

LONG-TERM PERSISTENCE OF CELLULAR HYPORESPONSIVENESS TO FILARIAL ANTIGENS AFTER CLEARANCE OF MICROFILAREMIA

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Abstract. The persistence of parasite-specific cellular hyporesponsiveness after clearance of blood microfilariae (mf) was studied in 18 individuals who had been treated with a single dose of ivermectin, diethylcarbamazine, or a combination 2-3 years previously and who had initially cleared their parasitemia. At recruitment into the present study, 50% were again mf+ and 50% remained mf-. There were no significant differences between the mf+ and mf- groups in the amount of interferon- γ (IFN- γ) produced by peripheral blood mononuclear cells in response to adult or microfilarial antigens, although IFN- γ production in response to purified protein derivative was greater in the mf+ group (geometric mean [gm] = 3,791 pg/ml; P = 0.02) than in the mf- group (gm = 600 pg/ml). These data suggest that although microfilaremic individuals may temporarily regain the ability to produce IFN- γ to parasite antigens post-treatment, they subsequently revert to a state of hyporesponsiveness to mf-containing antigens that appears to be independent of the recurrence of microfilaremia and the response to nonparasite antigens.

The clinically asymptomatic microfilariae (mf)-positive group of *Wuchereria bancrofti*-infected individuals has been of particular immunologic interest because of the relatively sparse inflammatory response seen despite the high intravascular parasite burden. Peripheral blood mononuclear cells (PBMCs) from the majority of individuals with subclinical microfilaremia display an inability to proliferate or produce interferon- γ (IFN- γ) to filarial adult, mf, or larval antigens.¹⁻⁴ These findings, combined with the elevated polyclonal IgE and antifilarial IgG4 levels found in these patients, suggests an alteration in the type 1 helper (Th1)/Th2 balance that may be mediated in part by interleukin-10 (IL-10).¹ Persistence of antigen^{5,6} and *in utero* exposure to microfilariae⁷ have been postulated as causes for the alteration of responses, as have mechanisms of peripheral tolerance such as antigen-induced apoptosis of parasite-specific T cells.⁸

Recent studies in lymphatic filariasis have demonstrated that changes in parasite antigen-specific proliferative responses and cytokine production may depend on the presence of active infection as measured by circulating filarial antigen (CAg) independent of clinical presentation,^{5,9} with the absence of CAg being associated with greater antigen-driven proliferation and production of IL-2 and IFN- γ .⁵ In addition, although cellular responses to parasite antigens vary and appear to correlate to a large degree with infection status, the production of IFN- γ in response to nonparasite antigens and mitogens appears to be independent of the stage and extent of disease, with PBMCs from infected patients maintaining a Th1-type response to purified protein derivative (PPD) in the face of nonresponsiveness to adult worm antigen.^{5,10}

Chemotherapy of the asymptomatic microfilaremic form of infection with *W. bancrofti*, however, markedly alters cellular responses to parasite antigens, with changes in responses to nonparasite antigens being variable. There is, however, considerable variation among published studies of post-treatment immunologic changes with regard to mode and duration of treatment, populations studied, geographic locales, and the time points at which immune responses were assayed. Studies of Bancroftian filariasis in Haiti^{11,12} and Ma-

layan filariasis in Indonesia¹³ have demonstrated increased T cell proliferation to parasite antigen at a variety of timepoints (3.9-12 months) following treatment with diethylcarbamazine (DEC) administered at a dose of 6 mg/kg/day or /week for 12 doses¹¹ or with ivermectin.¹² Of interest, PBMCs from a population in the Cook Islands infected with *W. bancrofti* displayed not only impaired cellular proliferation to *Brugia malayi* worm extract at baseline, but ongoing suppression of proliferation for up to two weeks following treatment with DEC.¹⁴

