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IMMUNE PROTEINS IN PERIPHERAL TISSUE FLUID-LYMPH IN PATIENTS WITH FILARIAL LYMPHEDEMA OF THE LOWER LIMBS

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ABSTRACT

Immune proteins and cytokine concentrations and activity were measured in skin tissue fluid-lymph and blood serum of patients with lower leg filarial lymphedema. High levels of lymph γ-globulins, α-1-acid glycoprotein and IgG lymph/serum ratio were found. Lymph from filarial patients had an elevated lymph stimulatory effect on blood mononuclear cell culture with phytohemagglutinin. There was also a high concentration and activity of IL-1β but γ-interferon was not detected. The deranged pattern of immune proteins and high activity of IL-1 suggest persistence of an ongoing local inflammatory process despite the absence of overt dermatitis. The high tissue-lymph IL-1 concentration and activity may signify a cytokine network promoting keratinocyte and fibroblast proliferation commonly seen on skin histology in patients with filariasis.

The tissue changes of lymphedema in patients infected with filarial (*W. bancrofti* or *B. malayi*) remains unclear. At least two factors seem to play a primary role—tissue fluid-lymph stasis and skin inflammation. The consequences of chronic lymph stasis are well documented in patients with obstructed lymphatics. These consist of peripheral edema with accumulation of plasma proteins, cellular metabolic byproducts, and trapped recirculating lymphocytes with proliferation of fibroblasts, keratinocytes, and exuberant collagen deposition. Longstanding lymphedema is characterized by repeated skin infection and eventually hyperkeratosis, verrucosis, and dermal fibrosis. This clinical pattern is also seen after removal and/or irradiation of regional lymphatics and nodes (e.g., radical mastectomy or groin dissection). In filarial infestations, the progression of lymphedema is often more severe, frequently complicated by overt dermatitis, with "overgrowth" of lymphedematous tissues and then relatively quickly "elephantiasis." In young patients, the size of the extremity increases with the skin texture typically remaining relatively soft; in older patients, by comparison, fibrotic skin changes dominate the process. The question arises, what is the mechanism of the rapid skin changes that take place in filariasis? Is it related to ongoing subclinical inflammation of the skin, and, if so, is inflammation evoked by the parasite and/or by secondary bacterial skin infection?

We recently observed (unpublished) using immunohistochemical methods, persistence of subacute inflammatory changes in skin of patients with grade II and III filarial lymphedema of the legs. Some of the changes such as proliferation of keratinocytes and fibroblasts and dividing mononuclear cells may result from an overproduction or accumulation of various cytokines. Therefore, we measured immune

proteins and cytokines (as a gauge of "inflammatory mediators" and cell-stimulator-proliferators) in the skin tissue fluid-lymph of patients with filarial lymphedema.

MATERIALS AND METHODS

Clinical Studies

Fifteen patients (age 18-55 yrs of both sexes), with grade IIa and IIIa filarial lymphedema of the legs of 2-15 years duration were studied. They neither had microfilaremia nor clinical dermatitis at the time of investigation. In each patient, filaria infection was demonstrated by blood serology and each was receiving diethyl-carbamazine (DEC) and long-acting penicillin (Benzathine PCN) according to a standard protocol.

Skin Tissue Fluid-Lymph Collection

The skin was incised 5cm above the medial malleolus using 2% xylocaine with adrenalin for analgesia. After 5-7 minutes of slight pressure on the wound to stop oozing, clear fluid with few erythrocytes flowed from cut dilated dermal lymphatics. An open lumen in a network of tortuous dermal lymphatics was visible under a dissecting microscope. If the lymph flow was minimal, the deeper lymphatics were located and opened. This technique of lymph collection was preferred to direct lymphatic cannulation to avoid extensive dissection, especially in these patients with sclerotic skin. One to 15ml of lymph were sampled within 15-30 minutes. The samples were collected in syringes with 50u of heparin and 10,000u of aqueous penicillin, spun down, and the supernatant stored in sterile plastic test tubes and frozen at -20°C.

Serum Collection

Blood was drawn from the cubital vein and the serum processed as described for lymph.

Lymph and Serum Proteins and Immunoglobulins

Total protein concentration of serum and lymph was measured using the biuret method, and globulin concentration with paper electrophoresis. IgG, IgM, α-1-acid glycoprotein, and α-2-macroglobulin levels were determined by radial immunodiffusion using NOR-Partigen plates (Hoechst, Germany).

