

Reversal in microfilarial density and T cell responses in human lymphatic filariasis

ERLIYANI SARTONO¹, CRISTINA LOPRIORE¹, YVONNE C.M.KRUIZE¹, AGNES KURNIAWAN-ATMADJA², RICK M.MAIZELS³ & MARIA YAZDANBAKHS¹

¹Department of Parasitology, Leiden University, Wassenaarseweg 62, Postbus 9605, 2300 RC Leiden, The Netherlands,

²Department of Parasitology, University of Indonesia, Jakarta, Indonesia and ³Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, UK

SUMMARY

This study reports reversals in microfilarial density and the accompanying changes in cellular immune responses to filarial antigens of 39 individuals (11 microfilaria-positives, 22 microfilaria-negatives and six converters) living in an area endemic for brugian filariasis. Microfilarial counts decreased from April, the end of the rainy season to July, middle of the dry season (g.m. 88 mf/ml and 38 mf/ml, respectively; $P=0.001$) and subsequently increased in November, the beginning of the rainy season ($P=0.088$). Whereas the proliferative responses remained low throughout the study period in microfilaraemic individuals, in amicrofilaraemics these responses changed in the opposite direction to that of microfilarial densities. In three converters, proliferation changed in the opposite direction to the presence or absence of microfilariae. Cytokine analysis in the converters revealed that interferon- γ was most affected by the shifts in microfilarial densities. In contrast, interleukin-4 responses showed little correlation with changes in parasite densities.

Keywords filariasis, *Brugia malayi*, microfilaraemia, T cell responses, Th1/Th2 cytokines

INTRODUCTION

Residents of areas endemic for filariasis are continuously exposed to mosquito-borne infective larvae (L3). Cross-sectional studies have shown that a proportion of these residents harbor no detectable microfilariae and have no clinical history or evidence of infection (Ottesen 1984, Ottesen 1989, Day 1991). These individuals variously termed asymptomatic amicrofilaraemics, endemic normals or putatively immune individuals, typically exhibit higher cellular immune responses in terms of filarial antigen-specific proliferation and interferon (IFN)- γ release. This is in contrast to microfilaria-positive individuals who generally show specific T cell unresponsiveness to parasite antigens (Ottesen *et al.* 1977, Steel *et al.* 1996, Sartono *et al.* 1997).

Our understanding of immunological events in filariasis is severely hampered by the lack of longitudinal data on responsiveness. To date, it has not been possible to measure both parasite density and cellular immunological reactivity over time without chemotherapeutic intervention. To begin to address this deficiency, we devised a study of short-term (7 months) duration of asymptomatic cases to follow the stability of infection and unresponsiveness.

In this study, without therapeutic intervention, we carried out three blood sampling surveys of an exposed population who were either microfilaria positive or negative but were clinically asymptomatic, living in an area endemic for brugian filariasis where the microfilarial rate was 35%. The cellular immune responses and their correlation with the microfilarial levels could thus be evaluated individually.

MATERIALS AND METHODS

Study population

During an extensive field study in Langaleso, Central Sulawesi, Indonesia, it was possible to carry out a subproject

