

THE EFFECTS OF UNGUENTUM LYMPHATICUM ON SKIN IN PATIENTS WITH OBSTRUCTIVE LYMPHEDEMA OF THE LOWER EXTREMITIES

W.L. Olszewski, U. Kubicka

Department of Surgical Research & Transplantology, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

ABSTRACT

Obstructive lymphedema of extremities in humans is characterized by swelling of tissues with lymph stasis and inflammatory infiltrates in skin and subcutaneous tissues. Treatment of the inflammatory component requires application of antiinflammatory drugs. We studied the effect of topical application of Unguentum Lymphaticum (UL) containing antiphlogistic compounds (digitalis, calendulin, hyoscyamine, colchicine and podophyllin) on lymphedematous skin in 33 patients with stage II postinflammatory obstructive lymphedema. A three-week treatment of swollen legs with UL brought about stimulation of epidermal cells with proliferation of keratinocytes, increased numbers of Langerhans cells, accumulation of macrophages in the dermis and activation of infiltrating cells and endothelia. Besides some foci of acanthosis, no degenerative changes were observed in the skin in patients treated for 12 weeks and no changes were observed in the placebo treated groups. Immunohistochemical evaluation of biopsy specimens of inguinal lymph nodes in patients treated for 12 weeks revealed reactive granulocyte and macrophage accumulation in the cortical and paracortical areas. Components of UL inhibited stimulation of blood mononuclear cells in in vitro cultures. UL did not change the spectrum of calf skin bacterial populations. The nonspecific stimulation of

skin-associated lymphoid tissue and putative elimination of factors responsible for lymphedematous skin inflammation appears to be responsible for the beneficial clinical effect of UL on legs with lymph stasis.

The physiopathological picture of lymphedema caused by obstruction of lymphatic pathways comprises edema, inflammation and progressive fibrosis of tissues. Skin of extremities affected by lymph stasis reveals microscopical signs of local inflammatory reaction (1). This phenomenon is observed in most patients with secondary lymphedema (postsurgical, posttraumatic, postinfectious) as well as in advanced cases of primary lymphedema. Hyperkeratosis, proliferation of fibroblasts, excessive deposition of collagen, and mononuclear infiltrates around dermal venules of the superficial capillary network and at the epidermo-dermal junction are observed (1). Each episode of dermatitis and lymphangitis, frequently seen in most patients, brings about progression in skin changes, with an increased density of cellular infiltrates and fibroblasts and proliferation of keratinocytes. The basic question is which pathological factors evoke and sustain the chronic inflammatory changes. Is it lymph stasis with increased interstitial fluid volume, accumulation of proteins in the interstitial compartment, retention of recirculating lymphocytes undergoing local activation in

the skin and lymphatics by tissue products, or bacterial antigens penetrating the epidermis and retained in the lymphedematous tissues? Irrespective of which factor dominates, three goals of therapeutic efforts should be reached: restoration of lymph flow from skin, control of the inflammatory reaction, and protection against bacterial colonization. The control of the inflammatory component should bring about attenuation of edema with decreased capillary permeability. Topical and systemic application of antibacterial drugs may prevent excessive bacterial penetration through the epidermis and colonization of tissues, lymphatics and lymph nodes facilitated by microinjuries of the foot (2,3).

One of the medicaments widely used along these lines for control of the inflammatory component of edema in lymphedematous skin is Unguentum Lymphaticum (UL). UL is a composition of extracts from medicinal plants and contains calendulin, hyoscyamine, digitalis, colchicine and podophyllin. All these components have been known as biostimulants and antiinflammatory agents (4-10).

In this paper we describe the results of immunohistochemical investigations of skin and lymph node biopsy specimens from lymphedematous legs of patients with obstructive lymphedema before and after topical therapy with UL. The effects of UL on calf skin bacterial flora were also studied.

MATERIALS AND METHODS

Patients

A group of 33 patients in stage II of lymphedema of lower limbs of 3 to 7 years duration was randomly selected.

