INCREASED FREQUENCY OF TH2-TYPE CYTOKINE-PRODUCING T CELLS IN MICROFILAREMIC LOIASIS

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Abstract. The frequency of cytokine-producing peripheral blood mononuclear cells was assessed in 28 subjects with microfilaremic loiasis and in 14 amicrofilaremic individuals. In addition, a subgroup of seven microfilaremic individuals coinfected with Plasmodium malariae was evaluated. By using flow cytometry for the intracellular detection of cytokines, a more pronounced T helper (Th)2 cell-type response with the expansion of interleukin (IL)-4, IL-10, and IL-13 expressing CD4+ cells in the microfilaremic compared with the amicrofilaremic group was noted. Expression of IL-5 was equivalent in both groups as was the frequency of Th2-type cytokines expressing CD8+ cells and of Th1-type cytokines (interferon [IFN]-γ, IL-2, IFN-γ/IL-2) producing CD4+ and CD8+ cells. Th0-type cytokine-expressing cells, represented by IL-4/IFN-γ, IL-10/IFN-γ, and IL-13/IFN-γ, were equally distributed within groups. Coinfection of P. malariae did not significantly alter the cytokine expression compared with microfilaremic individuals without P. malariae infections. By identifying a large panel of cytokine-producing T cell subpopulations, a Th2-driven immune response in microfilaremic Loa loa patients was noted.

Loiasis is a parasitic infection caused by the filarial nematode Loa loa, which is endemic in rain forest areas of Central and West Africa. Infective larvae are transmitted by diurnal bites of flies of the genus Chrysops and, over a period of several months, develop into mature, adult worms that may liberate microfilariae into the bloodstream. Among individuals infected with L. loa, clinically distinct subgroups can be identified with manifestations ranging from asymptomatic microfilaraemia to a hyperresponsive state characterized by more frequent episodes of local angioedema (Calabar swellings) and pronounced eosinophilia in the absence of microfilariae. In endemic areas, only a minority of infected people have detectable microfilariae in blood. However, the majority of infected subjects, including most visitors to endemic areas, remain amicrofilaremic and are more likely to have a hyper-responsive syndrome. The apparent differences in clinical presentation are thought to reflect the diversity of the immune response of the host to filarial antigen, with diminished humoral and cellular reactivity in the presence of circulating microfilariae. Most previous results on L. loa-host relationship were derived from studying temporary residents of areas of endemicity. These amicrofilaremic patients had elevated serum parasite-specific immunoglobulin (IgE and IgG) levels, augmented filaria-specific lymphocyte proliferative responses, and an increase in the ratio of CD4+/CD8+ T cells.

Immune mechanisms activated in individuals continuously exposed to infection have been studied far more extensively in other filarial species. In particular, the categorization of T helper (Th) cells into at least two functionally different subtypes on the basis of their cytokine profiles has provided a useful framework for the understanding of immune regulation of lymphatic filariasis and onchocerciasis, although somewhat conflicting results have been reported. In general, asymptomatic microfilaremic individuals demonstrated impaired lymphocyte proliferation and diminished filarial antigen–driven interleukin-2 (IL-2) and interferon-γ (IFN-γ) release, indicative of the Th1 down-regulatory processes. In some studies, expansion of Th2 cells with increased IL-4 and IL-5 production, subsequent B cell development, and a matching antibody profile was observed. Immunoglobulin switching predominantly to the IgG4 class has been associated with filarial disease. While the Th2 cytokines IL-4 and IL-5 clearly are responsible for eosinophilia and IgE and IgG4 isotypes, down-regulation of TH1 responses has been more frequently linked to IL-10 in both onchocerciasis and lymphatic filariases.

Most recent data concerning the cytokine profile in loiasis are derived from mandrills and rhesus monkeys experimentally infected with L. loa and from one study of immune responsiveness in human loiasis. These studies appear suitable for illustrating some aspects of the infection; however, the lack of a detailed analysis of cytokine-producing T cells in human loiasis is still evident.

