The Presence or Absence of Active Infection, Not Clinical Status, Is Most Closely Associated with Cytokine Responses in Lymphatic Filariasis

Adriana B. de Almeida, Maria Carmelita Maia e Silva, Maria Amélia Maciel, and David O. Freedman

Division of Geographic Medicine, University of Alabama at Birmingham; Centro de Pesquisas Aggeu Magalhães, Recife, Brazil

Twenty-eight Brazilians from an area in which *Wuchereria bancrofti* is endemic were classified as asymptomatic microfilaremia or having clinical filariasis with active infection or without current active infection. Total accumulation of antigen-specific interleukin (IL)-4 and IL-5 in 48 h peripheral blood mononuclear cell supernatants was not significantly different between groups. However, when cytokine kinetics were examined, responses segregated according to infection status. Sustained production of IL-4 and IL-5 beyond the first 24 h of stimulation and production of interferon-γ were seen only in the group with clinical filariasis without active infection. CD8 T cells were the major source of IL-5 production in this group, while CD8 production of IL-5 was undetectable in any subject with active infection (asymptomatic microfilaremia or with clinical filariasis and active infection). These findings indicate that active infection, rather than clinical status, is most closely associated with cytokine patterns in lymphatic filariasis.

Lymphatic filariasis, which is caused by the helminths *Wuchereria bancrofti* and *Brugia malayi*, affects ~80 million people worldwide [1]. The pathogenesis of the characteristic lymphatic damage is thought to involve three components: mechanical damage by motile parasites [2, 3], local immunologic responses to parasite antigen [4], and bacterial superinfection in previously damaged vessels [5]. The relative contribution of each of these components is poorly defined.

Based on the concept that differences in clinical manifestations reflect differences in the host immune response to the parasites, two polar groups have been used for classification: persons with asymptomatic microfilaremia (MF), who manifest relative immunologic hyporesponsiveness to filarial antigen [6-8], and those with chronic pathology, who have been generally assumed to be amicrofilaremic and have relatively increased filarial antigen-specific lymphocyte blastogenesis, T and B lymphocyte precursor frequency, and serum IgG levels [9-11] compared with asymptomatic MF subjects. With this bipolar paradigm, cytokine data from the few available studies have been interpreted as being suggestive of an association between a Th2-like response and MF.

The long-held concept that chronic pathology is uniformly associated with microfilaremia has, however, been dispelled by a recent elegant metaanalysis of 25 studies done between 1945 and 1982, which shows that, in fact, MF and amicrofilaremic persons are equally likely to have pathologic manifestations of disease [12]. Moreover, with the recent use of circulating antigen assays as a sensitive determinant of the presence of adult parasites, 15%–60% of those in Haiti, Tahiti, and Brazil with clinical pathology have been shown to be actively infected [13-16]. The presence of active infection has profound effects on the immune response [17]. For this reason, classification of patients according to infection status as well as clinical status rather than clinical status alone becomes imperative.

In the present study, we examined the nature and kinetics of cytokine production in antigen-stimulated lymphocytes in persons in the following groups: asymptomatic MF, clinical filariasis (Dis-positive) with active infection as manifested by the presence of circulating antigen (CAg-positive), and Dis-negative without CAg (CAg-negative). Cytokine production by CD8 T cells is important in other parasitic infections [18, 19]. Because the earlier studies in lymphatic filariasis examined only cytokine production by unfractionated peripheral blood mononuclear cells (PBMC), we also examined cytokine production by antigen-stimulated CD8 T cells.

Methods

Study population. Standardized histories were obtained and physical examinations were done on 28 study participants resident in two communities of Recife, Brazil, in which *W. bancrofti* is endemic. In this area, microfilaria rates are higher in males than in females [13]. Microfilaria counts by Nuclepore (Corning-Costar, Cambridge, MA) filtration of 3 mL of night blood were as described [20]. Subjects were classified into 3 groups: MF, that is, MF subjects who had no current or previous history of adenolymphangitis, erysipelas, cellulitis, or limb swelling; Dis-positive CAg-positive, that is, subjects with a clinical spectrum of lymphatic pathology ranging from acute filarial fever to chronic lymphedema or elephantiasis who had current active infection as determined by CAg in serum (TropBio, Australia) [21]; Dis-positive CAg-negative, that is, subjects with grade II or greater irreversible lymphedema [1], elephantiasis, or hydrocele who were cur-

Received 14 September 1995; revised 18 January 1996.
All subjects gave written informed consent according to human experimentation guidelines of the US Department of Health and Human Services. The protocol was approved by the Institutional Review Board, University of Alabama at Birmingham.

