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Transmission Intensity Determines Lymphocyte Responsiveness and Cytokine Bias in Human Lymphatic Filariasis[1]

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Humans living in areas where filariasis is endemic vary greatly in their exposure to mosquito-borne infective third-stage larvae (L3) of these parasitic helminths. Because the intensity of exposure to Ags affects T cell differentiation and susceptibility to parasitic infections in murine models, we compared T cell and cytokine responses in 97 residents of two villages in Papua New Guinea, where transmission intensity of *Wuchereria bancrofti* differed by 63-fold (37 vs 2355 L3 per person per year). Residents of the high transmission village had 4- to 11-fold lower proliferation and IFN-γ responses to filarial Ags, nonparasite Ag, and PHA by PBMC compared with the low transmission village ($p < 0.01$) even when subjects were matched for intensity of infection. In contrast, filarial Ag-driven IL-5 production was 5.5-fold greater ($p < 0.001$), and plasma IL-4 and TGF-β levels were 4-fold and 34% higher, respectively, in residents of the high transmission village. IL-4 and IL-10 responses by PBMC differed little according to village, and increased production of the counterregulatory cytokines IL-10 or TGF-β by PBMC did not correlate with weak proliferation and IFN-γ responses. Plasma IL-5, IFN-γ, and IL-10 levels were similar in the two villages. These data demonstrate that the intensity of exposure to L3 affects lymphocyte responsiveness and cytokine bias possibly by a mechanism that alters APC function. ***The Journal of Immunology*****, 2001, 166: 7427–7436.**

Brugia malayi and *Wuchereria bancrofti* are filarial helminths that infect ~120 million residents of the tropics. They are major causes of elephantiasis and hydroceles in Africa, Latin America, Asia, and various islands in the Pacific Ocean (1). Infection is initiated when infective third-stage larvae (L3)[3] are inoculated into the skin during blood feeding by the mosquito vector. Over a period of several months, L3 develop into sexually mature adult worms that live in afferent lymphatic vessels draining the extremities and genitalia. Fecund female worms release embryonic first-stage larvae (microfilariae or mf) into the bloodstream, from where they may be ingested by mosquitoes and continue development to L3. Individuals with blood-borne microfilariae (mf^+) and/or circulating filarial *W. bancrofti* Ag (CAg^+) exhibit strong type 2 cytokine production (e.g., IL-4, IL-5, and IL-13) and weak type 1 Ag-specific immunity (lymphocyte and IFN-γ proliferation). In contrast, uninfected (mf^- CAg^-) individuals who are nevertheless presumably repeatedly exposed to mosquito-borne L3 characteristically have strong type 1 immunity with prominent $CD4^+$ T cell IFN-γ responses (2–4). T cell hyporesponsiveness (weak filarial Ag-specific lymphocyte proliferation and cytokine responses) by mf^+ individuals has been attributed to active suppression by the counterregulatory cytokines IL-10 and TGF-β (5, 6). This spectrum of T cell cytokine responses may contribute to lymphatic pathology because, at least in some endemic areas, mf^+ persons with depressed lymphocyte proliferation and poor IFN-γ responses tend to be free of clinically overt elephantiasis (7–10).

The variables that favor induction and maintenance of type 2 immunity have been the subject of many investigations. Based in large part on studies of murine systems, these may include Ag affinity for the TCR (11), the dose or route by which Ag is administered (12, 13), costimulatory molecule interactions (14), the prevailing in vivo cytokine milieu (15), and the nature of the APC that initially encounters a microbial pathogen or new Ag (e.g., dendritic vs B cell) (16). Helminth Ags themselves have been shown to selectively differentiate naive human T cells toward a type 2 response (17). Examination of immunologically intact and genetically modified mice exposed to Ags of parasitic helminths and protozoa has led to fundamental insights into how the cytokine milieu and interaction with APC contribute to the differentiation and maintenance of type 1 and type 2 patterns of T cell immunity (18, 19). However, the in vivo factors that underlie induction and maintenance of analogous immune responses in human parasitic infections have been more difficult to define because multiple variables that may affect host immunity cannot readily be controlled. These include genetic heterogeneity between and within human populations, age-related changes in Ag-specific immunity, immune modulation that may occur during the course of chronic infections that persist for years, neonatal immune tolerance or sensitization resulting from maternal infection during pregnancy, and variability in exposure to infective-stage parasites (20–27).

Our investigations of bancroftian filariasis in East Sepik Province, Papua New Guinea have been aimed at understanding how heterogeneity in transmission influences infection and disease burdens and the efficacy of mass chemotherapy as a control strategy. Examination of residents of this area may have several advantages for discerning the evolution of filarial-specific immunity and its

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[3] Abbreviations used in the paper: L3, infective third-stage larvae of *W. bancrofti*; mf, microfilaremia; CAg, circulating *W. bancrofti* Ag; MFE, microfilarial Ags; BmA, *Brugia malayi* adult worm Ags; SLO, streptolysin O.

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relationship to infection and lymphatic pathology. First, human populations in this and other remote areas of Papua New Guinea have been and remain culturally and linguistically isolated (28, 29). This limited admixture with other populations has maintained genetic homogeneity relative to many other filariasis endemic areas of the world. Second, access to anti-filarial drugs and bed nets has until recently been limited, so self-treatment and inconsistent exposure to L3 are less likely to obfuscate interpretation of immunologic studies. Third, given the fact that transmission of *W. bancrofti* among various villages in East Sepik Province is heterogeneous (30, 31), the relationship between this ecologic variable and host immunity can be appreciated. We previously reported that transmission intensity, quantified as the annual transmission potential (the number of L3 to which an individual is theoretically exposed per year) of the local mosquito vector *Anopheles punctulatus*, correlated positively with the village-specific prevalence and intensity of microfilaremia (31). Although transmission intensity varies during the year depending on rainfall, the overall level of transmission in a community probably remains relatively stable over years because the local ecology determines the patterns of transmission (30). High transmission villages are located near rivers and streams that form good breeding habitats for the anopheline vector. Residents of low transmission villages often live on hilltops away from water.

In this study, filarial Ag-specific T cell proliferation and cytokine responses by residents of two villages separated by a distance of <20 km were compared. Residents of both villages belong to the same linguistic group and include both children and adults. Because residents in both villages could be identified with similar parasite burdens estimated by mf and CAg levels, the major difference between the two villages relevant to *W. bancrofti* infection was that transmission intensity differed by 63-fold.