Sartono and others¹⁵ have shown that in addition to T cell proliferative responses, production of IFN- γ in response to *B. malayi* crude adult antigen increased significantly one year after treatment in a study population in Indonesia, an area endemic for Brugian filariasis; however, patients in this study were treated with DEC every week for the entire year prior to reassessment of their cellular responsiveness. Diethylcarbamazine, in addition to its antifilarial activity, has measurable antifungal and antibacterial properties¹⁶ and has been shown to exert an effect on the response to mf *in vitro* independent of the effector cell population.¹⁷

Because strategies of single-dose administration of antifilarial chemotherapeutic agents (ivermectin or DEC) are becoming the norm, the present study was designed to investigate long-term alterations in cellular responses in previously microfilaremic individuals who had been treated 2-3 years prior to recruitment with single-dose DEC, ivermectin, or a combination of the two with an eye toward understanding the long-term immunologic consequences of single-dose therapy.

PATIENTS, MATERIALS, AND METHODS

Patients. The study was carried out at the Government General Hospital in Madras, India under a protocol approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD) and the Ethics Committee of the participating institute in Madras. Patients were identified from a cohort of individuals followed for a prior study of mf clear-

TABLE 1
Study population*

Mf status	Treatment	Mf count/ml		CAG status
		Pretreatment	2-3 years posttreatment	
Negative	D	625	0	Negative
Negative	S	335	0	Negative
Negative	D	520	0	Negative
Negative	D	285	0	Negative
Negative	D	115	0	Positive
Negative	D	208	0	Positive
Negative	D	445	0	Positive
Negative	D	150	0	ND
Negative	D	100	0	ND
Positive	D	150	40	Positive
Positive	D	240	15	Positive
Positive	D	398	6	Positive
Positive	S	230	400	Positive
Positive	S	105	220	Positive
Positive	D	102	200	Positive
Positive	NA	> 100	150	ND
Positive	S	350	90	ND
Positive	D	375	41	ND

* Mf = microfilariae; CAG = circulating filarial antigen; D = DEC + ivermectin; S = DEC or ivermectin; ND = not determined; NA = not available.

ance after treatment with single-dose ivermectin (400 kg/kg), single-dose DEC (6 mg/kg), or single-dose combinations of ivermectin and DEC at various doses. From the original group, 18 individuals were identified, each of whom had cleared their mf within the first year following treatment (Kumaraswami V, unpublished data). Each had been treated between two and three years prior to the present study, and all had remained in endemic areas in and around Madras. Individuals had received no further treatment with antifilarial drugs and were all healthy by history and physical examination. Informed consent was obtained from all patients.

Blood collection and treatment. A venous blood sample was collected in heparin between 9:00 PM and 10:30 PM. One milliliter was filtered through a 0.2- μ m Millipore (Bedford, MA) filter for assessment of mf counts. These individuals returned the next morning at 10:00 AM for a second peripheral venous blood sample for immunologic studies. These times were chosen because of the nocturnal periodicity of *W. bancrofti*; by 10:00 AM, peripheral blood was expected to be free of circulating mf in southern India. Blood was collected in EDTA Vacutainer[®] tubes (Becton Dickinson, Franklin Lakes, NJ) and used for flow cytometry studies and whole blood cell culture. Filtration of blood for mf counts was done by a single individual who was not involved in the immunologic studies, and the mf status of each individual in the study was not disclosed until all data had been collected. All patients were given a single dose of DEC (6 mg/kg) after the morning blood sample was taken.