Inclusion criteria. (1) Patients in stage II (pitting) lymphedema of lower limb (affecting foot and lower calf) of the post-inflammatory type, (2) at least one episode of dermatolymphangioadenitis (DLA) in the past, (3) on lymphoscintigraphy delayed absorption of the

tracer from the injection site, late accumulation in the inguinal lymph nodes, dermal backflow in the foot and calf, (4) on color Doppler investigation no thrombotic changes in calf and thigh veins.

Exclusion criteria. (1) Acute DLA, (2) hyperkeratosis of skin, (3) lymph oozing from the skin surface, (4) chronic venous insufficiency, and (5) edema of systemic origin.

Study groups. The study was divided into 8 groups according to the protocol of administration of UL and the effect of UL on patient blood leukocytes *in vitro* (Table 1). During the entire observation period patients were not given any additional therapy including antiinflammatory drugs and antibiotics, or the wearing of stockings or elastic bandages. All patients signed an informed consent to the study including drug application and biopsies of skin and nodes. The study was approved by the Institute's Ethical Committee.

Application of Unguentum Lymphaticum

UL was spread on the entire skin of foot and calf and rubbed in for 5 minutes. Then, the leg was wrapped in gauze soaked in UL and a bandage was loosely applied circumferentially. This procedure was repeated daily. During the first day of treatment, each patient had a feeling of "warmer skin." No complaints were reported.

Clinical Evaluation of Skin Tonicity (Edema)

The skin was pressed on the anterior aspect of tibia for 5 seconds with index finger to produce a pit, and time of its disappearance was measured. It ranged between 45-60 seconds before treatment. The test was performed at 4:00-5:00 pm, at the time of full return of swelling after night rest. The sensation of increased warmth of skin in the lymphedematous limb was reported by all patients. Measurement of skin surface

TABLE 1
Study Groups in Unguentum Lymphaticum-Treated Patients

Group	No. of Patients	Type of Study	Specimen	Follow-up Period
1	7	UL applied on skin 1 time daily	skin	5 days
2	5	UL applied on skin 1 time daily in 3 sessions for 5 days	skin	3 weeks
3	3	UL applied on skin 1 time daily in 12 sessions for 5 days	skin	12 weeks
4	3	Placebo applied on skin 1 time daily in 3 sessions for 5 days	skin	12 weeks
5	3	Placebo applied on skin 1 time daily in 3 sessions for 5 days	skin	12 weeks
6	12	UL applied on skin 1 time daily 12 sessions for 5 days	lymph node	12 weeks
7	10	UL applied on skin 1 time	bacteriologic swabs	3 days
8	5	Effect of UL ingredients on blood mononuclear cells in culture	blood	–

temperature using a thermocouple provided irreproducible data, and the method was discarded. Patients assessed the changes of skin surface temperature with dorsum of their own hand.

Skin Biopsy

Skin specimens were taken before and after treatment in patients from groups 1 to 5 (Table 1). A routine procedure, used in our department in lymphedema complicated by DLA, for bacteriological skin culture was applied. Biopsy was taken before and 3 days after termination of topical therapy with UL. A 3x4 mm skin fragment was excised under local anesthesia with 2% Xylocaine from the antero-medial aspect of leg 5 cm above the medial malleolus. Skin was stitched. The healing process was uneventful.

Lymph Node Biopsy

Lymph node specimens were taken before and after treatment from patients in group 6 (Table 1). A minimally invasive techniques used by us for diagnostic biopsy of enlarged nodes was applied. A 1cm incision was made in the inguinal fossa over the lymph nodes. A fragment of 4x4 mm of a lymph node located medially was harvested. Skin was stitched. After completion of therapy the same protocol was used with incision made laterally from the site of the previous biopsy. In all cases the wound after first biopsy was healed. The skin and node specimens were coded.