Mitogen Assay of Peripheral Blood Monocytes (PBM) Mixed Culture with Lymph and Serum

PBM were cultured in 200μl of RPMI 1640 containing 10% FCS at 2x10 cells/well in round-bottom 96 microtiter plates (Costar). Phytohemagglutinin (PHA) H15 (Wellcome) was added at final concentrations of 18 and 90μg/ml. Cultures were supplemented with 20% lymph or serum (serum protein concentration adjusted to that of lymph). Cultures were pulsed at 48h with 3H TdR (specific action 2μCi/ml) and harvested at 72h.

Interleukin 1 (IL-1) Bioassay in Lymph and Serum

C3H-HeJ thymocytes (1.5x10/well) were cultured in 96-well, round-bottom micro-culture plates for 72h in 100μl RPMI 1640 containing 5% FCS, antibiotics, $2.5x10^{-5}$M 2-mercaptoethanol, and 90μg/ml PHA. Dilutions of lymph or serum samples were added at 100μl volumes. Cultures in triplicates were harvested and incorporation of thymidine was measured in a Beckman liquid scintillation counter. Results were expressed as units of activity. One unit was half-maximum counts per minute of test preparations compared with IL-1 standard assigned an arbitrary activity of 1μ/ml.

Interleukin-1-β and Interferon-γ Assays

In order to measure the concentration of IL-1 the ELISA (Cistron) and RIA kits (Bender) were used. For measurement of

TABLE 1
Proteins (Mean±SD) in Lymph of Patients with Filarial Lymphedema (n=11) Compared with Healthy Volunteers (n=20)

Protein	Filariasis	Normal
Total protein (g/dl)	2.37±0.5	2.39±1.3
L/S	0.48±0.1	0.43±0.18
γ-globulins %	33.5±6.5*	17.5±3.0
L/S	1.2±0.2	1.1±0.17
α-1-acid glycoprotein (mg/dl)	37.7±6.2*	22.1±0.6
L/S	0.38±0.06	0.45±0.01
α-2-macroglobulin (mg/dl)	41.3±8.0*	64.7±7.7
L/S	0.11±0.02	0.18±0.02
IgG (mg/dl)	252±35*	355±9.3
L/S	0.42±0.06*	0.28±0.007
IgM (mg/dl)	24.5±8.9	23.6±1.3
L/S	0.22±0.08	0.20±0.01

*p<0.05
L/S=lymph/serum

interferon-γ the ELISA (Genzyme) kit was employed.

Control Data

The control data of protein levels were obtained from previous studies on volunteers (1,2). For measurement of control cytokine levels, lymph and serum from recently cannulated leg lymphatics was used. Sampling and storage of control lymph and serum was slightly different from that used in patients with filarial infection (30-60 min and 3-4 h, respectively), but were minimal and unlikely to influence the readings of protein concentrations. No wound complications occurred in the earlier control (normal) volunteers. To examine responsiveness of PBM to lymph from filarial patients, control data from simultaneously performed tests with peripheral lymph from normal volunteers were used.

Lymph studies were approved by the hospital ethical committee.

RESULTS

Tissue Fluid-Lymph proteins (Table 1)

The lymph total protein concentration was 23.7g/l (L/S ratio 0.48) and was not different from control subjects. The absolute level of α-1-acid glycoprotein was higher in lymph but the lymph/serum (L/S) ratio was similar to normal. Both the concentrations of α-2-macroglobulin and the L/S ratio were lower in lymph than in controls whereas the IgG level in mg/ml was also lower in lymph but the LS ratio was higher. The concentrations of IgM were within normal limits.

Responsiveness of PBM to PHA in Culture with Lymph

The responsiveness of PBM in cultures supplemented with lymph and serum from filarial patients was greater than from control subjects using a high concentration (90μg/ml) but not low concentration