Cell culture. Whole blood (1.5 ml) collected in EDTA was diluted 1:2 in RPMI 1640 medium (BioWhittaker, Walkersville, MD) with 10% fetal calf serum, gentamicin (1.6 mg/L), 1% HEPES, and 1% L-glutamine, and 200 μ l of the resulting solution was placed in each well of a 96-well cell culture plate (Costar, Cambridge, MA). Wells were set up in triplicate for each condition, and a media control in triplicate was used for each patient on each plate. Phytohemagglutinin (PHA) at a concentration of 10 μ g/ml was used as the mi-

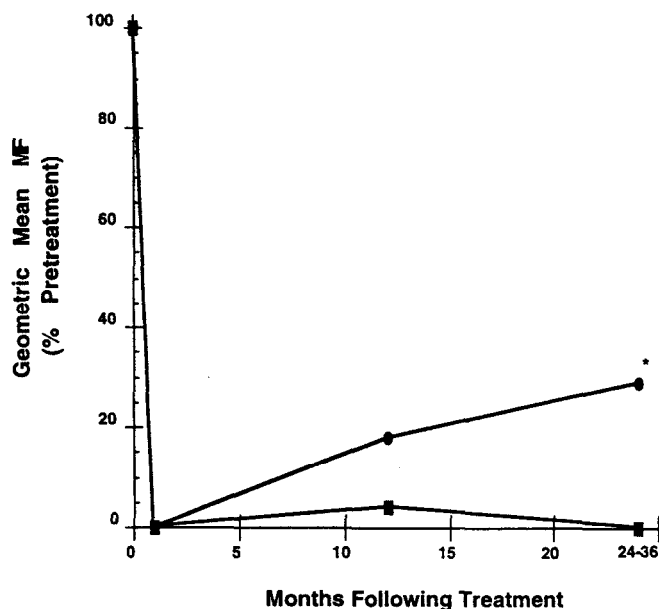


FIGURE 1. Microfilarial status as a function of pretreatment levels in the microfilariae (mf)+ (■) and mf- (●) groups over the study period in months. The asterisk indicates statistically significant differences between the two groups.

togen, while the antigens used were *B. malayi* crude adult antigen (BmA, 10 μ g/ml), *B. malayi* male antigen (BmM, 5 μ g/ml), *B. malayi* microfilarial antigen (mf, 1 μ g/ml), and *Mycobacterium tuberculosis* PPD (10 μ g/ml). Supernatants from the media control wells and the PI-IA-stimulated wells were collected at 24 hr, while supernatants from media control wells, BmA, BmM, mf, and PPD wells were collected at 72 hr. Supernatants were stored at -70°C until assayed.

Cytokine analysis. Interleukin-5 and IFN- γ were measured in supernatants obtained from whole blood culture by an ELISA as described previously.¹⁸ Four supernatants could not be analyzed due to contamination in the plate. Values in pg/ml were obtained by interpolating from a standard curve; the sensitivity of the IL-5 and IFN- γ assays were 7.8 pg/ml and 39 pg/ml, respectively.

Circulating filarial antigen assays. Thirteen of 18 plasma samples from patients were assayed quantitatively for the presence of *W. bancrofti* CAG using a *W. bancrofti* antigen detection kit (TropBio; JCU Biotechnology Pty., Ltd., Townsville, Australia).

Statistical analysis. Statistical analyses were performed using the Mann-Whitney paired test and Spearman rank correlation.

RESULTS

Eighteen male patients were identified from the records of a previous multi-arm treatment study at Government General Hospital, Madras (Kumaraswami V, unpublished data). A summary of previously collected and current parasitologic and treatment data is shown in Table 1. All patients had pretreatment microfilarial counts that ranged between 100 and 625 parasites/ml, and the groups did not differ in this regard. All patients initially cleared their circulating mf (Figure 1), and by one year post-treatment, the currently mf+

