LONG-TERM FOLLOW-UP OF TREATMENT WITH DIETHYLCARBAMAZINE ON ANTI-FILARIAL IgG4: DOSAGE, COMPLIANCE, AND DIFFERENTIAL PATTERNS IN ADULTS AND CHILDREN

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Abstract. We have followed a population in an area endemic for Brugia malayi for three years after intensive treatment with diethylcarbamazine (DEC). Microfilariae were cleared from the circulation within four months in all eligible study participants (n = 60). There appeared to be a strong correlation between the maximum reduction in specific IgG4 and the number of days drug was taken under supervision (ρ = 0.41, P < 0.001), indicating that high total dosage of DEC is necessary for optimal reduction of active infection. In individuals with good compliance (at least 180 mg/kg of body weight, n = 34), we observed variable IgG4 patterns. All pre-treatment IgG4+ children (9–14 years old) and 40% of the IgG4+ adult population (≥ 15 years old) showed a gradual decrease in anti-filarial IgG4; 53% of these showed complete clearance of worm burden by the end of the study. In contrast, another group of male IgG4+ adults showed IgG4 patterns that started to increase between nine months and two years after treatment, indicating either a partial efficacy of DEC that allowed recovery of resident adult worms or reinfection.

INTRODUCTION

Lymphatic filariasis is a vector-borne disease caused by tissue-dwelling nematodes of Brugia and Wuchereria species, and is estimated to affect 120 million people worldwide. Filarial parasites are a major cause of morbidity and thereby hinder socioeconomic development in parts of Asia, Africa, and the western Pacific. Current treatment strategies include therapy with diethylcarbamazine (DEC), which does not only kill microfilariae, but is also the only drug available with macrofilaricidal potential if prolonged treatment is sustained.

Although DEC has been widely used for the past 50 years, knowledge on the long-term effects of treatment on filarial infection is limited. Evidence for killing of adult parasites by DEC comes from in vitro experiments,5 experiments using ultrasound,6 and studies measuring the decrease in circulating filarial antigens after treatment.7,8 Presently, field-applicable detection of adult worm burden by either ultrasound or circulating antigen assays is restricted to detection of Wuchereria bancrofti infections and cannot be used for Brugia malayi. Another impediment to interpretation of long-term chemotherapy studies has been that DEC is usually administered as mass treatment with no documented records of compliance for each participant.

The present study was performed to gain more insight in the long-term effects of DEC on infection with Brugia malayi by measuring changes in microfilaria load and anti-filarial IgG4 after intensive treatment. Anti-filarial IgG4 can be a useful marker to help overcome the lack of assays for adult worm burden since it is an indicator of active filarial infection and correlates well with the presence of adult worms. Further, several studies have demonstrated that levels of specific IgG4 decrease after treatment with DEC, reinforcing its value as a diagnostic marker of active infection.

Volunteers from a village in southern Sulawesi in Indonesia (microfilaria prevalence = 32%) were treated with three 12-day courses of DEC. The drug was taken under direct supervision of field staff to ensure compliance. Medicated individuals were followed for three years and post-treatment IgG4 patterns were analyzed as a function of age and gender by taking into consideration the treatment compliance.

MATERIALS AND METHODS

Description of the study population. The study was conducted in desa Karondang in Budong-budong, a district of Mamuju Regency in southern Sulawesi, Indonesia, which is endemic for nocturnal, periodic Brugia malayi transmitted by the vectors Anopheles barbirostris and Mansonia uniformis. Karondang has approximately 400 inhabitants and is located five kilometers inland from the sea. Most of the houses are situated along the main road, which follows the Budong-Budong River into the sea and houses are spread over a distance of approximately four kilometers. The major occupation of the villagers is subsistence farming. In cooperation with the head of the village, teachers of primary and secondary schools, and the medical doctor and nurses of the local District Health Center, all residents of the village seven years of age and older were invited to participate in the study. After physical examinations were conducted, individuals exhibiting contraindications for therapy with DEC (pregnant women, individuals with weak physical conditions, or subjects with proteinuria) were excluded from the study. Informed consent was obtained from all study participants or parents of underage children before parasitologic studies and blood withdrawal in accordance with the guidelines of the Indonesian Department of Health and Human Services.