Processing of Skin and Lymph Node Specimens

Specimens were frozen immediately after harvesting in acetone at the temperature of -70°C , kept in a deep-freeze for 3 days, then sectioned on cryotome. A routine method for fixation and staining was applied. In order to characterize the phenotypes of cells, monoclonal antibody - immune peroxidase staining technique was applied. The monoclonal antibodies were: anti-CD1a (M721, DAKO, Denmark) specific for Langerhans cells, anti-HLA DR (M746, DAKO) visualizing all cells expressing class II antigens (macrophages, dendritic cell, endothelial cells, activated lymphocytes and keratinocytes), CD68 anti-macrophage antigen (M718, DAKO), CD3 (T-cells, DAKO), neutrophil elastase (MO 752, DAKO), CD22 (B cells), factor VIII-related antigen (DAKO), and anti-vimentin (M725, DAKO), identifying vimentin present in all mesenchymal cells.

Quantitative Evaluation of Immunohistochemical Pictures

Epidermis. The thickness of hematoxylin-eosin stained epidermis was measured and expressed in numbers of keratinocytes counted vertically in one row at 10 different locations of the specimen. Epidermal CD1a Langerhans cells were counted in 10 microscopical fields at x200 magnification and expressed in number per linear mm of epidermis. These cells were located mostly suprabasally, and their distribution along this layer best reflected their density.

Dermis. On skin sections, the cell size and intensity of expression of factor VIII-related antigen and HLA DR antigen on capillary endothelium, density of mononuclear cells around dermal papillary capillaries, density of perivenular and intervenular and epidermo-dermal infiltrates were measured. Increased size of endothelial cells was assessed by their protrusion into the capillary lumen and expression of factor VIII-related antigen was expressed in + to

+++ . In the dermal perivenular, intervenular and epidermo-dermal junction regions the number of CD1a+, CD68+, HLA DR+ macrophages, CD3+ lymphocytes, and neutrophil elastase-positive/ CD15+ cells was expressed in + to +++ , where + denoted accumulation of <10, ++ 10-20, and +++ >20 infiltrating cells around a venule or in the intervenular area (between two venules in papillary dermis). The number of vimentin positive cells at the epidermo-dermal junction was measured per linear mm. Counting was carried out in 10 microscopical fields at x200 magnification.

On lymph node sections, areas of distribution of CD3+ and CD22+ lymphocytes (paracortical, follicular, medullary regions), location and density of CD1a+, CD68+ and HLA DR+ cells and density of blood vessels filled with neutrophils were estimated.

Cell phenotype counting was performed using the automated system Microimage (Olympus, version 3.0.00.00 for Windows 95/NT) by two independent investigators. The sections were coded.

Skin Bacteriological Culture

In another 10 patients with stage II lymphedema of the leg, skin swabs were taken from the anterior aspect of calf above the ankle joint. Then, UL was spread on an area of 10 sq.cm and covered with adhesive tape. Seventy-two hours later the tape was removed and another swab was taken. Swabs were placed in a transport medium and then transferred to Hemoline (bioMerieux, France). Isolates were identified by standard procedures using the Api identification system (bioMerieux).

In Vitro Blood Cell Culture

To study the effect of chemical components of UL on lymphocyte responsiveness, peripheral blood mononuclear cells (PBM) obtained from 5 healthy volunteers were cultured in 96-well plates in a concentration of $10^4/100\mu\text{l}$ cells in RPMI (Gibco, USA)

TABLE 2
Epidermal Cell Frequency and Phenotypes in Skin of Unguentum Lymphaticum-Treated Patients

Group	Keratinocytes*				Intraepidermal CD1a+ cells** (Langerhans cells)		Morphological changes***	
	Before number	HLADR ⁺	After number	HLADR ⁺	Before	After	Before	After
1	5-8 (6)	-	5-8 (6)	-	10-12 (11)	10-13 (11)	-	Acanthosis+
2	5-8 (6)	-	12-18 (13)	-	10-12 (11)	25-30 (24)	-	Acanthosis+
3	4-9 (6)	-	12-20 (14)	-	10-14 (11)	25-40 (28)	-	Acanthosis+
4,5	5-8 (6)	-	5-8 (6)	-	10-12 (11)	10-13 (11)	-	-

