

Evolution of Immunologic Responsiveness of Persons Living in an Area of Endemic Bancroftian Filariasis: A 17-Year Follow-up

Cathy Steel¹ and Eric A. Ottesen²

¹Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; ²Lymphatic Filariasis Elimination (Department of Control, Prevention, and Elimination), World Health Organization, Geneva, Switzerland

On an island in which bancroftian filariasis is endemic, 29 microfilaremic and 16 “endemic normal” (EN) subjects initially studied in 1974–1975 were reevaluated 17 years later. Eleven persons remained microfilaremic, whereas 18 had cleared both microfilaremia and antigenemia. Despite decreased infection on the island, antibody levels remained relatively constant for the subjects with persistent microfilaremia (Mf^{+/+}), in contrast to sharp decreases for both EN subjects and subjects with cleared microfilaremia (Mf^{-/-}). Although clinically indistinguishable from the EN subjects, the Mf^{-/-} group had antibody levels (IgG, IgG4, and IgE) significantly lower than those of the EN subjects. Lymphocyte responses to parasite antigens were marginally greater in Mf^{-/-} than in Mf^{+/+} subjects, but both groups remained less cell responsive (as measured by proliferation, interleukin-5, interleukin-10, interferon- γ , and granulocyte-macrophage colony-stimulating factor) than did the EN subjects. These findings suggest that, for microfilaremic persons, complete clearance of infection is not sufficient to restore “normal” immune responsiveness; filarial infection may induce very long-term deficits in the ability to respond to parasite antigens.

Because of the difficulty inherent in carrying out longitudinal analyses of human populations, there are relatively few studies on the natural evolution of lymphatic filariasis in treated or untreated persons, and even less is known about changes in immunologic parameters that accompany this evolution. With the advent of new technologies to study lymphatic filarial infections (e.g., antigen detection [1], ultrasound [2], and lymphoscintigraphy [3]), our concepts of the progression of chronic filarial infection have changed greatly, particularly for persons categorized as “asymptomatic microfilaremic,” who have been the focus of many recent cross-sectional immunologic studies, primarily because of their general lack of T cell responsiveness to parasite antigens [4–6].

In the present study, we evaluated the immunologic and clinical changes that occurred in a group of microfilaremic persons

in an area in the South Pacific that is endemic for bancroftian filariasis 17 years after they were initially studied and 5 years after a single round of mass treatment with diethylcarbamazine (DEC) was administered to this island population. All these persons were microfilaremic in 1975 and, although ~40% remained microfilaremic in 1992, the rest had eliminated their infection during the 17-year period. Humoral and cellular immune responses of these 2 groups of subjects (“persistent microfilaremic” [Mf^{+/+}] and “cleared microfilaremic” [Mf^{-/-}]) were compared both with each other and with the responses of a population of “endemic normal” (EN) subjects (i.e., persons who were uninfected both in 1975 and in 1992).

Methods

Study population. The study population comprised 45 adults, all permanent residents of the *Wuchereria bancrofti*-endemic island of Mauke (Cook Islands). All subjects had been thoroughly evaluated 17 years earlier (1974–1975) [7] and were classified for the present study according to strict clinical criteria, on the basis of observations from both 1975 and 1992 when most ($n = 356$ in 1975) or all ($n = 627$ in 1992) of the island population was examined. Those evaluations included clinical (history, physical examination, and complete blood count), parasitologic (filtration of 1 mL of blood through a Nuclepore 3- μ m polycarbonate filter to identify circulating microfilariae), and serologic (see below) studies. Serum samples from all study subjects in 1975 and 1992 were examined for circulating filarial antigen (CAg) [8]. Five years before the present study, mass treatment with DEC (5 days, 6 mg/kg/day) was administered to everyone on the island ≥ 5 years old, including all persons in the present study. Likely as a result, the prevalence

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Informed consent was obtained from subjects and from parents of children (with assent of children). Procedures were explained in English or the local language (Maori). The study was done under protocols approved by the Cook Islands Health Department and the National Institutes of Health.