Recently, flow cytometric analysis of cytokine-producing cells has been developed, making it possible to detect a particular cytokine or coexpression of two cytokines at the single cell level in well-defined cell populations. By using this technique, we sought to characterize the phenotypes and frequencies of cytokine-producing T cells in human microfilaremic loiasis in comparison with amicrofilaremic individuals. The influence of concomitant infection with Plasmodium malariae on the Th1/Th2 cell dichotomy in microfilaremic disease was studied in a subgroup of patients.

MATERIALS AND METHODS

Patients. The study was carried out at the Albert Schweitzer Hospital in Lambarene, Gabon, an area endemic for L. loa infection. Subjects fulfilling the following criteria were enrolled in the study: 1) asymptomatic microfilaremic loiasis, age ≥ 20 years, no prior treatment with either ivermectin, diethylcarbamazine, or albendazole, no concomitant systemic parasitic diseases; 2) asymptomatic coinfection with microfilaricmic L. loa and P. malariae, age ≥ 20 years, no prior anti-filarial treatment (see above) or recent malaria treatment; 3) amicrofilaremic subjects, age ≥ 20 years, no prior anti-filarial or recent malaria treatment, no overt sys-
temic parasitic disease, yet chronic exposure to *L. loa* infection.

Study subjects (both microfilaricmic and microfilaricmic) were mainly blood donors or relatives of children attending the outpatient clinic of the hospital, who were willing to participate in the study. Amicrofilaricmic subjects were strongly considered to be exposed to infection with *L. loa* since all study participants were residents of the same small rain forest–located villages. In addition, occult loiasis in the amicrofilaricmic group was suspected on clinical grounds (history of episodes of local swellings combined with intense pruritus). Microfilaricmic loiasis was diagnosed, or in the case of controls, excluded by Giemsa-stained thick blood smears, leukoconcentration, and Nucleopore® (Costar Europe, Ltd., Badhoevedorp, The Netherlands) filtration methods. Since *L. loa* exhibits a pronounced diurnal periodicity, parasites were counted between 10:00 AM and 2:00 PM. Concomitant *P. malariae* infection was diagnosed by Giemsa-stained thick and thin blood smears. All patients provided informed consent, and the study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital in Lambarene.

Peripheral blood mononuclear cell (PBMC) cultures and intracellular cytokine detection by flow cytometry. Flow cytometric assessment of T cell cytokine production was performed essentially according to the technique described by Jung and others and modified by Willheim and others. The PBMCs were isolated from heparinized blood by ficoll-diatrizoate centrifugation. Cells were then cultured in Ultra Culture Medium (Bio Whittaker, Walkersville, MD) supplemented with L-glutamine (2 mM; Sigma, St. Louis, MO), gentamicin (170 mg/L; Sigma), and 2-mercaptoethanol (3.5 μL/L; Merck, Darmstadt, Germany) and stimulated with phorbol 12-myristate 13-acetate (10 ng/ml; Sigma) and ionomycin (1.25 μM; Sigma) in the presence of monensin (1 μM; Sigma) for 4 hr at 37°C in 5% CO₂. Cells were then harvested on ice, washed twice in phosphate-buffered saline (PBS), and fixed with 2% formaldehyde (1 ml per 2 × 10⁶ cells; Merck) for 20 min. After two additional washes in PBS, cells were resuspended in Hanks’ balanced salt solution supplemented with 0.3% bovine serum albumin and 0.1% sodium azide and stored at 4°C in the dark until staining. Fixed cells were washed twice with PBS and made permeable with saponin (0.1%; Sigma), resuspended in 50 μl of saponin buffer–diluted antibodies, and incubated for 25 min at room temperature in the dark. The following monoclonal antibodies (MAbs) were used: cytokine-specific mouse anti-human MAb (IFN-γ [clone: B27], fluorescein-isothiocyanate [FITC]-labeled) and rat anti-human MAb (IL-2 [MQ1–17H12], phycoerythrin [PE]-conjugated; IL-4 [MP4–25D2], PE-labeled; IL-4 [MP4–25D2], FITC-labeled; IL-5 [TRFK5], PE-labeled; IL-10 [JES3–9D7], PE-labeled; IL-13 [JES10–5A2], PE-labeled). All MAbs were obtained from Pharmingen (San Diego, CA). The anti-CD4 MAb and anti-CD8 MAb were labeled with allophycocyanin and peridinin chlorophyll, respectively (Becton Dickinson, Mountain View, CA). Four color staining was performed and at least 10⁶ cells were analyzed on a FACSCalibur (Becton Dickinson) equipped with two laser system (488 nm and 630 nm wavelength, respectively). All cytokine combinations (IL-2/IFN-γ, IL-4/IFN-γ, IL-10/IFN-γ, IL-13/IFN-γ, IL-4/IL-5) were stained in conjunction with CD4 and CD8. Figure 1 shows the respective dot plots of one patient gated on the CD4⁺ cell population. Data were analyzed with CELLQuest software (Becton Dickinson), and results were expressed as the percentage of cytokine-producing cells in each CD4⁺ or CD8⁺ cell population.

