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Th1-Like Antifilarial Immune Responses Predominate in Antigen-Negative Persons

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To characterize immune responses associated with the putatively immune state in bancroftian filariasis (that is, both microfilaria and antigen free), humoral and cellular responses were compared among antigen- and microfilaria-negative, antigen-positive and microfilaria-negative, and microfilaria-positive individuals. Antifilarial isotype levels were measured by enzyme-linked immunosorbent assay. Peripheral blood mononuclear cell responses were measured by proliferation, by bioassay for interleukin-2 (IL-2) and IL-10, and by reverse transcription-PCR for IL-4, IL-5, and gamma interferon. The absence of circulating filarial antigen was associated with Th1-like responses, including significantly higher proliferative ($P < 0.001$) and IL-2 ($P = 0.008$) responses and a higher prevalence of gamma interferon ($0.02 < P < 0.1$) responses. Significantly elevated antifilarial immunoglobulin G4 (IgG4) levels ($P = 0.0035$) were associated with antigenemia, whereas microfilaremia was associated with significantly decreased antifilarial IgG2 levels ($P = 0.0014$). IL-4 mRNA levels were not significantly different among the three groups; however, there was a subpopulation of microfilaremic individuals who did not make detectable levels of IL-4 mRNA and who produced low antifilarial IgG4 levels compared with those of individuals who had detectable levels of IL-4 mRNA. IL-5 mRNA levels also were not significantly different among groups; however, more microfilaremic individuals produced IL-5 mRNA in response to adult filarial antigens, and total parasite-specific IL-4 and IL-5 mRNA levels were significantly correlated ($P = 0.05$). Although longitudinal data are not currently available, the elevated Th1-like responses in antigen- and microfilaria-negative individuals are consistent with the hypothesis that these responses contribute to protection in putatively immune individuals.

Serologic assays and epidemiologic studies provide strong evidence that nearly all individuals living in regions of endemicity for lymphatic filariasis have been exposed to filarial parasites (3, 41). Despite lifelong exposure to infection, however, there are individuals, variously termed "endemic normal" or "putatively immune," with no detectable microfilaremia and no clinical history or evidence of infection (6, 14, 23, 36). In terms of filarial antigen-specific proliferation and B-cell responses, these endemic normals exhibit greater immune responsiveness than that of microfilaremic individuals, often comparable to that of individuals with chronic lymphatic dysfunction (9, 23, 36, 38–41).

Protective immunity to filarial parasites has been induced in animal models with radiation-attenuated infective larvae, with resultant immunity apparently directed against the L3 larvae (8, 29, 35, 49). Evidence also exists for the development of immune-mediated resistance in humans to filarial parasites (6, 7, 11, 27, 28). The question remains, however, as to whether those individuals who do not harbor parasites are truly immune or are temporarily infection free. It has been suggested (45) that there are two populations in areas where filariasis is endemic, i.e., those who remain uninfected and therefore microfilaria negative and asymptomatic and those who become infected and eventually develop chronic lymphatic dysfunction.

Characterizing the nature of antifilarial immunity in endemic normal persons has been complicated by our inability to ascertain the true infection status of microfilaria-negative individuals. Undoubtedly, these individuals represent a heterogeneous group, including individuals truly free of adult worms, individuals with single-sex infections, and individuals with pre- or postpatent infections (36). The use of antigen detection assays that are specific for *Wuchereria bancrofti* microfilarial and adult worm antigen (4, 24, 32, 47) has made it possible to identify two populations of microfilaria-negative individuals, i.e., those who are antigen positive and presumably carry adult worms and those who are antigen negative and are presumed to be infection free. By using the Og4C3 assay to more carefully define infection status, the present study was designed to examine humoral and cellular antifilarial immune responses in endemic normal and microfilaremic individuals and to characterize responses associated with antigen-negative, as well as antigen-positive, and microfilaria-positive states. Our data show that Th1-like antifilarial responses predominate in antigen-negative individuals and that Th2-like responses may be down-regulated in a subpopulation of microfilaria-positive individuals.

MATERIALS AND METHODS

Study population. Individuals from the coastal community of Leogane, Haiti, who attended clinics at the Ste. Croix Hospital were recruited for participation in this study. Participants were divided into three groups (Table 1) on the basis of parasitologic evaluation and the presence or absence of circulating *W. bancrofti* antigen: (i) microfilaria and antigen negative ($n = 57$), (ii) microfilaria negative and antigen positive ($n = 27$), and (iii) microfilaria and antigen positive ($n = 27$). No individuals with overt signs or symptoms of chronic filarial lymphatic dysfunction were included in the study nor were individuals who had been previously treated with either ivermectin or diethylcarbamazine. Proliferation data were generated for 71 individuals, who were selected to approximate equal numbers per group. Plasma samples from 62 of these individuals were also used for serologic assays. Since sufficient cell numbers were not available to perform all cytokine assays for each individual, subsets of individuals for whom proliferation and serologic data were obtained were used for cytokine assays. With few

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TABLE 1. Characteristics of the study population

Population subgroup[a]	*n*	No. of males/no. of females	Mean age (yr)	Age range (yr)	Microfilaremia range/ml (mean/ml)
Asymptomatic amicrofilaremic					
Ag−	57	18/39	27	8–70	
Ag+	27	12/15	32	10–60	
Asymptomatic microfilaremic	27	13/14	29	6–66	5–7,200 (1,146)

[a] Ag, antigen.

exceptions, all reverse transcription (RT)-PCRs were performed on the same subset of individuals.