Grant support: National Institutes of Health (AI-31552).
Reprints or correspondence: Dr. Adriana B. de Almeida, Division of Geographic Medicine, UAB Station, BBRB 544, Birmingham, AL 35294-2170.

The Journal of Infectious Diseases 1996;173:1453-9
© 1996 by The University of Chicago. All rights reserved. 0022-1899/96/7306-0020$01.00
currently uninfected but had positive antifilarial IgG. Also, for the interleukin (IL)-4 mRNA experiment, another cohort of 29 persons classified as either MF or Dis-positive CAg-negative by the above criteria was included.

Detection of antifilarial IgG4. Antifilarial IgG4 was measured in serum as described [22]. The results are expressed as a comparison of the means of the optical density values obtained for the 3 groups.

Isolation and stimulation of PBMC. PBMC were isolated by ficoll-diatrizoate gradient centrifugation from heparinized venous blood. PBMC (2 x 10^6/mL) were cultured for 48 h in complete RPMI (RPMI 1640 with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/mL streptomycin) at 37°C and 5% CO2 with or without 8 µg/mL adult filarial antigen (BmA), prepared as described [23]. In some experiments, CD8 T cells that were immunomagnetically positively selected (Dynal, Great Neck, NY) from 24 h media or BmA-stimulated PBMC, as well as identically stimulated unfraccionated PBMC, were transferred to fresh media for a further 24 h. All supernatants were snap-frozen in liquid nitrogen and subsequently kept at −70°C until use. Cultured T cells were treated with RNAzol B (Tel-Test, Friendsworth, TX), and RNA was extracted with chloroform, precipitated with isopropanol, and frozen as above.

Cytokine ELISA. Supernatants were assayed by ELISA. For interferon (IFN)-γ and IL-4, kits were from Biosource (Camarillo, CA); the IL-5 kit was from R&D Systems (Minneapolis). Limits of detection were 15.6, 7.8, and 7.8 pg/mL, respectively. Percentage of cytokine production in the second 24 h of cell culture for each sample was calculated as follows: 100 X (production during the second 24 h/48 h cumulative production).

Detection of IL-4 mRNA. mRNA transcripts for IL-4 and for the housekeeping gene hypoxanthine-guanine–phosphoribosyl transferase (HPRT) were measured by a semiquantitative reverse transcriptase (RT)–polymerase chain reaction (PCR) technique essentially as described [24]. Briefly, 1 µg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies GIBCO BRL, Gaithersburg, MD). One-tenth of the sample was then used for specific amplification of IL-4 or HPRT using Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Primers and probes were as described [25], and 35 and 32 PCR cycles were used for IL-4 and HPRT, respectively. PCR products were separated on agarose gel, Southern-transferred to a nylon membrane (Hybond N; Amersham, Amersham, UK), probed with an internal fluorescein-labeled, cytokine-specific oligonucleotide, and visualized with a chemiluminescent detection system (ECL; Amersham). The chemiluminescent signals were quantified with a GS-670 imaging densitometer (Bio-Rad, Her­nocles, CA) [24], and results were expressed as a ratio of the IL-4 to the HPRT signal.

Statistical analysis. Statistical analysis was done by using the Mann-Whitney test. P < .05 was considered significant. All values are expressed as geometric means.

Results

Study population. Subjects were classified as MF (n = 15; 8 female, 7 male), Dis-positive CAg-positive (n = 6; 1 female, 5 male; 5 subjects were microfilaremic), or Dis-positive CAg-negative (n = 7; 6 female, 1 male). As previously reported in this area of Brazil [16], Dis-positive CAg-negative subjects were older (median age, 41.2 years) than MF or Dis-positive CAg-positive subjects (median ages, 33.2 and 28.8 years, respectively), although this difference was not significant. Despite the long-held assumption that persons with clinical disease are currently uninfected, subjects classified as having active infection had statistically indistinguishable levels of circulating antigen compared with MF subjects (figure 2A). Similarly, levels of antifilarial IgG4, another marker of active infection [22, 26], was significantly elevated (P = .01) in the Dispositive CAg-positive group compared with the Dis-positive CAg-negative group (figure 1B). In fact, IgG4 levels in the Dis-positive CAg-positive subjects were significantly higher than those in MF subjects (P = .05).