Study set-up. After clinical examination and pre-treatment blood withdrawal, volunteers without contraindications received treatment with DEC (6 mg/kg of body weight for three consecutive 12-day courses). Intervals between courses lasted two weeks, and during each of these periods DEC was administered once a week to all study participants. The DEC tablets were provided between 7 AM and 11 AM at several distribution points in the village. Children attending primary or secondary school received DEC at school. Study participants who did not show up for treatment were visited at home. If patients were absent due to work, illness, travel, or other circumstances, medicine was left with a family member or friend and it was made sure that DEC reached the partici-
pents the same day. Each day was registered whether or not medicine was taken under supervision. During treatment periods all participants were regularly checked by a physician and adverse reactions, as well as other infections or illnesses, were treated when necessary. After completion of treatment with DEC all treated individuals were followed for three years. Venous blood was drawn after the first DEC course (two weeks post-treatment), after completion of all three courses (six weeks), and subsequently at four and nine months and two and three years post-treatment. After each blood withdrawal study participants were interviewed and clinically examined; individuals with circulating microfilariae or signs of acute filariasis received new treatment with DEC and were excluded from further study. During the whole study period the place of residence of each study participant was registered and individuals traveling to filarial endemic areas for periods of more than a week and participants staying in filarial non-endemic areas for more than one month (thus temporarily not exposed) were excluded from the study.

**Blood collection.** Pre-treatment and post-treatment venous blood samples (10 mL) were collected between 9:00 PM and midnight into tubes, and EDTA was then added (final concentration = 0.05 M). The tubes were centrifuged and plasma was stored at -20°C for several months before shipment to The Netherlands, where it was stored at -70°C until use.

**Parasitologic examination.** After centrifugation and removal of plasma, 10 mL of distilled water was added to the blood pellet and mixed. The next day this suspension was filtered through a Millipore® (Bedford, MA) membrane (pore size = 5 μm). The filters were air-dried, fixed with methanol, stained with Giemsa, and examined with a light microscope for the presence of microfilariae.

**Parasite antigen.** Adult *Brugia malayi* worms were obtained from TRS Laboratories (Athens, GA). Female worms were freeze-dried, ground to a powder, dissolved in phosphate-buffered saline (PBS), homogenized, and slowly stirred overnight at 4°C. The protein concentration was determined by 2,2'-biquinoline-4,4'-dicarboxylic acid disodium salt hydroxide (BCA) method before storage at −20°C.

**Enzyme-linked immunosorbent assay for detection of specific IgE and IgG4.** An enzyme-linked immunosorbent assay for detection of specific IgG4 was performed as previously reported.21 Duplicate samples of patient sera (100 μL/well) diluted 1:100 and 1:500 in IgG4 assay buffer (PBS containing 5% fetal calf serum and 0.05% Tween 20) were incubated overnight at 4°C in microtiter plates coated with *Brugia malayi* antigen. Bound IgG4 was detected with anti-human IgG4 monoclonal antibodies precisely as described before.21 The optical density value of patient plasma was converted into arbitrary units by extrapolation of a standard curve of a positive control plasma as previously described.21 A cut-off value for *B. malayi*-specific IgG4 antibodies was calculated by taking the mean + 3 SD IgG4 reactivity in arbitrary units of blood samples of 20 healthy Dutch donors at the Blood Bank in Leiden; this value was 4.23 for log10 specific IgG4.

**Statistical analysis.** Statistical analysis was performed using SPSS for Windows version 8.0 (SPSS, Inc., Chicago, IL). The chi-square test was applied for comparison of proportions. Fisher’s exact test was used if otherwise indicated. For analysis of the specific IgG4 levels a log10 transformation was used to obtain normally distributed data; throughout this paper specific IgG4 levels are presented as transformed log10 values. The Student’s *t*-test was used to compare log10 microfilaria levels and log10 IgG4 levels between different groups; a paired *t* test was used to compare anti-filarial IgG4 in the same individuals at different time points. For post-treatment blood samples, the percentage of specific IgG4 compared with pre-treatment levels was calculated as follows: 100 × (post-treatment IgG4/pre-treatment IgG4); the Mann-Whitney test was used for comparison of specific percentages of IgG4 after treatment in different groups. The Spearman rank correlation was calculated for testing concordance between data sets with a non-linear relationship.

