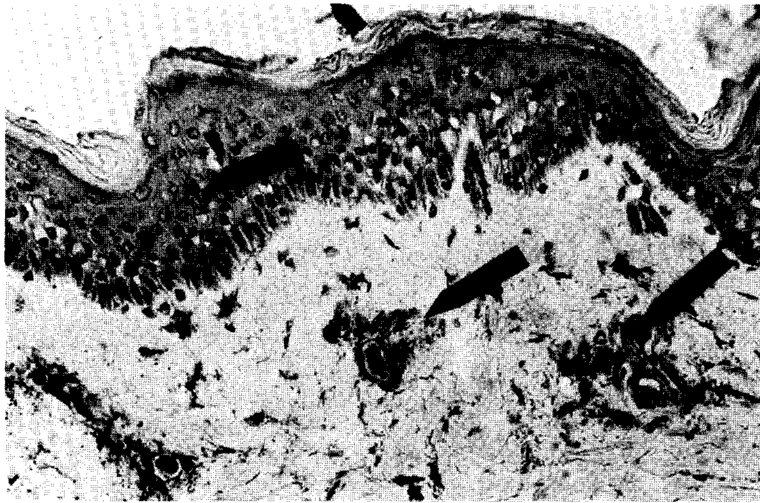

Fig. 4. The same specimen as in Fig. 3 *labeled with anti-HLA DR monoclonal antibody. Epidermal Langerhans cells are weakly stained, whereas in some infiltrates of the skin endothelium and many fibroblasts stain densely (markers). This appearance is typical of an inflammatory reaction with expression of Ia antigens.*

layer of skin. Also, no B (CD19), transferrin, C3bR and IL2-R-positive cells were detected. This arrangement is in keeping with patterns described for normal skin. The CD1-positive cells were found in epidermis in greater numbers than in healthy epidermis *(Fig. 3)*, with some around skin blood vessels. In contrast, fewer epidermal Langerhans cells stained with anti-HLA DR antibody. In the skin, mononuclear infiltrates, blood endothelial cells and many fibroblasts were consistently strongly HLA-DR positive *(Fig. 4)*. Skin specimens obtained from patients with longstanding elephantiasis and skin hyalinosis and those with hyperplastic lymphedema displayed poor staining of large cells in the epidermis and skin with anti-HLA DR antibody. Occasionally, some DAKO Mf-positive macrophages were observed in the skin. M4 (polymorphonuclears)-positive granulocytes were not found in skin tissue although many were marginated in dermal blood capillaries *(Fig. 5)*.

DISCUSSION

One of the most interesting findings was a high concentration of lymphoid cells in stagnant lymph with the number in two patients approaching the concentration of lymphocytes in blood. These cells either gradually accumulated or proliferated in the obstructed lymphatics with impairment of lymph drainage. High autotransformation rate of cultured lymphocytes and their high responsiveness to mitogens suggest an active proliferative tendency of this cell population, perhaps stimulated by latent infection. Inflammatory changes in skin are observed clinically in the majority of patients with obstructive lymphedema. High concentration of erythrocytes in lymph of the patients studied here also supports the concept of ongoing chronic inflammation in lymphedematous tissues. Although inflammation facilitates extravasation of erythrocytes, the pathways of migrant streams of erythrocytes from blood capillaries to initial lymphatics are unclear as histology failed to reveal large numbers of extravasated erythrocytes in the interstitial space of the skin.

In contrast to lymph, edema fluid obtained from lymphedema patients contained very few lymphocytes and tissue

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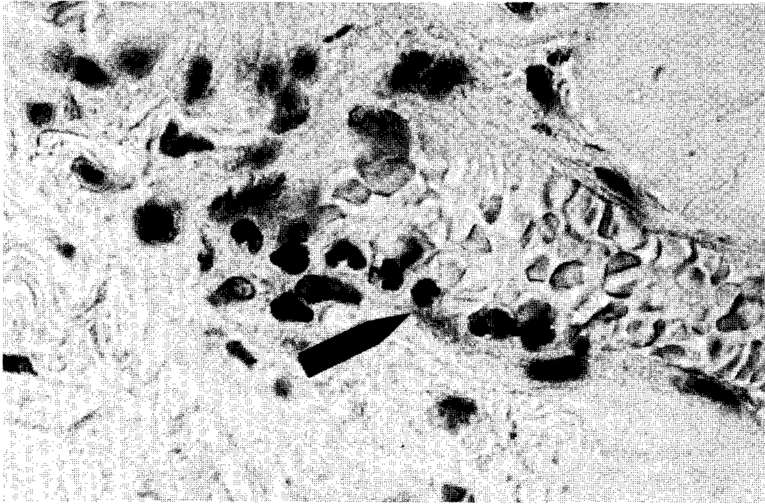

Fig. 5. Skin of a patient with obstructive lymphedema. Note blood vessel with marginated granulocytes located near an obliterated lymph collecting trunk (marker). Margination of PMNs is rare in normal skin.

macrophages and no granulocytes. There was, however, a high number of erythrocytes. In all instances the lymphocyte/erythrocyte ratio was higher than in blood whereas the hematocrit was only a fraction of 1%. This combination suggests that the collected sample was tissue fluid contaminated with blood and not merely blood from damaged capillaries. A lack of granulocytes further supports this view. A paucity of lymphocytes in tissue fluid but a high concentration in peripheral lymph suggests that extravasated lymphocytes possess the ability of directional migration toward lymphatics, probably along ground matrix "channels," with absence of spread in the mobile tissue fluid.