Correspondence: M.Yazdanbakhsh

Received: 5 February 1999

Accepted for publication: 28 May 1999

studying, longitudinally, 39 residents of this anthropophilic *Brugia malayi* endemic area (Partono & Purnomo 1987). Informed consent was obtained from all patients before clinical and parasitological study and blood withdrawal was performed in accordance with the guidelines of the Indonesian Department of Health and Human Services. Blood sampling was carried out at three time points in April, July and November 1993. Blood collection for parasitologic examination took place between 20:00 h and 23:00 h, in accordance with the periodicity of the microfilariae (mf) in this area (Partono & Purnomo 1987). The microfilaria prevalence rate was assessed by Nuclepore (Nuclepore Corporation, Pleasanton, CA, USA) filtration of 1 ml blood. The filtration and counting of the microfilariae was done by the same person on all three occasions. The residents of this village had never taken diethylcarbamazine (DEC) before this study. On completion of the study, all were treated with a standard dose of DEC (WHO Expert Committee on Filariasis, 1992). The participants of this study were asymptomatic and free of filarial symptoms (lymphadenopathy and/or pitting oedema) or disease, and the protocol required that any person who developed symptoms during the study would be given DEC treatment and excluded. Individuals were categorized into two groups, amicrofilaraemics and microfilaraemics. Amicrofilaraemics ($n = 22$) had no detectable microfilariae in 1 ml night blood at all three time surveys, while 11 microfilaraemics had circulating peripheral blood microfilariae at all three time surveys. Interestingly, six individuals had microfilariae in the peripheral blood at one or two sampling times but were negative at others (Table 1). These individuals were termed 'converters'.

Antigen and mitogen

B. malayi adult worm antigen (BmA) used for this study was prepared as described before (Yazdanbakhsh *et al.* 1993). Phytohemagglutinin (PHA; Wellcome Diagnostics,

Dartford, UK) and purified protein derivative of *Mycobacterium tuberculosis* (PPD; Statens Seruminstitut, Copenhagen, Denmark) were used in parallel cultures. Antigens and mitogen for the whole study were all from the same batch.

Proliferation assay and cytokine production *in vitro*

Heparinized venous blood was collected, transported by road and air to Jakarta where peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll isopaque density gradient, as previously described (Yazdanbakhsh *et al.* 1993; Sartono *et al.* 1997). PBMC were either cultured directly for *in vitro* cytokine production or cryopreserved in liquid nitrogen and transported to Leiden University until use. Proliferation assays were done as described previously (Yazdanbakhsh *et al.* 1993). Cryopreserved cells were cultured in the presence of PHA (2 µg/ml), PPD (10 µg/ml) or BmA (12.5 µg/ml). Cultures for *in vitro* cytokine production from July and November samples used fresh PBMC at 5×10^6 cells in 24 well plates in 1 ml of Iscove's medium containing 5% fetal calf serum in the presence of 12.5 µg/ml BmA or with medium only. Lack of facilities during our first field trip in April meant that cells had to be cryopreserved and examined in Leiden. The cryopreserved cells were cultured at 10^6 in round bottom microtitre plates in a total volume of 200 µl. To be able to directly compare cytokine release from April samples, which was from cryopreserved cells, with those of the July samples, we also cultured the July samples for cytokine release using frozen cells (10^6 cells in 200 µl). Supernatants were collected on days 3 and 5 [optimal time points for interleukin (IL)-4 and IFN-γ release, respectively] and stored at -70°C until assayed.

Assessment of cytokines in supernatants

Cytokine concentrations were determined using commercial kits (Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam, The Netherlands) with

Table 1 Summary of study population

Category	No.	Male	Female	Mean age	Age range	IgG4* (µg/ml)	IgG4† (µg/ml)	IgG4‡ (µg/ml)
Amicrofilaraemic	22	7	15	28	19–40	438	401	369
Microfilaraemic	11	5	6	29	19–44	1054	1097	1148
Converters	6	4	2	29	17–40	157	184	252
Total	39	16	23					

*, †, ‡, geometric means of IgG4 in April, July and November, respectively.

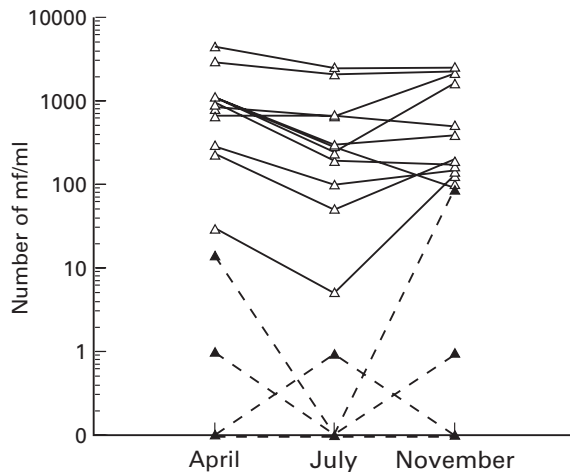


Figure 1 Microfilarial count per ml night blood in 11 microfilaraemics and six converters at three time points of April, July and November. Each line represents one individual. The dashed lines represent 'converters'.

thresholds of 3 pg/ml and 3 U/ml for IL-4 and IFN- γ , respectively.