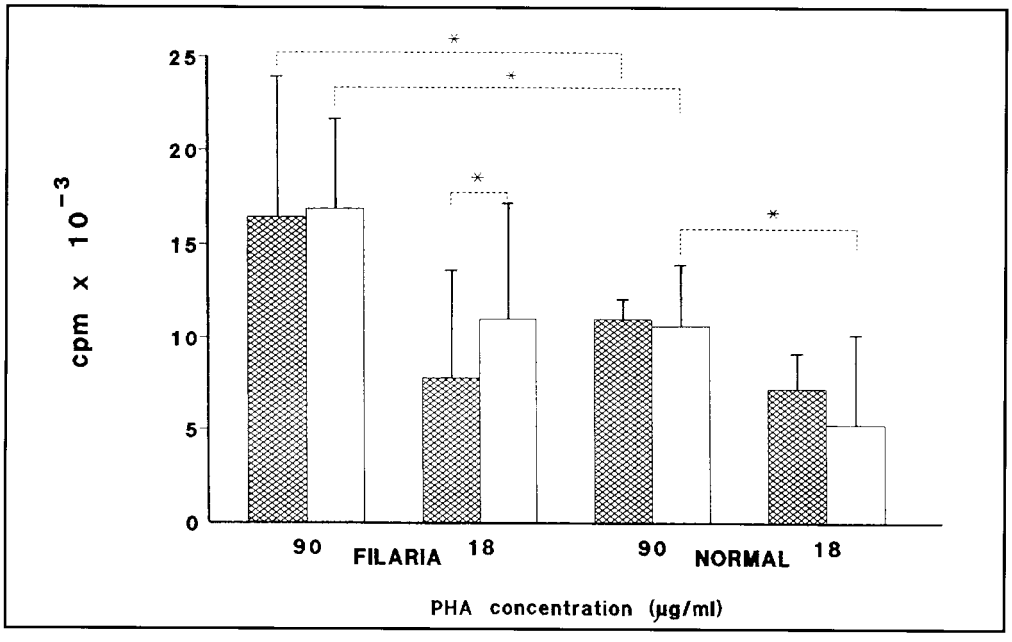

*Fig. 1. The response of skin lymph (mean±SD; n=9) from filarial patients on peripheral blood monocytes (PBM) in culture using 20% lymph (open bar) or serum (grid) and two concentrations of PHA (18 and 90µg/ml). Note that lymph and serum from filarial subjects stimulated PBM more than normal lymph and serum. On the other hand, lymph of filarial patients had a slight although non-significant inhibitory effect compared with serum of filarial subjects, opposite to that observed with normal lymph. *p<0.05*

(18µg/ml) of PHA *(Fig. 1)*. When the PBM response of lymph was compared to that of serum, no difference was seen in filarial patients with high dose of PHA but there was inhibition by lymph at the lower PHA dose. Normal peripheral lymph usually has a slight additive stimulatory influence with PHA.

IL-1 Activity in Lymph in Thymocyte Assay

Lymph showed thymocyte co-stimulating activity in the presence of PHA, notably higher than serum *(Fig. 2)*. The mean peak IL-1 activity was 22.3u/ml at lymph dilution 1:20. The peak serum value was 8.8u/ml at dilutions 1:5 and 1:10.

Interleukin 1-ß in Lymph

The level of IL-1ß in lymph from filarial patients was 207±7pg/ml and in serum was 375±70pg/ml. In normal lymph, these values were 44±8pg/ml, and in serum 30-40pg/ml.

Interferon-γ in Lymph

The level of interferon-γ was below 100pg/ml both in lymph from filarial patients and normal subjects.

DISCUSSION

This study yielded the following information: a) increase in lymph γ-globulin, α-1-acid glycoprotein level and IgG lymph/serum ratio; b) stimulatory effect of filarial lymph and serum in the presence of PHA on PBM over control lymph; c) slight inhibition of filarial lymph compared with filarial serum on PBM stimulation with PHA; d) high lymph IL-1 activity and concentration.

It is often stated but not confirmed by us (5) that stagnant lymph with lymphedema has a high protein concentration. In