### RESULTS

**Microfilaria prevalence after treatment.** Pre-treatment blood samples were drawn from 199 individuals who had lived in Karondang for at least five years prior to the study; microfilaria prevalence in this group was 32% (64 of 199). A group of 155 villagers without contraindications for DEC therapy agreed to treatment. Analysis of post-treatment microfilariae and IgG4 antibody levels were restricted to 60 in-

### Table 1

Description of the study population of the large (A, n = 60) and the small study group (B, n = 34; at least 30 treatment days under supervision)*

<table>
<thead>
<tr>
<th>A</th>
<th>Number</th>
<th>Males/Females</th>
<th>MF %</th>
<th>GM mf count</th>
<th>Mean IgG4 (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children (7–14 years old)</td>
<td>24</td>
<td>9/15</td>
<td>5/24 (21%)</td>
<td>549</td>
<td>4.77</td>
</tr>
<tr>
<td>Adults (≥15 years old)</td>
<td>36</td>
<td>22/14</td>
<td>8/36 (22%)</td>
<td>90</td>
<td>5.23†</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>31/29</td>
<td>13/60 (22%)</td>
<td>181</td>
<td>5.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Number</th>
<th>Males/Females</th>
<th>MF %</th>
<th>GM mf count</th>
<th>Mean IgG4 (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children (7–14 years old)</td>
<td>15</td>
<td>5/10</td>
<td>4/15 (27%)</td>
<td>372</td>
<td>4.70</td>
</tr>
<tr>
<td>Adults (≥15 years old)</td>
<td>19</td>
<td>13/6</td>
<td>6/19 (32%)</td>
<td>58</td>
<td>5.56†</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>18/16</td>
<td>10/34 (29%)</td>
<td>122</td>
<td>5.18</td>
</tr>
</tbody>
</table>

* Male/female, pretreatment microfilaria (mf) prevalence, geometric mean (GM) mf in MF+ individuals, and mean pretreatment IgG4 levels are given for children (7–14 years old) and adults (≥15 years old).

† Significantly different from children *P* < 0.05.
individuals who met the following criteria: 1) participation in the whole DEC treatment program, 2) four or more collected blood samples, including at least one of the two year or three year post-treatment samples, and 3) residence in the study village throughout the period of follow-up. A summary of the population characteristics of this group is shown in Table 1A. Pre-treatment microfilaria prevalence among this group was 22%, which was lower than the prevalence measured in the whole population. This was caused by a large study dropout rate among microfilaria-positive adults.

There was a rapid reduction in microfilaria prevalence and microfilaria levels after treatment with DEC (Figure 1). After the first course of DEC, microfilaria prevalence in the whole population dropped from 22% (13 of 60) to 12% (7 of 60). After completion of treatment (six weeks), only one adult still harbored circulating microfilariae (2%, 1 of 60). At four and nine months after treatment, all examined study participants remained free of circulating microfilariae (0 of 28 and 0 of 24, respectively). After two years, microfilariae were again detected in one adult male (2%, 1 of 56) who had been previously microfilaria-positive. This person was treated with a new course of DEC and was excluded from further analysis. Three years post-treatment no new cases with microfilariae were detected (0 of 33).

Anti-filarial IgG4 and compliance of treatment. During treatment periods, it was made sure that every study participant received DEC each day. Whether or not DEC was taken under direct supervision was registered and the number of days in which medicine was definitely taken was counted for each participant (maximum of 40). The post-treatment maximum reduction in specific IgG4 levels for each individual was calculated as a percentage as follows: 100 - ((lowest achieved post-treatment specific IgG4 level/pre-treatment IgG4 level) \times 100).