Statistical analysis

Lymphocyte proliferative responses, cytokine production and microfilariae counts of the same individuals in various surveys were compared using the Wilcoxon matched-paired signed rank test.

RESULTS

Microfilarial count

Of the 39 individuals participating in this study, 22 were amicrofilaraemic at all three sampling times, while 11 were microfilaria-positive throughout. The remaining six subjects showed a conversion during the study. For example, three persons designated microfilaria-negative at the first survey were found to be positive 3 or 7 months later. Three additional cases reverted from the microfilaria-positive to microfilaria-negative state during the same period (Figure 1, dashed lines). Remarkably, the overall intensity of infection also varied over time. The number of mf/ml blood decreased significantly from April (g.m. 88 mf/ml) to July (g.m. 38 mf/ml; $P=0.001$), and then increased again between July and November (g.m. 77 mf/ml; $P=0.088$) (Figure 1).

Lymphocyte proliferation

The T cell proliferative responses in each group (amicrofilaraemics, microfilaraemics and converters) were then assessed against parasite antigen (BmA), bystander antigen

(PPD), and mitogen (PHA). The microfilaria-positive group remained unresponsive to BmA throughout the study period (Figure 2b). Interestingly, the PPD response in this group changed in inverse proportion to the microfilarial density (Figure 2e) although this was not statistically significant. Among the microfilaria-negative group, there was a parallel upwards shift in T cell responsiveness, both to BmA and to PPD, at the July sampling point when parasite densities in microfilaria-positive individuals were lowest. Thus proliferation to BmA and PPD increased significantly between April and July ($P=0.02$ for BmA, $P=0.03$ for PPD), and then declined between July and November ($P=0.004$, $P=0.046$, respectively). The individual responses are plotted in Figure 2a,d.

The six converters showed varying individual patterns and three cases remained unresponsive throughout in terms of T cell proliferation. In the other three cases, however, there was an inverse relationship between the presence of microfilariae and the ability to mount parasite-antigen-specific T cell proliferative responses (Figure 2c). One subject (\blacktriangle , \triangle) who was microfilaria-positive in July failed to proliferate to BmA at that time but when tested in November, when no microfilariae could be found, responded by proliferating to parasite antigen. A second subject (\square , \blacksquare) who had undetectable microfilariae in July showed positive PBMC response to BmA, but when tested in November he was microfilaria positive and his PBMC failed to respond to BmA. The third case (\circ , \bullet) had a low level of proliferative response in April, when 1 mf/ml was detected, but dramatically higher responses to BmA when PBMC were tested in July or November, at times that no microfilariae could be found in the circulation.

Thus as shown in Figure 2c, we captured eight conversion events. Of these, five were in individuals who remained entirely unresponsive to BmA. Of the three observed increases or decreases in proliferation between April to July and July to November, each changed inversely to the loss or gain of microfilariae, i.e. proliferation increased or decreased, respectively.

Mitogen responses in these patients followed a different pattern, with lower blastogenic responses observed in the July samples, when antigen-specific responses were highest. The shift in PHA responses reached statistical significance (microfilaria-negative, July versus November $P=0.004$; microfilaria-positive April versus July $P=0.05$, July versus November $P=0.019$) (Table 2).