Materials and Methods

Study population

Ninety-seven residents of two villages in East Sepik Province, Musendi and Yauarang, were enrolled in the study. Musendi and Yauarang are located in a geographically isolated tropical rainforest where paved roads, telecommunications, and public power or sanitation sources do not exist. Major human activities include subsistence agriculture and work on cooperative coffee plantations. Residents of both villages belong to the Urat linguistic group (http://www.sil.org/ethnologue/countries/Papu.html). Based on mitochondrial DNA sequence polymorphisms, Urat speakers are thought to have originated from an ancestral founder population in the highlands of Papua New Guinea (Ref. 29 and our unpublished data).

Demographic information (age, sex, household) was collected as described (30–33). Physical examination for clinical signs of lymphatic disease attributable to *W. bancrofti* infection was performed according to recommendations outlined by the World Health Organization (34). Anti-filarial drugs or bed nets were not available before this study was instituted.

The procedure for informed consent and ethical clearance for the study were approved by the Medical Research Advisory Committee of the Government of Papua New Guinea and the Human Investigations Institutional Review Board of University Hospitals of Cleveland and Case Western Reserve University. All subjects were treated with a single dose of the anti-filarial drug diethylcarbamazine (6 mg/kg body weight) after blood was drawn for immunologic studies.

Measurement of infection status

Microfilaremia was determined by Nuclepore filtration (Nuclepore, Pleasanton, CA) of a 1-ml blood sample obtained between 10 p.m. and 2 a.m. (35). The results were log transformed and expressed as the geometric mean number of mf/ml blood per village. To estimate worm burden independent of microfilaremia, the level of CAg was determined by sandwich ELISA based on mAb Og4C3 (TropBioMed, Townsville, Australia) (36). This assay is specific for *W. bancrofti* and does not cross-react with Ags of common geohelminths. Results are expressed as the OD of triplicate determinations of plasma diluted 1/4 or 1/40 (the higher dilution was used if results were positive at a 1/4 dilution).

Entomologic monitoring

Anopheles punctulatus and *A. koliensis* are the only vectors of *W. bancrofti* in this area of East Sepik Province (30). The annual transmission potential in each village was quantified by dissection of human-biting mosquitoes for *W. bancrofti* L3 for 4 nights per month for 1 year as described (30–33). *A. punctulatus* was the only mosquito species found to harbor L3.

Filarial Ags

Brugia malayi adult worm (BmA) and microfilarial Ags (MFE) were prepared as saline extracts of parasites harvested from jirds (37). The concentration of endotoxin in these preparations was <0.5 ng/ml, which is 5- to 50-fold less than that required for LPS stimulation of cytokine production by human lymphocytes.

In vitro cytokine assays

All studies were performed using freshly isolated PBMC separated from heparinized venous blood by density gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, 25 mM HEPES, and 80 μg/ml gentamicin (BioWhittaker, Walkersville, MD; C-RPMI). PBMC were cultured at 2×10^6 cells/ml in C-RPMI in a total volume of 1 ml. In a subset of individuals, 1, 5, 10, 20, and 25 μg/ml BmA and 1, 5, 10, and 20 μg/ml MFE were used to stimulate PBMC from individuals from endemic areas ($n = 6$) and nonendemic areas ($n = 5$) from the same lots of Ag. Optimal concentrations (maximal cytokine production by sensitized individuals and minimal nonspecific production by control subjects) were 20 μg/ml BmA and 10 μg/ml MFE. The nonparasite Ag streptolysin O (SLO, 5 μg/ml) and mitogens PMA (50 pg/ml) plus ionomycin (1 μg/ml; Calbiochem, La Jolla, CA) or PHA (2 μg/ml; Burroughs-Wellcome, Durham, NC) were added to duplicate or triplicate cultures depending on the availability of cells. Cells were incubated at 37°C in 5% CO_2. Supernatants were collected at 48 and 72 h and immediately frozen at −70°C for subsequent determination of cytokine production.

Cytokines were measured by ELISA and expressed in picograms per milliliter by interpolation from standard curves based on recombinant lymphokines using Abs and methods described previously (5, 24, 25). Ab pairs for capture and detection, respectively, were as follows: IL-5, TRFK5 and 5D10 (PharMingen, San Diego, CA); IL-4, 8D4 and 25D2 (PharMingen); IFN-γ, M-700, and M-701 (Endogen, Cambridge, MA); IL-10, 18551D and 18652D (PharMingen). All detection Abs were biotinylated. The limits of detection were: IL-5, 18 pg/ml; IL-4, 16 pg/ml; IFN-γ, 10 pg/ml; and IL-10, 16 pg/ml. Lymphocyte proliferation was measured using quadruplicate aliquots of cells at 2×10^5/200 μl culture medium (RPMI 1640 containing 10% pooled human sera and 80 μg/ml gentamicin). TGF-β1 was assayed as follows: the coating Ab was mAb MAB240 (R&D Systems, Minneapolis, MN) at 2 μg/ml followed by the detecting biotinylated mAb BAF24 at 0.1 μg/ml (R&D Systems). Before assay for TGF-β, samples were activated by a 10-min incubation with 10 μl of 1 N HCl per 50 μl sample followed by neutralization with 1.2 N NaOH/0.05 HEPES. [^{3}H]Thymidine incorporation was measured by addition of the radiolabel (1 μCi/well) for the final 18 h of a 96-h incubation period at 37°C in 5% CO_2 in air.

Ag-driven cytokine production is expressed as net production (cultures with Ag minus no Ag cultures). Because the constitutive cytokine production was high in many individuals, significant net cytokine response was considered positive only if the net value was at least 50% greater than the mean value of spontaneous cultures. If the replicate spontaneous cultures were discordant by >25%, then an Ag-driven value was considered positive only if it exceeded the no-Ag controls by >2-fold.

Plasma cytokine levels

Cytokine levels were measured in plasma diluted 1/1 and 1/5 with RPMI 1640. Ab pairs used to quantify cytokines in plasma by two-site ELISA were identical with those for culture supernatants.

