Transmission Intensity Affects Both Antigen-Specific and Nonspecific T-Cell Proliferative Responses in *Loa loa* Infection

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T-cell proliferative responses were studied in two villages in Gabon with different levels of *Loa loa* transmission. The first village (Okoumbi) had an annual transmission potential (ATP) of ~9,000 infective larvae (L3)/person/year (high transmission village), while the second village (Ndjokaye) had an ATP of ~1,000 L3/person/year (low transmission village). Proliferation and cytokine assays were performed on peripheral blood mononuclear cells (PBMC) from individuals aged 18 years and over using either mitogens (concanavalin A or phytohemagglutinin), antigens (purified protein derivative [PPD], irrelevant antigen), or soluble extracts of L3, microfilariae, or adult *L. loa*. PBMC from individuals in the low transmission village responded better to stimulation with adult antigen and to PPD than did PBMC from individuals in the high transmission village (*P* = 0.0031 and *P* = 0.0012, respectively). These data suggest that high levels of transmission of *L. loa* depress both specific and nonspecific T-cell proliferative responses in infected humans.

*Loa loa* is a human filarial parasite that infects an estimated 13 million people in equatorial west and central Africa. The disease is characterized by a range of clinical manifestations including Calabar swellings, pruritus, and the ocular passage of the adult worm causing eye inflammation (24). In the endemic population pathologies such as hydrocele in males and encephalopathy are common (4). Other complications, including pulmonary abnormalities, renal disease, and cardiomyopathy, have variously been reported (14, 21). Most immunological studies on *L. loa* infection have concentrated on analysis of antibody responses (1, 6, 10), with the consequence that our understanding of cellular immune responses is limited (14, 22). In one study carried out in the same area of Gabon as the present study, microfilariae were reported to display impaired antigen-specific proliferative responses compared to amicrofilaric (3), a situation similar to that observed in lymphatic filariasis (23, 29). In lymphatic filarial infection, the original studies correlated the proliferative defect with the presence of microfilariae (MF) (23), but more recent studies have demonstrated that proliferative responses are suppressed in a percentage of all infected or exposed individuals (29). In parallel with defective T-cell proliferative responses, gamma interferon (IFN-γ) levels are significantly reduced in MF-positive individuals while interleukin-4 (IL-4) levels are similar between different clinical groups (18).

However, the spectrum of infection with *L. loa* in this area of Gabon is quite different from that usually observed with the lymphatic filarial worms. For *L. loa* in Gabon, most infected individuals are amicrofilaric (>70%), while in lymphatic filariasis a significant percentage of infected individuals are usually microfilaric. Despite this fundamental difference in the parasitological status of infected individuals, there are similarities between the immune response elicited by the lymphatic filariae and by *L. loa*. For example, immunoglobulin G4 (IgG4) levels are elevated during active infection with both lymphatic filarial worms and *L. loa*, while amicrofilaric individuals have elevated levels of other parasite-specific IgG subclasses (1, 2, 15).

Studies that have been carried out in other filarial infections with the aim of defining the mechanisms underlying proliferative suppression (17, 25) have shown that no one single factor consistently restores proliferation in vitro. Some studies have shown an antiproliferative effect for IL-10 (17), while a more recent study with *Onchocerca volvulus*-infected individuals proposed that the proliferative defect is a consequence of the development of Th3 cells secreting the antiproliferative cytokines IFN-γ and transforming growth factor β (9). Paradoxically, few studies to date have attempted to investigate the impact of external factors such as levels of transmission on immune responses in filarial infection, and yet herd immunity, which seems to be an important mechanism in human infection, was shown to be dependent upon vector biting rates (20).

In this study we present preliminary data on immune regulation in *L. loa* infection by comparing levels of proliferation and cytokine secretion in peripheral blood mononuclear cells (PBMC) from individuals from two villages in an endemic zone of Gabon that differed only in the intensity of transmission.

**MATERIALS AND METHODS**

**Villages and study population.** Two villages were designated for entomological follow-up: Okoumbi and Ndjokaye are situated in the forest and savannah areas of Gabon, respectively. Details of the entomological studies are reported elsewhere (J. P. Akue, E. Devaney, G. Wahl, and H. Moukana, submitted for publication). Briefly, *Chrysops* were captured in these two villages over a period of 1 year and dissected for *L. loa* infective larvae (L3). The daily biting rate was determined and the annual transmission potential (ATP) was calculated as the daily transmission potential × the percentage of infected *Chrysops* × the mean number of L3/infective *Chrysops*. The ATP was defined as the number of L3 potentially infecting an individual per year. Individuals in the two villages donated blood after giving their informed consent and the project was approved by the Ethical Committee of the International Center for Medical Research of Gabon.

