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SEROLOGICAL EVALUATION OF THE MACROFILARICIDAL EFFECTS OF DIETHYLCARBAMAZINE TREATMENT IN BANCROFTIAN FILARIASIS

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Abstract. An M_r 200,000 phosphorylcholine-containing antigen (PC-Ag) of predominantly adult worm origin was found in the sera of humans infected with *Wuchereria bancrofti*. This paper describes results of a longitudinal study of changes in levels of PC-Ag in response to diethylcarbamazine (DEC) therapy as measured by two-site immunoradiometric assay (IRMA) and Western blotting. One hundred thirty-two residents of a bancroftian filariasis-endemic area of Papua New Guinea (PNG) were treated with a 72 mg/kg dose of DEC. A macrofilaricidal effect was seen with this dose of DEC as 34% of the treated subjects had localized side effects and long-term decreases in microfilariae (mf) counts were observed 12 months after treatment. The PC-Ag levels were reduced to 72%, 52%, and 51% of pretreatment values at 21 days and at six and 12 months after treatment. These decreases, observed by IRMA, were specifically associated with loss of the M_r 200,000 PC-Ag detected by immunoadsorption and Western blotting. From drug treatment data, the maximum half-life of PC-Ag in circulation was calculated to be 50 days, assuming a first-order decay process. This maximum half-life indicates that persistent antigenemia observed in the majority of treated subjects could only result from the survival of adult worms. In the absence of methods to directly demonstrate *W. bancrofti* adult worms, detection of serum PC-Ag levels provides a sensitive indirect measure of the dynamics of adult worm populations. This serological measurement may be useful in optimizing the macrofilaricidal and therapeutic effects of DEC and in assessing the macrofilaricidal action of new antifilarial drugs and immunological interventions.

Lymphatic filariasis is caused by infection with the tissue-dwelling nematodes *Wuchereria bancrofti, Brugia malayi,* and *B. timori.* The World Health Organization estimates that >100 million people are infected with these parasites and the number of infected individuals is increasing.[1] To date, diethylcarbamazine (DEC) has been the drug of choice for the treatment and control of lymphatic filariasis. When used as currently recommended, DEC effectively kills blood-borne microfilariae (mf) that transmit infection via mosquito vectors. Inability to quantitate lymphatic-dwelling adult worms that produce mf and cause the lymphatic pathology associated with filarial infection has made it impossible to directly assess the ability of DEC to kill this stage in humans. However, a number of observations in treated humans provide indirect evidence that DEC kills adult worms of the lymphatic filariae.[2] Dying worms have been observed by histological examination of biopsy material from treated patients. Long-term decreases in mf levels and resolution of symptoms in treated patients are also consistent with the view that DEC is at least partially macrofilaricidal when used in conventional doses.

Optimization of the macrofilaricidal and therapeutic effects of DEC requires a method to quantify adult worm burdens in humans. Immunodiagnostic tests to detect products of adult worms in the blood or urine of infected humans may provide an indirect means to quantify relative changes in adult worm burdens due to chemotherapy. Antigen detection methods to assess the efficacy of antifilarial drugs have already been described for evaluation of both the embryocidal effects of benzimidazoles in *Onchocerca gibsoni*-infected cattle[3] and various anti-filarial drugs in *Dirofilaria immitis*-infected dogs.[4]

Recently, a number of workers have characterized an M_r 200,000 phosphorylcholine (PC)-containing antigen (PC-Ag) in the circulation of

W. bancrofti-infected humans.[5-7] This antigen, not detectable in sera of individuals infected with other species of filarial and nonfilarial nematodes, has been identified by Western blotting using monoclonal antibodies (Mabs) to PC[5,6] and non-PC[7] epitopes of this antigen. These PC-Ags have also been described in the sera of *W. bancrofti*-infected silver leaf monkeys (*Presbytis cristatus*.[8] While a large number of PC-Ags have been found in somatic extracts and excretory/secretory products of all life cycle stages of both human and animal filariae,[7-11] only a limited number of these antigens have been found in the circulation of infected hosts. A single PC-Ag of M_r 90,000 has been described in the circulation of *B. malayi*- and *B. pahangi*-infected animals.[9,10] The dominant PC-Ag in the sera of *W. bancrofti*-infected humans is of M_r 200,000.[5,6]

