

Microfilaraemia, filarial antibody, antigen and immune complex levels in human filariasis before, during and after DEC therapy : a two-year follow-up

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Objekttyp: **Article**

Zeitschrift: **Acta Tropica**

Band (Jahr): **45 (1988)**

Heft 3

PDF erstellt am: **08.08.2024**

Persistenter Link: <https://doi.org/10.5169/seals-314082>

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Microfilaraemia, filarial antibody, antigen and immune complex levels in human filariasis before, during and after DEC therapy

A two-year follow-up

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Summary

A study on the effect of DEC therapy on microfilaraemia and the immune status in 27 patients with *W. bancrofti* infection was carried out for two years. Persistence of microfilaraemia was observed in 4 out of 27 cases after one course of DEC therapy and were treated again for one week. On further follow-up, none was microfilaraemic upto Day 60. The mean filarial antibody titres of IgM and IgG showed a gradual decrease as assessed by enzyme linked immunosorbent assay (ELISA). The mean titres of circulating microfilarial excretory-secretory (ES) antigens and immune complexes (ICs) showed an initial increase during therapy, followed by a gradual fall upto Day 60. Filarial antigen was detected in urine of all the carriers during therapy. Excretion pattern of antigen in urine showed correlation with DEC dose. Reappearance of microfilariae (mf) in circulation in 12 patients after a year showed that DEC had temporary attenuating effect on adult worms or no effect on developing larvae, suggesting further treatment and follow-up of patients. Parasitological and immunoscreening at the end of 2 years showed that the presence of mf ES antigen in blood correlated with the appearance of microfilariae in blood.

Key words: *Wuchereria bancrofti*; DEC treatment; immune status.

Introduction

Diethylcarbamazine (DEC) is a potent microfilaricide in vivo. However, in all the studies carried out so far, the persistency of microfilariae in infected

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human population had been determined by relatively insensitive technique of examining stained blood film of 20 mm³ of capillary blood obtained by finger prick. Increased use of chemotherapeutic treatment has demonstrated the need for a sensitive diagnostic procedure to detect low microfilaraemia as well as establish the effectiveness of chemotherapy. A change in the immunological parameters following therapy may give more sensitive marker for evaluating the microfilaricidal effect of DEC in microfilaraemia patients. Several attempts were made to study the levels of antibody and antigen during chemotherapy. Oliver-Gonzales (1953) observed that positive intradermal tests fell down from 99% to 87% after treatment of filarial patients. Sawada et al. (1968), Katiyar et al. (1974) and Murthy et al. (1978) have reported the suppression of skin reaction in filarial patients following DEC therapy. Desowitz et al. (1978) have shown a rapid decrease of precipitating and reaginic antibodies in *D. immitis* infected dogs following treatment with DEC. A decrease in IHA titres and disappearance of some usual precipitin bands in gel diffusion test in sera of DEC treated albino rats infected by adult *L. carinii* was observed by Mishra et al. (1982). Several investigators have demonstrated the presence of circulating filarial antigen (Kaliraj et al. 1979; Hamilton et al., 1984; Dissanayake et al., 1984; Forsyth et al., 1985; Paranjape et al., 1986; Zheng et al., 1987; Weil and Liftis, 1987; Lal et al., 1987) and immune complexes (Dissanayake et al., 1982; Prasad et al., 1984) in bancroftian filariasis. However, reports are scanty on the circulating antigen and immune complex levels during and after DEC therapy. Carme et al. (1982) could observe release of free circulating antigens in 73% of cotton rats infected with *L. carinii* after DEC treatment. Earlier studies from this laboratory showed a gradual decrease in the mean antibody titre (Malhotra et al., 1983) and an initial increase followed by a gradual decrease in the mean antigen levels (Malhotra and Harinath, 1984) following DEC therapy in bancroftian filarial cases. This communication reports the levels of microfilariae, antigen, antibody and immune complexes in filarial patients during and after DEC therapy followed for two years.

Materials and Methods

DEC treatment

A total number of 27 patients with *W. bancrofti* infection who had been identified by wet blood smear examination were treated with diethylcarbamazine (DEC, Wellcome-Bombay) for 14 days under close supervision.

Drug schedule

Day 1 – DEC 1 mg/kg body wt. (single dose) + Aspirin,
Day 2 – DEC 2 mg/kg body wt. (divided doses) + Aspirin,
Days 3–14 – DEC 6 mg/kg body wt. (single dose).

The dose of aspirin was adjusted according to the age. The patients with persistent microfilaraemia, after one course of treatment were continued on treatment for 21 days. Further, a single dose of DEC 6 mg/kg body wt. was given to all the 27 cases on Days 360, 540 and 720.