IFN- γ and IL-4 release by PBMC

Cytokine release was determined in a subset of individuals from whom sufficient PBMCs were available. In amicrofilaraemics and microfilaraemics, no significant

Table 2 Proliferative T cell responses*

Category	PHA			PPD			BmA		
	April	July	November	April	July	November	April	July	November
Amicrofilaraemics	677	364	914 [‡]	3.9	10.7	2.7 [†]	1.7	3.7	1.5 ^{†‡}
Microfilaraemics	763	245	899 [‡]	2.6	4.3	2.4	1.3	1.3	1.3
Converters	823	547	282	3.0	14.3	6.2 [†]	1.6	3.0	3.0

*Proliferative responses are given as stimulation indices (SI). [†]Significantly ($P < 0.05$) different between April and July; [‡]significantly ($P < 0.05$) different between July and November.

shifts were seen when comparing antigen-specific responses between April and July (Figure 3a,b,d,e). Over the following 4-month interlude, an overall reduction was observed in IFN- γ responses to BmA (significant in microfilaria-negative subjects, $P = 0.03$).

When considering converters in the three sampling times we recorded seven increases or decreases in IFN- γ (Figure 3C) and, of these, four were consistent with the notion that presence of microfilariae is associated with low IFN- γ release and absence of microfilariae with high IFN- γ production. Thus the two microfilaria-positive subjects who

became microfilaria-negative in July ($\bullet\circ$, $\bullet\circ$) showed a sharp increase in IFN- γ release, whereas one individual who was microfilaria-negative in April but became microfilaria-positive in July ($\Delta\blacktriangle$) exhibited exactly the reverse switch with a decreased IFN- γ response. Between July and November one conversion ($\Delta\blacktriangle$) from microfilaria-negative to microfilaria-positive was associated with a decrease in IFN- γ release.

No consistent changes were recorded in the IL-4 response except for an increase in IL-4 production between July and November in microfilaria-positive subjects ($P = 0.07$). In