* Range and median number of cells counted vertically from surface to epidermo-dermal junction

** Range and median number of cells per linear mm

*** Acanthosis of epidermis + focal, ++ diffuse

supplemented with 5% of fetal calf serum, 4.5 and 9.0 µg/ml of PHA (phytohemagglutinin), and 5 µl of mixture of calendulae, hyoscamine, digitalis, colchicine and podophyllin or oleum petrae (vehiculum) for 72h at 37°C. Incorporation of ³HTdR into activated lymphocytes was measured in a beta-scintillation counter (Beckman, USA) and expressed in cpm/min.

RESULTS

Clinical Evaluation

All patients in group 2, 3 and 6, but not in group 1, reported softening and cooling of the previously warm and tender skin. In four patients of group 3, three of group 3 and ten of group 6, the disappearance time of skin pit decreased to 30-45 seconds. This effect was clinically evident even if all the reservations toward the method were taken into account. All patients reported subsidence of the warmth sensation of the swollen skin. This observation was subjective and difficult to grade. There were no significant changes in limb girth. No untoward clinical effects of UL on skin were observed.

Immunohistochemical Evaluation

Skin evaluation data are presented in Tables 2 to 4.

Group 1. 5 days of topical therapy with UL did not bring about histological changes in any of the 7 investigated patients. In 2 of them, acanthotic foci in the epidermis and slight accumulation of dendritic-like cells (CD68) were noted at the epidermo-dermal junction. In one patient, fewer Langerhans cells (CD1a) were seen in the epidermis.

Group 2. 21 days of treatment produced recruitment of immune cells into the epidermis and dermis.

Epidermis: The number of keratinocyte layers increased in 3 out of 5 specimens from 5-8 (median 6) to 12-18 (median 13); in some, focal protrusions of epidermis into dermis were observed. They did not become HLA DR+. The number of Langerhans cells increased from 10-12 (median 11) to 25-30 (median 24) per linear mm of epidermis (Figs. 1a,b). Focal acanthosis was observed.

Dermis: The density of cells

TABLE 3
Cellular Changes in Dermis of Unguentum Lymphaticum-Treated Patients

Group	Number of cells around papillary capillaries		Endothelial cells factor VIII*		Perivenular						Intervascular and epidermo-dermal junction												
	Before		After		CD 1a		HLA DR		CD 68		CD 3		CD 15		HLA DR		CD 68		CD 3		CD 15		
	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	2-3	4-5	+	++	-	-	+	+++	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+
2	2-3	5-8	+	+++	-	-	+	+++	+	+	-	-	+	+	+	+	+	+	-	-	+	+	++
3	2-3	5-8	+	+++	-	-	+	+++	+	+	-	-	+	+	+	+	+	+	-	-	+	+	++
4,5	2-3	4-5	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	-	-	+	+	+

*Intensity of staining, protrusion into lumen
 **B - before, A - after therapy
 ***density of cells + <10, ++ 10-20, +++>20, around one venule, between two venules in papillary layer, under epidermis per one linear mm.

extravasating from papillary capillaries increased on the average from 2-3 to 5-8. The endothelial cells in capillaries and venules increased in size, their bodies bulging into the lumen. They strongly expressed factor VIII-related antigen and were HLA DR+. The number of mononuclear cells around the papillary capillaries rose to 5-8 cells. There were several Langerhans cells visible around the papillary capillaries, a phenomenon not usually observed in normal skin. The density of perivenular infiltrates increased, as did the density of intercapillary cells in the papillary layer of dermis. The number of HLA DR positive cells increased focally from + to +++, especially in the subepidermal regions of the dermis (Figs. 2 a,b). The number of intercapillary CD68+ macrophages increased from 160-335 (median 215) to 216-340 (median 288)/sq mm., and cells were intensively stained (Fig. 3 a,b). Staining with anti-vimentin antibody revealed accumulation of mononuclear, sometimes dendritic-like cells at the epidermo-dermal junction. The number per linear mm rose from 15-18 (median 16) to 25-40 (median 32). The neutrophil-elastase and CD15+ cells appeared in the dermal venules.