**Statistical methods.** Statistical analysis was performed using a standard statistical package (SPSS 7.5 for Windows;
RESULTS

Study population. Characteristics of the study population are shown in Table 1. Thirty-five individuals with asymptomatic microfilaremic loiasis (MF) were studied; seven of them were coinfect ed with *P. malariae*. They were compared with 14 amicrofilaremic (AM) controls. Microfilaremic individuals were significantly older than the amicrofilaremic ones (*P < 0.01*). Age was similar in the *L. loa* and *P. malariae* coinfect ed subjects compared with those infect ed with *L. loa* alone. Neither gender nor the microfilarial count were correlated with the percentages of cytokine-expressing cells.

Frequency of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes in MF patients and AM controls. A mean of 50% (range = 27–66%) of cells within the lymphocyte scatter gate in MF patients consisted of CD4<sup>+</sup> T cells, while 17% (range = 8–37%) were CD8<sup>+</sup>. The AM individuals displayed equal percentages of CD4<sup>+</sup> (mean = 51%; range = 24–63%), but significantly higher CD8<sup>+</sup> (mean = 22%, range = 15–30%; *P < 0.01*). No significant differences were found when MF patients were compared with the *P. malariae*-coinfect ed sub-group (CD4<sup>+</sup>: mean = 52%, range = 42–62%; CD8<sup>+</sup>: mean = 21%, range = 5–41%).

Th<sub>1</sub> cytokine production of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in MF patients and AM controls. Ex vivo IL-2, IFN-γ-produc tion, and coexpression of both cytokines within CD4<sup>+</sup> and CD8<sup>+</sup> T cells were studied. Results are shown in Table 2. No difference was observed in the frequency of IFN-γ-producing CD4<sup>+</sup> cells between MF patients and AM controls. This was also true for CD4<sup>+</sup> cells, which contributed substantially to the overall number of IFN-γ-producing cells. The percentage of CD8<sup>+</sup> cells expressing IFN-γ was significantly higher in MF patients (*P < 0.05*), while cells coexpressing IL-2 and IFN-γ were similar in both groups. With regard to the expression of IL-2 in both CD4<sup>+</sup> and CD8<sup>+</sup> cells, no differences between groups were found.