Collection of peripheral blood mononuclear cells (PBMC) and plasma. Venous blood (10 ml) was drawn between 2000 and 2200 h for use in immunologic assays. Venous blood was also collected from North American volunteers to provide PBMC and plasma controls. PBMC were isolated by centrifugation over a Ficoll-Hypaque gradient and cryopreserved. Plasma samples were frozen for later use.

Determination of microfilaremia and antigenemia. One milliliter of venous blood was used to determine microfilaremia levels by the Nuclepore (Pleasanton, Calif.) filtration technique. Levels of circulating *W. bancrofti* antigens were measured in plasma samples by an enzyme-linked immunosorbent assay (Og4C3 ELISA) developed by More and Copeman (32) (JCU Tropical Biotechnology Pty Ltd., Queensland, Australia) as described in the kit instructions.

Preparation of parasite antigens. Saline-soluble extracts of *Brugia pahangi* adult worms were prepared as described previously (25). Parasites were provided by the U.S.-Japan Cooperative Medical Sciences Program, National Institute of Allergy and Infectious Diseases.

Serologic assays. Levels of parasite-specific immunoglobulin were quantified by ELISA as described previously (1, 17). Plasma samples from North American controls ($n = 10$) were assayed in parallel. Reference serum (provided by Eric Ottesen, National Institutes of Health, Bethesda, Md.) was used to standardize levels of isotype-specific antifilarial antibodies. Levels of total immunoglobulin E (IgE) and IgG4 were also assayed by ELISA as described previously (30).

Proliferation assays. Proliferative responses of PBMC to optimal concentrations (10 μg/ml) of *B. pahangi* crude adult extract, purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark; 10 μg/ml), and phytohemagglutinin (PHA; Difco, Detroit, Mich.; 10 μg/ml) were measured in vitro as described previously (9).

In vitro IL-2 production. A bioassay using the interleukin-2 (IL-2)-dependent mouse T-cell clone HT-2 (provided by K. Ziegler, Emory University) was used to measure IL-2 in supernatants of PBMC cultured with *B. pahangi* adult antigens, PPD, and PHA as described above. After 24, 48, and 72 h of culture, 50-μl volumes of supernatants were transferred to new round-bottom microtiter plates (Flow Laboratories, McLean, Va.), and these were stored at −20°C before being assayed for the presence of IL-2. IL-2 production was found to be optimal at 72 h, with 3.13 U/ml as the limit of detection for the assay.

For use in the assay, HT-2 cells were harvested at 48 h, washed twice in RPMI 1640, counted, and resuspended at 3×10^4/ml in complete culture medium. Plates containing culture supernatants were thawed, and HT-2 cells were added at 100 μl per well. Twofold serial dilutions of human recombinant IL-2 (400.00 to 3.13 U/ml) were included on each plate to standardize the assay. These cultures were incubated at 37°C, under 5% CO_2, for 24 h and then cultured overnight in the presence of tritiated thymidine (1 μCi per well). Cells were harvested on glass-fiber filters, air dried, and counted on a Matrix direct beta counter (Packard Instrument, Meriden, Conn.).

In vitro IL-10 production. A bioassay which uses the mouse cell line H-7 (provided by K. H. Grabstein, Immunex R & D Corporation, Seattle, Wash.) was used to measure production of IL-10 in response to *B. pahangi* antigens as described previously (9). The limit of detection for the assay was 0.061 U/ml.

RNA isolation and RT. Patient PBMC were cultured in duplicate at 10^5 cells per well in the presence of *B. pahangi* crude adult antigens or PHA for 24, 48, and 72 h. PBMC from duplicate wells (2×10^5) were then collected and washed twice in sterile phosphate-buffered saline (PBS), and pellets were stored in 1.5-ml Eppendorf tubes in liquid nitrogen before RNA isolation. Total cellular RNA was isolated from cell pellets by the method of Chomczynski and Sacchi (5) and reverse transcribed into cDNA as described in the GeneAmp RNA PCR kit instructions (Perkin-Elmer, Branchburg, N.J.) with oligo$(dT)_{16}$ as a primer. RT was carried out at 42°C for 1 h. Reverse transcriptase was inactivated by incubation at 99°C for 5 min, and the mixture (total volume, 20 μl) was stored at 4°C until used for the PCR.

PCR analysis of cytokine transcripts. PCR amplification was performed as described in the GeneAmp RNA PCR instructions (Perkin-Elmer), with 2 μl of RT reaction mixture per PCR rather than the entire 20-μl mixture. The total volume of the PCR mixture was 100 μl. The sequences of oligonucleotide primer pairs, 5′ and 3′, are as follows: β-actin, 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′; IL-4, 5′-GCCTCCAAGAACACAACTGAG-3′ and 5′-TTCCTTCACAGGACAGGAATT-3′; gamma interferon (IFN-γ), 5′-AGTTATATCTTGGCTTTTCAG-3′ and 5′-TACCGAATAATTAGTCAGCTT-3′; IL-5, 5′-ATGAGGATGCTTCTGCATTTG-3′ and 5′-TCAACTTTCTATTATCCACTCGGTGTTCATTAC-3′. For β-actin, IL-4, IFN-γ, and IL-5 transcripts, 30 cycles of PCR (previously determined to be optimal, that is, within the linear range) were performed on a TRIO-Thermoblock (Biometra, Tampa, Fla.) with the following temperatures: 2 min of denaturation at 95°C followed by 30 s at 94°C, 45 s of annealing at 55°C, and 1.5 min of extension at 72°C. For IFN-γ, the protocol was modified to separate *Taq* polymerase and the cDNA sample from the rest of the reaction mixture with Ampliwax (Perkin-Elmer), so that the annealing temperature would not drop below 55°C. The specificity of PCR products was confirmed by Southern blot analysis and the Southern Light Chemiluminescent Detection System (TROPIX, Inc., Bedford, Mass.). The only modification to this procedure was in the hybridization buffer, to which a final concentration of 5× Denhardt's solution was added. The sequence of biotin-labeled probes used was as follows: β-actin, 5′-ATCGAGCACGGCATCGTCACCAAC-3′; IL-4, 5′-CACAAGCAGCTGATCCGATTCCTG-3′; IFN-γ, 5′-GAGAGTGACAGAAAAATAATGCAG-3′; IL-5, 5′-CTGAGGATTCCTGTTCCTGTACAT-3′. Control amplifications, including primers with no RNA template, RNA template without primers, positive and negative control RNA, and non-reverse-transcribed RNA, were performed for each cytokine analyzed.

