Lymphocyte subpopulations in Bancroftian filariasis: activated (DR$^+$) CD8$^+$ T cells in patients with chronic lymphatic obstruction

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SUMMARY

To examine the relationship between lymphocyte phenotypes and states of activation in patients with Bancroftian filariasis, dual colour flow cytometry and concurrent *in vitro* cell culture were performed on normal individuals (NV: $n = 15$), and on patients with either asymptomatic microfilaraemia (MF; $n = 12$) or elephantiasis (CP: $n = 11$). In contrast to findings by others in a population with Brugian filariasis, the percentages of total B lymphocytes (CD19). T lymphocytes (CD3), helper inducer T lymphocytes (CD4), and suppressor/cytotoxic T lymphocytes (CD8) in both patient groups were found to be within the range defined by clinically normal individuals. Furthermore. there were no differences among the groups in the expression of the IL-2 receptor (CD25) on T cells. There was, however, a significantly greater proportion ($P < 0.01$) of 'activated' cytotoxic suppressor lymphocytes (defined by co-expression of CDS and HLA-DR) in patients with elephantiasis ($16.4 \pm 5.6\%$) than in the MF ($8.9 \pm 2.6\%$) or NV ($8.3 \pm 2.9\%$) groups. Further, when the expression of this activation antigen was examined in parallel with *in vitro* mitogen responsiveness, an inverse correlation between the percentage of CD8$^+$ HLA-DR$^+$ lymphocytes and pokeweed mitogen-induced proliferation was seen *($r = -0.54$; $P < 0.001$)*. These data provide further definition of the immunoregulatory abnormalities seen in human filarial infections and suggest that activated CD8$^+$ T lymphocytes may be involved in the pathogenesis of the chronic obstructed lymphatic form of this disease.

Keywords Lymphatic filariasis   lymphocyte phenotype CD8 lymphocytes

INTRODUCTION

The lymphatic filariases are chronic infections of humans caused by the nematode parasites *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori.* A striking feature of lymphatic filariasis is the variety of clinical manifestations seen among those living within the same endemic environment (Ottesen, 1980); these include chronic lymphatic pathology manifesting as elephantiasis (CP), recurrent episodes of filarial fevers or adenolymphangitis (FF), tropical pulmonary eosinophilia (TPE), and a clinically asymptomatic condition characterized by persistent microfilaraemia (MF).

These differing clinical manifestations of infection have been found to be associated with differences in immune responsiveness to parasite antigens (Ottesen, 1984). For example, lymphocyte proliferation, lymphokine generation and *in vitro* and in

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vivo antibody production in response to the parasite are all much greater in patients with CP than in those with MF (Ottesen, Weller & Heck. 1977; Ottesen et al., 1982; Piessens *et al.,* 1980a; Hussain *et al.* 1981; Nutman, Kumaraswami & Ottesen, 1987a). Though these findings point directly to an immunological abnormality which is parasite specific, the exact role of immune processes in the pathogenesis of obstructive filariasis is still uncertain. Indeed, many features of the immune alterations themselves remain undefined; for example, the specific cell types involved in the immunologic defect and the possible differences in cell subset activation between those with asymptomatic infection and those with chronic disease. To address these issues directly, we have utilized multiparameter flow cytometry (FACS) analysis to provide a highly sensitive and specific approach to phenotypic analysis of the human mononuclear cell subsets. Further, in order to examine the relation between disease expression and the state of cellular 'activation' we have focussed on the expression of HLA-DR and the interleukin 2 receptor (IL-2R) on the T cell subsets in these

Table 1. South Indian patient population *(Wuchereria bancrofti)*

Diagnosis	No.	Age*(years)	Sex (M/F)	MF/ml*
Filariasis				
Lymphatic obstruction (CP)	11	45 (37-65)	3/8	0
Asymptomatic microfilaraemia (MF)	12	27 (19-47)	11/1	430 (4-1350)
Normal controls	15	34 (21-48)	9/6	0

1 Median (and range).

patients with filariasis. Immune responsiveness to mitogens was evaluated concurrently in an effort to identify any general functional abnormality associated with the altered lymphocyte, phenotypes observed.