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Reprints or correspondence: Dr. Cathy Steel, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Dr., Rm. 4/126, Bethesda, MD 20892 (csteel@niaid.nih.gov).

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of microfilaremia on the island decreased from 30% in 1975 to 5% in 1992, although transmission was still ongoing [9].

Subjects were categorized into 3 clinical groups (table 1). $Mf^{+/+}$ subjects were those who were microfilaremic during both 1975 and 1992 ($n = 11$). Two of these persons developed lower limb lymphedema between 1975 and 1992, 1 developed hydrocele, and 1 had a persistent hydrocele during both evaluations. $Mf^{+/-}$ subjects were microfilaremic and CAG^+ in 1975 but had become amicrofilaremic and CAG^- by the 1992 evaluation ($n = 18$). Three of these persons had developed lower limb lymphedema since their evaluation in 1975, and 2 had persistent hydroceles. (Three others who had converted to Mf^- since the 1975 study but were still CAG^+ in 1992 were not included in the study.) Microfilaremia levels in 1975 were not statistically significantly different between the $Mf^{+/+}$ and $Mf^{+/-}$ groups ($P = .234$). The EN group ($n = 16$) had no evidence of current or past filarial infection, despite a lifetime of natural exposure [9]. All these subjects had negative clinical and parasitologic findings for filariasis both in 1975 and in 1992 and were CAG^- in serum samples from both time points.

Antigens. Parasite antigens were saline extracts of adult (BmA), Mf, and infective larval (L3) stages of the related filarial parasite *Brugia malayi* and were prepared as described elsewhere [10]. Antigen concentrations used in lymphocyte stimulation tests were predetermined as values to which healthy North American control subjects did not respond (5 or 1 $\mu\text{g/mL}$ for BmA, 1 $\mu\text{g/mL}$ for Mf, and 0.25 $\mu\text{g/mL}$ for L3). Nonparasite antigens (NPAG) included tuberculin-purified protein derivative (PPD; Connaught Laboratories) used at 10 $\mu\text{g/mL}$ and streptolysin O (SLO; Difco) used at a final concentration of 1:100.

Immunologic assessments. Serum IgG, IgG4, and IgE responses to parasite antigens were determined by ELISA, as described elsewhere [11, 12], including preabsorption of serum samples with protein G sepharose (Pharmacia) for IgE analysis. To compare antibody levels in 1975 and 1992, serum samples for each patient from both time points were run together on the same plates (all serum samples had been stored in liquid nitrogen or at -70°C since the time of collection).

Proliferation and cytokine responses to parasite and NPAG were assessed in peripheral blood mononuclear cells (PBMC) isolated from 80 mL of heparinized blood, as described elsewhere [9]. In brief, for evaluation of proliferation, 10^5 PBMC were cultured in the presence or absence of antigens in 0.2-mL volumes for 5 days, after which [^3H]thymidine incorporation was compared and was expressed as stimulation indices: mean counts per minute of stimulated cultures/mean counts per minute of unstimulated (medium alone) cultures. For assessment of cytokine responses, 2×10^6 PBMC were cultured in 1-mL volumes for 2 days (interleukin [IL]–2

and IL-4) or 5 days (IL-5, IL-10, interferon [IFN]– γ , and granulocyte-macrophage colony-stimulating factor). Supernatants were determined by ELISA, and responses were quantified by using standardized curves based on recombinant cytokines [9]. For analysis, media backgrounds (cytokine production in the absence of antigen) were first subtracted.

Statistics. The Mann-Whitney U test was used for comparison of unpaired data, and the Wilcoxon signed rank test was used for comparison of paired data. We used Fisher's exact test to compare response rates between groups and the Spearman's rank test for correlation of paired data. For data analysis, the higher of the 2 responses to the 5- or 1- $\mu\text{g/mL}$ concentrations of BmA was used for each subject; similarly, the greater of the 2 responses to SLO or PPD was used to assess NPAG responses.