It was first verified that the plasma cytokine ELISA detected authentic cytokine and not nonspecific reactivity with plasma proteins. Aliquots of plasma from filariasis subjects with the highest detectable cytokine levels ($n = 5$ for each cytokine) were preincubated overnight at 4°C with 10 μg/ml biotinylated polyclonal anti-human cytokine Ab (anti-IL-4, anti-IL-5, anti-IL-10, and anti-IFN-γ; R&D Systems). Streptavidin-coated magnetic beads (Pierce, Rockford, IL) were then added to remove the polyclonal Ab. In all cases, cytokine in plasma was no longer detectable by ELISA. Parallel experiments were performed using samples from three North American subjects who did not have detectable cytokine in their plasma. When aliquots of plasma from these individuals were preincubated

Table I. *Transmission intensity and filarial infection status of residents of Musendi and Yauarang villages in East Sepik Province, Papua New Guinea*

	Musendi	Yauarang
Annual transmission potential	37 L3/person/yr	2355 L3/person/yr
No. of subjects	48	49
Mean age (range) in years	30 (9–63)	33 (7–57)
No. of mf^+ persons	18 (37%)	36 (73%)
Geometric mean mf/ml	6	58
Range of microfilaremia	11–6151	1–5161
No. of CAg^+ persons	28 (58%)	47 (96%)
Geometric mean ± SD CAg level (OD)	0.096 ± 0.402	0.813 ± 0.272

with anti-cytokine Ab and subsequently spiked with recombinant IL-4, IL-5, IL-10, or IFN-γ (1 ng/ml and serial 2-fold dilutions thereof), cytokine was detectable. These results indicate that anti-human cytokine Abs used in the preincubation step did not interfere with the ability of the two-site ELISA to detect authentic cytokine.

IL-4 bioactivity in plasma

One hundred microliters of various dilutions of plasma was added to the IL-4-dependent CT.4S human T cell clone (provided by Alan Levine, Case Western Reserve University) suspended at 2.5×10^4 cells/200 μl C-RPMI. [^{3}H]Thymidine incorporation was measured by addition of the radiolabel (1 μCi/well) for the final 18 h of a 72-h incubation period at 37°C in 5% CO_2 in air. Ten micrograms per milliliter of neutralizing anti-human IL-4 (mAb 25D2; PharMingen) and an Ig isotype-matched control were added in parallel to triplicate cultures. Radioactivity incorporation was measured with a Packard Matrix 96 gamma counter (Meriden, CT).

Statistics

The significance of association between infection status (mf and CAg level) and transmission intensity was determined by the χ^2 test. The association between cytokine responses and infection status or transmission intensity was determined by Student's *t* test using log-transformed values. Values of $p < 0.05$ were considered significant.

Results

Infection and disease status in residents of low and high transmission villages

The annual transmission potential of *W. bancrofti* in Musendi and Yauarang differed by 63-fold (37 vs 2355 L3/person/year, respectively). The mean age of study subjects from the two villages was similar. A lower proportion of mf^+ and CAg^+ persons lived in

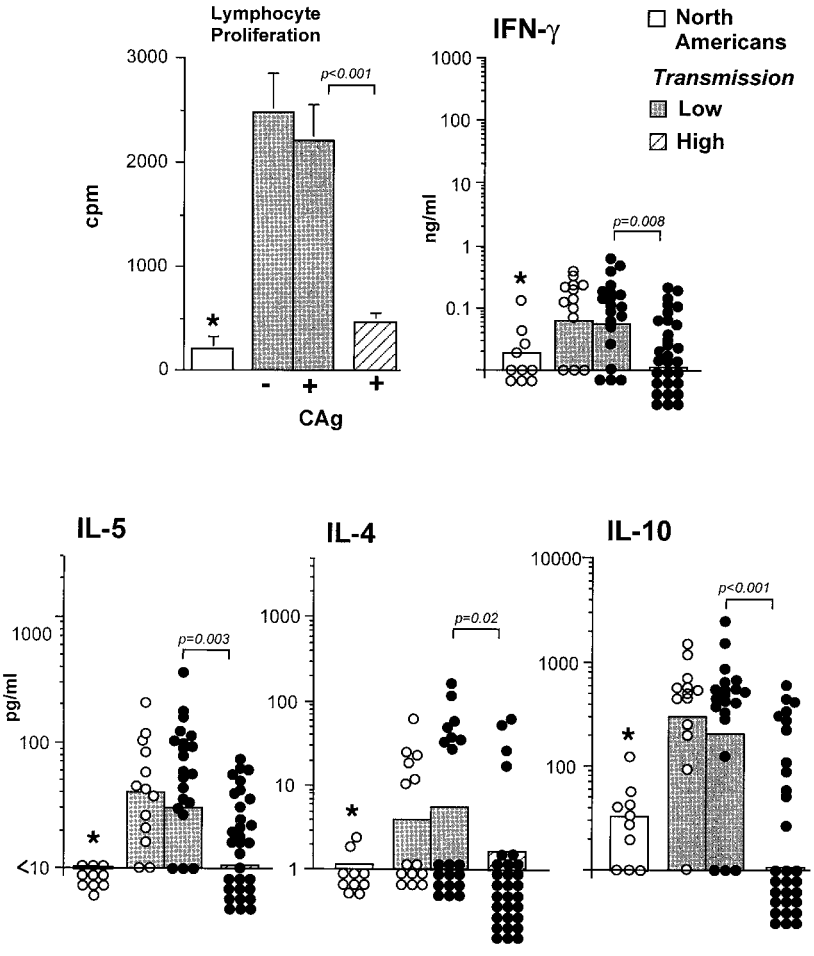

FIGURE 1. Papua New Guinean residents of the high vs low transmission village have diminished basal [^{3}H]thymidine uptake and constitutive cytokine production. Open bars represent adult North Americans who have never traveled to filarial endemic regions, solid bars represent individuals from the low transmission village (Musendi), and hatched bars represent residents from the high transmission village (Yauarang). For cytokine production, open circles (○) denote CAg^- (uninfected) individuals, and solid circles (●) denote CAg^+ (infected) individuals. Each circle represents the mean value of duplicate or triplicate cultures from a single individual as described in *Materials and Methods*. Differences in spontaneous lymphocyte proliferation and cytokine production between residents of the low transmission village and North Americans and residents of the high transmission village are highly significant ($p < 0.001$). Basal proliferation response in residents of the high transmission village is 2-fold higher than that in North Americans ($p < 0.05$).

Musendi than Yauarang, 37 vs 73% and 58 vs 96%, respectively (Table I). The average parasite burden estimated by the level of circulating Og4C3 Ag was higher for residents of Yauarang. Only two individuals in this village were not infected, i.e., mf^- CAg^-. Three adults in Yauarang had grade II-III lymphedema (elephantiasis) of the leg. No other disease manifestations of filariasis were observed.

Constitutive lymphocyte proliferation and cytokine production are suppressed in the high transmission village

Basal [^{3}H]thymidine uptake and constitutive cytokine production (medium alone) were evaluated as a measure of in vivo lymphocyte activation to filarial infection (Fig. 1). Basal [^{3}H]thymidine uptake and constitutive cytokine production were elevated in the majority of residents of the low transmission village compared with North American controls. In contrast, constitutive [^{3}H]thymidine uptake and IFN-γ, IL-5, IL-4, and IL-10 production by residents of the high transmission village were uniformly lower than those of subjects from the low transmission village. There was no difference in the amount of basal [^{3}H]thymidine uptake and constitutive cytokine production when individuals were stratified by the intensity of infection. This reduced basal [^{3}H]thymidine uptake and constitutive IFN-γ and IL-10 production are consistent with diminished responsiveness to filarial Ag, but stand in contrast to the enhanced BmA-driven IL-5 production in parallel cultures of PBMC from individuals residing in the high transmission village (Fig. 2).