Lymph smears in lymphedema patients were similar to normal lymph. More mitosis was apparent in stagnant lymph, but this was expected in light of the exceptionally high lymphocyte concentration. A lower number of Langerhans cells staining with T6 antibody as well as of lymphocytes expressing the IL2 receptor in stagnant than in normal lymph was ascribed to functional blockage of these determinants rather than to their decreased density on the cell surface. A high percentage of B-cells in patients with obstructive lymphedema was a new observation. This finding was confirmed by staining with anti-HLA DR antibody which binds to the Ia molecule present on B cells along with macrophages and activated T cells. What is the origin of these cells in lymph? Normally, not more than 2% of B cells are detected in lymph (7). B lymphocytes are generally thought to extravasate at a lower rate than T cells and move sluggishly through the tissue space. In stagnant lymph they probably proliferate but the signal for this process remains unknown. Most likely B cells respond to bacterial or viral antigens in stagnant lymph although the immunoglobulin-producing activity of lymph B cells was not tested. Interestingly, no B cells were detectable in skin of patients with high levels of lymph B cells.

Lack of cells with epithelial membrane antigen in stagnant lymph contrasts sharply with normal lymph. Since the role that EMA-positive cells play in normal lymph remains obscure, further studies are needed for proper interpretation of both positive and negative findings.

Culture of cells in stagnant lymph with mitogen revealed a high level of responsiveness when compared to blood lymphocytes, a finding previously described in normal lymph (8). The level of

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lymph lymphocyte reactivity is likely regulated by the presence of Langerhans cells functioning in an accessory role. Supplementing blood lymphocyte cultures with Langerhans cells spectacularly increases the lymphocyte responsiveness to lectins and alloantigens (9). There was a clear difference in responsiveness between lymph and blood cells to high doses of PHA and ConA, much greater than in normal subjects (8). Mitogens may act as secondary stimulators after the primary signal has been received by the cells in tissue fluid or lymph. The primary stimulator may be bacterial or viral protein. Taken together these findings favor that stagnant lymph cells retain considerable reactivity.

Comparison of phenotypes of migrating immune cells in lymph and in skin and epidermis uncovered unexpected observations. One was a limited expression of CD3, CD4, and CD8 phenotypes on skin lymphocytes including those accumulating around blood capillaries. Lack of expression of CD19 (B cells), transferrin and C3b receptors and IL2-R on lymphocytes and macrophages when stained with monoclonal antibodies was probably due to involvement of these determinants in local tissue processes. These processes were not necessarily pathological as similar findings were observed in normal skin. Staining of skin was performed on frozen sections, a technique which does not damage surface receptors. Thus, the described findings can not be attributed to technical shortcomings. Labeling cells with anti-CD1 (Langerhans cells) and anti-HLA DR antibodies produced distinct patterns. Multiple CD1-positive cells were found in the epidermis and some in the Malpighian layer (especially in post-inflammatory lymphedema) a number higher than in normal skin. Besides Langerhans cells, anti-HLA DR antibody marked endothelial cells, fibroblasts, and mononuclear cells in skin infiltrates. High density of Ia molecules on these cells as well as on the non-cellular structures pointed to the presence of a chronic inflammatory process. In support of this concept was the finding of granulocytes marginated around dermal blood capillaries, a phenomenon rarely observed in normal skin.

Taken together, lymphoid cell composition of stagnant lymph and lymphedematous skin in patients with obstructive lymphedema reveals considerable differences compared with normal lymph and skin. These include high concentration of lymph lymphocytes, with greater numbers of B-cells, increased CD1 positive Langerhans cells in epidermis and in skin, high density of Ia determinants on extravasated mononuclear and endothelial cells and fibroblasts. Thus far, no functional deficiency at the cell level was found as the immune responsiveness to mitogens exceeded normal values. The changes in lymph and skin immune cells point to the presence of a chronic inflammatory process with obstructive lymphedema. Lack of effective drainage of immune cells from local tissues to regional lymph nodes is probably the primary factor responsible for inefficient removal of tissue foreign antigens with persistent chronic inflammation in peripheral tissues with lymph stasis.

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