A number of experiments have provided evidence that the restricted numbers of PC-Ags found in the circulation of hosts infected with lymphatic filariae predominantly reflect adult worm burdens. First, both *W. bancrofti* male and female adult worms release an M_r 200,000 PC-Ag during in vitro culture,[7] with female adult worms producing the largest amounts.[7] Second, a positive correlation between mf density and circulating PC-Ag levels has been reported for subjects infected with *W. bancrofti* from Papua New Guinea (PNG).[5] Presumably, mf density and adult worm burden are related in some density-dependent fashion. A positive correlation between levels of circulating PC-Ag and adult worm mass, in particular female worm numbers, has been observed in vivo by screening sera of *B. malayi* infected jirds.[10] Fourth stage larvae (L4) release PC-Ag in circulation of *B. malayi*-infected jirds, but amounts were significantly less (i.e., 80–90% less) than those released by adult worms.[10] The most compelling evidence for the predominantly adult worm origin of these antigens comes from an experiment in which *B. malayi*-infected jirds were treated with a large dose of DEC (the drug does not kill adult worms in this host).[10] The DEC treatment cleared mf one day after the commencement of therapy, but had not significantly altered levels of PC-Ag in circulation 15 days after treatment began. In contrast, administration of a partially macrofilaricidal dose of DEC to 12 human subjects with *W. bancrofti* microfilaremia resulted in long-term decreases in levels of circulating PC-Ag 6–12 months after treatment began.[12] Given the likely adult worm origin of circulating PC-Ags, measurement of such antigens may provide a means to assess the macrofilaricidal effects of chemotherapeutic and immunological interventions. The goal of this study was to test this hypothesis.

MATERIALS AND METHODS

Study population

The study was undertaken in Nanaha village near Dreikikir in East Sepik Province (ESP), PNG, where bancroftian filariasis is endemic.[13] Geographic and cultural details of this area have been described previously.[13] Nanaha is a rural village with a population of 172 individuals, most permanent residents. Individuals who consistently migrated in and out of the study area were excluded from the study.

Study design

Residents of the village of Nanaha were treated with the anti-filarial drug DEC during June 1985. The study aimed to compare efficacy of mass treatment of a community with either a conventional course of DEC of 72 mg/kg or a higher dose of 144 mg/kg over a 12-day period. Clinical, parasitological, and antigenemia data were collected on 156 residents of Nanaha prior to treatment. One hundred thirty-seven subjects >5 years of age commenced treatment with DEC. Pregnant women and some elderly members of the community were excluded from the study. Villagers were given DEC twice daily for 12 days. Supervised administration of treatment occurred at 6:00AM, before villagers went to work in their gardens, and at 6:00PM, when they returned to the village for the evening. Occurrence of systemic and localized side effects was checked twice daily (at the time of drug administration) and individuals were given a physical examination five and 21 days after commencement of treatment. The treatment regimen was as follows: all subjects began treatment on a 3 mg/kg dose of DEC twice a day for five days. On days 6 and 7, 68 subjects were given a 6 mg/kg dose twice a day. The others continued to receive 3 mg/kg twice a day for another seven days. Side reactions to treatment, such as nausea, vomiting, dizziness, and headache due to the pharmacological toxicity[1] of the drug, were reported in 34% of subjects within 36 hr of commencement of the

6 mg/kg twice daily dosage of DEC. In contrast, none of these side reactions were observed in the group on the 3 mg/kg twice daily dosage of DEC. Due to these side reactions, the daily dosage of all of these subjects was reduced to 3 mg/kg twice daily. Of the 137 subjects who commenced treatment, 132 completed a 72 mg/kg course of DEC over a 12-day period.

Efficacy of the DEC intervention was evaluated by comparing clinical, parasitological, and antigenemia measurements prior to treatment (T_0) and 21 days (T_1), 6 months (T_2), and 12 months (T_3) after treatment. Parasitological and antigenemia data are analyzed in this paper. Clinical data will be reported separately. This study was approved by the Medical Research Advisory Committee of PNG.

Parasitology

Ten milliliter night blood samples were collected into EDTA from individuals in the study population between 10:00PM and 2:00AM at T_0, T_1, T_2, and T_3. Two milliliter blood volumes were filtered by Nuclepore filtration using 5 μm filters; these were later methanol-fixed and Giemsa-stained. These filters were examined microscopically to determine mf densities. The other 8 ml of blood were used for serum collection and subsequent antigen analysis.