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Francoise (CIRM/F). Blood was collected into EDTA and 1 ml was examined for the presence of MF by the Knotts technique. The remaining blood was used for isolation of PBMC and plasma.

**Parasite materials and antigen preparation.** Adult worms of *L. loa* were removed by an ophthalmologist during ocular passage. MF were obtained from heavily infected patients and purified on a Percoll gradient, as previously described (28). *L3* were obtained by dissection of naturally infected *Chrysops* species caught in an *L. loa*-endemic village. Parasites were stored in liquid nitrogen until required. Adult worms and *L3* were washed and then homogenized in RPMI, while MF were disrupted by sonication in the same buffer. Antigens (Ag) were left to extract on ice for 1 h. The solutions were then centrifuged at 13,000 × g for 10 min and sterilized by passage through a 0.2-μm-pore-size filter (Millex; Millipore, Molsheim, France). A 20-μl aliquot of Ag solution was removed for protein assay using the Bio-Rad method (5), and the remainder was stored at −70°C until use.

**Separation of PBMC.** PBMC were separated by Ficoll-Hypaque centrifugation from whole blood diluted 1:1 with RPMI 1640. Cells were then washed twice with RPMI containing 10 μg of gentamicin/ml and cryopreserved in RPMI containing 10% dimethyl sulfoxide and 20% human AB serum. For use, cells were thawed in 10% dimethyl sulfoxide and 20% human AB serum. For use, cells were thawed from whole blood diluted 1:1 with RPMI 1640. Cells were then washed twice with RPMI containing 10 μg of gentamicin/ml and cryopreserved in RPMI containing 10% dimethyl sulfoxide and 20% human AB serum. For use, cells were thawed in 10% dimethyl sulfoxide and 20% human AB serum. For use, cells were thawed

**RESULTS**

**Characteristics of the village and study population.** The details of the ATP calculations have been reported elsewhere (Akue et al., submitted). The village of Okoumbi had an estimated ATP of ≈9,300 *L3* per person per year and was defined as a high transmission village, whereas Ndjokay, with an ATP of ≈1,300 *L3* per person per year, was defined as a low transmission village. In the original survey, 111 individuals were examined in Okoumbi (91% of the population) and 73 were examined in Ndjokay (82% of the population). Similar percentages of the population were microfilarial in both villages (21% in Okoumbi and 22% in Ndjokay; *P* = 0.870). The incidence of reported ocular passage was greater in Okoumbi (43.4%) than in Ndjokay (15.7%; *P* = 0.001), while there was no significant difference in the incidence of reported Calabar edema (31.3% in Okoumbi compared to 28.2% in Ndjokay; *P* = 0.734).

Blood samples from 37 adults from Okoumbi and 30 adults from Ndjokay were used for immunological analysis. There was no difference in age or sex of the study populations in the two villages (Table 1). All individuals participating in the study were infected with *L. loa*, and another filarial parasite, *Mansonella perstans*, was present in the majority of patients (Table 1). *L. loa* infection was defined by one or more of the following: presence of *L. loa* MF in the blood; elevated levels of IgG4 (greater than the mean plus 1 standard deviation of 11 sera from Gambian individuals infected with *M. perstans* alone); the ocular passage of an adult worm; or the clinical signs of Calabar edema. MF levels and levels of *L. loa*-specific IgG4 tended to be higher in the high transmission village, but these differences did not reach significance. The details of the participating adults are summarized in Table 1.

**Proliferation assay.** PBMC from the participating patients were used for the proliferation assay in order to compare responses between the two villages. Mitogens (PHA or ConA) and antigens (PPD, L3, MF, adult Ag) were used to stimulate cells from each individual. Cells from most individuals responded to both mitogens, and there was no significant difference in the means per minute incorporated by PBMC from individuals in the high or low transmission village in response to ConA (Fig. 1A) or PHA (Fig. 1B). ConA stimulation resulted in a mean counts per minute of 15,559 ± 2,718 in the high transmission village compared to 17,373 ± 2,455 in the low transmission village (*P* = 0.2538); the corresponding values for PHA were 36,401 ± 5,405 and 26,218 ± 3,983 (*P* = 0.2591) for the high and low transmission villages, respectively. Interestingly, when proliferative responses to a nonparasite Ag (PPD) were compared between the two villages (Fig. 1C), there was a significant difference, with PBMC from the low