Collection of blood and urine samples

Blood samples were collected both on glass slides and filter papers (Whatman No. 3) between 21 to 24 h before treatment (Day 0), during treatment (Day 7) and after treatment (Days 15, 30, 60 and 360). Thereafter once at the end of 2 years. Urine samples were collected before treatment (Day 0), during treatment (Days 2, 3, 4, 5 and 7) and afterwards as above.

Microfilarial excretory-secretory (ES) antigens

W. bancrofti microfilariae were isolated by nucleopore membrane filtration from microfilaraemia blood samples and maintained in medium 199 supplemented with organic acids and sugars of Grace's medium at 28° C for 24 h (Kharat et al., 1982). The culture fluid containing microfilariae was centrifuged at 4° C and the antigenic protein in the supernatant was isolated by Sephadex G25 filtration.

Isolation of filarial serum immunoglobulin-G (FSIgG)

Human filarial serum immunoglobulin (FSIg) was separated from pooled clinical filarial sera showing antimicrofilarial antigen antibody by CIE, by 35% ammonium sulphate saturation. The IgG fraction was isolated from FSIg by DEAE-cellulose (Whatman DE 52) column chromatography (Reddy et al., 1984).

Anti human C3

Monospecific antihuman C3 raised in goat was obtained from Immunodiagnostics, New Delhi, India.

Rabbit anti urinary filarial antigen (UFA) serum immunoglobulins

A rabbit weighing 3 kg was injected (sc) with 1 ml of UFA (1 mg/ml) along with 1 ml of complete Freund's adjuvant. The same dose was given on Day 7 with Freund's adjuvant and on Day 14 without adjuvant. Blood samples were collected after a booster dose on Day 40. The antiserum was absorbed with normal human urine and then immunoglobulins were isolated by 35% ammonium sulphate saturation (Malhotra et al., 1985). Preimmunized blood sample was used as control.

Enzyme linked immunosorbent assay (ELISA)

Conjugation of antihuman IgM, IgG, ES antigen as well as FSIgG and penicillinase was achieved by the method of Avrameas (1969) using glutaraldehyde. Substrate in ELISA consisted of soluble starch (150 mg) in 27.5 ml of 0.25 M sodium phosphate buffer (SPB), pH 7 containing 10.64 mg of penicillin V and 100 µl of 0.08 M iodine in 3.2 M potassium iodide solution. The solution was prepared fresh before use.

Indirect ELISA for the filarial antibody was carried out as described by Kharat et al. (1982). The microtitre plate (Dynatech, Singapore) was coated overnight at 4° C with 100 µl of mf ES antigen (3.5 ng/ml) diluted in 0.06 M carbonate buffer, pH 9.6 to each well and then saturated with 3% bovine serum albumin (BSA). Hundred µl of filter paper eluates serially diluted (4 fold) starting with 1:300 dilution, were then added and incubated at 37° C for 3 h. After addition of antihuman IgM or IgG penicillinase conjugate the plate was further incubated as above. The plate was washed after every incubation with 0.01 M phosphate buffer saline pH 7.2 containing 0.05% Tween (PBS-T). Finally the plate was incubated with 100 µl of starch iodine penicillin substrate to observe immune reaction. The decolorization of blue color denoted the positive reaction while negative reaction was confirmed by the persistence of blue color.

Inhibition ELISA for filarial mf ES antigen was carried out as described by Malhotra and Harinath (1984). The first coating was done using FSIgG (50 µg/ml) in inhibition ELISA. After blocking with 3% BSA, optimally diluted filter paper eluates (1:300) were added, and incubated. After washing, final incubation was done using ES antigen penicillinase conjugate. The persistence of blue color indicated positive reaction in inhibition ELISA.

Anti C3 ELISA was carried out as described by Prasad et al. (1984). The plate was initially saturated with anti C3 (1 μ l/ml). The second incubation was done with filter paper eluates. The final incubation was with FSIgG-penicillinase conjugate. The decolorization of the substrate indicated the presence of immune complexes in the filter paper eluates.

Double antibody sandwich ELISA was carried out as described by Malhotra et al. (1985). Gamma globulin fraction of anti-UFA (1 μ g/ml), undiluted and serially diluted human urine samples and FSIgG penicillinase conjugate were used in the assay system. Anti rabbit UFA antibody was used to catch the antigen and then FSIgG was used as detecting antibody. The disappearance of blue color denoted a positive reaction.