Residents of the high vs low transmission village have a bias toward Th2-type immune response

The dramatic difference in transmission intensity likely corresponds to many more developing larvae in the skin and draining lymph nodes of residents in the high transmission village (31). To examine the impact of these larvae and their Ag products on host immune responses, filarial and nonfilarial Ag-driven lymphocyte proliferation and cytokine production by residents of the two villages were compared (Fig. 2). BmA-driven lymphocyte proliferation was greatest in uninfected (CAg^-) residents of Musendi, the low transmission village (Fig. 2, *upper left panel*). Proliferation responses decreased progressively among Musendi residents who were mf^- CAg^+ and mf^+ CAg^+, respectively. The lowest values for BmA-driven proliferation were observed for residents of Yauarang, the high transmission village, regardless of whether they were mf^- or mf^+ (all shown were CAg^+). To exclude the possibility that the filarial Ag preparations may nonspecifically stimulate cytokine production, PBMC from North American residents who had not been exposed to filariasis (the same subjects shown in Fig. 1) were cultured with the same Ag preparations shown in Fig. 2. No significant cytokine production was observed (data not shown).

With respect to the nonfilarial Ag SLO (Fig. 2, *lower left panel*), lymphocyte proliferation responses were uniformly lower in residents of the high transmission village. PBMC proliferation responses to BmA or SLO for the three individuals with elephantiasis were similar to other residents of the high transmission village without clinical signs of lymphatic disease.

Results for IFN-γ and IL-5 responses are presented in Fig 2. (*right panels*). For this comparison, individuals were grouped together solely on the basis of CAg status because the levels of cytokines produced were equivalent among CAg^+ persons regardless of whether they were mf^- or mf^+ (data not shown). BmA- (*upper panels*) and SLO-driven (*lower panels*) IFN-γ production

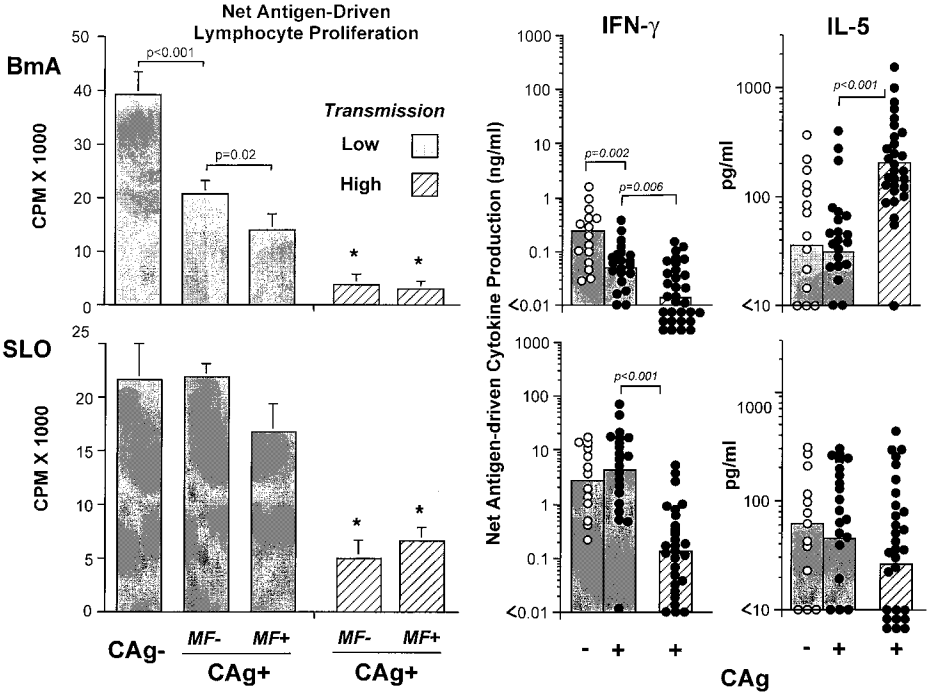

FIGURE 2. Residents of the high vs low transmission village have impaired lymphocyte proliferation responses and IFN-γ release and increased filarial Ag-specific IL-5 production. Solid bars represent individuals from the low transmission village (Musendi) and hatched bars denote residents from the high transmission village (Yauarang). Infection status was determined by measurement of CAg (Og4C3 sandwich ELISA) (35, 36), and mf level by Nuclepore filtration (34). Net proliferation responses (cultures with Ag minus no Ag cultures) of quadruplicate aliquots of PBMC stimulated with BmA or SLO were measured as described in *Materials and Methods*. Bars represent the mean ± SE of the group. The mean cpm for PBMC from residents of the high transmission village was significantly less (denoted by an asterisk) than that of every group from the low transmission village ($p < 0.001$–0.01). For net (Ag-driven minus spontaneous) IFN-γ and IL-5 production, open circles (○) denote CAg^- (uninfected) individuals, and solid circles (●) denote CAg^+ (infected) individuals. Each circle represents the mean value of duplicate or triplicate cultures from a single individual as described in *Materials and Methods*. Bars indicate geometric means. Significance of differences between groups is shown in the figure.

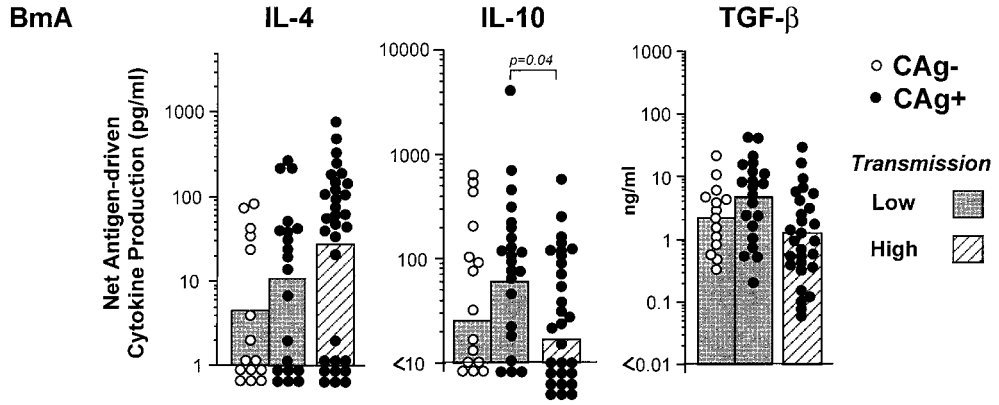

FIGURE 3. The intensity of exposure is not associated with levels of filarial Ag (BmA)-driven IL-4, IL-10, and TGF-β production by Papua New Guinea study subjects. Filled bars represent individuals from the low transmission village (Musendi), and hatched bars denote residents from the high transmission village (Yauarang). Values represent net cytokine production as described in Figs. 1 and 2. Any significance difference between groups is shown in the figure.