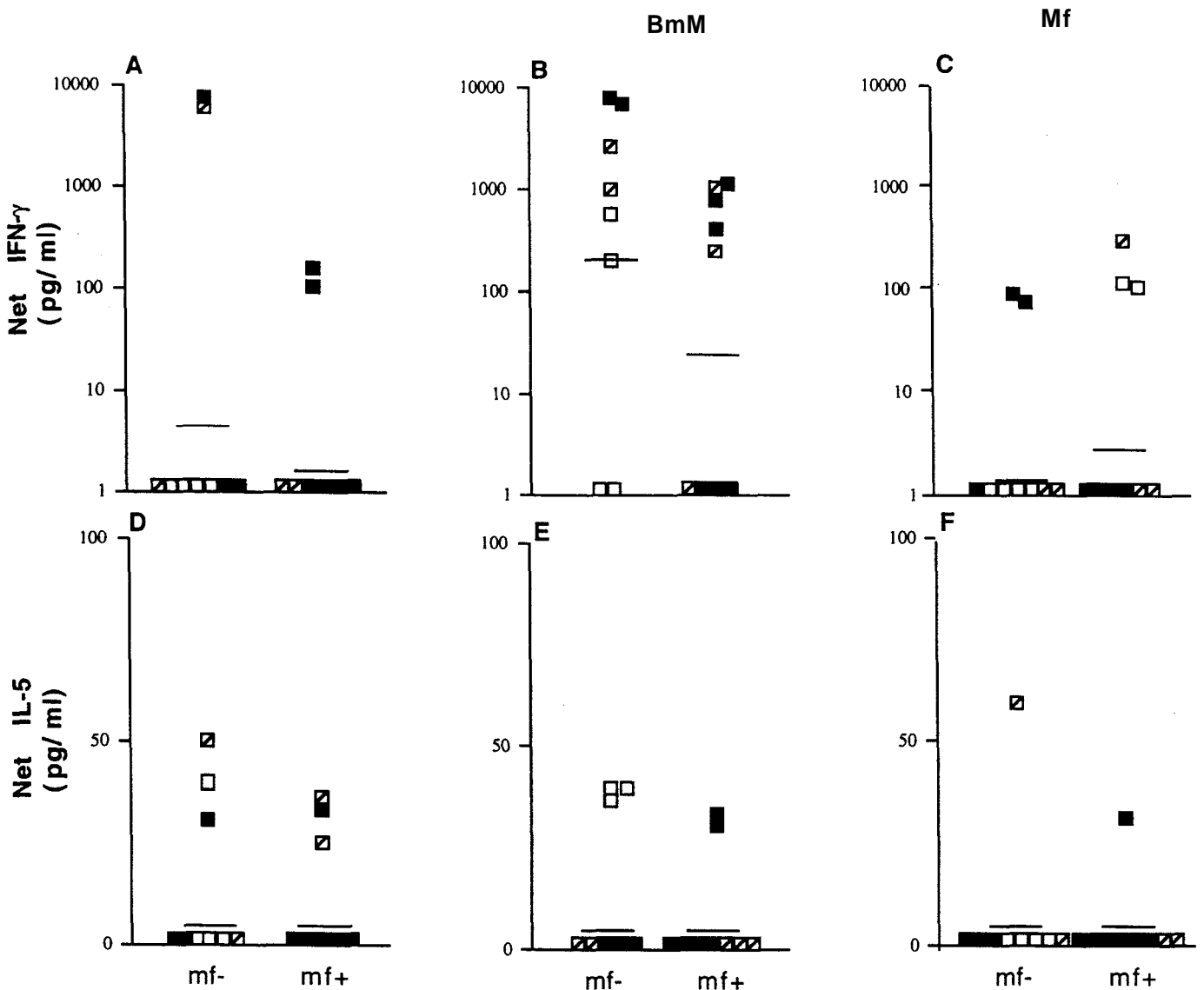


FIGURE 2. Net interferon- γ (IFN- γ) (A-C) and interleukin-5 (IL-5) (D-F) production in response to *Brugia* mixed adult antigen (BmA; A and D), adult male antigen (BmM; B and E), and microfilarial antigen (Mf; C and F). Each square represents an individual patient, and the horizontal bars are the geometric means. The shaded symbols indicate circulating filarial antigen (CAg) positivity, open symbols indicate CAg negativity, and striped symbols indicate those in whom CAg assays were not performed.

group had higher levels of blood mf (geometric mean [gm] percentage pretreatment = 18.8% versus 4.2%). This difference was more apparent at the 2-3-year timepoint ($P < 0.0001$) (Figure 1). Of the 18 individuals in this follow-up study 2-3 years after initial treatment, nine were mf+ (range = 6-400 mf/ml), and nine were mf- by filtration of 1 ml of peripheral blood (Table 1).