**Th2 cytokine production of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in MF patients and AM controls.** The frequencies of IL-4-, IL-5-, IL-10-, and IL-13-producing T cells were assessed. In addition, the capacity of CD4<sup>+</sup> and CD8<sup>+</sup> cells to produce both IL-4 and IL-5 was studied (Table 3). Patients with MF generally exerted somewhat higher frequencies of Th2 cytokine-producing cells both in CD4<sup>+</sup> and CD8<sup>+</sup> cells than the AM controls. However, significant differences were restricted to the CD4<sup>+</sup> subpopulation. An expansion of CD4<sup>+</sup> cells exclusively producing the respective cytokine was detected in MF patients compared with the AM controls (IL-10<sup>+</sup>/IFN-γ<sup>+</sup>: 2.5% versus 1.5%; *P < 0.01*; IL-4<sup>+</sup>/IFN-γ<sup>+</sup>, 8.9% versus 6.6%; *P < 0.05*; and IL-13<sup>+</sup>/IFN-γ<sup>+</sup>, 8.5% versus 6.2%; *P < 0.05* (Table 3).

Although not significantly more expanded in MF patients when compared with the AM controls, our *ex vivo* data confirm earlier results of a relevant subset of CD4<sup>+</sup> cells co-producing IL-4 and IL-5 in helminth disease.**

**Effect of concomitant *P. malariae* infection on cytokine production in MF patients.** To identify possible influences of a malarial parasite on lymphocyte phenotypes in MF patients, a subgroup analysis comparing MF patients and *P. malariae*-coinfect ed MF patients was undertaken. By applying the same staining protocol as above, an overall lower cytokine responsiveness was observed within CD4<sup>+</sup> cells of the coinfect ed group but differences were not significant.
### TABLE 3

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<th>IL-4 (ng/mL)</th>
<th>IL-5 (ng/mL)</th>
<th>IL-10 (ng/mL)</th>
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<td><strong>Study group (n)</strong></td>
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<tr>
<td>Asymptomatic microfilaric (28)</td>
<td>8.9² (4.1±1.7)</td>
<td>0.7 (0.2±1.0)</td>
<td>0.7 (0.2±1.0)</td>
<td>2.3 (1.1±2.4)</td>
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<tr>
<td>Asymptomatic amicrofilaric (14)</td>
<td>7.3 (2.8±1.7)</td>
<td>0.8 (0.1±2.0)</td>
<td>0.7 (0.1±1.9)</td>
<td>1.7 (0–1.7)</td>
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<tr>
<td>Microfilaric/coinfected (7)</td>
<td>8.5² (3.7±1.8)</td>
<td>0.7 (0.2±1.0)</td>
<td>0.5 (0.1±2.5)</td>
<td>2.1 (0.4±3.9)</td>
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**Significant differences between asymptomatic microfilaric and asymptomatic amicrofilaric subjects (P < 0.05) by the Mann-Whitney U-test.**

**Influence of age on the CD4⁺/CD8⁺ ratio and the frequency of cytokine-producing cells in MF patients.** By calculating Spearman’s correlation coefficient, a significant decrease in CD4⁺ cells within the lymphocyte scatter gate as an age-related phenomenon was observed (r = −0.440, P < 0.05). The most striking age-related modification was the significant increase of IFN-γ-expressing cells and the significant decrease of exclusively IL-2-expressing cells in the CD8⁺ subset with age (r = 0.627, P < 0.001 and r = −0.532, P < 0.01, respectively). Thus, the IL-2 and IFN-γ production capacity was inversely correlated within the CD8⁺ subset (r = −0.653, P < 0.001). The IFN-γ-producing CD8⁺ cells were significantly more frequent in microfilaric patients than in the amicrofilaric subjects (Table 2; P < 0.05). However, this was clearly an age-related modification since differences were no longer significant when adjusted for age. The frequency of all other cytokine-producing cells was not correlated with age.

### DISCUSSION

Differential profiles of T cell-derived cytokines have frequently been associated with immunopathology and clinical outcome of human filarial infection. The cellular hyporesponsiveness of asymptomatic microfilarial disease has been shown to be parasite specific, with responses to nonparasite-derived antigens remaining intact. This finding has recently been substantiated in a study of human loiasis, which showed no differences between microfilaric and amicrofilaric individuals with respect to lymphoproliferation and cytokine production when PBMC were stimulated with nonspecific mitogens. In addition, in a longitudinal study of *L. loa*-infected rhesus monkeys, blastogenic responses of PBMC and IL-2 production capacity decreased only after stimulation with filarial antigen. By applying nonspecific (non-filarial) stimulants in our flow cytometric assay, it was nevertheless possible to elaborate differences between microfilaric loiasis and the amicrofilaric group, with an expansion of IL-4-, IL-10-, and IL-13-expressing CD4⁺ cells within the microfilaric individuals.