Quantitation of PCR products. To quantify IL-4 products, a modified version of the PCR ELISA described by Sabbatini et al. (42) was performed with a biotinylated capture probe and a digoxigenin-labeled detection probe (5′-CATGAGAAGGACACTCGCTGCCTG-3′). Immulon microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated with streptavidin (Sigma) at 10 μg/ml in 0.1× PBS (pH 7.0) for 1 h at room temperature. The plates were washed three times with buffer A (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20), blocked with a solution of 0.5% nonfat dry milk and 100 μg of salmon sperm DNA per ml in buffer A for 45 min at room temperature, and washed three times with buffer A. The biotinylated capture probe was then added to the plate at a final concentration of 0.75 pM per well (0.015 μM) in 1× hybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 mM Tris-HCl [pH 7.5], 5 mM EDTA) for 1 h at room temperature. Capture of PCR products involved liquid-phase hybridization of PCR products to digoxigenin-labeled probes, followed by solid-phase hybridization of the digoxigenin-labeled product to the biotinylated capture probe on the plates. For liquid-phase hybridization, 20 μl of PCR product was combined with 29 μl of H_2O, 1 μl of 0.4 μM digoxigenin-labeled probe, and 50 μl of 2× hybridization buffer. Each sample was then denatured by boiling for 10 min, cooled immediately on ice, and left at room temperature for 20 min to 1 h. Samples were added in duplicate (50 μl per well) to the plates and hybridized at 55°C for 1 h. Quantitation was performed as described previously (42), with a 1:2,000 dilution of the alkaline phosphatase-conjugated anti-digoxigenin antibody.

As a positive control and for use in standard curves, cDNA was prepared from Hut-78 cells that had been stimulated for 5 h with PHA (10 μg/ml). Twofold serial dilutions from a single preparation of IL-4 product amplified from this cDNA were used to generate standard curves in each assay, and values were expressed as a fold increase over control.

To quantify β-actin, IL-5, and IFN-γ products, Southern blot and chemiluminescent detection (Southern Light DNA detection kit [Tropix]), followed by densitometric analysis, were used. Films were scanned on a Scanjet II cx (Hewlett-Packard, Greeley, Colo.) and analyzed with the SigmaScan Image program (Jandel Scientific Software, San Rafael, Calif.). For β-actin, values were expressed in densitometric units. IL-5 and IFN-γ cDNA products were expressed in densitometric units and standardized against twofold serial dilutions of one preparation of either IL-5 or IFN-γ product amplified from Hut-78 cell cDNA. All PCR products were normalized with respect to the constitutively produced gene β-actin, to control for possible differences in the amount of mRNA isolated and reverse transcribed.

Statistics. Analysis of variance was performed on log-transformed data to determine the significance of differences in proliferative responses, antibody responses, and cytokine production between groups. For RT-PCR products, the significance of differences between groups in the prevalence of positive responses to adult filarial antigens was determined by χ^2 analysis.

RESULTS

Study population. Characteristics of the study population are shown in Table 1. In terms of the distribution of microfilaremia and antigenemia, the study population was representative of asymptomatic persons in the community (24). Although females were slightly overrepresented in the antigen-negative group, the difference was not significant. All microfilaremic individuals and 32% of asymptomatic amicrofilaremic individuals participating in the study were positive for circu-

TABLE 2. Geometric mean parasite-specific antibody levels

Immunoglobulin	Antibody level (μg/ml)			
	Asymptomatic amicrofilaremic[a]		Asymptomatic microfilaremic (n = 26)	NA[b] control (n = 10)
	Ag− (n = 22)	Ag+ (n = 14)		
IgG2	56.2	44.4	16.0[c]	1.0
IgG4	4.3[c]	45.2	25.1	0.2

[a] Ag, antigen.
[b] NA, North American.
[c] Significantly different from values of other clinical groups.

TABLE 3. Adult filarial antigen-induced cytokine production

Cytokine	Cytokine produced (U/ml [range])[a]		
	Asymptomatic amicrofilaremic		Asymptomatic microfilaremic
	Ag−[b]	Ag+	
IL-2	18.24[c] (3.65–35.99)	2.92 (0.00–13.45)	3.08 (0.06–23.85)
IL-10	0.04 (0.00–0.30)	0.10 (0.02–0.54)	0.07 (0.00–0.57)

[a] Values are geometric mean units per milliliter minus control values.
[b] Ag, antigen.
[c] Significantly different from values of other clinical groups.

lating *W. bancrofti* antigen. There were no significant differences in the mean age of individuals among groups.