In the present study, CAg assays were performed on samples from the 13 of 18 patients from whom plasma was available. Of these 13, seven were mf- by filtration; four of the mf- group were also CAg- and three were CAg+ (Table 1). The remaining six were mf+ and CAg-.

The production of IL-5 was equivalent between the mf- and mf+ groups, with very low geometric means in response to parasite antigens, net production being ~3.4 pg/ml and 3.7 pg/ml to BmA, 4.0 pg/ml and 2.2 pg/ml to BmM, and 1.6 pg/ml and 1.5 pg/ml to Mf antigen in the mf- and mf+

groups, respectively (Figure 2D-F). When the groups were analyzed by CAg status, there were no significant differences in amount of IL-5 produced by the mf- CAg- group compared with either the mf- CAg+ group or the mf+ CAg+ group.

The IFN- γ response to BmA (mixed female and male), BmM, and mf antigens did not differ between the groups (Figure 2A-C). The PBMCs from only two of nine mf- patients and two of eight mf+ patients responded to BmA with production of IFN- γ (gm = 6.1 pg/ml and 2.3 pg/ml, respectively). The response to mf antigens was similarly muted, with only two of nine mf- and three of nine mf+ patients producing IFN- γ (gm = 1.7 pg/ml and 4.4 pg/ml, respectively), while the response to BmM was much more pronounced, with six of eight mf- patients (gm = 249.1 pg/ml) and five of nine mf+ patients (gm = 34.3 pg/ml) producing IFN- γ . The differences between the mf+ and mf-