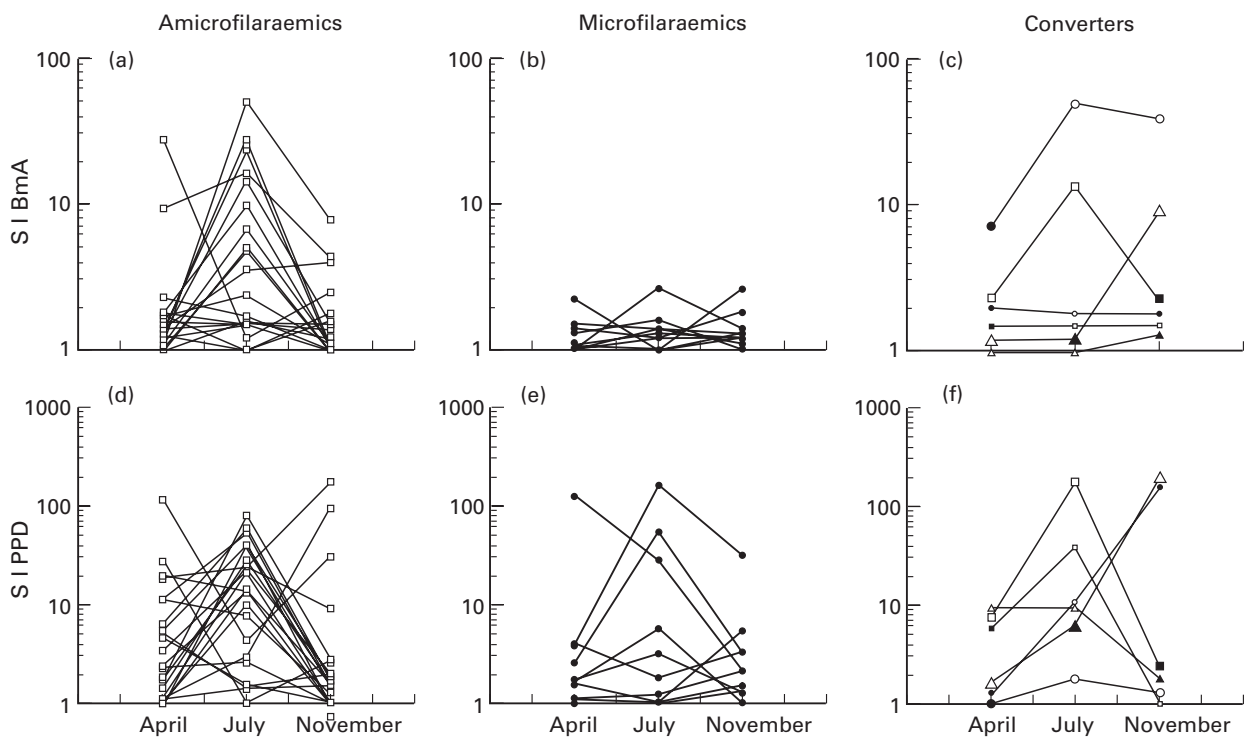


Figure 2 PBMC responses to BmA and PPD in amicrofilaraemics (Figure 2a,d, respectively), microfilaraemics (Figure 2b,e, respectively) and converters (Figure 2c,f, respectively) at three survey times, April, July and November. Open and closed symbols represent amicrofilaraemic and microfilaraemic status, respectively. Each line represents one individual.

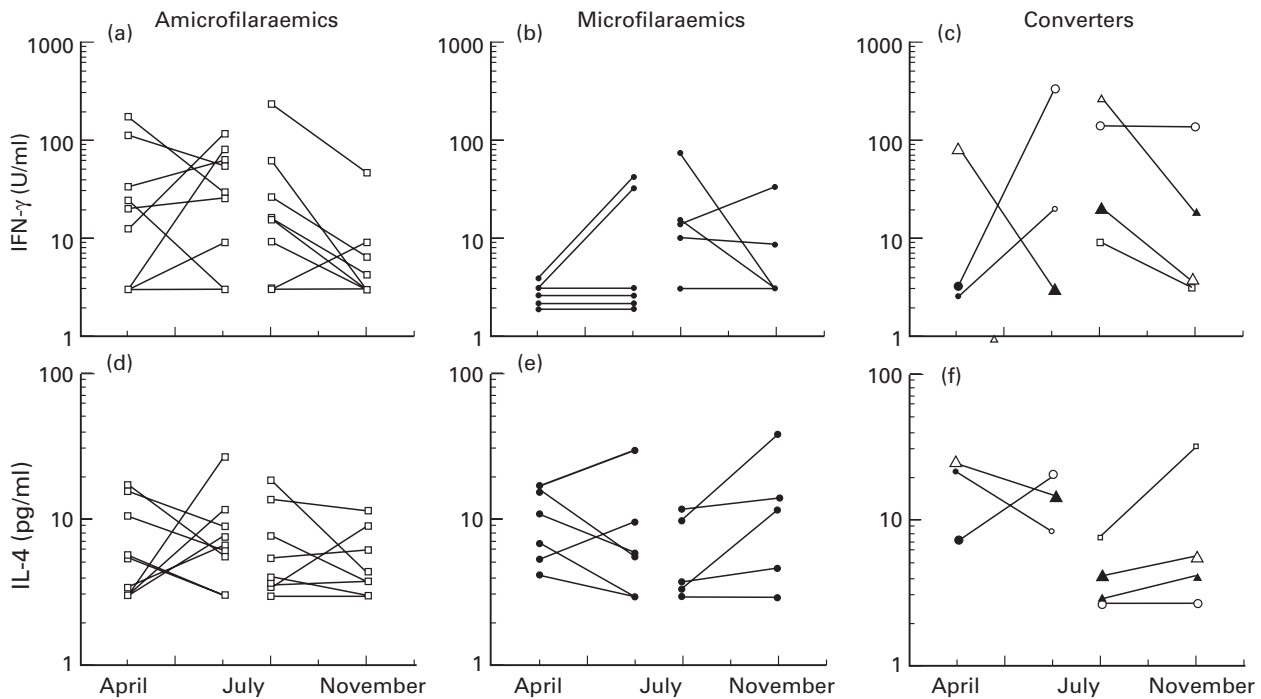


Figure 3 IFN- γ and IL-4 production in response to BmA in amicrofilaraemic (Figure 3a,d, respectively), microfilaraemic individuals (Figure 3b,e, respectively), and converters (Figure 3c,f, respectively) at three survey times, April, July and November. Open and closed symbols represent amicrofilaraemic and microfilaraemic status, respectively. Each line represents one individual.

converters, the increase and decrease recorded in IL-4 showed no link to microfilarial status.