Serologic assays. Levels of antifilarial antibodies as well as polyclonal IgG4 and IgE were compared among a randomly selected subset of individuals from each patient group. There were no significant differences in the levels of anti-filarial IgG1 or IgG3 (data not shown). Serum levels of anti-filarial IgG2 were significantly lower (P = 0.0014) in microfilaria-positive than microfilaria-negative individuals (Table 2). In contrast, anti-filarial IgG4 was associated with the presence of circulating *W. bancrofti* antigens, with levels significantly lower (P = 0.0035) in microfilaria- and antigen-negative individuals. Although differences in total IgG4 were not significant (P = 0.0972), geometric mean levels were higher in serum samples from amicrofilaremic antigen-positive (n = 16; 642 μg/ml) and microfilaria-positive (n = 17; 412 μg/ml) individuals than in those from antigen-negative individuals (n = 15; 182 μg/ml). The same trend was observed for total IgE levels (data not shown). In addition, both total and antifilarial IgG4 levels were directly correlated with total IgE levels (P = 0.01; data not shown).

Proliferative responses. Proliferation in response to mitogen (PHA) and nonparasite antigen (PPD) was not significantly different among the three groups (data not shown). However, the geometric mean proliferative response to adult filarial antigen was significantly higher ($P < 0.001$) in PBMC of antigen-negative individuals (geometric mean = 32) than in those of amicrofilaremic antigen-positive (geometric mean = 9) or microfilaria-positive individuals (geometric mean = 6) (Fig. 1).

Cytokine levels in PBMC culture supernatants. To further characterize patterns of cellular responses in each patient group, levels of IL-2, a cytokine produced by Th1 cells (33), and IL-10, a counterregulatory molecule known to down-regulate Th1 responses (22, 31, 44), were measured in supernatants of cultured patient PBMC. Neither IL-2 nor IL-10 produced in response to PHA or PPD differed significantly among the three patient groups (data not shown). As with proliferation, IL-2 production to adult filarial antigens was significantly (P = 0.008) higher in cultures of PBMC from antigen-negative individuals (Table 3). In addition, IL-2 levels were well correlated with proliferative responses to adult antigens (P = 0.01). There were no significant differences in net IL-10 production to adult filarial antigens (Table 3) nor were there significant differences in spontaneous IL-10 production (data not shown).

Cytokine mRNA production. Because of limitations in cell numbers and quantities of culture supernatants, RT-PCR was used to examine other markers of immune responsiveness. Levels of IL-4 mRNA induced by PHA stimulation were not significantly different among the three groups. Cells from all individuals produced IL-4 mRNA in response to PHA stimulation, with geometric mean levels for each group exceeding those of the positive control (data not shown). IL-4 mRNA produced in response to adult filarial antigen was not significantly different among the three groups (Fig. 2A) nor was it well correlated with antifilarial IgG4 production (data not shown). Interestingly, however, there was a subpopulation of microfilaria-positive individuals (8 of 18 or 44% of those examined) who made no detectable IL-4 mRNA in response to

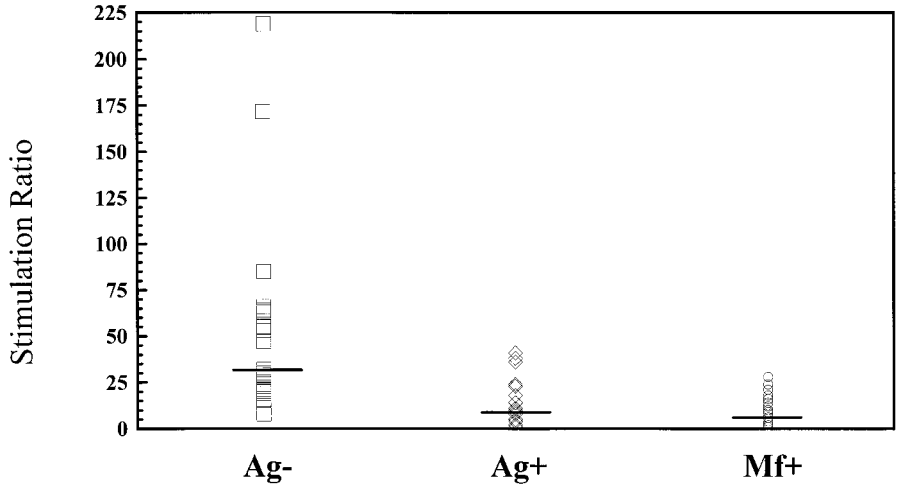

FIG. 1. Proliferative responses of PBMC to adult filarial antigens, from individuals who were antigen and microfilaria negative (Ag−, n = 25), antigen positive and microfilaria negative (Ag+, n = 21), and antigen and microfilaria positive (Mf+, n = 25). Responses are shown as the stimulation ratio for each individual. Geometric mean levels are represented by a solid bar for each group.