Group 3. The immunohistochemical pictures of skin biopsies in patients treated with UL for 12 weeks did not differ from those observed after 3 weeks. There were only more acanthotic foci in the epidermis. The perivenular infiltrates persisted.

Group 4 and 5. In both placebo groups no changes in the density and phenotype of cells accumulating around the capillaries were seen. Moreover, no acanthotic foci were observed in the epidermis.

Lymph node cell phenotypes are shown in Table 4.

Group 6. A wedge-shape biopsy was made in order to assess the cellular changes in all functional regions of lymph nodes. All 12 nodes were mostly fibrotic with islands of lymphoid tissue in paracortical and follicular areas surrounded by a wall of fibroblasts. After treatment, the most striking change was

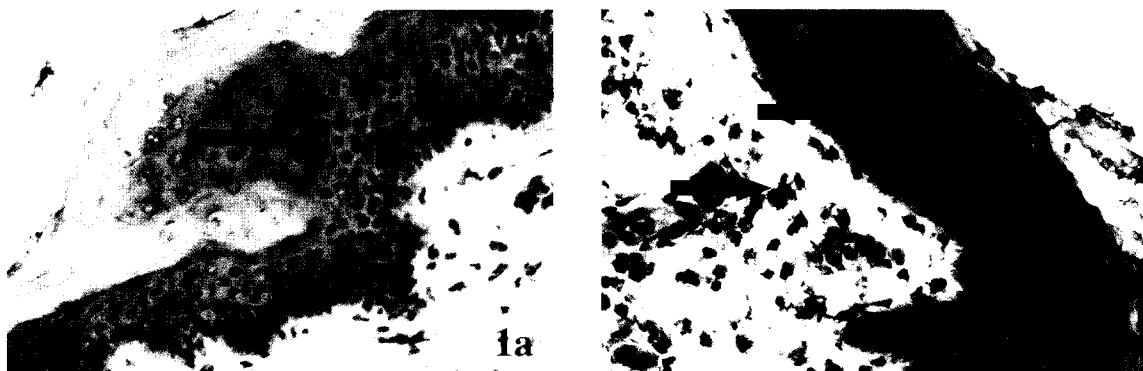


Figure 1 a,b. Skin specimen stained with monoclonal antibody against CD1 showing spider-shape Langerhans cells in epidermis (arrow). The number of Langerhans cells before (a) therapy was normal and increased after 3-week treatment with UL (b) (upper arrow). Langerhans cells appeared in the papillary dermis (lower arrow). Intensive proliferation of epidermal cells (b). Magnification (a) x 250, (b) x400.