MATERIALS AND METHODS

Study population
Thirty-eight adult volunteers, living in the area of Madras. India, where filariasis with periodic *W. bancrofti* is endemic. were studied (Table 1). Eleven patients had CP with no circulating microfilariae, 12 had a variable number of circulating microfilariae (MF) with no symptoms of disease, and 15 were normal volunteers with no parasitological or clinical evidence of infection. Eight of the 12 MF patients had received a single dose of the anti-filarial drug ivermectin one year prior to this study. All the blood samples were collected and processed in Madras, India.

Monoclonal antibodies
The following monoclonal antibodies (MoAb) were used in this study: Leu4 (anti-CD3, pan T-cell antigen), Leu2a [anti-CD8, suppressor/cytotoxic T-cell antigen (Engleman *et al.,* 1981)]. Leu3a [anti-CD4, helper/inducer T-cell antigen (Engleman *et al..* 1981)], Leu12 [anti-CD19, B cell associated antigen (Clark. Shu & Ledbetter, 1985)], L_{243} [anti-HLA-DR (Reinherz *et al..* 1979)], and 2A3 (anti-CD25, IL-2R (Uchiyama, Broder & Waldman, 1981)]. All but L_{243} were IgGl; L_{243} was an IgG2a. Irrelevant isotype-matched antibodies (mouse IgGl and mouse IgG2a) were included as controls for non-specific immunofluorescence. All the MoAbs were directly conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and were purchased from Becton Dickinson Immunocytometry (Mountain View, CA).

Isolation of peripheral blood mononuclear cells (PBMC)
The collected heparinized blood was diluted with an equal volume of RPMI-1640 (M. A. Bioproducts, Walkersville, MD). and lymphocytes were obtained by sedimentation on a Ficoll-Diatrizoategradient (LSM, Litton Bionetics, Rockville, MD) at 400 g for 40 min at room temperature by standard procedures (Boyum, 1968). The cells at the interface were collected, washed three times in RPMI-1640 at 400 g for 10 min at 4° C. The cell concentration was adjusted after trypan blue enumeration.

Analysis of lymphocyte subsets
The choice of antibodies and the method for antibody staining of human lymphocytes which permit storage of these cells for two or more weeks before assay have previously been described (Lal, Edison & Chused, 1988). Briefly, one million cells were stained with a combination of saturating amounts of directly labelled MoAbs to human lymphocyte differentiation antigens for 30 min at 4°C. After three washes in phosphate buffered saline, 400 µl of 1% freshly made paraformaldehyde were added to the cell pellet and in&bated for 30 min at 4°C. Four millilitres of cold Hank's balanced salt solution supplemented with 0.1% bovine serum albumin (BSA) and 3 mM sodium azide (HBSS) were added to each tube, and the tubes were centrifuged at 400 g for 7 min at 4°C. After two more washes, the final cell pellet was resuspended in 0.5 ml of cold HBSS with 1% BSA. These suspensions were kept at 4°C in the dark and flown to the NIH within 12 days, where flow cytometric analysis was performed.

Flow cytometry (FACS) analysis
The cells were analysed by a Coulter Epics V flow cytometer. The data were collected per 10 000 lymphocytes for each tube, and parameter data (abscissa, FITC fluorescence and ordinate, PE fluorescence) were displayed as contour maps indicating increasing number of cells in a defined area of display. In every case, the percentage of cells showing positive staining was calculated by first acquiring results for an isotype matched non-binding control antibody coupled to the appropriate fluorochcome (FITC or PE). The lower channel was then set to a threshold which indicated 2% of the cells showing fluorescence above the threshold. Results for experimental samples were then collected and the percentage of cells showing positive fluorescence determined as the number of cells exceeding the set threshold. divided by the total number of cells analysed in the cytogram gate, multiplied by 100. For double stained populations, the percent was calculated by dividing the number of cells stained by a particular monoclonal antibody by that of the total number of cells gated. Each of the monoclonals to lymphocyte subsets was used in at least three independent combinations for each person, and the mean of these readings (always extremely close) was used to enumerate the percentage of each phenotype.