Microfilarial antigen–driven and even spontaneous release of IL-10 was found to be elevated in microfilaric individuals and correlated with impaired lymphocyte proliferation as well as lower IL-2 and IFN-γ production in studies of lymphatic filariases. In onchocerciasis, IL-10 was also spontaneously released from PBMC, and levels were higher in the microfilaric group than in a putatively immune (microfilaric) population. Another study, however, showed no differences in both adult filarial antigen-induced and spontaneous IL-10 production between microfilaric and amicrofilaric *Wuchereria bancrofti*-infected patients. An additional study performed by the same group revealed even lower *Brugia pahangi* antigen-driven IL-10 levels in microfilaric subjects than in patients with chronic lymphatic dysfunction. These studies and a recently published murine model indicated that IL-10 is not responsible for the induction of proliferative suppression of T lymphocytes by filarial parasites. However, recombinant filarial proteins exhibiting biological activity as protease inhibitors have been identified as potent inducers of IL-10, but not IFN-γ production, in human PBMC and murine spleno-
cytes.25, 26 The first evidence of a role of IL-10 in microfilaremic loiasis was derived from a study in mandrills experimentally infected with human *L. loa.*15 During early prepatency, a hyper-responsive state was noted, as indicated by increases in lymphocyte proliferation, IL-2 and IL-5 production, as well as expression of IL-2, IL-5, IL-10, and IL-12 mRNAs in PBMC.15 Concurrent with the appearance of microfilariae, both lymphoproliferation and cytokine responses were suppressed, with only strong IL-10 expression remaining unaffected.12 In human loiasis, however, the same research group described significantly higher IL-10 production in amicrofilaric than in microfilaric subjects and concluded that this cytokine is not responsible for the Th unresponsiveness observed in the microfilaric group.14 The apparent divergences concerning the contribution of IL-10 to microfilaria-specific hyporesponsiveness cannot be fully resolved by our data because we concentrated on analyses of cytokine production and T cell subpopulations and did not perform lymphoproliferation assays. In all, the expansion of the IL-10-producing lymphocyte subpopulation in our microfilaric subjects did not significantly influence the frequency of Th1 cytokine-expressing cells represented by intracellular staining of IFN-γ, IL-2, and IFN-γ/IL-2. Interestingly, one-third of all IL-10-producing cells in our study coproduced IFN-γ, and the frequency of these cells was strongly correlated with the Th2 response, but not to IFN-γ expression. It has been hypothesized that the IL-10/IFN-γ subset might result in indirect feedback inhibition of IFN-γ expression since IL-10 suppresses antigen-presenting cell function by down-regulating class II major histocompatibility complex antigens, costimulatory signaling through CD80, as well as by blocking IL-12 production.27-29