PC-antigen detection

Serum samples were analyzed by immunoradiometric assay (IRMA) using the PC-specific Mab designated Gib 13 as described previously.[5] Due to intra- and inter-assay variation, serum samples from the same individual at T_0, T_1, T_2, and T_3 were assayed in duplicate on the same plate. Results of assays were expressed as an antigen index (AI).

$$\text{AI} = \frac{\text{cpm bound with test serum}}{\text{cpm bound with control serum pool}}$$

The control serum pool was made up of 10 sera from residents of Melbourne, Australia who had never visited a filariasis-endemic area. Sera were also screened for 200,000 PC-Ag by immunoadsorption and Western blotting. Briefly, 100 μl aliquots of PNG sera were reacted with 200 μl of a 50% suspension of Gib 13 coupled to cyanogen bromide activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's specifications. Gib 13-Sepharose was diluted in mouse tonicity phosphate-buffered saline (MTPBS). After 3 hr incubation at room temperature, unbound material was removed by centrifugation at 100 × *g*. This procedure was repeated three times with a total volume of 30 ml of MTPBS. Washed Gib 13-Sepharose pellets were solubilized in an equal volume of sodium dodecyl sulfate (SDS) sample buffer containing 0.125 M Tris, 100 mM dithiothreitol, and 4.6% SDS (pH 6.8). Samples were centrifuged at 100 × *g* and 40 μl of supernatant loaded per track of a 7.5% gel and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to nitrocellulose at 50 V for 16 hr at 4°C. The nitrocellulose filter was then blocked with 5% non-fat skim milk in MTPBS and probed with ^{125}I-labeled Gib 13 in 5% milk in MTPBS. The filter was then washed twice with 200 ml volumes of 5% milk in MTPBS and examined by autoradiography.

RESULTS

Pretreatment parasitological data

Table 1 defines the age-specific rates and densities of both microfilaremia and antigenemia in the study population 3–4 days prior to commencement of DEC treatment. Clearly, *W. bancrofti* infection occurs at an early age and is highly endemic in this village. The prevalence and intensity of both microfilaremia and antigenemia increased with age. When mf and antigenemia rates were compared, there were individuals in all age classes who were mf-negative as judged by Nuclepore filtration of a 2 ml blood volume, but antigen-positive by Gib 13 IRMA. The converse was not found.

Post-treatment microfilarial data

Changes in the microfilaremia status of those subjects who completed a 12 day course of 72 mg/kg DEC and who participated in any of the three post-treatment surveys are shown in Table 2. Twenty-one days (T_1) after commencement of treatment, the mf rate had been reduced from 66% to 39% and the mean mf density of subjects mf-positive before treatment was reduced by 96%. Comparable reductions in mf rates and densities were also observed at T_2 and T_3 in relation to T_0 measurements, indicating that DEC had pro-

TABLE 1

Age-specific rates and densities of both microfilaremia and antigenemia for Nanaha residents prior to DEC treatment

Age (years)	Mf rate*	Mean mf density ± SEM	Antigenemia rate*†	Mean AI ± SEM
5–14	17/42 (41)	19.3 ± 1.9	37/42 (88)	3.9 ± 0.6
15–29	25/34 (74)	111.3 ± 1.3	32/34 (94)	4.7 ± 0.6
30–44	23/29 (79)	110.9 ± 1.7	29/29 (100)	6.4 ± 0.6
≥45	27/32 (84)	269.7 ± 1.5	32/32 (100)	6.4 ± 0.6
Total	92/137 (67)	104.3 ± 1.3	131/137 (96)	4.9 ± 0.5

* Percentages given in parentheses.
† Samples were considered antigen-positive if they had AI values ≥2.

duced a long-term reduction in mf densities consistent with the macrofilaricidal action of the drug.[2] The efficacy of DEC treatment to reduce microfilaremia densities was examined in relation to pretreatment mf densities. Subjects with >200 mf/ml blood before treatment showed a poor rate of complete clearance of mf at T_3; i.e., >80% had persistent microfilaremia at T_3, whereas no subjects with <200 mf/ml at T_0 had detectable microfilaremia at T_2 and T_3.

Localized side reactions to treatment

Further evidence of the macrofilaricidal effect of DEC was provided by clinical examination of the treated population for localized side reactions. These side reactions include lymphadenitis, lymphangitis, and transient lymphedema due to the death of adult and immature *W. bancrofti* worms.[2] Thirty-four percent of the treated population had such localized reactions within three weeks of beginning treatment.