was significantly lower in residents of the high transmission village. The opposite pattern was observed for BmA-driven IL-5, i.e., CAg^+ residents of the high transmission area had the strongest responses, indicating an expanded population of filarial Ag-reactive lymphocytes producing this type 2 cytokine. SLO-driven IL-5 production was similar among the three groups. IFN-γ and IL-5 production in response to MFE showed a similar pattern to that for BmA, although the amounts of cytokine produced were lower (data not shown). These observations show that intense exposure to filarial larvae or their Ag products (i.e., residency in the high transmission village) is associated with a bias toward filarial Ag-driven IL-5 and away from IFN-γ production and lymphocyte proliferation.

To examine the basis for impaired lymphocyte and IFN-γ responses by infected CAg^+ individuals from both villages, production of the immunoregulatory cytokines IL-4, IL-10, and TGF-β was evaluated (Fig. 3). BmA-driven IL-4 and TGF-β were statistically equivalent for infected individuals from both villages. In contrast, BmA-driven IL-10 production was lower among infected subjects in the low transmission village. SLO-, PMA/ionomycin-, and PHA-driven IL-4, IL-10, and TGF-β production were similar among the groups (data not shown) except for SLO-driven IL-4 release, which was lower for infected individuals in the high vs low transmission village (geometric mean $\pm$ SE = 12 $\pm$ 6 vs 3 $\pm$ 9 ng/ml, $p = 0.02$). Therefore, these results indicate that impaired lymphocyte proliferation and IFN-γ responses in residents of the high transmission village do not correlate with increased production of the putative cross-regulatory cytokines IL-4, IL-10, or TGF-β.

Relationship of lymphocyte proliferation and in vitro cytokine production to intensity of infection

Because the CAg levels are greater in residents of the high transmission village, it is possible that the weak lymphocyte proliferation and IFN-γ production following stimulation with both filarial and nonfilarial Ags may be associated with high parasite burdens. To examine this possibility, responses by residents of the low and high transmission area with similar mf intensities and CAg levels

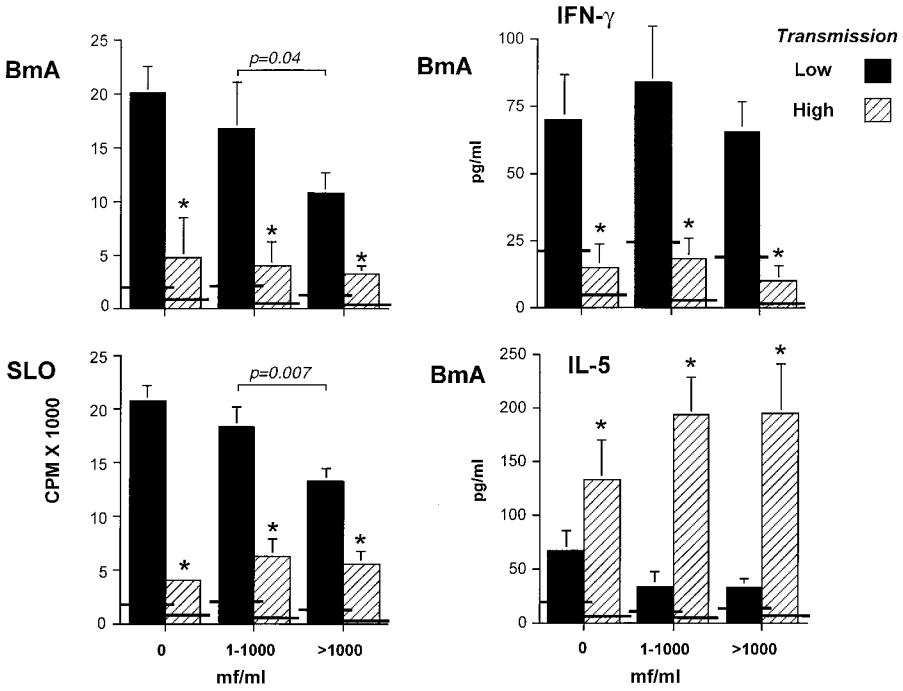

FIGURE 4. Increased parasite worm burdens estimated by peripheral blood microfilaremia level does not account for bias toward filarial Ag-driven IL-5 and away from IFN-γ production and lymphocyte proliferation to filarial BmA and SLO in the high vs low transmission village. Solid bars represent individuals from the low transmission village (Musendi), and hatched bars indicate residents from the high transmission village (Yauarang). Bars represent mean $\pm$ SEM for lymphocyte proliferation and geometric mean $\pm$ SEM for cytokine production. Horizontal bars through each column represent mean spontaneous lymphocyte proliferation or geometric mean spontaneous cytokine production. CAg^+ persons were categorized according to mf levels of 0, 1–1000, or >1000 parasites per milliliter. Asterisks (*) denote a significant difference between the two groups ($p < 0.001$ for all comparisons of lymphocyte proliferation and $p <$ 0.05–0.001 for cytokine production). Values of p are given for significant differences between groups in the low transmission village stratified according to mf levels.

were compared. When levels of microfilaremia were stratified as 0, 1–1000, and >1000 parasites/ml, BmA- and SLO-driven lymphocyte proliferation responses were consistently lower for residents of the high transmission village (Fig. 4, *left panels*). Similarly, BmA-driven IFN-γ production was reduced among individuals in the high transmission village irrespective of mf intensity (Fig. 4, *upper right panel*). SLO-driven IFN-γ production was 2- to 8-fold lower among subjects residing in the high vs low transmission village, although statistical significance was observed only for individuals with >1000 mf/ml (geometric mean = 842 ± 103 vs 111 ± 129 ng/ml, $p = 0.01$). In contrast to IFN-γ, BmA-driven IL-5 production was consistently greater for residents of the high transmission village, particularly among mf^+ individuals (Fig. 4, *right panels*). When subjects were stratified according to CAg level (OD <0.60, 0.60–1.00, or >1.00), lymphocyte proliferation and IFN-γ responses were also consistently weaker and BmA-driven IL-5 responses stronger in the high vs low transmission village (data not shown). Therefore, the bias toward filarial Ag-driven IL-5 and away from IFN-γ production and lymphocyte proliferation in residents of the high compared with low transmission village was independent of host parasite burden as judged by mf and CAg status.