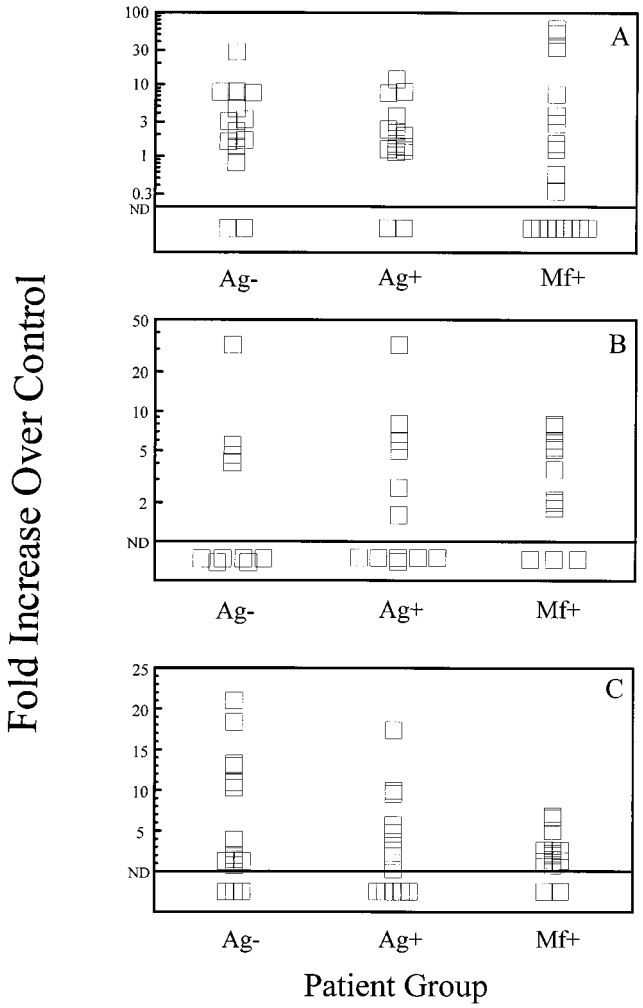

FIG. 2. Production of adult filarial antigen-induced mRNA, as measured by RT-PCR for IL-4 (A), IL-5 (B), and IFN-γ (C) in PBMC of individuals who were antigen and microfilaria negative (Ag−), antigen positive and microfilaria negative (Ag+), and antigen and microfilaria positive (MF+). Responses are shown as a fold increase over control (that is, spontaneous transcription) levels for each individual. All values were normalized with respect to β-actin levels. Responses of individuals from whom no cytokine mRNA was detected (ND) are shown below the horizontal line in each graph.

adult filarial antigen (Fig. 2A). This subpopulation was also characterized by significantly lower ($P < 0.01$) anti-filarial IgG4 responses (geometric mean = 8.63 μg/ml) compared with those who did produce detectable levels of IL-4 mRNA (30.53 μg/ml).

As a further measure of Th2-like responses, IL-5 mRNA production was examined. PHA-stimulated IL-5 production was not significantly different among the three groups (data not shown) and was approximately fourfold higher than that of adult filarial antigen-stimulated IL-5 mRNA production. The prevalence of adult filarial antigen-induced IL-5 mRNA production was higher in microfilaremic individuals than in either antigen-negative or antigen-positive individuals (shown in Fig. 2B as a fold increase over control, that is, spontaneous transcription levels); however, these differences were not significant ($P = 0.09$). The three microfilaremic individuals who did not make IL-5 mRNA in response to adult antigen also did not make IL-4 mRNA; however, they did produce IL-5 in response to PHA. In addition, total IL-4 and IL-5 mRNA responses (unadjusted for control values) to adult filarial antigens were significantly correlated ($P = 0.05$).

IFN-γ, a Th1 cytokine known to down-regulate Th2 responses (12), was also analyzed by RT-PCR. As with IL-4, high levels of IFN-γ mRNA were produced by cells from all individuals in response to PHA, with no significant differences observed between groups (data not shown). Production of IFN-γ mRNA in response to adult filarial antigen (Fig. 2C) was highest with PBMC from antigen-negative individuals and lowest with PBMC from microfilaria-positive individuals. These differences were found to be marginally significant ($0.02 < P < 0.1$).

DISCUSSION

In the absence of definitive longitudinal data, a cross-sectional approach to examining immunity, with clearly defined patient groups, can be used to identify potential correlates of protective immunity (34). This approach has been used to define antigens that are preferentially recognized by putatively immune individuals (14) and, in studies of onchocerciasis, to define patterns of T-cell reactivity (11, 46). Recognizing the importance of distinguishing between infected and uninfected microfilaria-negative individuals, we used a sensitive and specific antigen detection assay to assess the infection status of microfilaria-negative persons. By using this technique, we have, for the first time, been able to characterize antifilarial antibody and T-cell responses that were associated with the presence or absence of *W. bancrofti* antigen, independent of microfilaremia.

Individuals differ in responsiveness to adult filarial antigens, based on microfilaria status (9, 23, 37, 38, 40), but these studies did not distinguish between antigen-negative and antigen-positive individuals. We have extended these observations by determining that increased proliferative responses to adult antigen are associated with the absence of circulating filarial antigen (Fig. 1). This observation may help to explain the heterogeneity in responsiveness observed in amicrofilaremic individuals, many of whom are antigen positive. This association has also been reported in patients with lymphedema and elephantiasis (1); thus, it would appear that increased proliferation is correlated with the absence of antigen rather than the presence of disease.

Cellular responses can be further defined on the basis of cytokine production by T helper cells, with T helper 1 (Th1) and Th2 representing proposed subgroups (33). While it is difficult to see a clear distinction between Th1 and Th2 subpopulations in vivo, characteristic patterns of Th1- and Th2-like cytokine responses have been observed (20), particularly in murine *Leishmania major* infections (48). In filariasis, T-cell responses of microfilaremic individuals are reported to be Th2-like (22, 37), whereas those of patients with chronic lymphatic dysfunction are described as being Th1-like (9, 22, 28, 37). In an effort to characterize the immune response in microfilaria-negative and disease-free individuals as a function of antigen status, T-cell responses were assessed by bioassay for IL-2 and IL-10 and by RT-PCR for IL-4, IL-5, and IFN-γ.

As with proliferation, adult filarial antigen-induced production of IL-2 was strongly associated with a lack of circulating filarial antigens. Similarly, although to a lesser degree, IFN-γ mRNA levels were highest in PBMC of antigen-negative individuals. Taken together, these results indicate that in the absence of circulating filarial antigens, Th1-like responses dominate. The presence of antigen is associated with a modulation of these responses. Given the counterregulatory nature of some Th1 and Th2 cytokines (13, 43), it would be reasonable to hy-

pothesize that this modulation would be associated with Th2-like responses. This hypothesis was not well supported by our data.