Results

Antibody responses. IgG, IgG4, and IgE antibody responses to filaria antigens first were assessed in serum samples collected in 1975 when all subjects from the $Mf^{+/+}$ and $Mf^{+/-}$ groups were microfilaremic. No significant differences between these 2 groups were found for any of the isotypes measured, although both groups, compared with the EN group, had generally lower IgG levels in 1975 to all 3 parasite antigen stages (data not shown) and significantly lower IgE levels to adult and microfilaria antigens ($P < .02$). In contrast, both the $Mf^{+/+}$ and $Mf^{+/-}$ subjects had significantly higher levels of IgG4 to adult antigen in 1975 than did the EN subjects ($P < .005$; data not shown); IgG4 to microfilaria and L3 antigens did not differ significantly by group.

Between the 2 time points (1975 and 1992), significant changes were observed in antibody isotype levels to all 3 parasite antigens (figure 1 shows representative responses to the microfilaria antigen). Even within the EN population, very significant decreases from 1975 levels were seen for every antibody isotype and for each parasite antigen stage studied ($P < .002$, all comparisons), which most likely reflects decreased filariasis transmission (and exposure to the parasite) because of the island-wide 1-time treatment with DEC in 1987. The $Mf^{+/-}$ subjects showed a similar decrease in all antibody isotype responses to every parasite stage; in contrast, the $Mf^{+/+}$ subjects showed no decreases at all for some responses (e.g., IgG to adult-stage antigens and both IgG4 and IgE antibodies to microfilaria antigen; figure 1) and generally lesser decreases in most other antibody responses to these parasite

Table 1. Bancroftian filariasis study population in the Cook Islands.

Patient group	No. female/ no. male	Age, years	Mf/mL, 1975	Mf/mL, 1992	Overt lymphatic pathology
$Mf^{+/+}$	3/8	67 (27–80)	743 (75–10,000)	28 (2–769)	4 Positive ^a
$Mf^{+/-}$	7/11	52 (20–77)	539 (2–7840)	0	5 Positive ^a
EN	13/3	55 (33–82)	0	0	0

NOTE. Data are median (range), except where noted. EN, endemic normal; Mf, microfilaremia; $Mf^{+/+}$, persistent microfilaremia; $Mf^{+/-}$, cleared microfilaremia.

^a See text for details.