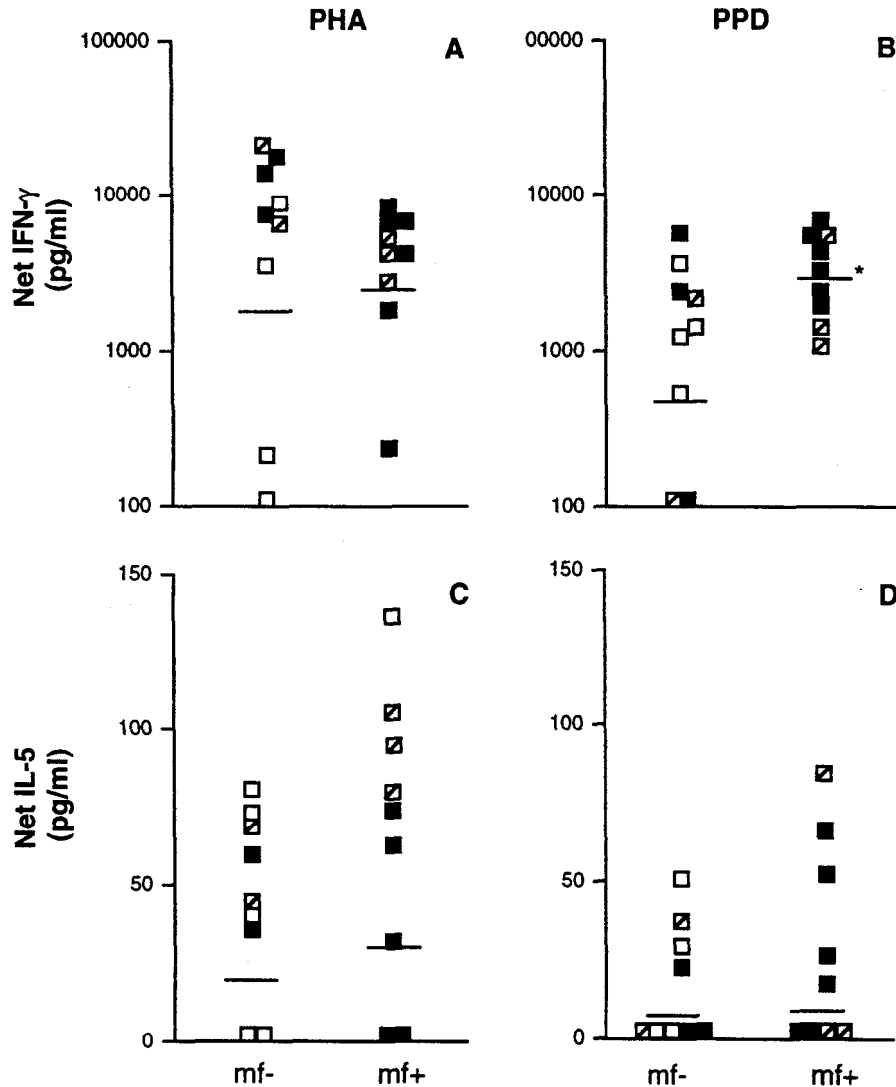


FIGURE 3. Net interferon- γ (IFN- γ) (A and B) and interleukin-5 (IL-5) (C and D) production in response to phytohemagglutinin (PHA; A and C) and purified protein derivative (PPD; B and D). Each square represents an individual patient, and the horizontal bars are the geometric means. The shaded symbols indicate circulating filarial antigen (CAG) positivity, open symbols indicate CAG negativity, and striped symbols indicate those in whom CAG assays were not performed. mf = microfilariae. The asterisk indicates a statistically significant difference between the groups.

groups as a whole were not statistically significant; when broken down by CAg status, the only significant difference was that the production of IFN- γ in response to BmM by the mf- CAg+ patient was greater than its production by the mf+ group ($P = 0.04$).

In contrast to the responses to BmA and mf antigen, measurable IFN- γ responses to nonparasite antigen and to mitogen were seen in all patients. Production of IFN- γ in response to PPD (Figure 3B) was significantly greater in the mf+ group (gm = 3,971 pg/ml; $P = 0.02$) than in the mf- group (gm = 600 pg/ml). When broken down by CAg status, there was significantly less production of PPD-driven IFN- γ by the CAg- mf- group compared with the CAg+ mf+ group ($P = 0.02$). Production of IL-5 (Figure 3D) in response to PPD was seen in more patients and to a greater degree than that in response to parasite antigen, although there were no differences between the groups based either on microfilarial status or on CAg status. Similarly, all pa-

tients who were capable of producing IFN- γ and IL-5 to parasite antigens produced more IFN- γ and IL-5 in response to PHA than to these antigens. There was no statistically significant difference in PHA responsiveness between the mf+ and mf- groups (Figure 3). When the data were analyzed based on CAg status, the only significant difference was that the mf- CAg+ group produced more IFN- γ in response to PHA (gm = 12,977 pg/ml) than did the mf+ CAg+ group (gm = 3,322 pg/ml; $P = 0.03$).

DISCUSSION

It is well established that most individuals with clinically asymptomatic microfilaremia are relatively unresponsive to adult¹⁻³ and microfilarial⁴ derived parasite antigens as measured by proliferative responses or production of IFN- γ or IL-2. The reasons for this appear to be multifactorial and include regulation by IL-10,¹⁹⁻²² genetic background, dura-

tion and intensity of infection,²² *in utero* exposure to parasite antigens,⁷ and the presence of soluble suppressive parasite products.²³ Each of these mechanisms has been implicated in skewing the immune response away from the production of IFN- γ and IL-2. By whatever mechanism it occurs, the skewed response reflects the markedly diminished frequency of antigen-reactive IFN- γ -producing cells rather than an increase in IL-4- and IL-5-producing cells.²⁴

Studies in lymphatic filariasis^{11-13,14} as well as in schistosomiasis²⁵ and in onchocerciasis²⁶⁻³⁰ have, however, demonstrated a partial reversal of cellular hyporesponsiveness to parasite antigens and enhancement of responses to nonparasite antigens following treatment of these infections. Most of these studies demonstrated an increase in IFN- γ and IL-2 production in a segment of the infected study population to parasite-specific antigen and mitogen. Although study conditions and parameters varied widely, these changes appeared to be maintained for six months to one year post-treatment, but by two years, in at least one study,²⁸ responses returned to baseline.