Post-treatment antigenemia data

Table 3 summarizes changes in individual PC-Ag levels (AI values) for those subjects who were antigen-positive (AI ≥ 2) at T_0, completed the 72 mg/kg course of DEC, and attended any of the three post-treatment surveys. Significant decreases were seen in PC-Ag levels in ≥75% of the subjects (i.e., change in AI > 1) at T_1, T_2, and T_3 relative to T_0. There were no significant increases in AI for any subjects noted between T_0 and T_1, T_2, or T_3, although a small percentage of treated subjects showed no significant change in AI values post-treatment. Generally, the pretreatment sera of these subjects gave weak positive signals in Gib 13 IRMA. This lack of change in post-treatment AI may be explained by the fact that T_0 antigen levels in these subjects were at the limit of sensitivity of Gib 13 IRMA. Seven percent and 13% of subjects showed significant increases in AI values at T_2 and T_3, respectively, relative to T_1 AI values.

Increases in AI values at T_3 relative to T_2 values were also observed in 20% of subjects. These increases may be due to reinfection with *W. bancrofti*, since entomological data showed that transmission of infection was not interrupted by the DEC intervention in Nanaha village (J. Bryan, University of Queensland, Brisbane, Australia, unpublished data). Alternatively, they may be due to the ambiguity of the PC specificity of the Gib 13 IRMA, as increases in circulating PC-Ag level may be due to infection with non-filarial pathogens.

Paired *t*-tests were performed on AI values for each subject who attended any of the three post-treatment surveys (Table 3). Differences between

TABLE 2

Changes in microfilaremia rates and densities after DEC treatment

Time after DEC treatment	Proportion mf-positive before treatment*	Proportion mf-positive after treatment*	Mean ± SEM mf density before treatment†	Mean ± SEM mf density after treatment†	Percentage reduction in mf density
T_1	83/126 (66)	49/126 (39)	94.6 ± 1.4	3.1 ± 1.2	96
T_2	81/125 (65)	52/125 (42)	107.6 ± 1.2	7.4 ± 1.2	93
T_3	78/119 (66)	45/119 (38)	112.7 ± 1.4	6.5 ± 1.3	94

* Percentages given in parentheses.
† Only subjects mf-positive before treatment were considered in these calculations.

TABLE 3

Comparison of differences between pre- and post-treatment AI values using paired t-test

Comparison of AI values*	Number of pairs examined	Number of pairs with decreased AI†	Number of pairs with increased AI†	Mean differences in AI ± SEM	*P* value
T_0 vs. T_1	98	73 (75)	0 (0)	1.6 ± 0.2	<0.0001
T_0 vs. T_2	96	86 (90)	0 (0)	2.9 ± 0.2	<0.0001
T_0 vs. T_3	100	83 (83)	0 (0)	2.8 ± 0.3	<0.0001
T_1 vs. T_2	95	51 (53)	7 (7)	1.2 ± 0.2	<0.0001
T_1 vs. T_3	99	54 (54)	13 (13)	1.2 ± 0.2	<0.0001
T_2 vs. T_3	97	26 (27)	19 (20)	0.0 ± 0.1	>0.09

* Only subjects whose pre-treatment AI values were ≥2 were considered in this analysis.
† Percentages given in parentheses.

AI values of paired samples at T_0 and T_1, T_0 and T_2, T_0 and T_3, T_1 and T_2, and T_1 and T_3 were highly significant. However, differences between paired AI values at T_2 and T_3 were not significant because the majority of subjects showed no further decreases in AI at T_3 compared to T_2. Mean differences in AI show that the general trend in the treated population was for PC-Ag levels to decrease after treatment up until T_2 and then remain stable. This trend is seen most clearly by calculation of percent reduction in AI values for 96 subjects who attended all four surveys (Table 4).

The relationship between pretreatment microfilaremia status and antigenemia at T_3 was also examined (Table 5). Subjects with persistent microfilaremia at T_3 (patient category 1) had a significantly greater mean mf density ($P < 0.001$) and PC-Ag level ($P < 0.01$) at T_0 compared to either microfilaremic subjects who were mf-negative 12 months after treatment (patient category 2) or subjects who were amicrofilaremic at T_0 (patient category 3). The presence of detectable although reduced levels of antigenemia in 86% of the subjects with persistent microfilaremia at T_3 was consistent with the view that the 72 mg/kg dose of DEC is not optimized to kill all worms in heavily infected individuals. The response to DEC treatment in terms of changes of antigenemia rates and densities was very similar in patient categories 2 and 3, suggesting that worm loads in these subjects were similar, but were lower than those in patient category 1.