DISCUSSION

Serological assays and entomological studies provide strong evidence that nearly all individuals living in regions endemic for lymphatic filariasis become exposed to filarial parasites (Day 1991, Bailey *et al.* 1995, Grove *et al.* 1997). In the face of continuous exposure, some individuals can remain amicrofilaraemic for decades without progressing to other forms of clinical expression, while others become microfilaria positive (Ottesen 1992). Some revealing parallels can be drawn between human filarial infection and the feline model of filariasis. In experimentally infected cats, a proportion of cats abruptly became amicrofilaraemic after repeated infection with L3 whereas a considerable proportion of animals remained microfilaria positive (Denham *et al.* 1992). However, our understanding of long-term infection in humans is still very poor.

To address this deficiency, 39 subjects classified as microfilaria negative or positive in an area endemic for brugian filariasis were followed at three time points without any drug intervention. In 15% (six of 39) of the study population, we observed changes in parasitological status within a 7-month period. Of these six 'converters', three

amicrofilaraemics became microfilaria positive, and three microfilaraemics returned to microfilaria-negative status.

The microfilarial intensity altered over the three time points studied, decreasing between April and July and increasing again between July and November. Thus the microfilarial density was lower in the dry season (July) than either of the wet season months. In the absence of entomological data, we cannot ascertain whether the fluctuation in microfilarial levels is associated with varying transmission. However, such a link has been reported in a study of bancroftian filariasis in which the microfilaraemia rate fell during the low transmission periods of the late dry season (Weil *et al.* 1988). Similarly, studies in dogs naturally infected with *Dirofilaria immitis* revealed that microfilarial concentration may vary seasonally, being higher in the summer, the mosquito season, than in the winter (Kume 1975, Sawyer 1975). It is conceivable that the variation in microfilarial density might be dependent on the biting activity of the mosquitoes, if parasites regulate production of microfilariae in accordance with the current vector availability. Alternatively, fluctuation in microfilarial density might be directly related to changes in adult worm burden, which would suggest that there is a rapid turnover of worms.

In previous studies, it has been shown that amicrofilaraemics and a proportion of asymptomatic amicrofilaraemics are hyporesponsive to filarial antigens, probably because

many designated amicrofilaraemics carry low levels of infection (Yazdanbakhsh *et al.* 1993, Bradley *et al.* 1995). In this study, we extend these findings by analysing the responses to BmA at three time points. All microfilaria-positive carriers were unresponsive to BmA and this condition was stable at the three survey times. Curiously, the responses to BmA shifted in amicrofilaraemics; it increased between April and July and then reversed between July and November. Moreover, these dynamic changes in proliferative responses to BmA in amicrofilaraemics were inversely related to microfilarial density in microfilaraemics; which was lowest in July (dry season). If changes in the levels of microfilariae in microfilaraemics also represent changes that occur in individuals with undetectable infections, then it has to be concluded that T cell responses in lightly infected subjects are balancing on the edge of being suppressed and any slight alteration in parasite burden will have visible effects on proliferation.

In parallel with proliferation, we found that IFN- γ release decreased in November compared to July in amicrofilaraemics, however, between April and July, unlike proliferation, IFN- γ production in response to BmA did not change significantly. It should be noted that changes in proliferation between July and November were more marked than between April and July.

We next examined the influence of the parasite density on immune responses in the converters. Our immediate observation was that IFN- γ is the most affected response whereas proliferation is less affected by the changes in parasite density. These findings suggest that proliferation is more profoundly suppressed than IFN- γ and that IFN- γ changes more readily with shifts in parasite density. In contrast to IFN- γ , IL-4 was found to be unaffected by infection and changes in IL-4 production appear to reflect true fluctuations in this immune parameter.