Levels of IL-10, a cytokine known to down-regulate Th1 responses (22, 31, 44), have been reported to be significantly higher in microfilaremic individuals than in patients with chronic lymphatic dysfunction (22). However, in separate studies, the use of anti-IL-10 antibody did not reverse unresponsiveness in microfilaremic individuals (18, 28). In the present study, no significant differences in spontaneous or adult filarial antigen-induced IL-10 production were observed between antigen-negative, antigen-positive, and microfilaria-positive individuals. In addition, considerable variability in antigen-specific IL-10 production was observed within each group. Not only were we unable to find evidence for a cross-regulatory role for IL-10, but when responses to antigen fractions were measured, IL-10 production was highest in patients with lymphatic dysfunction and lowest in microfilaremic individuals (9).

Filarial antigen-induced production of mRNA of the Th2 cytokine IL-4 was also not significantly different among the three groups examined. These data emphasize that Th2-like cytokine responses are not absent in antigen-negative persons. The absence of a difference in IL-4 production by microfilaria status has been reported previously, but no information on antigen status was available from microfilaria-negative patients in this study (28).

Interestingly, we observed two distinct response patterns for microfilaremic individuals. Of those examined, 44% had undetectable levels of adult filarial antigen-induced IL-4 mRNA by RT-PCR as well as very low levels of anti-filarial IgG4 (8.63 μg/ml). In contrast, those individuals who produced IL-4 in response to filarial antigen mounted significantly higher antifilarial IgG4 responses (30.53 μg/ml; $P < 0.01$) than did either IL-4 nonresponders or antigen-negative individuals. In parallel studies, we showed that microfilaremic persons with low antifilarial IgG4 responses had significantly higher antigenemias than did persons with high antifilarial IgG4 responses (30). Although the explanation remains speculative, these findings may reflect a tolerizing effect of high antigen loads in vivo on Th2 cells. In support of this hypothesis, microfilaremic individuals who did not produce parasite-specific IL-5 also did not produce IL-4. On the basis of the absence of significant differences in IL-4 mRNA production between infected and uninfected persons, it seems unlikely that IL-4 is playing a regulatory role in Th1 responses. Furthermore, treatment of PBMC cultures from microfilaremic individuals with neutralizing anti-IL-4 antibody did not lead to enhanced proliferation to filarial antigens (10). In contrast, where Th1-like responses are present (e.g., antigen-negative persons), Th2-driven responses (i.e., IgG4) are decreased.

It has been suggested that regulation of IL-4 and IL-5 may be linked in helminth infections (26). Our data support this hypothesis in that adult filarial antigen-induced IL-4 production and IL-5 total mRNA production were directly correlated ($P = 0.05$). Although more microfilaremic persons produced IL-5 mRNA in response to adult filarial antigens than did either antigen-positive or antigen-negative persons, the differences were not significant. It is as yet unclear what is responsible for regulating either IL-5 or IL-4 production; moreover, we did not find any direct association between IL-2 and IL-5 production as has been reported with individuals putatively immune to *Onchocerca volvulus* infection (46).

Previous studies have reported that microfilaria-positive individuals mount poor antifilarial IgG1, IgG2, and IgG3 responses but very high antifilarial IgG4 responses (19, 37). An examination of antifilarial isotype responses by ELISA indicated that for Haitians, antifilarial IgG4 was associated with the presence of filarial antigens, not microfilaria, an observation previously made of Haitian individuals with chronic lymphatic dysfunction (1). In contrast, antifilarial IgG2 was associated with absence of microfilaria and not influenced by the presence of antigen. Although no significant differences were observed between groups in levels of total IgG4 or IgE, microfilaremic and amicrofilaremic antigen-positive individuals produced higher levels of both antibodies than did antigen-negative individuals. Thus, both total IgG4 and antifilarial IgG4 levels were directly correlated ($P \leq 0.01$) with total IgE levels.

The results of this study indicate that there are strong associations between the antigen-negative state and Th1-like responses, specifically, elevated proliferative, IL-2, and IFN-γ responses. Antigenemia and microfilaremia were associated with the absence of Th1-like responses and with elevated IgG4 and IL-5 mRNA levels, respectively, responses typically described as Th2-like. However, not all Th2-like responses measured were elevated in microfilaremic or antigenemic individuals. For example, IL-4 was not significantly elevated in either of these groups compared with that of antigen-negative individuals. This should not be surprising, however, in light of a recent reevaluation of Th1 and Th2 subsets (20). While responses of T cells and clones conform to patterns of Th1- and Th2-like responses, it may be an oversimplification to divide T cells into discrete subsets. In fact, there is increasing evidence that cytokine production by T cells and clones is continuously distributed and that different cytokine genes may be regulated independently (2, 20, 21).

In the absence of longitudinal data, it is impossible to say whether these Th1-like responses are associated with maintenance of the antigen-negative state. There is also no way of distinguishing between those who were exposed but never infected, those who were infected but have since overcome the infection, and those who are currently uninfected but will later become infected. In the absence of information about these possibilities, two explanations for the distribution of infections in disease-endemic populations have emerged. According to the immunologic paradigm, the immune response dictates the outcome of infection (37). Alternatively, the distribution of infections may be determined by rates of parasite acquisition and clearance (15). Perhaps a combined approach that takes into account dynamics of infection and immunity as well as differences in transmission intensities among different populations would represent a useful alternative (15).

The best evidence for protective immunity is seen in individuals with lymphedema and elephantiasis, who are more likely to be antigen negative than age-matched persons without overt clinical manifestations (1, 16, 24). The fact that immune responses in antigen-negative individuals are very similar to those of antigen-negative patients with lymphatic pathology (9) is consistent with the hypothesis that protective immunity is related to Th1-like responses. If this is true, then it raises the question of whether asymptomatic antigen-negative individuals who were previously infected are at greater risk of developing pathology than are antigen-positive persons.