Results

Effects of DEC on microfilaraemia status

Four of the 27 subjects showed the persistence of microfilaraemia at the end of the treatment i.e. on Day 15 and one was microfilaraemic on Day 30. However, none was positive for microfilariae on Day 60. Parasitological examination of all the subjects on Day 360 demonstrated reappearance of microfilaraemia in 12 cases of which 6 were persistent for microfilariae till Day 720. In addition, 2 cases who were amicrofilaraemic on Day 360, revealed microfilaraemia on Day 720 (Fig. 1). However, density of pretreatment parasitaemia (9.3/20 mm³) was reduced to 2.3 on Day 360 which further declined to 1.6 at the end of the study.

Effect of DEC on the immune status

All the 27 cases were tested for IgM, IgG antibody and ES antigen levels by ELISA before and after DEC therapy.

Effect of DEC on IgM and IgG antibody level

Out of 27 cases, seven persons for IgM and three for IgG antibody showed an initial increase in antibody titre, followed by a decrease. However, the mean reciprocal antibody titres of IgM (7133) and IgG (3266) showed a gradual decrease till Day 60 to 505 and 408, respectively (Fig. 2). Reappearance of microfilariae after a year showed a slight increase in the mean antibody titre of IgM (1359) and IgG (1071). Analysis of IgM and IgG antibody levels on Day 720 showed reciprocal antibody titre of 1100 and 1586, respectively. Four cases for IgM and two cases for IgG showed absence of antibodies on Day 60. Reduction of antibodies to undetectable levels was found after a year in ten cases for IgM and six for IgG. At the end of 2 years 18 persons for IgM and 13 for IgG antibody showed negative results.

Effect of DEC on mf ES antigen level

Excepting two cases all the patients showed mf ES antigen before treatment with a mean antigen titre of 732. The mean titre raised to 1633 on Day 7. Thereafter the antigen titre showed a gradual decrease to 1411 on Day 15, 732

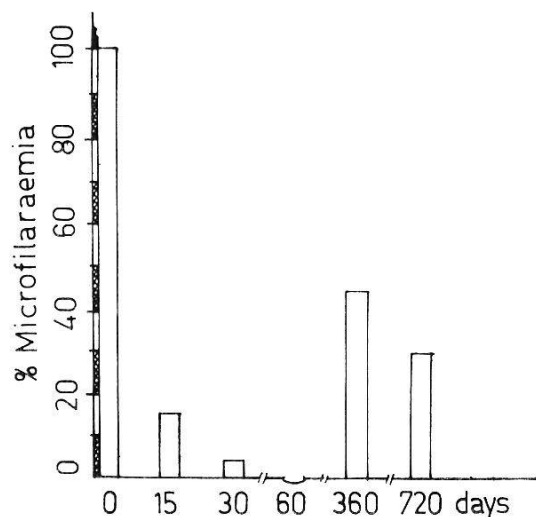


Fig. 1

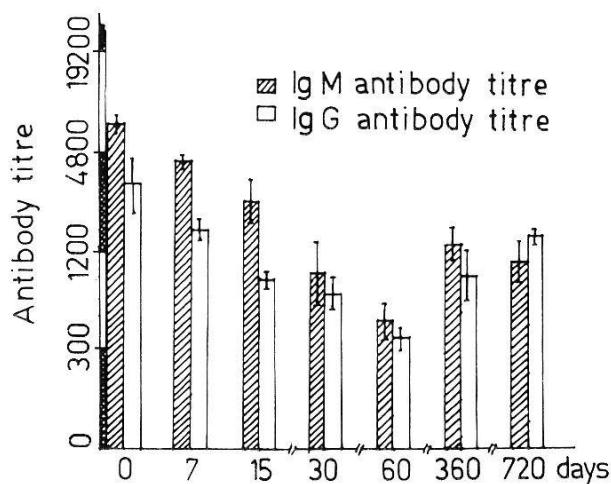


Fig. 2

Fig. 1. Percentage of microfilaraemia in microfilaraemic cases before, during and after DEC therapy.

Fig. 2. The mean IgM and IgG antibody titre in microfilaraemic cases before, during and after DEC therapy.

on Day 30. A further fall of antigen titre to 450 was observed on Day 60 (Fig. 3). Only one case on Day 30 and ten on Day 60 showed absence of antigen. However, the mean titre of ES antigen enhanced to 1024 after a year when microfilariae reappeared in 12 cases. Of these 12 cases 10 were antigenically positive on Day 60. Four cases who had no antigen on Day 60 showed antigen on Day 360. Out of these four two were found to be microfilaraemic. Parasitological and immunoscreening on Day 720 showed antigenaemia in 9 cases of which 8 were parasitologically confirmed.