<table>
<thead>
<tr>
<th>Village</th>
<th>ATP</th>
<th>No. of subjects</th>
<th>Mean age (yr)</th>
<th>No. of MF* (range Mf/ml)</th>
<th>No. of MF* (IgG4 in MF*)</th>
<th>M. perstans incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okoumbi</td>
<td>9,300</td>
<td>37 (20/17)</td>
<td>41.7 ± 3.5</td>
<td>16 (2-86,000)</td>
<td>21 (0.744 ± 0.147)</td>
<td>30/37</td>
</tr>
<tr>
<td>Ndjokay</td>
<td>1,300</td>
<td>30 (12/18)</td>
<td>42.7 ± 3.9</td>
<td>8 (44-5620)</td>
<td>22 (0.440 ± 0.092)</td>
<td>28/30</td>
</tr>
</tbody>
</table>

* MF*, amicrofilaric but infected based on levels of specific IgG4 greater than mean plus 1 standard deviation of the mean of 11 Gambian subjects infected with *M. perstans* and/or the ocular passage of an adult worm and/or Calabar edema. IgG4 levels were not significantly different between villages (*P* = 0.082).
transmission village incorporating significantly more counts per minute (21,770 ± 3,941) than PBMC from the high transmission village (11,799 ± 3,233; *P* = 0.0012).

When PBMC were stimulated with adult *Loa* Ag (Fig. 2B), mean levels of proliferation were significantly higher in the low transmission village (13,312 ± 3,377) than in the high transmission village (5,036 ± 2,071; *P* = 0.0031). A similar trend was noted with both L3 (Fig. 2A) and Mf (Fig. 2C) Ag, but the differences fell just short of statistical significance (*P* = 0.0786 and *P* = 0.0797, respectively). It was notable that levels of spontaneous proliferation were elevated under conditions of high transmission (mean, 1,998 cpm versus 256 cpm in the low transmission village; *P* = 0.232), and the proportion of individuals having spontaneous counts >1,000 cpm was increased
Cytokine assays. In parallel with the proliferation assays, cytokine secretion was also measured in a small subset of individuals from both villages (8 individuals from Ndjokaye and 19 from Okoumbi) in response to in vitro restimulation with adult, Mf, or L3 Ag. The results are summarized in Table 3. There was no significant difference in levels of any cytokine in response to stimulation with L. loa Ag between the high and low transmission villages. However, levels of IL-4, IL-2, and IFN-γ were two to three times greater in the high transmission village than in the low transmission village in response to adult Ag, while levels of IL-5 were similar between villages. With Mf Ag the picture was similar to that observed with adult Ag, with higher levels of both IL-4 and IFN-γ in the high transmission village than in the low transmission village, while levels of IL-2 and IL-5 were similar in both villages. Interestingly, both adult and Mf Ag elicited more IL-4 and IL-5 than did L3 Ag in the high transmission village, and these differences reached statistical significance (Table 3).

Despite the absence of statistical differences between the villages for any cytokine, it was noted that there were differences between the villages in the number of people who secreted Ag-specific cytokines. For example, in Okoumbi, the high transmission village, a significantly greater proportion of individuals secreted IFN-γ to L3 Ag than in Ndjokaye ($\chi^2 = 10.503$; degrees of freedom [df] = 1; $P = 0.001$). Likewise, an increased proportion of individuals secreted IL-4 in Okoumbi compared to that in Ndjokaye in response to Mf Ag ($\chi^2 = 29.302$; df = 1; $P = 0.0001$) or to adult worm Ag ($\chi^2 = 13.52$; df = 1; $P = 0.0001$). Attempts to measure IL-4 and IFN-γ in plasma that had been stored at $-20^\circ$C were unsuccessful (data not shown).

**DISCUSSION**

The key finding of this study is that the level of transmission of *L. loa* affects both parasite-specific and nonspecific T-cell proliferative responses in an area of endemicity in Gabon. PBMC from infected individuals from a village with a high level of transmission proliferated significantly less well than PBMC from a low transmission village. This result was most clear-cut when using adult worm Ag, but a similar trend was also noted with Mf and L3 Ag. Previous studies in the same area (3) correlated reduced Ag-specific proliferative responses with the presence of Mf, but in the present study this correlation only held true for the high transmission village when the

### TABLE 3. Cytokine levels in PBMC from individuals from the high and low transmission villages in response to stimulation with adult *L. loa* Ag

<table>
<thead>
<tr>
<th>Ag</th>
<th>IL-2 (U/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>L3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>13.9 ± 10.1</td>
<td>8.12 ± 5.82</td>
<td>67.4 ± 23.2</td>
</tr>
<tr>
<td>Adult</td>
<td>32.5 ± 23.3</td>
<td>65 ± 35.8</td>
<td>50 ± 25.8</td>
<td>121.3 ± 45.8</td>
</tr>
<tr>
<td>Mf</td>
<td>112.5 ± 85.4</td>
<td>130.5 ± 51.5</td>
<td>51.9 ± 28.5</td>
<td>138.7 ± 57</td>
</tr>
</tbody>
</table>

a A single individual in each group secreted IL-4 over background levels.