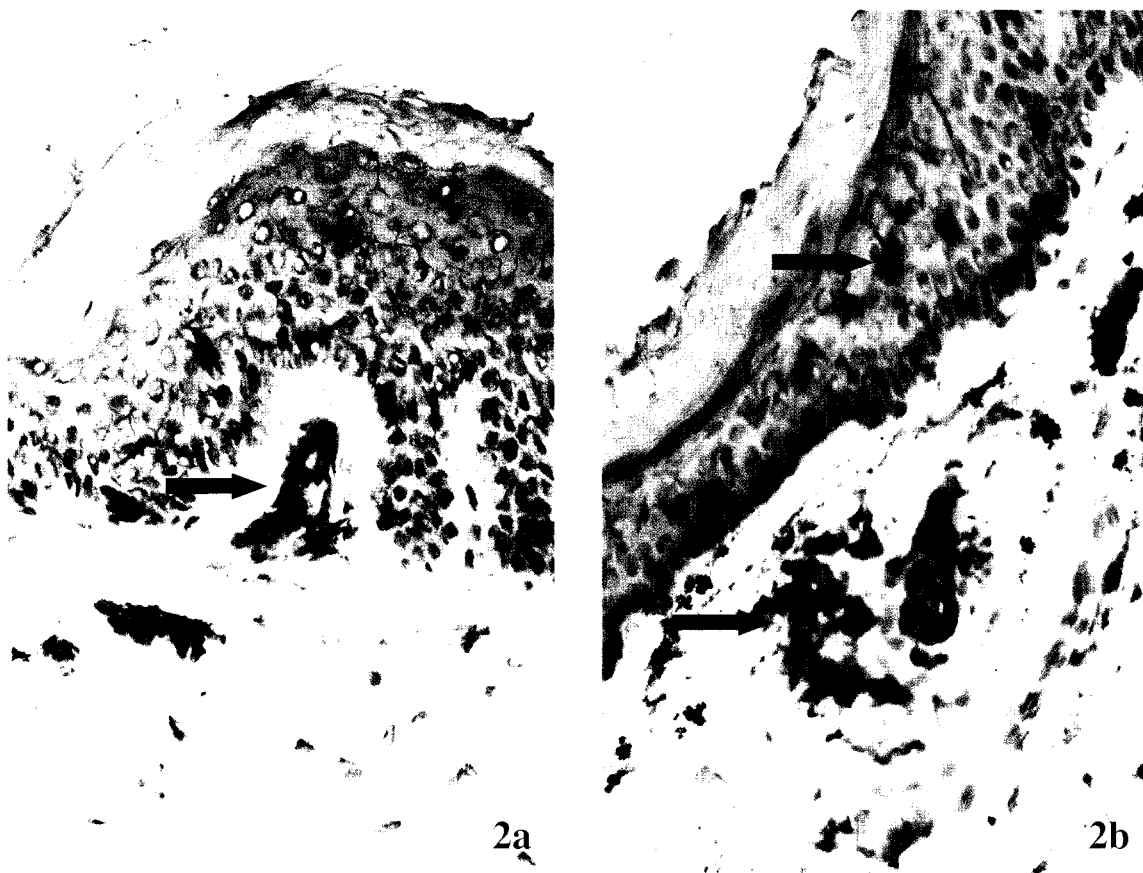


Figure 2 a,b. Specimen as on Fig.2, stained with antibody against HLA DR antigens (arrow). Before treatment (a). After 3-week treatment more Langerhans cells in epidermis (upper arrow) and more intensive staining of perivascular cells in the papillary layer (lower arrow) (b). Magnification (a) (b) x250.

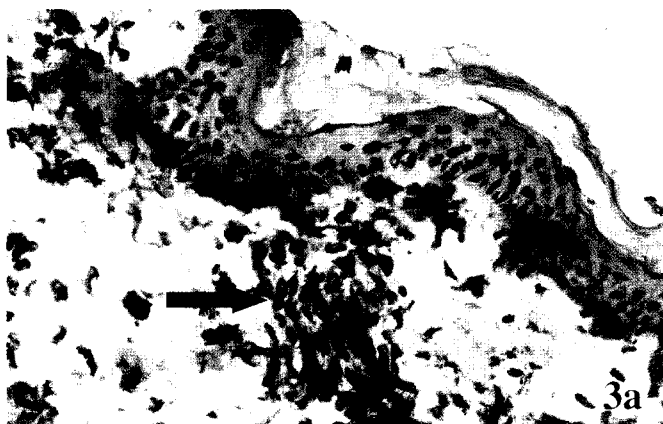


Figure 3 a,b. Specimen as on Fig.2, stained with monoclonal antibody against macrophages (arrow). Before treatment (a). After 3-week treatment (b) the density of macrophages increased (upper arrow) (b) with more of these cells around blood vessels (lower part of the figure) (lower arrow) (b). Magnification (a)(b) x250.