Kinetics of filaria-specific cytokine production. Existing studies of cytokine production in filarial infections have examined only cumulative levels in supernatants after a single fixed incubation time. To determine more precisely a possible pathophysiologic role for differential cytokine production in the 3 patient groups, the kinetics of IFN-γ, IL-4, and IL-5 production were examined. Cumulative production of IL-4 and IL-5 over 48 h in response to BmA was equivalent in all 3 groups (MF, 13.97 and 25.57 pg/mL, respectively; Dis-positive CAg-positive, 20.08 and 126.58; and Dis-positive CAg-negative, 28.35 and 163.7; figure 2B, C). Cumulative IFN-γ production was detectable only in the Dis-positive CAg-negative group (139.02 pg/mL; figure 2A). As expected, Brazilian controls produced undetectable BmA-specific IFN-γ, IL-4, and IL-5 (data not shown). That the differences in cytokine production are filaria antigen–specific was shown by the finding that phytohemagglutinin-stimulated IFN-γ mRNA production by PBMC did not differ between groups (data not shown).

When cytokine levels during only the second 24 h of culture were measured, production of IL-4 was significantly higher (figure 2E) in the Dis-positive CAg-negative group compared with the MF group (P = .02) or with the MF and Dis-positive CAg-positive groups combined (all infected subjects; P = .02). Production of IL-4 in the second 24 h accounted for a mean of 69.4% of the cumulative production in the Dis-positive CAg-negative group compared with only 35.3% in the MF group. Dominant IL-5 production in the second 24 h also occurred in the Dis-positive CAg-negative group (mean, 83.7%) and was significantly higher than that in either the MF group (P < .01) or the MF and Dis-positive CAg-positive groups combined (P < .01; figure 2F). Production of IFN-γ by Dis-positive CAg-negative subjects in the second 24 h was 94.33 pg/mL (figure 2D) and accounted for 51% of cumulative levels. Levels of individual cytokines did not correlate with levels of CAg or with absolute blood levels of microfilariae (data not shown). The only microfilaremic subject in the Dis-positive CAg-positive group had a cytokine profile similar to that found in subjects who were microfilaremic and positive for CAg (includes MF subjects and the remaining Dis-positive CAg-positive subjects).

To support the finding that most IL-4 production in Dispositive CAg-negative persons occurs later than in infected
subjects, expression of IL-4 mRNA in 29 subjects at 4 and 24 h was examined (figure 3A, B). At 4 h, the IL-4/HPRT ratio was equivalent in Dis-positive CAg-negative subjects and MF subjects (0.042 and 0.057, respectively). In contrast, at 24 h, which would be expected to correspond to cytokine production in the hours subsequent to that, the IL-4/HPRT ratio was significantly higher in the Dis-positive CAg-negative group ($P < .01$) than in the MF group (0.48 and 0.06, respectively).

**Cytokine production by CD8 T cells.** CD8 T cells can be significant sources of cytokine production, and in the area studied in which filariasis is endemic, persons with clinical pathology have a dominant CD8 T cell infiltrate in tissue [4]. To examine the contribution of CD8 T cells to total cytokine production, IL-4 and IL-5 production by positively selected BmA-stimulated CD8 T cells was compared with production by unfractonated T cells in parallel cultures. In all 4 Dis-positive CAg-negative subjects examined, CD8 T cells were the major source of IL-5 (>50% of total production), but CD8 T cells produced no detectable IL-5 in MF or in Dis-positive CAg-positive subjects (figure 4B). CD8 T cells accounted for the majority of total IL-4 production (>50%) in 3 of 4 Dis-positive CAg-negative subjects but in only 2 of 13 MF subjects (figure 4A). Of interest, 2 of 3 Dis-positive CAg-positive subjects had dominant IL-4 production by CD8 T cells. No production of IFN-$\gamma$ by CD8 T cells was detected in any study subject (data not shown). To confirm that no CD4 T cells were present in the CD8 T cell cultures, RT-PCR using CD4-specific primers (Clontech, Palo Alto, CA) and mRNA extracted from those cultures gave no detectable signal in any case (data not shown).