While no differences were shown for cells expressing cytokines in a Th0-type manner (IL-4/IFN-γ, IL-13/IFN-γ) between microfilaric and amicrofilaric subjects, an expansion of exclusively IL-4- and IL-13-producing CD4+ cells was shown in the microfilaric group. Some of our results corroborate those of a previous study, which determined IL-4, IL-5, and IFN-γ coproducing cells in a small group of healthy individuals and helminth-infected patients.15 By sorting for CD4+ /CD27+ and CD4+ /CD27- cells, a distinct distribution of Th0/Th1/Th2 cytokine-producing cells was identified in this study. While the majority of cells exclusively expressed the respective cytokine, less cells produced both Th1 and Th2 cytokines. In a recent study of Bancroftian filariasis, no differences between asymptomatic microfilaric subjects, antigenemic patients, and non-antigenemic patients with clinical filariasis were observed by staining negatively selected CD4+ cells with IL-4, IL-5, and IFN-γ.30 Analysis of unfractinated PBMC showed that microfilaric subjects displayed significantly less IL-4- and IFN-γ-producing cells than non-antigenemic patients with clinical filariasis. The investigators suggested that non-CD4 T cells, presumably CD8 T cells or even non-CD3 cells, would have accounted for the differences observed between these groups. Direct comparisons of our data with these results appear inappropriate because different filarial species were studied and the included amicrofilaric (antigen-negative) subjects in the *Brugia* study had clinical filarial disease. Generally, much lower frequencies of IL-4- and IL-5-producing CD4+ cells were detected in this study from Brazil, possibly reflecting differences in the overall antigenic load in comparison to our African study population. The data of our 49 study participants provided no evidence that CD8 cells contributed substantially to the overall production of IL-4, IL-5, IL-10 and IL-13, although this T cell subset was clearly capable of producing the whole array of cytokines. Interestingly, most IL-4-producing CD8+ cells coexpressed IFN-γ, which would identify a Tc0 subset not previously reported in helminth disease. The difference in IFN-γ production by CD8+ cells between microfilaric and amicrofilaric subjects was clearly an age-related modification since the frequency of IFN-γ-producing CD8+ cells was significantly increased in older study participants, irrespective of the presence or absence of microfilariae. Cytokine expression in both T cell subsets was otherwise not correlated with age. Therefore, the older age of the microfilaric group should have not accounted for the observed differences in cytokine-producing cells, except for IFN-γ production within the CD8+ subset. Further age-related alterations consisted of a decrease of the CD4+ subset with a concomitant increase of CD4+/CD8+ cells. These cells were most likely of the natural killer (NK) cell type with substantial IFN-γ production, but negligible type-2 cytokine expression (Winkler S and others, unpublished data). Similar decreases of lymphocyte subsets and enhancement of NK cell activities have been reported in older people.31 These findings indicate that age, which implies a variable long exposure to antigens (not only of helminthic origin), should be considered in all studies dealing with lymphocyte subsets and their respective cytokines. This has been pointed out by a study of Brugian filariasis, which showed increases in IL-4 production with age.32

As expected, the expression of IL-13, which shares most of its activities with IL-4, was strongly correlated with IL-4. This confirmed the pronounced Th2 response in microfilaric individuals. Recently, IL-13 has been recognized as more important than IL-4 in the expulsion of gastrointestinal nematodes, which supports a unique role for IL-13 in Th2-mediated immune responses.33,34
The inclusion of a small subgroup of Plasmodium coin- 
fected microfilaricidal subjects was triggered by the idea of a possible interaction of two different parasites, both providing high and chronic antigen loads, on cytokine responses in peripheral blood. Although the pattern of cytokine-pro- 
ducing cells resembled more that of the amicrofilaricidal group, no significant differences to the cytokine response observed in microfilaricidal loiasis alone were found. Indeed, a recent epidemiologic study of Bancroftian filariasis has not shown any interaction between microfilaria and P. falci- 
parum parasitemia. Clearly, our data concerning the relationship of Plasmodium spp. and L. loa are limited. Never- 
theless, the pronounced Th2 response in our microfilaricidal subjects and the finding that cerebral malaria in mice was prevented by the simultaneous infection with B. pahangi out- 
line the importance of studying the influence of concomitant 
filarial infection on clinical manifestations of malaria in ar- 
as where both parasites are hyperendemic.

Several factors including age, clinical status, presence of circulating antigens, stage-specific differences, as well as geographic variations have all been shown to influence the immune responsiveness to filarial infection. Considering the complexity of this host-parasite relationship, it appears ob- 
vious that the simple classification into Th1 or Th2 catego- 
ries is not appropriate. In fact, we identified a surprisingly 
large panel of cytokine-producing T cell subpopulations 
that showed a more pronounced Th2-driven bias in the presence of microfilariae.

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