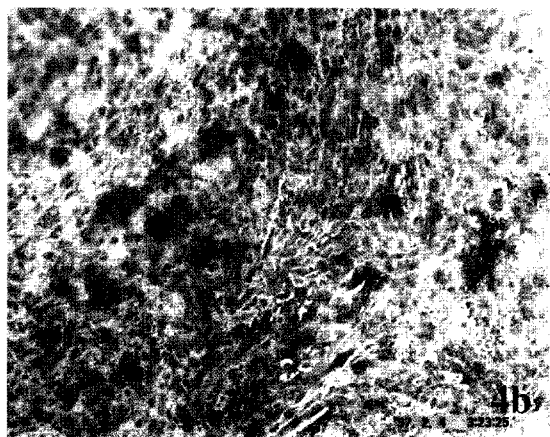
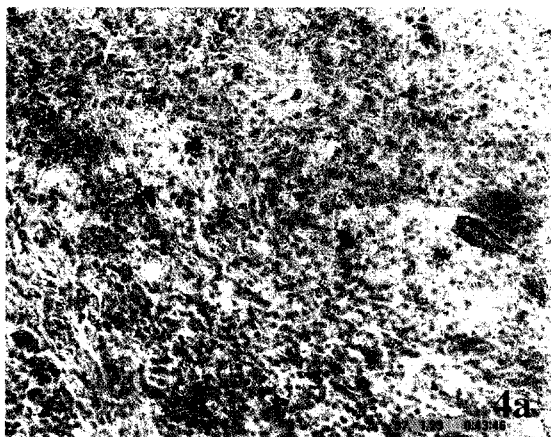


Figure 4 a,b. Inguinal lymph node stained with monoclonal antibody against granulocytes (CD15). Before treatment (a) single granulocytes marginating in the lumen of node blood vessels in the paracortical area. After 3 months treatment (b) evidently more margination of granulocytes. Magnification x400.

accumulation of CD68+ (+++) and HLA DR (+++) cells compared with (+) in controls. Another finding was formation of neutrophil aggregates (+++) in the blood capillaries in the cortical and paracortical areas compared with the pretreatment specimens (+). There

were no CD1a+ cells detected. No changes were seen in the density of CD3 and CD22 lymphocytes.

Group 7. Blood cell culture. The UL chemical substances totally inhibited the PBM response to PHA, compared with

TABLE 4
Distribution of Cell Phenotypes in Inguinal Lymph Nodes
in Unguentum Lymphaticum-Treated Patients

	Paracortical*		Follicular		Medullary	
	Before	After	Before	After	Before	After
CD1a	-	-	-	-	-	-
CD3**	+	+	+	+	scattered cells	
CD22	-	-	focal	focal	-	-
CD68	single dispersed	focal aggregates	single dispersed	focal	single	
HLA DR	as CD 68		as CD 68		single	
Neutrophil-elastase	single in blood vessels	aggregates of 3-8 cells in vessels	-	aggregates of 3-8 cells in blood vessels	single in blood vessels	

* In most specimens distorted lymph node architecture
** Cell accumulation as in normal lymph nodes (+)

controls. The viability of cells after 72h culture with Podophyllin was 60%, with Calendulae 36%, oleum petrae 70% and all ingredients 36% (control 72%).

Group 8. Bacteriological studies. UL did not produce major alterations in the skin surface bacterial flora after 3 days of application. In 10 patients a total of 17 strains were cultured before and 21 after treatment. There was 61% of Bacilli, 16% of coagulase-negative Staphylococci, 11% of Acinetobacter, 5% of Micrococci and 5% of Corynebacteria before treatment and 80%,9%,5%, 5% and 0% after treatment, respectively.