**Discussion**

Antigen-specific cytokine production is an important determinant of pathology in a number of helminth infections. For example, a polarized Th2-type response has been associated with deleterious immunopathology in murine models of schistosomiasis and onchocerciasis [24, 27]. Unfortunately, no suitable animal model of bancroftian filariasis exists, so relationships between cytokine profiles and induction of immunopathology have been less clear.

In human lymphatic filariasis, just one study each in areas in which *W. bancrofti* or *B. malayi* is endemic has examined the production of both Th1 and Th2 cytokines simultaneously in response to filarial antigenic stimulation [28, 29]. Together these studies show that the frequency of IL-4-secreting cells and the production of IL-4 on antigenic stimulation are not significantly different when persons classified as having MF and those classified as having chronic pathology are compared. In contrast, MF persons have a significantly lower frequency of IFN-$\gamma$-secreting cells and produce less IFN-$\gamma$ than do those with chronic pathology. The antigenic specificity of this finding has been demonstrated by the finding that polyclonal activation ($\alpha$-CD2 and recombinant IL-2) of PBMC from persons classified into these 2 clinical groups stimulates equivalent amounts
of IFN-γ [30]. The increased antigen-specific IL-4/IFN-γ ratio [28] in MF subjects has been interpreted as indicating a predominant Th2-type response in these patients, even though their production of the Th2 cytokine IL-4 is no different from that in persons with chronic pathology. Our present data suggest that both the classification of filariasis patients into 2 groups based on clinical findings alone and examination of cumulative cytokine production in PBMC supernatants, as has been done previously, may have been overly simplistic.

The presence of active infection has profound effects on the immune response [17] in filariasis. Persons with clinical manifestations of lymphatic filariasis are as likely as not to have active infection, so should not be assumed to be an immunologically homogeneous group. For this reason, classification of persons according to infection status as well as clinical status, rather than clinical status alone, becomes imperative.

In the present study from an area in which W. bancrofti is endemic in Brazil, subjects were classified into 3 groups: MF, Dis-positive CAg-positive (includes microfilaremic and amicrofilaremic subjects), and Dis-positive CAg-negative. Parasite-specific cumulative production of IL-4 and IL-5 over 48 h was statistically indistinguishable between the 3 groups. Infected subjects (MF and Dis-positive CAg-positive together) were unable to produce IFN-γ in response to parasite antigen.

Figure 2. Production of interferon (IFN)-γ (A, D), interleukin (IL)-4 (B, E), and IL-5 (C, F) in adult filarial antigen (BmA)-stimulated peripheral blood mononuclear cells from subjects with lymphatic filariasis: asymptomatic microfilaremic (MF+), clinical disease with circulating antigen (Dis+CAg+), and clinical disease without CAg (Dis+CAg−). A–C, 48 h cumulative production; D–F, production during second 24 h only. Limits of detection were 15.6 pg/mL for IFN-γ and 7.8 pg/mL for IL-4 and IL-5 (shading); horizontal bars denote geometric means.
Thus, when interpreted within the less-well-defined framework of patient classification used for the earlier published studies, these IL-4 and IFN-γ data agree with those findings. Parasite-specific IL-5 had not been previously examined in persons in areas in which W. bancrofti is endemic.

To examine in more detail the kinetics of cytokine production, levels of IFN-γ, IL-4, and IL-5 were measured only during the second 24 h of stimulation. Only Dis-positive CAg-negative subjects produced IFN-γ, and production appeared to be equivalent in the first and second 24 h. Production of both IL-4 and IL-5 in the second 24 h was significantly higher in Dis-positive CAg-negative than in CAg-positive subjects. We interpret this to indicate that while total accumulation of IL-4 and IL-5 in supernatants over time in all 3 groups appears to be similar...
(figure 2B, C), when kinetics are taken into consideration, CAg-negative subjects differed from CAg-positive subjects. Dispositive CAg-negative subjects appeared able to mount a sustained IL-4 and IL-5 response that in the second 24 h increased from initially low levels, while CAg-positive subjects mounted a brief response that waned after the first 24 h (figure 2E, F). This concept is supported by the finding that expression of IL-4 mRNA was significantly increased at 24 h versus 4 h of antigen stimulation in the Dis-positive CAg-negative group but remained at low levels in the MF subjects (figure 3). This pronounced difference in IL-4 mRNA expression between the MF and Dis-positive CAg-negative subjects at 24 h clearly reflects a real difference in kinetics. The peak of IL-4 mRNA expression in MF (asymptomatic) subjects likely occurred between 4 and 24 h, the only two time points examined. This would explain why despite the differences in mRNA kinetics, cumulative IL-4 production at 48 h is statistically indistinguishable between MF and Dis-positive CAg-negative subjects.