There was a significant correlation between percentage reduction in specific IgG4 and the number of days DEC was taken under observation (n = 60; r = 0.41, P = 0.001) (Figure 2). The post-treatment reduction in specific IgG4 in study participants with 10–19, 20–29 and 30 or more treatment days under supervision is shown in Table 2. Individuals with 30 or more supervised treatment days (total DEC dosage of at least 180 mg/kg) showed a higher overall reduction in specific IgG4 levels than persons with 10–19 or 20–29 treatment days. These results show not only that compliance was less in the absence of field staff, but also that a longer treatment period is needed for optimal reduction of worm burden, despite a rapid clearance of microfilariae from the circulation. However, even in individuals with 30 or more days of treatment under supervision, there was a large variation in reduction of specific IgG4 after treatment.

Anti-filarial IgG4 after treatment. The changes in post-treatment IgG4 were analyzed in 34 individuals who had received at least 30 days of treatment under supervision. Population characteristics of this group are shown in Table 1B. Levels of anti-filarial IgG4 in this population were reduced considerably following therapy with DEC. Levels of specific IgG4 (log_{10} IgG4 = 5.20) started to decrease at six weeks post-treatment (log_{10} IgG4 = 4.80; P = 0.07 compared with pre-treatment levels, n = 34), and lowest levels were reached nine months after treatment (log_{10} IgG4 = 4.60; P < 0.001, compared with pre-treatment levels, n = 25).

Interestingly, three different patterns could be distinguished in the dynamics of change in specific IgG4 considered at the individual level (Figure 3). The first group of 15 individuals (pattern A) showed a reduction in anti-filarial IgG4 levels of at least 50% (although not immediately obvious in Figure 3, which is log-scaled), and specific IgG4 continued to decrease until two years or three years post-treatment in the

<table>
<thead>
<tr>
<th>Treatment days under supervision</th>
<th>Number</th>
<th>Specific IgG4 reduction</th>
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<tbody>
<tr>
<td>10–19</td>
<td>6</td>
<td>49.5%</td>
</tr>
<tr>
<td>20–29</td>
<td>20</td>
<td>50.9%</td>
</tr>
<tr>
<td>30–40</td>
<td>34</td>
<td>72.0%†</td>
</tr>
</tbody>
</table>

*Note that DEC was supplied to all study participants each day, but the number of days are presented in which medicine was taken under supervision of field staff.
†Significantly different from 20–29 treatment days (P = 0.02, by Mann-Whitney test).
majority (87%) of these cases. In more than half of these subjects (53%, 8 of 15) specific IgG4 levels were less than the cut-off level after three years, suggesting considerable clearance of filarial worms. In contrast, a second group showed a completely different picture (pattern B) with either no change or an initial decrease in anti-filarial IgG4, followed by a later increase, which could be detected between nine months and three years after administration of DEC. The increase in specific IgG4 (which followed the initial decrease) was at least 50% of pre-treatment IgG4 value. In a third group (n = 10) with anti-filarial IgG4 levels near or less than the cut-off level, no changes were recorded during the observational period (pattern C).

Individuals with pattern B had higher pre-treatment anti-filarial IgG4 (IgG4 = 6.18) than participants with pattern A after treatment (IgG4 = 5.58; P = 0.06). At nine months post-treatment, before an increase of specific IgG4 could be observed in part of the study population, the reduction in specific IgG4 levels was highest in persons with pattern A (41%) compared with individuals with pattern B (16%) (P = 0.02, by Mann-Whitney test). There was no difference in number of days treatment was provided under supervision between individuals with patterns A or B (33.3 days versus 33.8; P = 0.18).

When groups with patterns A and B were compared, age seemed to be an important factor. There was a significant difference in post-treatment IgG4 patterns between IgG4+ adults and children (P < 0.01), since all nine study participants with pattern B (increase in specific IgG4) were 15 years of age or older. Moreover, there was a significant influence of gender on patterns of change of IgG4 post treatment within the IgG4+ adults, since all nine individuals exhibiting pattern B were male (versus three males with pattern A), whereas all three adult females showed a decrease of IgG4 (pattern A) (P = 0.04). In contrast, no difference in IgG4 patterns was observed between pre-treatment microfilaria-positive and microfilaria-negative study participants.