TABLE 4

Mean AI for 96 subjects examined at T_0, T_1, T_2, and T_3

Time	Mean AI ± SEM	Percent reduction in AI after DEC treatment
T_0	6.1 ± 0.2	0
T_1	4.4 ± 0.2	72
T_2	3.2 ± 0.2	52
T_3	3.1 ± 0.2	51

Sera from subjects in patient category 1 (Table 5), taken at T_0, T_1, and T_2, were also screened by immunoadsorption with Gib 13-Sepharose and Western blotting to determine whether the changes in levels of circulating PC-Ag observed by Gib 13 IRMA reflected changes in intensity of the *W. bancrofti*-specific M_r 200,000 Pc-Ag. Consistent with Gib 13 IRMA, data in Figure 1 shows specific loss of this M_r 200,000 Pc-Ag as early as 21 days after DEC treatment of three subjects from patient category 1. The M_r 200,000 band was not detectable in these three patients six months after treatment, although there was a detectable signal by Gib 13 IRMA. This presumably reflects greater sensitivity of antigen detection by IRMA compared to immunoadsorption and Western blotting.

Maximum half-life of PC-Ag

The mean PC-Ag level of the population at T_0 was decreased to 72% and 52% of pretreatment levels at T_1 and T_2, respectively. This result, if it was assumed that all worms were killed by DEC (clearly not the case in all treated subjects), would indicate that the half-life of the PC-Ag after drug treatment is between 21 days and six months. A maximum estimate of the half-life can be calculated if we assume that the total worm population was killed and that the decay of PC-Ag in circulation was a first order process described by the equation

$$P(t) = e^{-at}$$

where P is the proportion of antigen remaining at time (t) and a is a rate constant. It is possible to use DEC treatment data to calculate the rate constant (a) and thereby solve the equation and

TABLE 5

Relationship between pretreatment microfilaremia status and persistent microfilaremia/antigenemia 12 months after treatment

Patient category	Number of subjects	Mean mf density ± SEM at T_0	Mean mf density ± SEM at T_3	Number with AI ≥2 at T_0*	Number with AI ≥2 at T_3*	Mean AI ± SEM at T_0	Mean AI ± SEM at T_3
(1) mf + T_0 mf + T_3	42	654 ± 1.3	32.1 ± 1.3	42 (100)	36 (86)	7.9 ± 0.1	4 ± 0.3
(2) mf + T_0 mf − T_3	34	12.9 ± 1.5	0	32 (94)	20 (59)	4.6 ± 0.1	2.4 ± 0.1
(3) mf − T_0 mf − T_3	37	0	0	30 (81)	19 (53)	3.5 ± 0.3	2.4 ± 0.4

* Percentages given in parentheses.

calculate the estimated maximum half-life of this antigen. Data from Table 4 shows that at T_1, 72% of antigen remains. If P(t = 21 days) = 0.72, a = 0.016.

Using this calculated value for the rate constant (a), the maximum half-life (when P = 0.5) of PC-Ag in circulation is 50 days. In the absence of adult worms, PC-Ag would persist at detectable levels (i.e., P = 0.33) by Gib 13 IRMA in the circulation for a maximum period of 69 days. Thus, the persistent antigenemia observed 12 months after treatment of the majority of the study population must be due to the presence of living adult worms.

DISCUSSION

A longitudinal population-based study of the kinetics of circulating PC-Ag levels was designed to determine whether this serological measurement was an indicator of the macrofilaricidal effects of DEC. The study design assumed, for the reasons outlined above, that PC-Ag levels predominantly reflect adult worm burdens. The 72 mg/kg dose of DEC chosen for the study clearly had a macrofilaricidal action, as localized side reactions were observed in 34% of the study population and long-term reductions in mf counts were observed 6–12 months post-treatment. Consistent with the fact that this DEC treatment regimen was partially macrofilaricidal, significant reductions in AI values, an indirect measure of adult worm burdens, were observed in the majority of the treated population. In addition, PC-Ag levels were reduced to 72%, 52%, and 51% of pretreatment values at 21 days, 6 months, and 12 months after treatment, respectively. The decreases observed by IRMA were associated