Although a clear relationship between microfilarial density and cellular immunological responses is shown in this study, it is not known whether the changes in immune responses cause the microfilarial levels to fluctuate or vice versa. If parasites dictate immunological status changes in microfilaria-positive subjects who harbor a high worm burden, then suppression of proliferative responses is more profound than of either IFN- γ or IL-4. The changes in parasite intensities have therefore more effect on cytokine production. It is also possible that deposition of L3 and L4 (in addition to adult worms) exerts suppressive effects on cellular responses, indeed recently subcutaneous injection of L3 has been shown to down regulate antigen-specific Th1 responses in mice (Osborne & Devaney 1999). In this case, an increase in incoming L3 (during high transmission) would not affect the already depressed proliferative responses in microfilaraemics whereas it would in

amicrofilaraemics whose immunological reactivity is less profoundly suppressed.

Studies in malaria provide strong evidence for seasonal variation in the proliferative responses to malaria antigens. These fluctuations have been shown to be due to seasonal variation in the suppressive effects of CD8⁺ cells. During the wet season, CD8⁺ cells have a better suppressive effect than cells isolated during the dry season (Riley *et al.* 1993, Theander *et al.* 1993). Reduced T cell responses during the transmission season have been suggested to reflect an appropriate feedback regulation of the immune system as antigenic challenge increases (Hviid & Theander 1993).

Lymphocyte proliferation to PPD followed responses to BmA: although fluctuating slightly in microfilaraemics, the changes were not statistically significant. In amicrofilaraemics, the proliferative responses changed over time in the same manner as did BmA responses. As responses to PHA did not follow the pattern observed with BmA or PPD, these results indicate that there is some degree of a spill-over suppression, as a result of filarial infection, affecting PPD responses (Sartono *et al.* 1995, Yazdanbakhsh & Sartono 1995).

In summary, we have shown that cellular immune responses of people living in areas endemic for brugian filariasis shift over time in a coordinated manner with microfilarial levels, which in turn may depend on varying transmission. By studying 'converters', we have shown that IFN- γ is more profoundly affected by changes in parasite density than proliferative responses whereas IL-4 production in response to filarial antigen is unaffected by levels of parasite infection.

ACKNOWLEDGEMENTS

We thank Heri Wibowo, Taniawati Supali, Rita Ekarina, Sudirman and Sudiono for help in organizing the field trip and the staff of Department of Pathology and Anatomy, University of Indonesia, Jakarta, for allowing us to use their facilities. Financial support was provided by the Scientific Directorate of the Commission of the European Communities under the Science and Technology for Development Programmes 3: EC-STD3 programme (Contract numbers TS3-CT91-0031 and TS3-CT92-0141) and EC-ALA programme (Contract number CII-CT91-0928) and The Netherlands Foundation for the Advancement of Tropical Research (WOTRO, grant no. W93-266).

REFERENCES

- Bailey J.H., Hightower A.W., Eberhard M.L. *et al.* (1995) Acquisition and expression of humoral reactivity to antigens of infective stages of filarial larvae. *Parasite Immunology* **17**, 617-623