DISCUSSION

This study has provided the following information: (1) Five-day therapy with UL did not evoke clinical or microscopic changes in the skin of patients with lymphedema of the lower limbs, (2) Three and 12 weeks therapy brought about clinical softening of the skin and on histology, proliferation of keratinocytes with protrusion into the dermis, recruitment of immune cells to the epidermis

and skin in the perivenular and subepidermal regions, with an increased number and intensity of immunostaining of C1a⁺ Langerhans cells and CD68⁺ macrophages. No such changes were observed in the placebo group. Furthermore, there was recruitment of granulocytes and macrophages in lymph nodes, not seen in the pre-treatment biopsied nodes, (3) lack of changes in the skin surface bacterial flora, (4) *in vitro* inhibition of lymphocyte activation by UL chemical components.

UL is a composition of plant extracts regulating cell metabolism and mitosis. Calendulin is a biostimulant with antiinflammatory activity. Hyoscyamine sulphate is an alkaloid, similar in action to atropine, with parasympatholytic properties. Colchicine is an alkaloid with antimetabolic activity and pain relieving anti-gout drug. Podophyllin is an antimetabolic substance halting cell division in metaphase. The mechanism of action of UL at the microscopic level has not been studied before. UL has been used in patients since 1969 when it was registered in Germany. It was not until 1983 when Casley-Smith (11)

published the first experimental results of UL on the edema controlling mechanism in skin with lymph stasis. Since that time, open clinical trials established the efficacy of UL in treatment of skin lymphedema and its complications (Kovach & Koller, Sterner & Grahwit, personal communications). Cluzan et al (12) showed an increase in clearance of radioisotope tracer on lymphoscintigraphic pictures of patients treated with UL. We decided to evaluate microscopically the effect of UL on leg skin and lymph nodes in patients with lymphedema of the lower limbs.

Our findings indicate that the substances present in UL stimulate keratinocytes and chemoattract migrating immune cells to skin and lymph nodes. An increase in density of macrophages was also found by Casley-Smith in his animal studies (11). How can one reconcile an increase in cellular infiltrates typical for inflammation with clinical improvement? What role could the accumulating Langerhans and macrophages play in attenuating the chronic inflammatory process routinely seen in skin with lymphedema? One concept is that the recruited cells eliminate factors responsible for inducing local inflammation (bacteria or their products). They produce and release cytokines such as interleukin 1 and 6, and TNF α (tumor necrosis factor), which activate other cells, especially keratinocytes, for recognition and immune contact with the invading pathogens. Each inflammatory reaction is balanced by the proinflammatory and antiinflammatory cytokines produced by immune cells. Recruited cells also produce the antiinflammatory interleukin 10 and transforming growth factor alpha (TGF α). These substances may attenuate changes seen in chronic inflammation characteristic for lymphedema. The lack of changes in the placebo group has clearly shown that the described cellular infiltrates are the effect of UL chemical ingredients. Mobilization of granulocytes and macrophages in mostly fibrotic lymph nodes point to a chemoattractive effects of UL components transported

from skin to the nodes. The infiltrating cells can presumably remove from lymph nodes the invading pathogens as well as "self" degradation products delivered in lymph. Total inhibition by UL substances of *in vitro* lymphocyte responsiveness to PHA can explain their regulatory effects. These substances attract immune cells and regulate their local activation level.

The observations of three-month UL application are of prime clinical importance. They suggest that long-term topical therapy with UL is safe and not burdened with any untoward skin changes.

The question whether UL may have an effect on foot and leg skin bacterial flora has been partly answered. Preliminary studies indicate that UL does not cause changes in prevalence of bacterial strains.

In summary, Unguentum Lymphaticum produces nonspecific stimulation of skin immune cells and mobilization of granulocytes and macrophages. These findings correlated with the post-treatment clinical improvement of the skin clinical picture and patient's subjective assessment, and suggest the usefulness of this compound in chronic therapy of lymphedema and its inflammatory complications.

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Waldemar L.Olszewski MD
Dept. of Surgical Research
& Transplantology,
Medical Research Center
Polish Academy of Sciences
02 116 Warsaw
Pawinski Str.5, Poland
e-mail: wlo@cmdik.pan.pl