PHA- but not PMA/ionomycin-driven lymphocyte proliferation and cytokine production are suppressed in the high vs low transmission villages

The depressed basal [^{3}H]thymidine uptake and constitutive cytokine production and impaired recall responses to nonfilarial Ags indicate a possible defect in APC function. To examine this possibility, T cell responses to the APC-dependent T cell mitogen PHA and APC-independent mitogen PMA/ionomycin were compared for CAg^+ residents of the high and low transmission village (Fig. 5). PHA-driven lymphocyte proliferation and IFN-γ production were ~10-fold lower in the high transmission village. Addition of a higher concentration of PHA, e.g., 10 μg/ml, did not reverse the depressed lymphocyte proliferation and IFN-γ production by PBMC from residents of the high transmission village (data not shown). There was no significant difference between the two villages in PHA-driven IL-5 production. In contrast, PMA/ionomycin-driven lymphocyte proliferation and cytokine production was equivalent for residents of both the high and low transmission villages. These results suggest a defect in the ability of APC to deliver costimulatory signals to Th1 type but not Th2 type lymphocytes.

Individuals from the high transmission village have elevated plasma IL-4 levels

It is possible that several of the cytokines examined in this study may be produced by cells that are absent from or poorly represented in PBMC (e.g., basophils and mast cells). Therefore, cytokine levels in plasma were measured to gain additional insight into in vivo responses. The geometric mean plasma levels of IL-10, IFN-γ, TGF-β, and IL-5 were generally higher in Papua New Guineans than North Americans (Table II). The only significant difference among the various groups was a 34% higher mean TGF-β level in mf^+ CAg^+ residents of the high transmission village. The mean IL-5 level was also lowest in mf^+ CAg^+ residents of the high transmission village, but this value was not significantly different from the other groups ($p > 0.05$). However, fewer residents of the high transmission village had detectable plasma IL-5 compared with persons living in the low transmission village (1 of 33 vs 22 of 37, $p < 0.001$). Three of 35 North Americans had IL-5 detectable in their plasma.

Results for plasma IL-4 are presented in Fig. 6. There were striking differences compared with the other cytokines. First, the mean IL-4 level of North Americans and uninfected (mf^- CAg^-) Papua New Guineans living in the low transmission village were similar. Less than 10% of subjects in either group had detectable IL-4. Second, infected (CAg^+ and mf^- or mf^+) residents of either the low or high transmission village had higher values than CAg^- Papua New Guineans or North Americans ($p < 0.001$). Third, among Papua New Guineans who were CAg^+, the mean plasma IL-4 level was 4-fold greater in residents of the high compared with low transmission village ($p = 0.02$).

To confirm that IL-4 measured in the two-site ELISA represented bioactive cytokine, plasma samples from three Papua New Guineans from the high transmission village with elevated IL-4, four CAg^+ subjects without detectable IL-4 (two infected subjects

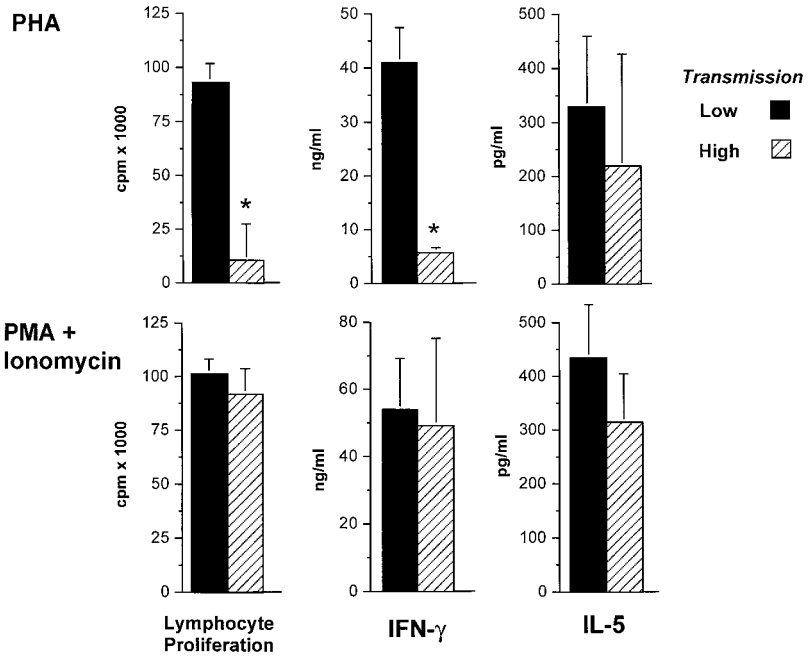

FIGURE 5. Papua New Guinean residents of the high transmission village have impaired PHA- but not PMA/ionomycin-driven lymphocyte proliferation responses and IFN-γ production compared with residents of the low transmission village. Solid bars indicate CAg^+ residents of the low transmission village, and hatched bars denote residents of the high transmission village. Bars represent net mean ± SEM (for lymphocyte proliferation) and net geometric mean ± SEM for cytokine production. Asterisks (*) denote a significant difference between two groups, $p < 0.001$ for both comparisons.

Table II. *Plasma IFN-γ, IL-5, IL-10, and TGF-β levels in North American and Papua New Guinea study subjects*

	IFN-γ (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	TGF-β (ng/ml)
CAg^- North Americans ($n = 35$)	1.2[a] (0–344)	1.5 (0–46)	6.4 (0–301)	4.8 (1.6–7.2)
CAg^- residents of low transmission village ($n = 14$)	67 (0–344)	16.1 (0–200)	217 (0–789)	7.9 (5.6–11.7)
CAg^+ residents of low transmission village ($n = 20$)	122 (0–704)	10.2 (0–315)	542[b] (54–3151)	8.0 (2.3–13.3)
CAg^+ residents of high transmission village ($n = 28$)	167 (0–863)	0.1[c] (0–27)	387 (108–4729)	10.7[d] (6.6–16.8)

[a] Results are presented as the geometric mean with range in parentheses.
[b] $p = 0.036$ vs CAg^- persons in low transmission village.
[c] $p < 0.05$ vs CAg^- or CAg^+ residents of low transmission village.
[d] $p = 0.007$ vs CAg^+ persons in low transmission village.

from the low transmission village and two from the high transmission village), and three North Americans without detectable IL-4 were examined for their ability to support proliferation of the IL-4-dependent CT.4S T cell clone. Only plasma from individuals with immunoreactive plasma IL-4 supported proliferation. Results of an experiment using plasma from one representative Papua New Guinean without immunoreactive IL-4 and another with immunoreactive IL-4 are described in Table III. These results suggest that increased IL-4 release from non-T cells contributes to altered immune responses observed among infected individuals living in the high transmission village.