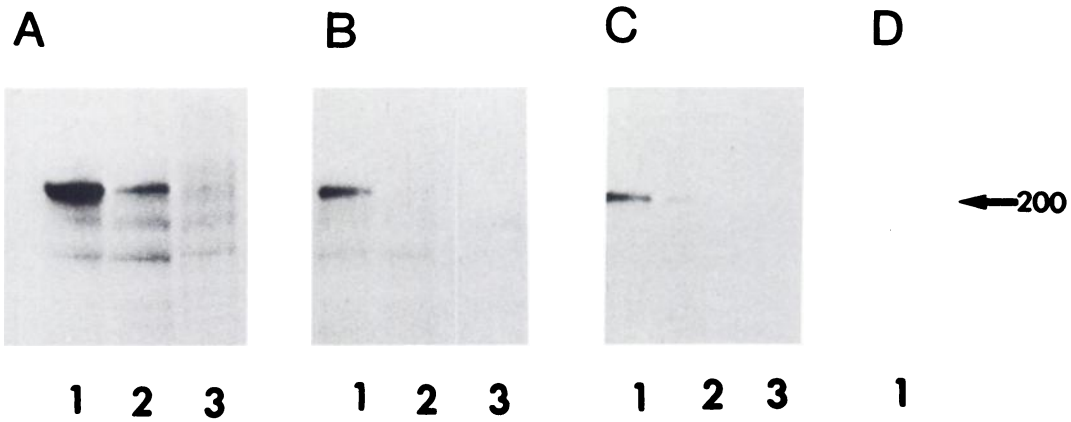

FIGURE 1. Autoradiographs of Western blots of Gib 13-reactive PC-Ag isolated from sera of microfilaremic subjects (**A, B, and C**) and from serum of a control subject (**D**). For all subjects, lane 1 is before DEC treatment (T_0), lane 2 is after 21 days of treatment (T_1), and lane 3 is after 6 months of treatment (T_2). AI values and mf densities for subjects A, B, and C for T_0, T_1, and T_2, respectively, were as follows: for A, AI = 15, 11, and 7.1, and mf/ml = 2400, 4, and 22; for B, AI = 13.4, 8.8, and 6.4, and mf/ml = 1200, 177, and 463; for C, AI = 5.8, 3.6, and 2.5, and mf/ml = 178, 2, and 13.

with specific loss of the M_r 200,000 PC-Ag from the circulation of infected individuals. These decreases occurred irrespective of age, immune status, or parasite load. In a longitudinal study of DEC treatment of 12 microfilaremic subjects, Weil and others[12] reported a similar loss of serum antigen reactivity by the Mabs AD 12.1 and DH 6.5, which recognize the *W. bancrofti* M_r 200,000 PC-Ag. Both studies clearly showed that measurement of serum levels of the *W. bancrofti* M_r 200,000 PC-Ag was a sensitive indicator of the dynamics of adult populations after chemotherapy.

From drug treatment data, the maximum half-life of PC-Ag was calculated to be 50 days, assuming a first-order decay process. This maximum half-life indicates that persistent antigenemia observed in the majority of treated subjects could only result from the survival of adult worms. When the PNG study population was stratified according to indirect measures of adult worm burden, i.e., mf density and AI values, the efficacy of DEC to clear microfilaremia related to pretreatment parasite load. Reduction of mean antigenemia levels to 50% of pretreatment values indicated that 72 mg/kg DEC given over 12 days had only a 50% macrofilaricidal action at the population level. Weil and others[12] found a similar percentage reduction in antigenemia using the same drug regimen.

Results presented in this paper suggest a number of applications for measurements of *W. bancrofti* M_r 200,000 PC-Ag levels. Firstly, the macrofilaricidal efficacy of DEC could be tailored to the infection load in a community by monitoring dosage schedules which result in total reductions in circulating PC-Ag levels in a population sample stratified according to indirect measures of worm burdens, such as PC-Ag and mf density. This serological assessment of drug efficacy would be quantitative and more rapid than waiting to observe mf densities at 12 months since the half life of PC-Ag is less than 50 days. Specificity of the measurement can be ensured by directly assessing changes in the M_r 200,000 PC-Ag by Western blotting. A second application would be to compare the efficacy of DEC given in low doses, as suggested by Partono and others,[14] as compared to high doses, using PC-Ag levels as the indicator of macrofilaricidal activity. Presumably, the reason for the long-term success of low dose treatment is the more effective macrofilaricidal action of this treatment regimen. Another application of longitudinal measurement of the M_r 200,000 PC-Ag levels would be to evaluate the efficacy of both new antifilarial drugs such as ivermectin and immunological interventions.

The results presented in this study and those reported previously[12, 15] show that indirect measures of adult worm burdens, such as PC-Ag levels, provide tools to study the dynamics of *W. bancrofti* infection in humans[15] and to evaluate control strategies in both experimental animals[10] and epidemiological situations.

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