Fifty percent of the 18 individuals in the present study who had cleared their microfilaremia following single-dose DEC or ivermectin or a combination of the two were found to be mf+ 2-3 years following treatment. Of interest, two patients who were mf+ at one year post-treatment were mf- at the time of the present study (Figure 1 and Table 1); this is likely reflective of the segment of an infected population that spontaneously clears infection with *W. bancrofti*.³¹

Of importance, however, regardless of recurrence of infection or CAg status, most of the responses to mf-containing antigens in the group as a whole were muted, whereas the responses to PPD and PHA were intact. Indeed, consistent with earlier findings,³ we found that the mf+ individuals produced significantly more IFN- γ than the mf-group in response to PPD regardless of antigen status (Figure 3B). In contrast, production of IFN- γ to mf-containing antigens (Figure 2) was equivalent but low in the mf- and mf+ post-treatment groups (also independent of antigen status), again suggesting the existence of host factors in the diminished cellular response displayed to mf containing antigens independent of parasite-derived factors. The relatively greater amount of IFN- γ produced in response to BmM in both groups (Figure 2) probably reflects the absence of gravid female worms with mf in the antigen preparation and corroborates earlier findings.²¹

Few patients in either group produced IL-5 in response to adult or microfilarial antigens (Figure 2), and those who did produced lower amounts of this cytokine than in response to PHA. This finding may reflect a lack of antigen-driven IL-2 production that may be required for optimal IL-5 production.³² It may also be a consequence of using whole blood rather than purified PBMCs, or perhaps a function of length of exposure (e.g., age) to the parasite, as has been recently suggested.³³

Our data support other studies^{5,10,34} indicating that responses to parasite and nonparasite antigens are differentially regulated in filarial-infected individuals. Even in the absence of Th1-like responses to parasite antigens, responses to PPD remained Th1-like, with significant amounts of IFN- γ produced by both the mf- and mf+ groups in response to this nonparasite antigen.

The results from this study differ somewhat from studies of Brugian filariasis,¹⁵ where the reversal of cellular response was maintained for periods of up to one year. However, our study looked at time points 2-3 years after treatment, assessed only cytokine production rather than proliferation, and used a different DEC regimen with lower total quantities of DEC. Furthermore, we found that the persistent hyporesponsiveness to parasite antigens appeared to be independent of CAg status, in contrast to the findings of Dimock and others,⁵ although the number of CAg+ mf- patients in our study was low.

In summary, our study shows that 2-3 years after treatment, individuals previously infected with *W. bancrofti* continue to display mf-specific cellular hyporesponsiveness regardless of their current mf status. That these patients are able to maintain a Th1 response to nonparasite antigens augers well for the formulation and implementation of health and immunization programs in these regions.

The fact that half of our study population reacquired mf positivity 2-3 years following their initial treatment underlines the importance of filariasis control programs and regular chemotherapy in areas of high disease prevalence. In the present study, five (62.5%) of eight mf+ patients had been treated with combination single-dose therapy, while 89% (8 of 9) of the mf- group were treated with combination therapy. This would suggest that although a single-dose of either drug appears to be completely effective in initial microfilarial clearance,³⁵⁻³⁷ the combination of ivermectin and DEC is more effective for long-term suppression of microfilaremia or cure.³⁸ Therefore, as has been proposed,³⁹ implementation of yearly single-dose therapy with DEC and/or ivermectin would be indicated to establish and maintain a decrease in the reservoir of infection.

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