Lymphocyte function
Lymphocyte function was determined using blastogenic responses to the following stimuli: phytohaemagglutinin (PHA; Wellcome Diagnostics, Dartford, England): concanavalin A (ConA: Ey Labs, San Mateo, CA), pokeweed mitogen (PWM; Gibco Laboratories, Grand Island, NY). Lymphocytes were adjusted to 1×10^{6}/ml in RPMI 1640 containing 10% human AB serum and cultured in 0.2 ml in 96-well, round-bottomed microtitre trays with or without PHA (2 and 1 µg/ml), ConA (20 and 10 µg/ml), and PWM (1/40, 1/80, and 1/160 dilution). Blastogenesis was determined by measuring the levels of DNA synthesis upon addition of 1 µCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) to each well 18 h prior to termination of the culture. The labelled cells were harvested with a multiple sample harvester on glass fibre filters and were counted by liquid scintillation spectroscopy. Data are expressed as the stimulation index (SI) obtained by dividing the ct/min (mean of triplicate cultures) of the mitogen-stimulated cultures by the mean ct/min of the control cultures.

Table 2. Cell populations in filariasis patients and normal controls according to monoclonal antibody binding*

Antigen cluster	Normal (n= 15)	Microfilaraemic (n= 12)	Elephantiasis (n =11)
CD3	79.3 ± 6.5	79.9 ± 5.0	72.9 ± 8.07
CD4	39.68 ± 7.9	39.9 ± 6.2	36.66 ± 9.3
CD8	29.63 ± 3.9	33.01 ± 5.2	31.3 ± 7.9
CD4/CD8	1.38 ± 0.48	1.26 ± 0.36	1.29 ± 0.64
CD19	13.72 ± 4.6	10.34 ± 3.78	11.22 ± 5.7
CD25	1.8 ± 0.66	1.86 ± 0.36	1.98 ± 0.92
HLA-DR	17.09 ± 5.2	14.56 ± 4.26	20.61 ± 7.34
$CD3^+ DR^+$	16.09 ± 4.2	14.40 ± 4.6	25.10 ± 10.45†
$CD19^+ DR^+$	79.24 ± 13.2	81.55 ± 7.64	85.56 ± 9.4

*Values are expressed as mean ± s.d.

† $P < 0.01$, when compared with normal or MF.

Statistical evaluation

The Student's t-test and the Spearman rank correlation coefficient were used for statistical evaluation as noted.

RESULTS

Lymphocyte subsets in filariasis

Lymphocyte subsets in all subjects were enumerated by one- and two-colour FACS analysis using multiple MoAbs. The percentage of total B lymphocytes (CD19), T lymphocytes (CD3), helper/inducer T lymphocytes (CD4), or suppressor/cytotoxic T lymphocytes (GD8) in either clinical group did not differ significantly from those of the normal subjects (Table 2). The total percentages of cells bearing surface IL-2R (CD25) or HLA-DR were also within the normal range; however, among the lymphocyte subpopulations examined. CD3 ⁺ T cells bearing the HLA-DR antigen were significantly more frequent in the patients with CP, being increased almost two-fold in comparison with either the normal controls or those with MF (Table 2). Total B cell populations bearing the HLA-DR antigen were within the normal range for all groups.

Distribution of HLA-DR antigen on T lymphocytes

To investigate further the T lymphocyte subset bearing the HLA-DR antigen, two-colour FACS analysis was performed using a PE-labelled antibody to CD4 or CD8 and a fluorescein-labelled antibody to HLA-DR. As seen in a representative analysis, an abnormal population of CD8⁺ lymphocytes bearing the HLA-DR antigen was identified in patients with CP (Fig. 1 b) when compared to normals (Fig. la) or patients with MF (data not shown). Additionally, there was only a slight increase (statistically not significant) in the HLA-DR⁺ CD4⁺ T lymphocytes (Fig. 1) in the same patients.