Effect of DEC on immune complex level

Twenty carriers were tested for immune complexes by anti C3 ELISA. Only three showed presence of filarial ICs, before treatment. A sudden increase of mean titre of 73,020 on Day 7, followed by a gradual decrease to 24,240 on Day 15, 7320 on Day 30 and 5820 on Day 60 was observed (Fig. 4).

Effect of DEC on urine antigen level

Urinary filarial antigen level was determined in DEC treated carriers by double antibody sandwich ELISA with appropriate controls of DEC treated healthy endemic normals. All the carriers excreted antigen in urine during the treatment with varying antigen titres in the range of 1:4 to 1:64. The antigen levels increased with increased dose of DEC. The day after receipt of 6 mg/kg body wt. i.e. on Day 4 most of the carriers showed high antigen titre of 1:64. Antigen titre gradually fell down on Days 5, 7 and 15, respectively. On Day 30 only one had antigen in urine who was microfilaraemic. None of the samples

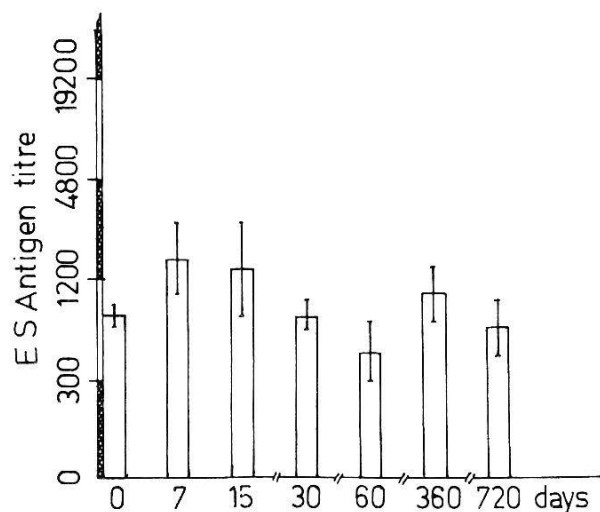


Fig. 3

Fig. 3. The mean microfilarial excretory-secretory antigen titre in microfilaraemic cases before, during and after DEC therapy.

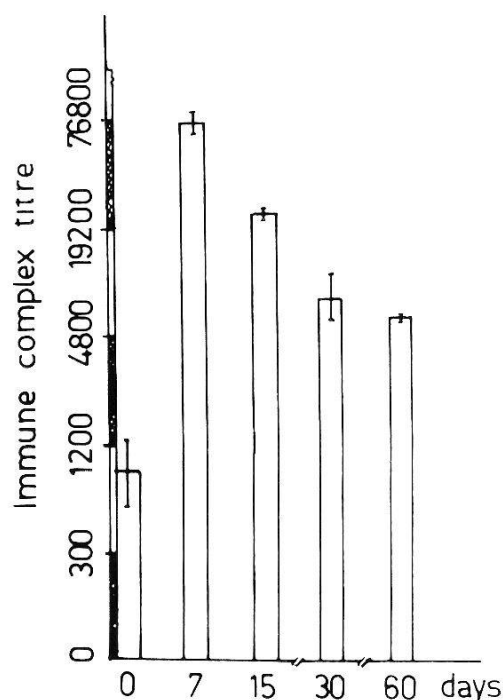


Fig. 4

Fig. 4. The mean immune complex titre in microfilaraemic cases before, during and after DEC therapy.

showed antigen on Day 60 (Fig. 5). At the end of a year 8 samples showed antigen in urine out of 12 cases showing mf in blood. Urinolysis at the end of the study (Day 720) showed antigen in $\frac{3}{8}$ microfilaraemic samples.

Discussion

Diethylcarbamazine, a well known filaricide, has been used since 1949 for control of bancroftian filariasis in various dose schedules. To minimise, the side reactions caused by it a low dose of DEC (1 mg/kg body wt. on Day 1 and 2 mg/kg body wt. on Day 2) was administered followed by a 12-day course. At the end of the treatment 4/27 (15%) cases had low microfilaraemia. When a total dose of 72 mg/kg body wt. was administered by Sethumadhavan et al. (1980) in monthly, weekly and daily doses, the percentage of mf carriers positive even after the treatment was 11, 21 and 14, respectively. The presence of mf in 20% post-treatment night blood smears of *Wb* infected patients who had consumed 30 mg DEC/kg body wt. was reported by Katiyar et al. (1974). Only one was microfilaraemic on Day 30 in the present study. However, none was microfilaraemic on Day 60. Reappearance of mf was observed in 12/27 (44%) cases, a year after the treatment. Sethumadhavan et al. (1980) observed the reappearance of mf in about 45% of DEC treated carriers 9 months after 72 mg/kg