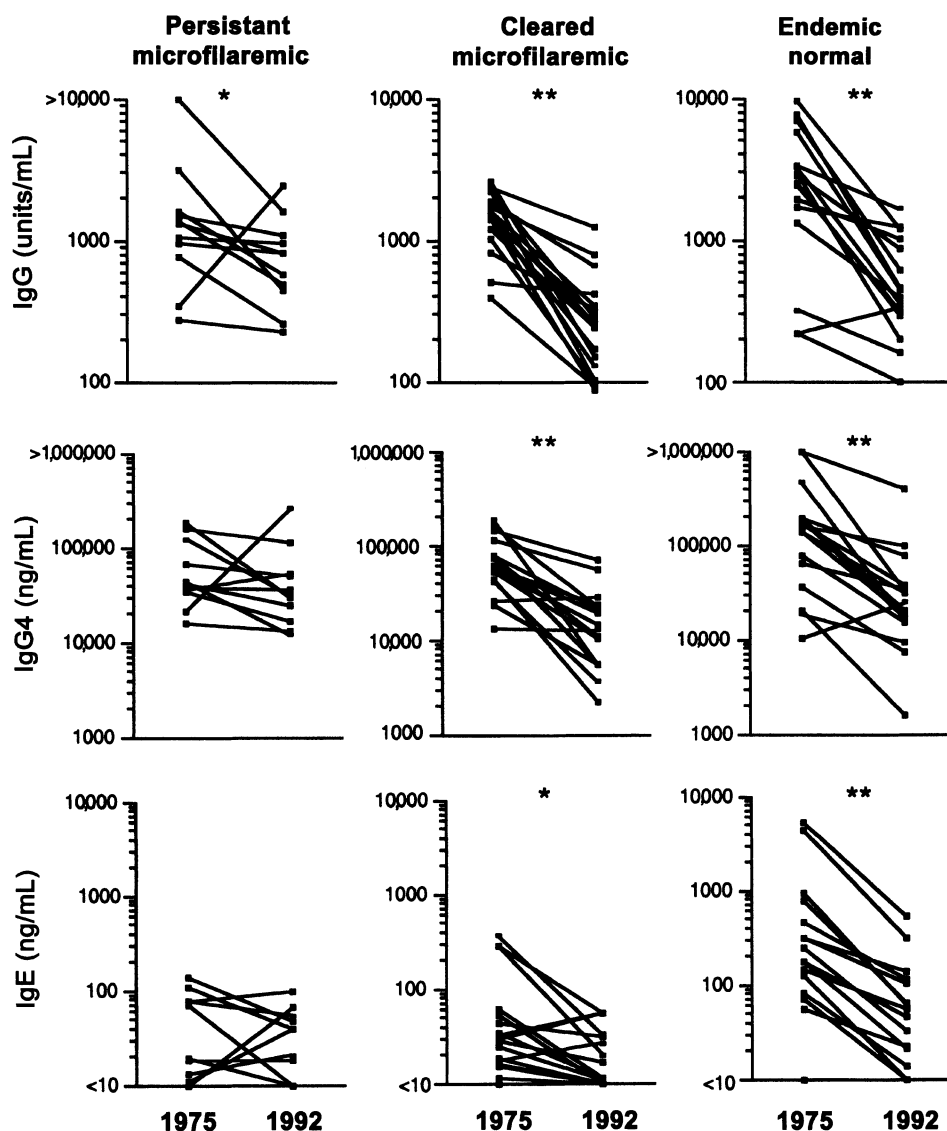


Figure 1. Changes in IgG, IgG4, and IgE antibody responses to microfilarial antigen in persistent microfilaremic ($n = 11$), cleared microfilaremic ($n = 18$), and endemic normal ($n = 16$) groups between 1975 and 1992. Each line represents 1 patient (some obscured by overlapping values); P values were calculated by the Wilcoxon signed rank test. * $P < .05$; ** $P < .001$.

antigens. As a result, in 1992, the antibody responses of the $Mf^{+/+}$ group had become significantly greater than those of the $Mf^{+/-}$ subjects (including the IgG4 levels for all 3 parasite-stage antigens [$P \leq .005$], IgG levels for microfilaria and L3 antigens [$P < .02$], and with trends in the same direction for both the IgG response to adult antigen and IgE responses to all antigens). Of interest, except for higher IgG4 levels to adult and L3 antigens, the $Mf^{+/+}$ group did not differ significantly in 1992 from the EN subjects (data not shown).

Of the 18 $Mf^{+/-}$ persons in 1992, 13 were clinically indistinguishable from the EN subjects (i.e., they had no overt pathology of lymphedema or hydrocele). When the 1992 antibody responses of this subset of patients were compared with those

of the EN group, the results were surprising. Despite their clinical and parasitologic similarities, the clinically normal, cleared microfilaremic group had lower antibody levels than those of the "true" EN group (responses to microfilaria antigen shown in figure 2; $P \leq .05$).

Cellular responses. The $Mf^{+/-}$ subjects had significantly higher lymphocyte proliferation responses to adult antigen (but not to microfilaria or L3 antigens) than did the $Mf^{+/+}$ persons; however, both the $Mf^{+/+}$ and $Mf^{+/-}$ groups had significantly lower responses to adult and microfilaria antigens than did the EN subjects (figure 3). Proliferation responses to NPAg and to the L3 antigen preparation did not differ by study group.