According to the thresholds defined by analysis of unlabelled cells, an increase in the percentage of HLA-DR⁺ CD8⁺ lymphocytes was evident in 7 of the 11 patients with CP (Fig. 2a) when compared to normal individuals (shaded area) or those with MF in whom only 2/12 had even a modest increase. Indeed, the mean percent of CD8⁺ HLA-DR⁺ lymphocytes in patients with CP was 16.4 ± 8.6% vs. 8.9 ± 2.6% in those with MF (P<0.01). There was only a slight increase (statistically not significant) in the mean percentage of HLA-DR⁺ CD4⁺ lymphocytes in CP patients (Fig. 2b).
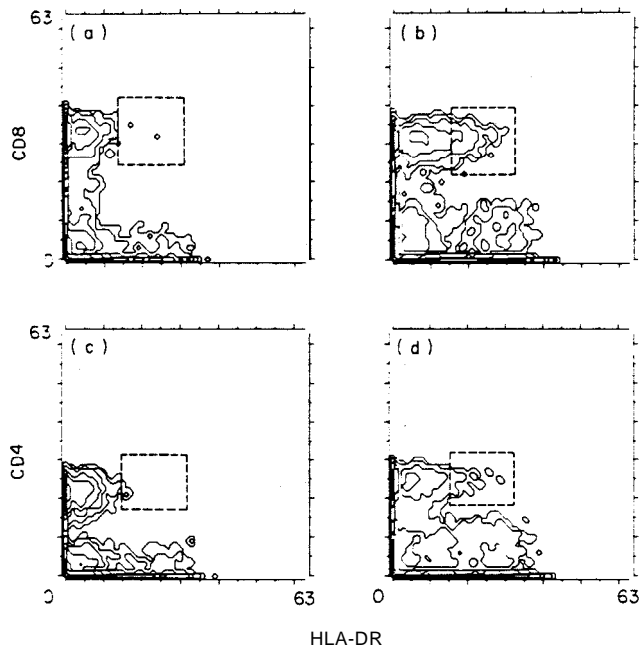

Fig. 1. Activated CD8⁺ T suppressor/cytotoxic lymphocytes in a patient with elephantiasis. Peripheral blood mononuclear cells from a. normal subject (a and c) and a patient with elephantiasis (b and d) stained with monoclonal antibodies to both HLA-DR and either CD8 (a and b) or CD4 (c and d) before flow cytometry. A population of doubly labelled HLA-DR⁺ CD8⁺ cells (12.41%) is apparent in the patient (panel b); HLA-DR⁺ CD4 cells (2.96%) in the same patient (d) were not statistically different from the normal control.
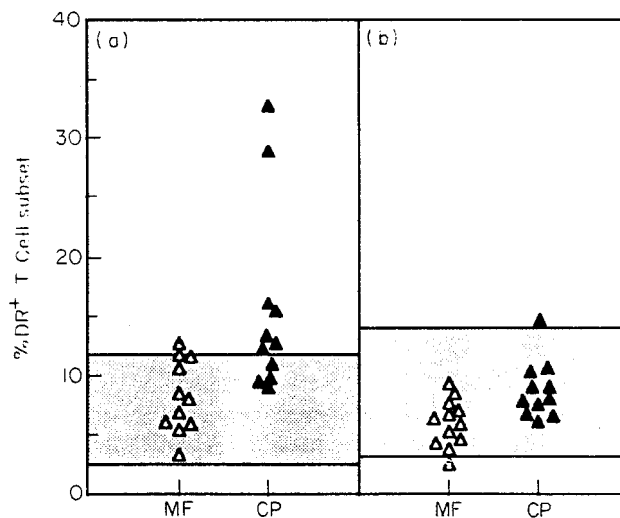

Fig. 2. Activated suppressor T lymphocytes (HLA-DR⁺ CD8⁺) (a) and activated helper T lymphocytes (HLA-DR⁺ CD4⁺)(b) in patients with elephantiasis (CP) or microfilaraemia (MF). The percentage of positive lymphocytes from each patient, determined by comparison of fluorescence of specifically labelled cells with that of unlabelled cells for each sample, is indicated by open (MF) or closed (CP) symbols. The mean percentage ± 2 s.d. of the 15 normal controls is defined by the shaded area.

Table 3. Lymphocyte proliferative response to mitogens in patients and normal controls

Mitogen	MF (n = 12) SI*	CP (n=9) SI	NL (n=12) SI
PHA	50.12† (39.2-73.2)	68.12 (22.2-140.5)	58.28 (44.5-92.5)
ConA	46.67 (27.5-79.2)	48.89 (27.2-93.3)	39.32 (21.0-85.4)
PWM	18.39 (3.2-59.2)	34.38 (20.1-68.2)	33.1 (17.26-73.6)

*Stimulation index:

$$\frac{ct/min \ in \ mitogen\text{-}stimulated \ culture}{ct/min \ in \ medium \ control}$$

† Geometric mean (95% confidence value).