Discussion

A complex sequence of interactions critical to the success of lymphatic filariae as human parasitic helminths occurs shortly after L3 are deposited on the skin during blood feeding by the mosquito vector. The larvae must quickly penetrate the dermis and establish residence in the local lymphatics, a tissue niche that is absolutely essential for their survival and subsequent development (38–41).

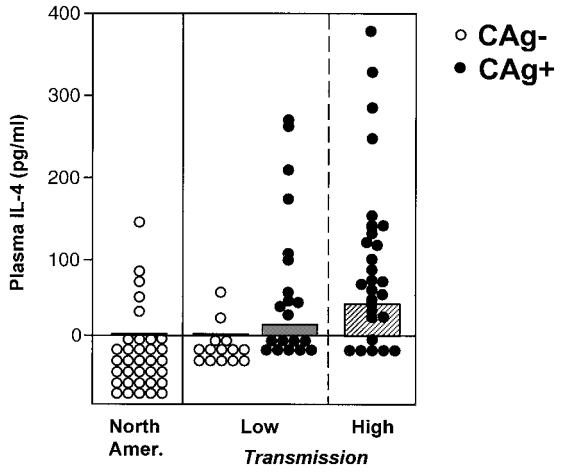

FIGURE 6. Plasma IL-4 levels are elevated in Papua New Guinean residents of the high compared with low transmission villages. Plasma IL-4 was detected by immunoassay as described in *Materials and Methods*. Open circles (○) denote uninfected (CAg^-) subjects. Solid circles (●) denote infected (CAg^+ and mf^- or mf^+) individuals. Each circle represents the mean value for two or more determinations of serial 2-fold dilutions of plasma from one individual. The significance of difference in the mean level for CAg^+ vs CAg^- subjects from the low transmission village and CAg^+ persons from the low vs high transmission village was $p < 0.05$.

Nine to 14 days later and after L3 have shed their cuticle (i.e., molted), fourth-stage larvae appear and differentiate over 6–9 mo into sexually mature adult worms. When an adequate number of female and male worms accumulate in the lumen of afferent lymphatic vessels, mf are produced and released by fecund female worms. The number of mf eventually increases to become detectable in the bloodstream. During the initial or prepatent period of infection, large amounts of soluble filarial Ags are excreted and secreted by developing parasites, especially during the molting process (42, 43). These Ags are presumably processed by APC in and near lymphatics draining the extremities and other anatomic sites where the parasites have migrated. In persons repeatedly exposed to L3, a situation that is presumably the norm for residents of endemic areas where transmission is perennial, T and B cell responses may be directed at one or more stages of the parasite life cycle, each of which has unique as well as shared antigenic determinants (44). Until recently, unequivocal ascertainment of infection status could only be obtained by the demonstration of bloodborne mf. However, assays that detect CAg in the absence of microfilaremia have recently become available and enabled more precise classification of human infection such that nonpatent (mf^- CAg^+) or light infections can be diagnosed (45, 46). Although it is not possible to quantify directly the relationship between CAg

Table III. *IL-4 bioactivity in plasma of Papua New Guinea study subjects without or with IL-4 detectable by immunoassay*

Plasma Dilution ± Anti-IL-4 mAb[a]	[^{3}H]Thymidine Incorporation by CT4.S T Cell Clone (cpm)	
	No immunoreactive IL-4 in plasma[b]	Immunoreactive IL-4 in plasma[b]
1/2 + rat IgG	442 ± 48[c]	1841 ± 144
1/2 + anti-IL-4	303 ± 61	637 ± 86
1/4 + rat IgG	255 ± 33	641 ± 66
1/4 + anti-IL-4	192 ± 17	223 ± 9
1/8 + rat Ig	117 ± 28	333 ± 54
1/8 + anti-IL-4	129 ± 45	355 ± 71

[a] Plasma diluted 1/2, 1/4, and 1/8 was mixed with 10 μg/ml rat isotype-matched Ig (IgG) or rat anti-human IL-4 mAb (25D2; Pharmingen). [^{3}H]Thymidine incorporation by the CT4.S T-cell clone was conducted as described in *Materials and Methods*.
[b] Plasma from a Papua New Guinean subject with no IL-4 and a subject with 383 pg IL-4/ml detectable by immunoassay were compared for their ability to support proliferation of the IL-4-dependent CT4.S T cell clone as described in *Materials and Methods*. Similar results were obtained with plasma from other Papua New Guinean subjects without and with immunoreactive IL-4 ($n = 3$).
[c] Mean ± SD of quadruplicate cell cultures.

level and filarial worm burden in humans, available data suggest that the former can be used as an indirect measure of the latter. First, the level of circulating phosphorylcholine-containing Ag, a carbohydrate moiety that is abundant in filariae and which was the basis of a first generation CAg assay (47, 48), correlates positively with the number of adult *B. malayi* worms recovered at necropsy of experimentally infected jirds (49). Second, the amount of serum Og4C3 Ag, which is specific for *W. bancrofti* infection, increases with mf density and age in residents of endemic areas (Refs. 50 and 51 and our unpublished data).