Despite the lack of longitudinal data, our results support the possibility that a Th1-like response protects antigen-negative individuals from (further) infection. The fact that immune responses in antigen-positive individuals were intermediate between those of antigen-negative and microfilaremic individuals would indicate that the antigen-positive state represents a transitional stage. It is impossible to say, however, whether these individuals have pre- or postpatent infections. Continued observation of infection status and immune responses in these individuals would help to clear up these issues. Likewise, an examination of immune responses to larval antigens in these

groups of individuals would help to determine whether immunity is directed against larval and/or adult antigens and whether it is stimulated by the presence of adult worms (concomitant immunity) or maintained in their absence.

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REFERENCES

1. **Addiss, D. G., K. D. Dimock, M. L. Eberhard, and P. J. Lammie.** 1995. Clinical, parasitologic, and immunologic observations of patients with hydrocele and elephantiasis in an area with endemic lymphatic filariasis. J. Infect. Dis. **171:**755–758.
2. **Assenmacher, M., J. Schmitz, and A. Radbruch.** 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-γ and in interleukin-4-expressing cells. Eur. J. Immunol. **24:**1097–1101.
3. **Bailey, J. H., II, A. W. Hightower, M. L. Eberhard, and P. J. Lammie.** 1995. Acquisition and expression of humoral reactivity to antigens of infective stages of filarial larvae. Parasite Immunol. **17:**617–623.
4. **Chanteau, S., J. P. Moulia-Pelat, P. Glaziou, et al.** 1994. Og4C3 circulating antigen: a marker of infection and adult worm burden in *Wuchereria bancrofti* filariasis. J. Infect. Dis. **170:**247–250.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Day, K. P.** 1991. The endemic normal in lymphatic filariasis: a static concept. Parasitol. Today **7:**341–343.
7. **Day, K. P., W. F. Gregory, and R. M. Maizels.** 1991. Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of Papua New Guinea. Parasite Immunol. **13:**277–290.
8. **Denham, D. A., P. B. McGreevy, R. R. Suswillo, and R. Rogers.** 1983. The resistance to re-infection of cats repeatedly inoculated with infective larvae of *Brugia pahangi*. Parasitology **86:**11–18.
9. **Dimock, K. A., D. G. Addiss, M. L. Eberhard, and P. J. Lammie.** 1994. Differential proliferative and interleukin-10 responses to fractionated filarial antigens: preferential recognition by patients with chronic lymphatic dysfunction. J. Infect. Dis. **170:**403–412.
10. **Dimock, K. A., and P. J. Lammie.** 1993. Unpublished observations.
11. **Elson, L. H., H. M. Calvopina, Y. W. Paredes, N. E. Araujo, J. E. Bradley, R. H. Guderian, and T. B. Nutman.** 1995. Immunity to onchocerciasis: putative immune persons produce a Th1-like response to *Onchocerca volvulus*. J. Infect. Dis. **171:**652–658.
12. **Farrar, M. A., and R. D. Schreiber.** 1993. The molecular cell biology of interferon-γ and its receptor. Annu. Rev. Immunol. **11:**571–611.
13. **Finkelman, F. D., E. J. Pearce, J. F. Urban, Jr., and A. Sher.** 1991. Regulation and biological function of helminth-induced cytokine responses. Immunoparasitol. Today **7:**A62–A66.
14. **Freedman, D. O., T. B. Nutman, and E. A. Ottesen.** 1989. Protective immunity in bancroftian filariasis: selective recognition of a 43-kD larval stage antigen by infection-free individuals in an endemic area. J. Clin. Invest. **83:**14–22.
15. **Grenfell, B. T., and E. Michael.** 1992. Infection and disease in lymphatic filariasis: an epidemiological approach. Parasitology **104:**S81–S90.
16. **Grenfell, B. T., E. Michael, and D. A. Denham.** 1991. A model for the dynamics of human lymphatic filariasis. Parasitol. Today **7:**318–323.
17. **Hitch, W. L., A. W. Hightower, M. L. Eberhard, and P. J. Lammie.** 1991. Analysis of isotype-specific antifilarial antibody levels in a Haitian pediatric population. Am. J. Trop. Med. Hyg. **44:**161–167.
18. **Hitch, W. L., and P. J. Lammie.** Unpublished observations.
19. **Hussain, R., M. Grogl, and E. A. Ottesen.** 1987. IgG antibody subclasses in human filariasis: differential subclass recognition of parasite antigens correlates with different clinical manifestations of infection. J. Immunol. **139:**2794–2798.
20. **Kelso, A.** 1995. Th1 and Th2 subsets: paradigms lost? Immunol. Today **16:**374–379.
21. **Kelso, A., P. Groves, A. B. Troutt, and K. Francis.** 1995. Evidence for the stochastic acquisition of cytokine profile by $CD4^+$ T cells activated in a T helper type 2-like response *in vivo*. Eur. J. Immunol. **25:**1168–1175.
22. **King, C. L., S. Mahanty, V. Kumaraswami, J. S. Abrams, J. Regunathan, K. Jayaraman, E. A. Ottesen, and T. B. Nutman.** 1993. Cytokine control of parasite-specific anergy in human lymphatic filariasis: preferential induction of a regulatory T helper type 2 lymphocyte subset. J. Clin. Invest. **92:**1667–1673.