- Bradley J.E., Elson L. & Tree. T.I.M. (1995) Resistance to *Onchocerca volvulus*: differential cellular and humoral responses to a recombinant antigen, OvMBP20/11. *Journal of Infectious Diseases* **172**, 831–837
- Day K.P. (1991) The endemic normal in filariasis: a static concept. *Parasitology Today* **7**, 341–343
- Denham D.A., Medeiros F., Baldwin C. *et al.* (1992) Repeated infection of cats with *Brugia pahangi*: parasitological observations. *Parasitology* **104**, 415–420
- Grove D.I., Cabrera B.D., Valeza F.S. *et al.* (1977) Sensitivity and specificity of skin reactivity to *Brugia malayi* and *Dirofilaria immitis* antigens in bancroftian and malayan filariasis in Philippines. *American Journal of Tropical Medicine and Hygiene* **26**, 220–229
- Hviid L. & Theander T.G. (1993) Seasonal changes in human immune responses to malaria. *Parasitology Today* **9**, 26–27
- Kume S. (1975) Experimental observations on seasonal periodicity of microfilariae. In *Proceedings of the Heartworm Symposium '74*, eds Morgan H.C., Otto G.F., Jackson R.F. *et al.*, pp. 26–31, Veterinary Medicine Publishing; KS Bonner Springs
- Osborne J. & Devaney E. (1999) IL-10 and antigen presenting cells actively suppress Th1 cells in BALB/C mice infected with the filarial parasite *Brugia pahangi*. *Infection Immunity* in press
- Ottesen E.A. (1984) Immunological aspects of lymphatic filariasis and onchocerciasis in man. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 9–18
- Ottesen E.A. (1989) Filariasis now. *American Journal of Tropical Medicine and Hygiene* **41**, 9–17
- Ottesen E.A. (1992) Infection and disease in lymphatic filariasis: an immunological perspective. *Parasitology* **104**, S71–S79
- Ottesen E.A., Weller P.F. & Heck L. (1977) Specific cellular immune unresponsiveness in human filariasis. *Immunology* **33**, 413–421
- Partono F. & Purnomo (1987) Periodicity studies of *Brugia malayi* in Indonesia: recent findings and a modified classification of parasite. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**, 657–662
- Riley E.M., Morris-Jones S., Taylor-Robinson A.W. *et al.* (1993) Lymphocyte proliferative responses to a merozoite surface antigen of *Plasmodium falciparum*: preliminary evidence for seasonal activation of CD8+/HLA-DQ-restricted suppressor cells. *Clinical Experimental Immunology* **94**, 64–67
- Sartono E., Kruize Y.C.M., Kurniawan A. *et al.* (1997) Depression of antigen-specific interleukin-5 and interferon- γ responses in human lymphatic filariasis as a function of clinical status and age. *Journal of Infectious Diseases* **175**, 1276–1280
- Sartono E., Kruize Y.C.M., van der Meide P.H. *et al.* (1995) Elevated cellular immune responses and interferon- γ responses after long-term diethylcarbamazine treatment of patients with human lymphatic filariasis. *Journal of Infectious Diseases* **171**, 1683–1687
- Sawyer T.K. (1975) Seasonal fluctuation of microfilariae in two dogs naturally infected with *Dirofilaria immitis*. In *Proceedings of the Heartworm Symposium '74*, eds Morgan H.C., Otto G.F., Jackson R.F. *et al.*, pp. 23–25, Veterinary Medicine Publishing; KS Bonner Springs
- Steel C., Guinea A. & Ottesen E.A. (1996) Evidence for protective immunity to bancroftian filariasis in the Cook Islands. *Journal of Infectious Diseases* **174**, 598–605
- Theander T.G., Hviid L., Abu-Zeid Y.A. *et al.* (1993) Activation of CD8+ suppressor cells during the malaria season. *Immunology Infectious Diseases* **3**, 97–102
- Weil G.J., Sethumadhavan K.V.P., Santhanam S. *et al.* (1988) Persistence of parasite antigenemia following diethylcarbamazine therapy of bancroftian filariasis. *American Journal of Tropical Medicine and Hygiene* **38**, 589–595
- WHO Expert Committee on Filariasis. (1992) Lymphatic filariasis: the disease and its control. *World Health Organization Technical Report Series* **821**, 1–71
- Yazdanbakhsh M., Paxton W.A., Kruize Y.C.M. *et al.* (1993) T cell responsiveness correlates differentially with antibody isotype levels in clinical and asymptomatic filariasis. *Journal of Infectious Diseases* **167**, 925–931
- Yazdanbakhsh M. & Sartono E. (1995) Diethylcarbamazine-related immunity. *Journal of Infectious Diseases* **172**, 1640