**DISCUSSION**

Despite the enormous body of literature on DEC treatment of lymphatic filariasis, studies that monitor the long-term efficacy of this drug are scarce, even though knowledge of this topic is of vital importance in filariasis eradication programs. Uncertainty about drug compliance and the difficulty in monitoring the adult worm burden, particularly for *Brugia* spp., are the main problems encountered when designing and interpreting reinfection data. In Brugian filariasis, anti-filarial IgG4 has proven to be a suitable marker of active infection with filarial worms.11–13 In the present study we have used not only microfilariae but also anti-filarial IgG4 to assess the long-term effects of treatment with DEC.

The results of our three-year follow-up showed a clearance of microfilariae from the periphery within four months after the beginning of treatment, and all individuals examined remained free of microfilariae until two years post treatment. Re-emergence of microfilaraemia was low (2% at two years, 0% at three years). Despite the rapid clearance of microfilaria in all individuals, the reduction of anti-filarial IgG4 after treatment was strongly dependent on drug compliance. This finding suggests that a longer treatment period is needed for optimal reduction and elimination of the worm burden. Within the group with good drug intake (compliance > 30 days), there was considerable variation in how specific IgG4 changed with time after treatment, which in turn depended on age and gender. All children and 40% of the IgG4+ adults showed a reduction of specific IgG4 after treatment, which continued to decrease even at two years and three years post-treatment (pattern A). In 53% of these
subjects, IgG4 levels were less than the cut-off value by the end of the study, indicating elimination of filarial worms. In contrast, in 60% of the IgG4+ adults, the levels of specific IgG4 started to increase again between nine months and two years after treatment (pattern B), suggesting either a revival of the adult worms, which survived treatment, or new infection.

Only few studies using a long course of DEC (at least 12 days) have examined the long-term effects of treatment on microfilaria prevalence for more than one year, but all of these have described a re-emergence of microfilariae between one year and five years post-treatment in a proportion of the treated individuals, which is in agreement with the present results. The initial decrease in specific IgG4 in the first nine months of our study is comparable with the results of earlier studies, which reported a decrease in specific IgG4 up to 18 months after treatment.

To overcome the uncertainty in drug compliance, which is encountered when interpreting data from community or mass treatments, we put considerable effort in ensuring and monitoring DEC intake in this study. However, due to (agricultural) work, travel, or illness during the 40 days of treatment, there was an average of 7.9 days per person in which participants were absent for treatment. Although medication was provided during these days of absence, the results showed a clear correlation between reduction of specific IgG4 after treatment and the number of days DEC was taken under supervision \( (n = 60; p = 0.41, P = 0.001) \), suggesting that compliance was less in the absence of field staff. These results indicate that a longer treatment period and/or high total DEC dosage of 180 mg/kg of body weight is needed for substantial and long-lasting reduction of the adult worm burden.

There are two possible explanations for the differential patterns of IgG4 obtained in individuals with good treatment compliance (at least 30 days under supervision). First, there could be a differential susceptibility to DEC, with either complete eradication/partial but sustained elimination of the worms in individuals with a persistent decrease in specific IgG4 (pattern A) or a poor response to drug in persons with a delayed increase in IgG4 post-treatment (pattern B). This reduced drug susceptibility clearly does not affect clearance of the microfilariae from the circulation, but instead leads to incomplete killing of the adult worms. Surviving worms may have temporary reduction in viability and fecundity. Low susceptibility to DEC has been reported in a study on Bancroftian filariasis in which the possibility could be excluded that treatment failure was due to incomplete drug intake or differences in serum levels of the drug between study participants. In addition, experiments using ultrasound have established that some of the adult worms of *W. bancrofti* in scrotal nests are resistant to DEC. In the present study, higher initial IgG4 levels in individuals showing pattern B were accompanied by a reduced capacity to eliminate adult worms, evident from the lower reduction in IgG4. Thus, it is also possible that a lower response to treatment is due to a density dependent killing of parasites, in which case the amount of medication provided to individuals in group B was just insufficient for complete elimination of the worm burden.