23. **King, C. L., and T. B. Nutman.** 1991. Regulation of the immune response in lymphatic filariasis and onchocerciasis. Immunoparasitol. Today **7:**A54–A58.
24. **Lammie, P. J., A. W. Hightower, and M. L. Eberhard.** 1994. Age-specific prevalence of antigenemia in a *Wuchereria bancrofti*-exposed population. Am. J. Trop. Med. Hyg. **51:**348–355.
25. **Lammie, P. J., and S. P. Katz.** 1983. Immunoregulation in experimental filariasis. II. Responses to parasite and nonparasite antigens in jirds with *Brugia pahangi*. J. Immunol. **130:**1386–1389.
26. **Mahanty, S., J. S. Abrams, C. L. King, A. P. Limaye, and T. B. Nutman.** 1992. Parallel regulation of IL-4 and IL-5 in human helminth infections. J. Immunol. **148:**3567–3571.
27. **Maizels, R. M., and R. A. Lawrence.** 1991. Immunological tolerance: the key feature in human filariasis? Parasitol. Today **7:**271–276.
28. **Maizels, R. M., E. Sartono, A. Kurniawan, F. Partono, M. E. Selkirk, and M. Yazdanbakhsh.** 1995. T-cell activation and the balance of antibody isotypes in human lymphatic filariasis. Parasitol. Today **11:**50–56.
29. **Maizels, R. M., and M. E. Selkirk.** 1988. Immunobiology of nematode antigens, p. 285–308. *In* P. T. Englund and A. Sher (ed.), The biology of parasitism. Alan R. Liss, Inc., New York.
30. **Marley, S. E., P. J. Lammie, M. L. Eberhard, and A. L. Hightower.** 1995. Reduced antifilarial IgG4 responsiveness in a subpopulation of microfilaremic persons. J. Infect. Dis. **172:**1630–1633.
31. **Moore, K. W., A. O. O'Garra, R. de Waal Malefyt, P. Vieira, and T. R. Mosmann.** 1993. Interleukin-10. Annu. Rev. Immunol. **11:**165–190.
32. **More, S. J., and D. B. Copeman.** 1990. A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. Trop. Med. Parasitol. **41:**403–406.
33. **Mosmann, T. R., and R. L. Coffman.** 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
34. **Nutman, T. B.** 1995. Immune responses in lymphatic-dwelling filarial infections, p. 511–523. *In* J. C. Boothroyd and R. Komuniecki (ed.), Molecular approaches to parasitology. Wiley-Liss, Inc., New York.
35. **Oothuman, P., D. A. Denham, P. B. McGreevy, G. S. Nelson, and R. Rogers.** 1979. Successful vaccination of cats against *Brugia pahangi* with larvae attenuated by irradiation with 10 krad cobalt 60. Parasite Immunol. **1:**209–216.
36. **Ottesen, E. A.** 1984. Immunological aspects of lymphatic filariasis and onchocerciasis in man. Trans. R. Soc. Trop. Med. Hyg. **78:**9–18.
37. **Ottesen, E. A.** 1992. Infection and disease in lymphatic filariasis: an immunological perspective. Parasitology **104:**S71–S79.
38. **Ottesen, E. A., P. F. Weller, and L. Heck.** 1977. Specific cellular immune unresponsiveness in human filariasis. Immunology **33:**413–421.
39. **Ottesen, E. A., P. F. Weller, M. N. Lunde, and R. Hussain.** 1982. Endemic filariasis on a Pacific island. II. Immunologic aspects: immunoglobulin, complement, and specific antifilarial IgG, IgM, and IgE antibodies. Am. J. Trop. Med. Hyg. **31:**953–961.
40. **Piessens, W. F., P. B. McGreevy, P. W. Piessens, M. McGreevy, I. Koiman, J. S. Saroso, and D. T. Dennis.** 1980. Immune responses in human infections with *Brugia malayi*: specific cellular unresponsiveness to filarial antigens. J. Clin. Invest. **65:**172–179.
41. **Piessens, W. F., P. B. McGreevy, S. Ratiwayanto, M. McGreevy, P. W. Piessens, I. Koiman, J. S. Saroso, and D. T. Dennis.** 1980. Immune responses in human infections with *Brugia malayi*: correlation of cellular and humoral reactions to microfilarial antigens with clinical status. Am J. Trop. Med. Hyg. **29:**563–570.
42. **Sabbatini, A. R. M., P. A. Werner, C. Guha, G. V. Paddock, and R. M. Galbraith.** 1993. The vitamin D-binding protein gene: quantitation of amplified nucleic acids by ELISA. BioTechniques **15:**706–713.
43. **Sher, A., and R. L. Coffman.** 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. **10:**385–409.
44. **Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann.** 1991. Production of IL-10 by $CD4^+$ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. J. Immunol. **147:**2713–2716.
45. **Srividya, A., S. P. Pani, P. K. Rajagopalan, D. A. P. Bundy, and B. T. Grenfell.** 1991. The dynamics of infection and disease in Bancroftian filariasis. Trans. R. Soc. Trop. Med. Hyg. **85:**255–259.
46. **Steel, C., and T. B. Nutman.** 1993. Regulation of IL-5 in onchocerciasis: a critical role for IL-2. J. Immunol. **150:**5511–5518.
47. **Weil, G. J., D. C. Jain, S. Santhanam, A. Malhotra, H. Kumar, K. V. P. Sethumadhavan, F. Liftis, and T. K. Ghosh.** 1987. A monoclonal antibody-based enzyme immunoassay for detecting parasite antigenemia in bancroftian filariasis. J. Infect. Dis. **156:**350–355.
48. **Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. Bloom, and R. L. Modlin.** 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **253:**277–279.
49. **Yates, J. A., and G. I. Higashi.** 1985. *Brugia malayi*: vaccination of jirds with ^{60}cobalt-attenuated infective stage larvae protects against homologous challenge. Am. Soc. Trop. Med. Hyg. **34:**1132–1137.

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