Table 4. Correlation between CD8$^+$ HLA-DR$^+$ lymphocytes and PWM responses in patients with elephantiasis

Patients	CD8$^+$ HLA-DR$^+$ (% positive)	PWM* (stimulation index)
1	8.93	85.62
2	9.37	10.26
3	11.42	29.17
4	12.06	53.00
5	12.19	32.41
6	13.05	80.17
7	15.46	51.07
8	28.94	11.79
9	32.75	10.90

*Spearman rank correlation coefficient = –0.54; P <001.

Distribution of IL-2R on T lymphocytes
Because IL-2R (CD25) can also be a marker of lymphocyte activation. its expression on T lymphocyte subpopulations was analysed but not found on either CD4 or CD8 positive lymphocytes (data not shown).

Lymphocyte function
The functional capacity of peripheral blood lymphocytes of the three study groups was next assessed by examining lymphocyte responses to T cell mitogens (PHA, ConA) and the T cell-dependent B lymphocyte mitogen (PWM) (Table 3). As seen, all groups responded equally well to PHA and ConA, whereas there was a slight (statistically not significant) diminution of the PWM-induced responses in those with MF compared to patients with CP or to normals.

Correlation between HLA-DR$^+$ T cells and lymphocyte function
To begin to examine the implications of the increased expression of HLA-DR on CD8$^+$ lymphocytes, correlations between expression of this antigen and *in vitro* lymphocyte responsiveness were examined. There was no correlation between HLA-DR expression on T lymphocytes and *in vitro* responses to PHA or ConA, but there was an inverse relation between the percentage of CD8$^+$ DR$^+$ lymphocytes and the PWM response (Table 4) in patients with CP (*r*= –0.54; *P* <0.001). The percentage of CD4$^+$ DR$^+$ lymphocytes did not correlate with the response to any of the three mitogens. In MF patients, there was no correlation between DR expression and mitogen responsiveness.

DISCUSSION

Patients with Bancroftian filariasis show a wide spectrum of immunological and clinical characteristics, often related to their degrees of immunological responsiveness (Ottesen, 1980). To understand the mechanisms underlying these immunological differences, we have examined the relationship between lymphocyte subsets and their states of activation by dual-colour flow cytometry and concurrent *in vitro* cell culture. The percentages of total B lymphocytes (CD19), T lymphocytes (CD3), helper/inducer T lymphocytes (CD4), and suppressor/cytotoxic T lymphocytes (CD8) in patients with either CP or MF were all within the ranges defined by clinically normal individuals taken from the same population. These results contrast with those of a previous report of patients with lymphatic filariasis caused by *B. malayi,* where increased numbers of CD8-positive T lymphocytes were observed in MF individuals (Piessens *et al.,* 1982). The reasons for this discrepancy are not certain; possibly, it could relate to differences in filarial species in the study populations (subperiodic Brugian filariasis in the earlier report and periodic Bancroftian filariasis in the present one) or to technical factors (indirect immunofluorescence with manual counting of cells in the earlier study and flow cytometry analysis in the present one).

Specific hyporesponsiveness of T cells to filarial parasite antigens, manifested by minimal antigen-driven T cell proliferation *in vitro* despite normal responses to mitogens and non-parasite antigen (Ottesen *et al.,* 1977; Piessens *et al.,* 1980a; Nutman *et al.,* 1987a), is well documented in the asymptomatic group of patients with IMF. Serum suppressor factors (Piessens *et al.,* 1980b), suppressor adherent cells (Piessens *et al.,* 1980b), and T suppressor cells (Piessens *et al.,* 1982) have all been implicated as causes of this parasite-specific immunosuppression. Our observations here of normal levels of CD4 and CD8 cells and lack of increased expression of activation markers on either T-cell subset of these MF patients point to a more complex immunoregulatory network than merely CD4/CD8 interactions and, in agreement with recent reports (Nutman *et al.,* 1987 a,b), continue to support the concept of 'acquired tolerance' to parasite antigen rather than one implicating global suppressor cell mechanisms (Piessens *et al.,* 1980b, 1982).