b Only three individuals secreted IL-4 at levels above the cutoff (10 pg/ml).

c In the high transmission village, IL-4 levels were significantly higher with adult Ag versus L3 ($P = 0.0017$) and with Mf Ag versus L3 ($P = 0.0015$); IL-5 levels were significantly higher with adult Ag versus L3 ($P = 0.0025$) and with Mf Ag versus L3 ($P = 0.0008$). In the low transmission village, IL-5 levels were significantly higher with Mf Ag versus L3 ($P = 0.0101$). Sensitivity was 10 pg/ml for IL-4 and IL-5, 7.8 pg/ml for IFN-γ, and 0.3 U/ml for IL-2.
population was analyzed as microfilaremic or amicrofilaremic. As noted previously, ≈70% of the infected population in this region is amicrofilaric but infected (as assessed by the ocular passage of an adult worm or elevated Ag-specific IgG4 levels). Despite these results, further analysis of the data did not reveal any correlation between Mf density and proliferative responsiveness at an individual level. Although there was a trend for higher proliferative responses to Ag in both microfilaremics and amicrofilaremics of the low transmission village compared to those in the high transmission village, these differences did not reach statistical significance.

Analysis of ATP in the respective villages revealed that individuals in Okoumbi were exposed annually to seven times the number of L3 compared to those in Ndjokaye. Whether the higher levels of L3 are translated into increased numbers of adult worms remains unclear, as there are no strictly quantitative tests available for measuring adult worm burden. The proportion of individuals who were microfilaric was similar in both villages, although Mf levels tended to be higher in the high transmission village. Consequently, it is not possible from the available data to definitively state whether the down-regulated proliferative response is driven by continued exposure to high numbers of L3, by the presence of increased numbers of adult worms, or by an increased Mf load in the high transmission village. Differences between the two villages in proliferative responses reached statistical significance only where adult worm Ag was used, not L3 or Mf Ag, although the same trend was apparent with these Ag. These results may suggest that the presence of adult worms is sufficient to suppress proliferative responses but that under conditions of high transmission, Mf also contribute to the down-regulation of proliferative responses. Further studies will be required to clarify this issue.

In a recent study on Wuchereria bancrofti infection in Papua New Guinea, it was possible to match patients from low and high transmission villages for adult worm burden on the basis of circulating Ag and Mf levels (13). That study showed that in patients with equivalent worm burdens, proliferative responses and IFN-γ levels were suppressed in individuals from the high transmission village and thus demonstrated that the down-regulated responses under conditions of high transmission were independent of adult worm or Mf burden.

One of the most interesting findings of the present study was that proliferative responses to PPD were also reduced in the high transmission village compared to the low transmission village. In the original studies with lymphatic filariasis, only Ag-specific responses were reported to be affected (23). However, there is a growing recognition that responses to other Ag and in some cases to mitogen may also be impaired, particularly under conditions of high transmission intensity (13). The mechanism by which bystander responses are down-regulated remains unclear, although in W. bancrofti infection high levels of serum IL-4 are correlated with reduced proliferation (13). It may be relevant that the differences in transmission levels were much more profound in the W. bancrofti study (∼63-fold) than in the present study. With O. volvulus, active infection is known to influence the immune response against non-specific stimuli (11), tetanus vaccine (7, 8), and mycobacterial antigen (27). Modulation of the immune response to irrelevant Ag may in fact be a general phenomenon in helminth infection (19).

While analysis of cytokine responses in a small number of individuals did not reveal any significant differences between the villages, a number of interesting trends were apparent. For example, L3 Ag elicited more IFN-γ and IL-2 in the high transmission village and a significantly greater proportion of individuals secreted IFN-γ to L3 Ag under conditions of intense transmission. In contrast, in vitro restimulation with Ag derived from Mf and adult worms elicited higher levels of IL-4 and IL-5 than did L3 Ag, suggesting that there are differences in the profile of cytokines elicited by specific life cycle stages. Previous studies with Brugia malayi Ag have revealed that Mf and mixed sex adult worm Ag elicited higher levels of IL-10 than adult male Ag (16). In most studies on lymphatic filarial infection, cytokine profiles are dominated by IL-4, IL-10, and IL-5 (for examples, see references 13, 18, and 29), while in L. loa infection the cytokine response appears to be less biased (3). Further studies with larger groups of L. loa-infected individuals will be required to determine whether these findings represent real differences in responses to infection.

In conclusion, our results suggest that the relationship between transmission, cellular immune responses, and the clinical or parasitological outcome of L. loa infection deserves further study. The findings of this and other studies are also of significance for vaccination programs in countries where filarial parasites are endemic (7, 8, 11, 27) and for increased understanding of the interrelationships between immune responses elicited by concurrent infections with different pathogens (12, 26).

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