In vitro cytokine production was not significantly different

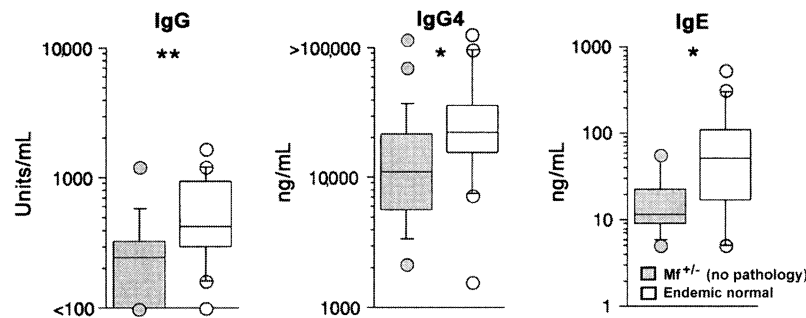


Figure 2. Comparison of IgG, IgG4, and IgE antibody levels to microfilarial (Mf) antigen in 1992 between persons who previously cleared their microfilaremia and had no overt lymphatic pathology ($Mf^{+/-}$; $n = 13$) and endemic normal subjects ($n = 16$). Each box shows 3 horizontal lines representing 25th, 50th (median), and 75th percentiles of the data. Vertical lines denote 10th and 90th percentiles; circles indicate values outside the percentiles. P values were calculated by the Mann-Whitney U test. * $P \leq .05$; ** $P \leq .02$.

between the $Mf^{+/+}$ and $Mf^{+/-}$ groups for any of the parasite antigens, although the $Mf^{+/-}$ persons tended toward higher responsiveness (table 2). As with the lymphocyte proliferation responses, however, both groups produced significantly lower levels of most cytokines in response to adult and microfilaria antigens than did the EN subjects; all groups produced similar cytokine levels in response to both L3 antigens (table 2) and NPAG (data not shown). Inclusion or removal of the 5 persons with overt lymphatic pathology in the $Mf^{+/-}$ group did not alter the significance of the conclusions for either the proliferation or cytokine results.

Although cytokine production did not differ between the $Mf^{+/+}$ and $Mf^{+/-}$ groups, there were significantly more IL-5 responders to microfilaria antigen in the $Mf^{+/-}$ group than in the $Mf^{+/+}$ group (71% vs. 27% of patients, respectively; $P = .05$, Fisher's exact test). In addition, within the $Mf^{+/-}$ group, there was a differential responsiveness to the antigen stages; that is, there were more IL-5 responders to adult (16/17) and microfilaria antigen (12/17) than to L3 antigen (2/16; $P \leq .001$). However, this differential antigen responsiveness in the number of IL-5 responders was not seen in the $Mf^{+/+}$ group. Furthermore, although the number of IFN- γ responders did not differ between the $Mf^{+/+}$ and $Mf^{+/-}$ groups to the different antigen stages, both groups had significantly more responders to L3 antigen than to microfilaria antigen (55% vs. 0% [$P = .006$ for the $Mf^{+/+}$ group] and 63% vs. 13% [$P = .009$ for the $Mf^{+/-}$ group]).

Relationship between changes in antibody levels and cellular proliferation. We examined the changes in antibody levels between 1975 and 1992 (assessed as a percent change for each patient between the 2 time points) in the patient population, to detect any correlation with the ability of persons to proliferate to parasite antigens in 1992. Of interest, when the entire study population was evaluated, changes in IgG, IgG4, and IgE to BmA were correlated significantly with proliferation responses to the same antigen, such that the greater the loss of antibody, the higher the proliferation response ($P \leq .01$, all

comparisons). Of further interest, the 1992 levels of IgG4 to BmA correlated negatively with proliferation to BmA, whereas IgE to microfilaria antigen was positively correlated with proliferation to microfilaria antigen ($\rho = -0.652$ and $\rho = 0.505$, respectively; $P \leq .001$).

Discussion

The principal goal of this study was to evaluate changes over time (17 years) in the parasitologic presentation and immune responsiveness to parasite and other antigens in persons living in an area endemic for bancroftian filariasis. Comparison of such changes could offer important insight into the mechanisms that underlie the altered immunologic and clinical manifestations of persons living in filariasis-endemic areas.