The mechanisms involved in the activation of peripheral blood lymphocytes following exposure to specific antigens or mitogens are complex. It has been shown clearly that certain cell surface determinants expressed only in low proportions or not at all on resting T cells are-expressed on activated T cells (Uchiyama et al., 1981 : Ko *et al.,* 1979). Among these 'T-cell activation antigens', the HLA-DR antigen, expressed primarily on B lymphocytes and monocytes (Winchester *et al.,* 1975) can be found in a significant proportion of T cells upon activation

(Reinherz *et al.,* 1979; Ko *et al.,* 1979). Another marker, the IL-2R, is similarly inducible on human T cells activated by mitogens, soluble antigens, or allo-antigens (Uchiyama *et al.,* 1981). It has been postulated that these antigens may be part of the network through which cell-cell signals are conveyed.

The significant increase in circulating T cells bearing the HLA-BR antigen indicates the presence of activated T cells in patients with chronic lymphatic pathology. Although the exact origin and significance of these HLA-DR$^+$ T cells remain to be determined, it is possible that these cells reflect direct activation by parasite products and. thus, may be intimately involved in the pathogenesis of lymphatic obstruction. Alternatively, these cells may be activated by host mediators released during the process of the inflammation that clearly accompanies this particular clinical manifestation of filariasis. Such mechanisms have been invoked to explain the increased levels of circulating HLA-DR$^+$ T ceils in several non-parasitic immunologically-mediated diseases (Yu *et al.,* 1980).

It is possible that these activated T cells in the chronic form of filariasis are responsible for suppressing the actions of other T cell subsets or B cells, as most of the HLA-DR$^+$ T cells in these patients were found on the suppressor/cytotoxic cells (CD8) and not on the helper/inducer cells (CD4). The regulatory interactions between CD8$^+$ HLA-DR$^+$ cells and other 7 and non-T cells are still poorly characterized. and further distinction between the subsets of CD8 cells using Leu-15 (Landay, Gartland & Clement. 1983) or anti-9.3 (Damle *et al.,* 1983) should provide additional clues about potential functional roles these cells might have in filariasis.

Though a population of CD8$^+$ cells was found that expressed one activation marker (HLA-DR) in patients with CP, these same cells did not show any enhanced expression of another activation marker (IL-2R) on their surfaces. While IL-2R$^+$ CD8 lymphocytes have been described both *in vivo* and *in vitro,* previous studies have indicated that the expression of the antigen on circulating T cells is likely to be a transient phenomenon even after antigenic challenge (Yachie *et al.,* 1983).

In an effort to study the functional implications of the HLA-DR$^+$ T cells in the patients with CP, we related its expression to mitogen stimulation. There was no correlation between the expression of HLA-DR on T cells and either the PHA or ConA responses, but there was an inverse correlation between the PWM responsiveness and HLA-DR$^+$ CD8$^+$ T cells in these patients. There are several possible mechanisms that might account for this pattern of reactivity:

1. Both PHA and ConA are known to activate T cells through the CD3 or CD2 pathways (Fleisher, 1984; Valentine *et al.,* 1985). Since response to both PHA and ConA was intact in patients with CP, it suggests that CD8$^+$ DR$^+$ lymphocytes do not exert their effect on these pathways of T cell activation.
2. PWM, on the other hand, has been shown to activate T cells through an alternative IL-2 independent pathway (Ceuppens *et al.,* 1986), in addition to the classical CD3 pathways (Puck & Rich, 1984). While CD3 pathways of T cell activation appear to be unaffected (based on the PHA/ConA data), it is possible that CD8$^+$ DR$^+$ lymphocytes exert an effect on the PWM-induced alternate pathway of activation.
3. Furthermore, PWM-induced proliferation of T cells appears to be regulated at two levels-one in which CD4 cells

respond directly to PWM thereby producing factors that drive CD8 cellular proliferation, and another in which PWM-activated CD8$^+$ cells suppress proliferation by CD4$^+$ cells (Puck & Rich, 1984; Yachie *et al.,* 1982). CD8$^+$ DR$^+$ lymphocytes in elephantiasis may indeed be involved in such a feedback suppression mechanism.

Our results support the concept that T cell mediated immune reactions arc operative in patients with the chronic lymphatic obstructive form of filariasis and are different from those in patients with asymptomatic microfilaraemia. This difference is reflected by increased number ofactivated T lymphocytes in the peripheral blood. Whether these activated T cells function favourably or unfavourably for the host, however, remains to be determined.

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