A second way to interpret the results is to assume that the increase in anti-filarial IgG4 after treatment in certain individuals is due to re-infection and acquisition of new worms. Previous reports, in which long term effects of DEC treatment on microfilariaemia were evaluated, have interpreted re-emergence of microfilariae after several years as re-infection. Although we cannot exclude the possibility of reinfection, we find this option less likely. First, although transmission would have continued after treatment, since a reservoir of microfilariae was left in the untreated part of the population, exposure rates to infective larvae would be expected to have been reduced considerably. In a follow-up study in an area endemic for *W. bancrofti* (pre-treatment microfilaria prevalence = 13%) in which four rounds of treatment with a single dose of DEC were performed in 66–75% of the population, infection in both resting and landing mosquitoes (*Culex quinquefasciatus*) was dramatically reduced after four years by 70% and 96%, respectively. This indicates that although transmission might continue after treatment, gain of (re)infection after intervention will occur at a much slower rate. Second, the fact that specific IgG4 was continuously detectable in individuals with a later increase in anti-filarial IgG4 (pattern B), despite efficient clearance of microfilariae, is evidence for the survival of adult worms instead of development of reinfection. In addition, the reduction in anti-filarial IgG4 levels at nine months post-treatment was significantly less in individuals with a later increase in the level of this antibody than in individuals with a stable decrease in the level of IgG4. Both findings argue in favor of a reduced susceptibility to DEC or an insufficient drug dosage in participants with increase of IgG4 (pattern B).

Interestingly, in contrast with adults, all pre-treatment IgG4+ children showed a continuing decrease in specific IgG4 levels after treatment. A possible explanation for this age-related variation could be physiologic differences that exist between adults and children, which allow adults to have greater worm burdens than children. This could result in more efficient elimination of worms in children when the equivalent dosage of DEC is provided. Pre-treatment anti-filarial IgG4 levels were indeed lower in children than in adults (specific IgG4 = 4.70 versus 5.56; \( P = 0.04 \)), which argues for differences in worm load. However, microfilaria prevalence was equivalent in children and adults (27% versus 32%; \( P = 0.53 \)) and microfilaria burden among microfilaria-positive children was even larger than in adults, although this difference did not reach statistical significance \( (P = 0.21) \). This was also true for the larger pre-treatment study population. Since the possibility of reinfection remains, a second clarification could be that the differential results between children and adults are due to differences in acquisition of new infections. In a study among a previously unexposed transmigrant population that settled in the same area, we have shown that infection established more rapidly in adults than in children, as measured by microfilaraemia and levels of anti-filarial IgG4, despite an equal length of exposure to filarial infection. This differential gain of filarial infection between adults and children could result from greater exposure in adults.

Previous studies have suggested that the differential susceptibility to treatment with DEC in a population could be due to genetic heterogeneity. Although our final data set is rather small to permit such investigations, we have determined whether patterns A and B are clustered in households or families, but were not able to show aggregation of the post-treatment IgG4 patterns. We did find clear differences in the patterns of specific IgG4 after treatment be-
between sexes. All pre-treatment IgG4+ adults with pattern B were male, whereas all females showed a consistent decrease in IgG4 after treatment (pattern A). Adult females, similar to the children in the study, may have lighter worm burdens than adult males and therefore respond better to treatment; alternatively, they may inherently be more resistant to reinfection.

The results of the present three-year follow-up study show that high total dosage of DEC (at least 180 mg/kg of body weight) is required for substantial reduction or elimination of the adult worm burden, and that drug compliance is very important if prolonged therapy with DEC is provided. However, the results also indicate that children and females respond different to treatment than adult males, who may need even higher total dosages of DEC than used in the present study for effective killing of the adult worms they harbor.

The World Health Organization currently recommends mass treatment of populations at risk with a yearly single dose of DEC; the hope for eradication of filariasis lies in the assumption that at very low microfilaria levels in the population, transmission cannot be sustained and will be interrupted after 4–6 years, when the infection level in the population will have dropped to zero. To achieve such a goal treatment compliance of the population will be crucial. The present results indicate that if a part of the population is reluctant to receive repeated treatment, the effect of previous treatment rounds might not be sustained, since adult worms might survive therapy due to an insufficient drug dosage. In this case, initial reductions in microfilariaemia achieved at the population level might not be sustained in the long run, leading to resurgence of filarial transmission.

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