All 45 study subjects had undergone thorough clinical and parasitologic evaluation 17 years before the present study, so any change in these parameters could be accurately defined. In addition, serum samples were obtained from all persons and were stored in liquid nitrogen or at -70°C since that time. Thus, direct comparison of humoral immune responses 17 years apart could be made. Two sets of detailed comparisons were possible: first, between persons who had remained microfilaremic (presumably for 17 years) and those who had cleared their microfilaremia during that time; and, second, between an EN population whose status had remained unchanged for 17 years and a group of amicrofilaremic persons who would have met the criteria for EN subjects in a cross-sectional study (i.e., clinically healthy, Mf^{-} , and CAg^{-}) but whose prior evaluation had shown them to be microfilaremic 17 years earlier. Previously, such very long-term longitudinal observations accompanied by detailed immunologic assessments have only been made in experimental animals [13].

The study design of this assessment was complicated by the fact that, in 1987 (5 years before the current study), an effort to treat the entire population with DEC for 5 days resulted in a very significant decrease of infection on the island. Indeed, a 30%

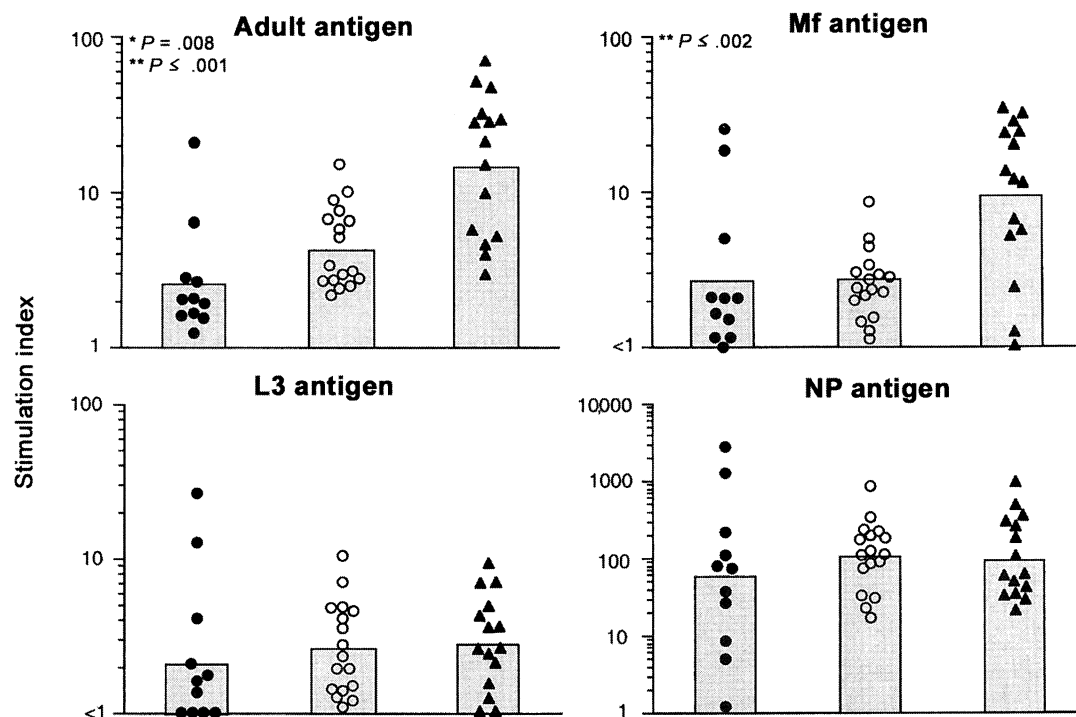


Figure 3. Lymphocyte proliferation responses (expressed as stimulation indices) to parasite (adult, microfilarial [Mf], and L3) and nonparasite (NP [purified protein derivative or streptolysin O]; see Methods) antigens among persistent microfilaremic (Mf^{+/+} [●]; *n* = 11), cleared microfilaremic (Mf^{+/-} [○]; *n* = 18), and endemic normal (EN [▲]; *n* = 16) subjects. Each symbol represents 1 patient; gray boxes are geometric means. *P* values were calculated by the Mann-Whitney *U* test. *Significance between Mf^{+/+} and Mf^{+/-} groups. **Significance between EN group and both Mf^{+/+} and Mf^{+/-} groups.

microfilaremia prevalence in 1975 fell to 5% in 1992, so that the “force of infection” to which the population was exposed decreased significantly, although transmission was still ongoing [9]. The effect that this decrease had on the specific immune responsiveness of the population is clearly reflected in the significant decreases in antibody responses (IgG, IgE, and IgG4) to specific parasite antigens in the uninfected EN population.