Studies conducted in India and Indonesia over the past 20 years demonstrated that weak lymphocyte proliferation and IFN-γ responses to filarial Ags characterize T cell immunity in mf^+ individuals with bancroftian and brugian filariasis. In contrast, mf^- persons generally have strong parasite-specific proliferation responses and type 1 immunity (7–10). More recent observations indicate that impaired lymphocyte proliferation and IFN-γ responses correlate more closely with infection status defined by the presence of Og4C3 CAg rather than microfilaremia (2–4). The observations reported here confirm the association between CAg status and filarial-specific proliferation and IFN-γ responses but also suggest that transmission intensity and not simply coexisting infection status is a major determinant of the latter aspects of host immunity (Figs. 1 and 2). Because lymphocyte hyporesponsiveness in residents of the high transmission village extended to the nonfilarial Ag SLO and the T cell- and APC-dependent mitogen PHA, the current findings raise the possibility that intense exposure to L3 and preadult stages of *W. bancrofti* up-regulate counterregulatory cytokines or production of immune complexes, both of which may suppress lymphocyte activation or impair the function of APC. Alternatively, excretory/secretory molecules of developing larvae may directly suppress the function of Ag-processing cells or accessory pathways of T cell activation. It is not yet clear whether either or both of these possibilities is operative. With respect to the first, there were not striking differences in BmA- or mitogen-driven IL-4, IL-10, or TGF-β production according to infection status or village of residence. This observation contrasts a previous report that showed that IL-10 and TGF-β contribute to lymphocyte hyporesponsiveness in mf^+ subjects (5), although other studies have also failed to demonstrate that IL-10 modulates T cell responses to filariae (52). In the context of modulation by immune complexes, we found that reduced constitutive production of IL-4 and IL-5 by PBMC from residents of the high transmission village was totally reversed by addition of filarial or nonfilarial Ags (Figs. 1 and 2). Addition of exogenous Ag may have increased the ratio of Ag to Ab and thereby displaced immune complexes from Fc receptors of APC (53). This mechanism could be examined by addition of high affinity anti-Fc receptor Abs to PBMC cultures (54) to determine whether constitutive cytokine production is enhanced. With respect to the alternative possibility whereby APC function is directly impaired by exposure to parasite larvae, filarial Ag-driven IFN-γ production and mitogen-induced lymphocyte proliferation were noted to be suppressed in mice inoculated with *Brugia* L3 (55). Because L3, fourth-stage larvae, and immature adult worms are obligatory parasites of lymphatic vessels, it is possible that molecules secreted by these tissue-invasive helminths subvert the function of local or even anatomically distant APC (56, 57). Further investigation of this issue will require isolation of APC from the skin of infected individuals and assessment of their level of activation and expression of costimulatory molecules such as CD40, CD80, and CD86. It may also be informative to determine whether filarial larvae themselves or molecules released during the molting process modify the function of APC isolated from the dermis of uninfected individuals.

The molecular basis of the propensity for filariae and other helminths to induce bias toward type 2 immunity is poorly understood. Helminths have abundant 'ladder' proteins with amino acid repeat sequences similar to those of environmental and venom allergens (58). They also contain carbohydrates that preferentially induce IL-10 production by innate immune cells and up-regulate CD28-CTLA4 or other costimulatory pathways that favor type 2 T cell differentiation (59–61). In this context, a secreted product of the animal filarial parasite *Acanthocheilonema viteae* denoted ES-62 (42) has been reported to signal murine dendritic cells to drive differentiation of OVA-specific TCR-transgenic T cells to the type 2 cytokine phenotype (62). In this study, we focused on evaluating the in vivo variables that favor differentiation of type 2 cells. First, the relationship between BmA-driven in vitro production of IL-4 and IL-5 by PBMC, infection status, and transmission intensity was examined. Whereas BmA-specific IL-4 and IL-5 responses by residents of the low transmission village were homogeneous and did not segregate according to infection status, persons in the high transmission village had stronger type 2 cytokine responses, particularly IL-5 (Fig. 2). Second, an estimate of the in vivo cytokine milieu was obtained by measurement of plasma cytokine levels. There were no differences in plasma IFN-γ, IL-5, and IL-10 levels between infected and uninfected study subjects, all of which were greater than uninfected North Americans (Table III). The one exception was IL-5, which was detectable in the plasma of fewer residents of the high than low transmission village (4 vs 59%). We speculate that the apparent dissociation between in vitro IL-5 production by PBMC and plasma IL-5 levels is due to an increased number of cells bearing receptors for this cytokine in residents of the high transmission village.

The small, but significant increase in plasma levels of TGF-β in subjects from the high transmission village may contribute to the suppressed lymphocyte proliferation and IFN-γ production observed in these subjects. TGF-β is a potent suppressant of lymphocyte proliferation and IFN-γ production. Although subjects from the high and low transmission villages produced similar amounts of TGF-β in lymphocyte cultures, this may not reflect its overall production because it can be produced by a variety of cell types (63). The small difference between populations should be interpreted with caution although an indirect finding suggests the elevated TGF-β may be biologically significant. Subjects from the high transmission village had significantly more basophils and tended to have fewer eosinophils in PBMC compared with the low transmission village (our unpublished observations). TGF-β in the presence of IL-3 suppresses eosinophil differentiation and enhances that of basophils (64).

The most striking finding related to plasma cytokine measurements was related to IL-4. The level of this cytokine was increased in infected subjects compared with uninfected individuals. The greatest elevation in plasma IL-4 was in residents of the high transmission village (Fig. 6). Interpretation of the significance of measurements of IL-4 in plasma may be problematic because this cytokine has a short in vivo half-life (IL-4 is a T cell growth factor and may thus be rapidly consumed). Moreover, immunoassays may not reflect the presence of biologically active cytokine. Therefore, we confirmed that plasma IL-4 detected by the two-site ELISA was able to drive the proliferation of an IL-4-dependent human T cell clone.

It is not yet known what cells contribute to plasma IL-4 and why residents of the high transmission village have the highest levels. Studies in which T cells isolated from uninfected persons were coincubated with filarial parasites indicate that mf Ags induce production of IL-4 and IL-5 by $CD4^+CD45RA^+$ T cells in the absence of exogenous cytokines or dendritic cells (17). *Brugia* L3

have also been shown to stimulate IL-4 production by APC of immunologically naive mice (65). By analogy with studies of atopic contact dermatitis in experimental animals and observations of humans with this disease (66–68), we speculate that mast cells and basophils in the dermis are important sources of IL-4. Both cell types are present in low numbers in peripheral blood but plentiful in dermal tissues where L3 are inoculated and larval development subsequently takes place. Persons exposed repeatedly to large numbers of L3 and preadult *W. bancrofti* may experience sustained increases in IL-4 production by activation and degranulation of cells located in the dermis, particularly mast cells bearing cytophilic filarial-specific IgE. Accordingly, current efforts are directed at determining whether basophils isolated from persons living in areas where *W. bancrofti* is endemic secrete IL-4 following incubation with filarial Ags.

An additional biologic feature of human filariasis that may predispose to the establishment of type 2 bias relates to the temporal profile of exposure to parasite Ags in the skin. Prolonged and continuous administration of soluble Ags into the s.c. tissue of genetically predisposed mice results in preferential induction of $CD4^+$ Th2 cells (12, 13). If the intensity or cumulative degree of exposure to L3 and developing larvae in the dermal lymphatics is an important determinant of the strength of type 2 immunity in human filariasis, such responses should wane following sustained reduction in transmission. Comparison of plasma IL-4 levels and T cell cytokine responses before and after reduction in transmission intensity should allow this hypothesis to be tested. Given the existing global plan to control lymphatic filariasis through mass chemotherapy that reduces or even eliminates mosquito-borne transmission of *W. bancrofti* (69), such studies may be feasible in the near future.

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