These differences in kinetics of cytokine responses possibly relate to frequency of antigen-reactive cells. Ongoing exposure to circulating parasite antigen in infected persons could lead to a relatively increased number of antigen-reactive PBMC available to mount a more immediately measurable cytokine response. In contrast, because of relatively small numbers of “dormant” antigen-reactive cells, Dis-positive CAg-negative subjects would be able to mount a measurable response only at a later time point. This possible variation in the frequency of CAg-specific lymphocytes in the CAg-positive subjects compared with CAg-negative subjects is currently being investigated in our laboratory at the single-cell level by a highly sensitive technique of intracellular staining for production of individual cytokines.

Correlations between absolute blood levels of microfilariae and measures of immune responses to filarial antigen have in general not been reported. This dissociation includes studies that have examined parameters such as filaria-specific IgG4 and IgE, stimulation index, and cytokine production [9, 31–33]. Similarly, in our studies, no correlation of levels of microfilariae with levels of CAg or cytokine production were found.

Though CD8 T cells are important sources of cytokines in infections such as leprosy and leishmaniasis [18, 19], published data in persons with filarial infections in areas in which filariasis is endemic are restricted to unfraccionated PBMC [9, 28, 33]. Persons with filarial pathology have elevated levels of soluble CD8 molecules and of CD8+ HLA-DR+ T cells in their circulation, and we have reported the striking and consistent finding of a CD8 T cell infiltrate in the tissue of pathology patients [4, 34, 35]. In the present study, CD8 T cells were the major source of IL-5 production (>50% of total production) during the second 24 h of stimulation in BmA-stimulated cells from all Dis-positive CAg-negative subjects, while no IL-5 production by CD8 T cells was detected in CAg-negative subjects. Because cumulative IL-5 production did not differ between patient groups, this suggests that a delayed expansion of IL-5–producing CD8 T cells may occur in currently uninfected persons with clinical disease, that is, those who have been able to clear adult parasites. IL-4 production by CD8 T cells was not significantly different when CAg-positive and CAg-negative subjects were compared.

Whether activated peripheral T cells, either from helminth-infected or -uninfected persons, can be classified into the Th1/Th2/Th0 subsets (originally developed from the study of T cell clones) is being increasingly called into question [36, 37]. In helminth-infected persons, this spectrum of in vivo cytokine profiles has now been demonstrated using intracytoplasmic staining of activated peripheral blood cells. From single patients, a distinct subpopulation of cells producing both IFN-γ and IL-4 at the same time, both IFN-γ and IL-5 at the same time, or only IL-5, only IL-4, or only IFN-γ have been described [36].

In conclusion, we have shown that in persons in an area in which filariasis is endemic, peripheral blood cytokine responses to parasite antigen do not appear to fall into the distinct Th1/Th2/Th0 paradigm. Infection status (circulating antigen–positive vs. –negative), rather than clinical status (clinical manifestations vs. none), appears more closely associated with the segregation of cytokine response patterns that do occur. The role of the cytokine responses as measured in peripheral blood in determining the clinical manifestations of the infection are as yet unclear. The relative contribution of IL-5 production by the CD8 T cell subset in mediating filarial disease is currently under investigation using tissue biopsies to assess local cytokine responses by PCR and in situ hybridization.

Acknowledgments

We thank Eridan Coutinho, Fred Abath, and André Furtado for facilitating the continuing support of Centro de Pesquisas Aggeu Magalhães in carrying out these studies, Mineo Nakasawa for technical assistance in Brazil, and Adam Plier for helpful discussions and support. We are indebted to the late Amaury Coutinho for his insight and patient discussions over the past 5 years.

References