Of particular interest, the Mf^{+/-} cleared microfilaremia group, who no longer had filarial infection to stimulate antibody responses from within, showed dramatic decreases in antibody levels very similar to those of the EN group. In contrast, the Mf^{+/+} group, with persistent microfilaremiases and persistent stimulation of their immune systems, showed either no change in the levels of antibody responses over the 17 years or lesser decreases than the other 2 groups. In experimental animal models, clearance of microfilariae is often accompanied by an increase in specific antifilarial antibodies [14, 15], but the Mf^{+/-} subjects of the present study showed no such increase (comparing their 1975 and 1992 values). One reason might be that the increase, if and when it occurred, was transient and was not detectable in the 1992 serum samples; alternatively, any relative increase in specific antibodies that might have been involved either in clearing the microfilaremia or in maintaining an amicrofilaremic state might have been masked by the si-

multaneously diminished responsiveness resulting from the decreased force of infection on the island in recent years and the concomitant lower level of stimulation to these subjects.

In contrast to the dramatic differences seen between antibody responses of the Mf^{+/+} and the Mf^{+/-} subjects, the down-regulation characteristic of responses in Mf⁺ individuals [4–6] appeared to be largely maintained in the Mf^{+/-} group (figure 3) even after the microfilaremia and infection had disappeared; for only 1 of the 3 parasite antigens tested did the Mf^{+/-} group have proliferation responses greater than those of the Mf^{+/+} group, and for none of the cytokine responses to any of the 3 parasite antigens were the levels of response between the 2 groups significantly different (table 2). Thus, it appears that the down-regulated T cell responses to parasite antigen, so characteristic of microfilaremic patients, persist long after the microfilaremia and active parasite infection (as defined by the circulating antigen assay) disappear. This finding contrasts with the increase in parasite-specific cellular responses seen in some posttreatment studies [16–18]; however, these studies were comparatively short-term and, in one study [16], the increase in responses was primarily seen in asymptomatic amicrofilaremic patients and in patients with elephantiasis rather than in microfilaremic persons. One possible explanation is that enhancement of cellular responses after drug treatment leads to an

Table 2. Cytokine responses to filarial parasite antigens in persistent microfilaremic (Mf^{+/+}), cleared microfilaremic (Mf^{+/-}), and endemic normal (EN) patients.

Cytokine, clinical group	Adult antigen, pg/mL	Mf antigen, pg/mL	L3 antigen, pg/mL
IL-2			
Mf ^{+/+}	20.5 (<10–1051) ^a	<10.0 (<10 to <10) ^a	24.3 (<10–347)
Mf ^{+/-}	72.2 (<10–1574) ^a	25.3 (<10–914) ^a	15.4 (<10–949)
EN	707.0 (146–2597)	221.9 (<10–1402)	21.9 (<10–227)
IFN- γ			
Mf ^{+/+}	25.7 (<10–447)	<10.0 (<10 to <10) ^a	83.2 (<10–4212)
Mf ^{+/-}	24.7 (<10–346) ^b	17.1 (<10–242) ^b	86.4 (<10–1169)
EN	107.8 (<10–3238)	52.0 (<10–421)	105.2 (<10–1126)
IL-5			
Mf ^{+/+}	29.1 (<3–490) ^a	5.8 (<3–672) ^a	3.2 (<3–36)
Mf ^{+/-}	74.0 (<3–934)	17.6 (<3–119) ^a	<3.0 (<3–34)
EN	153.1 (<3–491)	137.0 (<3–804)	3.1 (<3–22)
IL-10			
Mf ^{+/+}	156.0 (<5–1369)	83.7 (<5–928) ^a	252.0 (<5–559)
Mf ^{+/-}	233.8 (29–1533)	165.1 (24–518) ^b	424.9 (94–815)
EN	383.9 (77–1121)	301.6 (72–1274)	395.2 (149–781)
GM-CSF			
Mf ^{+/+}	11.7 (<5–89) ^a	4.0 (<5–78) ^a	12.5 (<5–118)
Mf ^{+/-}	25.8 (<5–196) ^a	8.1 (<5–102) ^a	11.0 (<5–165)
EN	87.9 (<5–359)	35.9 (<5–355)	19.0 (<5–79)