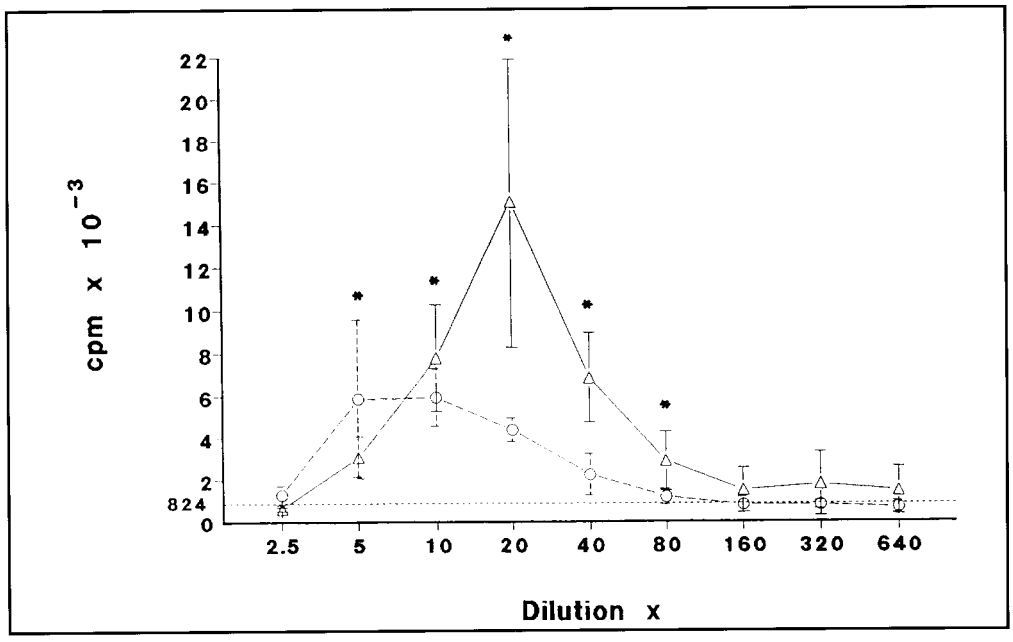

*Fig. 2. The IL-1 assay (mean±SD; n=5) with C3H/HeJ thymocytes in lymph (triangle) and serum (circle). Small dashed horizontal line represents the mean level of background incorporation in the absence of either lymph or serum. At low dilutions, a slight inhibitory effect of test lymph and serum samples is seen. At higher dilutions, lymph shows higher activity than serum. *p<0.05*

this study as earlier, the lymph-tissue protein level was no different from normal subjects. If a higher than normal protein concentration accumulates in the tissues, the resultant high oncotic pressure would in turn attract water and thereby quickly redilute the protein concentration. There may be, however, changes in proportions of concentration of lymph proteins of different molecular size (6). In this study, the peripheral lymph IgG L/S ratio was higher than normal although its total level was lower. Each filarial patient had decreased serum total protein and this abnormality impacted on the lymph level. A high ratio of lymph to serum protein level suggests local production of a particular protein. This phenomenon typically occurs in tissue infiltrated with immune cells, which is characteristic of filarial lymphedema.

Filarial lymph and serum stimulated PBM cells in the presence of PHA much more than normal lymph and serum. This finding may relate to high concentration of IL-1 in both fluids. In normal subjects, lymph costimulates PBM with PHA more than serum (3). In patients with filarial lymphedema, lymph showed a slight inhibitory effect. This response was not due to interferon-γ as its levels were unaltered, but theoretically may emanate from locally produced prostaglandins.

Unusually high levels of IL-1 were detected in lymph from filaria-infected swollen limbs. IL-1 is constitutively present in normal lymph at levels similar or higher to that of serum (3,4). In our group of patients both their serum and lymph levels were significantly elevated. This finding suggests a systemic immune reaction to the parasite and/or bacterial antigens in the lymphedematous skin and perhaps other compartments. Cytokines play a prominent role in host responsiveness to filarial

antigens (7-9), and may also be responsible for stimulation of skin parenchymal cells and their excessive proliferation.

A discrepancy was observed between higher stimulatory properties of lymph than serum in the IL-1 thymocyte assay and lower lymph than serum concentration of IL-1ß in ELISA assay. This disparity suggests the presence in lymph of additional stimulatory factors which may have an additive effect to the action of IL-1.

Filariasis is a systemic disease. The changes in immune proteins and cytokines were observed both in serum and lymph, although more pronounced in lymph. Investigation of lymph from the contralateral non-swollen leg would have allowed evaluation of the intensity of the local inflammatory reaction caused by the parasite. This, however, was not done as most patients had some swelling on the contralateral leg, whereas others were reluctant to undergo cannulation of an apparently healthy leg.

Taken together, the distorted pattern of immune proteins and immunoresponsiveness in tissue fluid-lymph from lymphedematous skin of filarial-infected patients points to persistence of a latent, ongoing inflammatory reaction. The high IL-1 concentration and activity may mediate an inflammatory cytokine network which results in keratinocyte and fibroblast proliferation.

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