NOTE. Data are geometric mean (range). GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon- γ ; IL, interleukin.

^a Significant difference with EN group ($P \leq .01$, Mann-Whitney U test).

^b Significant difference with EN group ($P \leq .05$, Mann-Whitney U test).

expansion of antigen-specific cells that is temporary in nature, reverting finally to the previous hyporesponsive state.

How such cellular down-regulation is maintained in patients despite the elimination of infection is unclear. A similar, long-lasting, down-regulated, parasite-specific T cell response has been seen in adolescents who have never been infected but who were exposed to the microfilarial stage in utero [19]. Furthermore, persistent down-regulated cytokine (IFN- γ) responsiveness has been described as long as 2–3 years after treatment of microfilaria-positive patients with either ivermectin or DEC [20]. It is intriguing to speculate that such persons might have developed and maintained a persistent population of regulatory T cells (Th3 and/or Tr1 cells) that produce IL-10 or transforming growth factor- β or otherwise interfere with antigen presentation and thereby cause a down-regulation of T cell effector functions [21]. Such Th3/Tr1 cell populations have been described in patients with chronic onchocerciasis [22].

An additional finding that emerged quite vividly from the current study was the clear distinction between the 2 groups of subjects whose presentation in 1992 would have been defined in any cross-sectional study as EN subjects (i.e., persons with no clinical or parasitologic evidence of infection and no CAg in their blood). Data from 1975 clearly showed that some of these persons had been microfilaremic and had changed to an EN clinical presentation between 1975 and 1992. Despite their clinical and parasitologic similarities in 1992, those who were infected in 1975 were very different immunologically from the true EN subjects. First, the antibody levels to microfilaria (fig-

ure 2) and other parasite antigens in the Mf^{+/-} individuals in 1992 were almost always lower than those of the EN group (the EN subjects were much more similar to the Mf^{+/+} group than to the Mf^{+/-} group). Second, cellular immune responses (both lymphocyte proliferation and cytokine production) were also significantly lower in the Mf^{+/-} group than in the true EN subjects. What these differences mean in terms of protective immunity or other clinical consequences of cellular immune responsiveness is not certain, but the immunologic differences between these clinically similar groups are so prominent that their implications need to be further studied.

It is rare to have the opportunity to carry out long-term follow-up studies of persons or populations endemic for filariasis, but such long-term studies often can be the most informative [23]. In the present study, although the force of infection did not remain constant on the island over the 17 years between detailed evaluations, the 3 study groups could serve as their own internal controls for many of the important comparisons to be made. Certainly, a most intriguing finding was the diminished cellular responsiveness that persisted in the Mf^{+/-} group of patients. Indeed, although these persons appeared to be essentially identical to the EN group in clinical, parasitologic, and CAg aspects, their persistently diminished cellular and humoral responses to parasite antigen indicated that the Mf^{+/-} subjects were a subgroup of patients distinctly different from either the Mf^{+/+} or the EN group. Unlocking the mechanisms that underlie these differences should reveal a great deal not only about the immunologic down-regulation so characteristic of chronic helminth infections and the specific immune responses that determine protective immunity to them but also about possible long-term vestiges of earlier infection that have left permanent imprints on the host's ability to respond immunologically to parasite antigens.

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