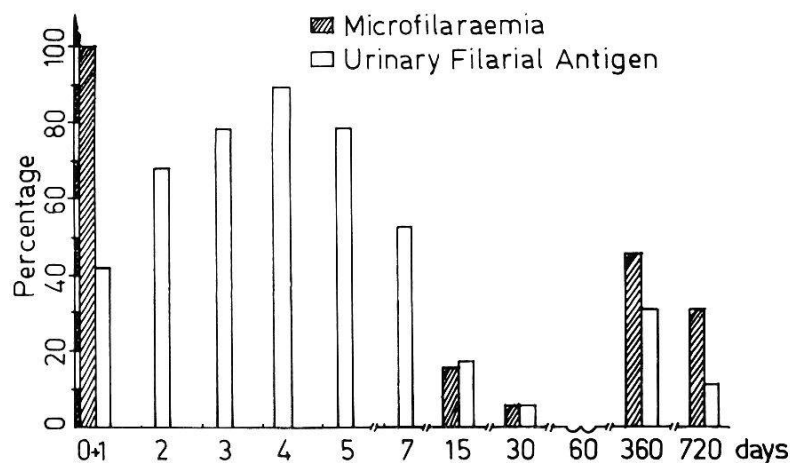


Fig. 5. The percentage of microfilaraemic cases showing filarial antigen in urine before, during and after DEC therapy.

regimen. Reappearance of mf after a year suggests that DEC had either only temporary attenuating effect on adult worms or no effect on the developing larvae, ruling out the possibility of reinfection and production of microfilariae within a year. Thereafter the same group was administered a single dose of DEC (6 mg/kg body wt.) at an interval of six months. There were eight carriers showing microfilaraemia at the end of two years. Treatment and follow-up for 2 years showed that DEC had only limited impact on adult worms, suggesting repeated treatment and long-term follow-up may be needed to determine optimum dosage of the drug and duration of treatment in the endemic areas. DEC has been shown to have no effect on embryogenesis by Schulz-Key et al. (1984) and Rivas-Alcala et al. (1981). Despite 30% of patients were microfilaraemic at final blood examination, the mean microfilarial count (1.6) was very much lower than pretreatment values (9.3).

A significant reduction in anti-filarial passive haemagglutinating antibody was recorded after treatment with DEC in bancroftian filariasis by Kaeuffer et al. (1976). Ambroise-Thomas and Kien Truong (1974) observed a temporary increase in fluorescent antibody titre following treatment and showed a gradual decline after treatment. Release of antigens was observed in *W. bancrofti* (Malhotra and Harinath, 1984) and *L. carinii* infection (Carme et al., 1982). But no relation had been reported between levels of antigen, antibody and parasite load in the infected cases. The present study was carried out to assess the effect of DEC on the immune status for a period of two years.

The gradual decrease of mean IgM antibody levels reached undetectable levels in one and four individuals on Days 30 and 60, respectively. Only two cases showed no detectable IgG antibodies on Day 60. The antibody analysis of all the subjects on Days 360 and 720, respectively, displayed absence of antibody in ten and eighteen samples for IgM and six and thirteen for IgG. Ottesen (1984) had determined IgG antibody levels in *W. bancrofti* patients using het-

erologous antigen in ELISA and reported a fall in the specific antifilarial antibodies following DEC treatment. Monitoring of IgM antibody levels to *Wb* mf ES antigen was found to be more informative than IgG owing to its high initial level and a rapid decrease from the circulation, to assess the effectiveness of DEC therapy. The IgG antibodies not only showed a low individual titre but also persisted for longer periods.

A significant rise in mf ES antigen level on Day 7 following by a gradual fall of titre reaching less than pretreatment level was observed on Day 60. One subject on Day 30 and ten on Day 60 showed no detectable antigen in blood. The appearance of serum antigens following treatment with DEC was observed by Carme et al. (1982) and the antigens remained detectable for two to three weeks after DEC therapy in cotton rats infected with *L. carinii*. In this study 3/20 mf carriers showed the presence of filarial immune complexes. However, all the samples showed ICs in circulation on Day 7 and 18/20 had maximum titre of 19,200. Formation of ICs might be attributed for the gradual drop of IgM and IgG antibodies in the circulation. Similar to the antigen, IC level also showed steady decrease till Day 30 and continued upto the Day 60. Out of 12 carriers showing microfilaraemia and antigenaemia on Day 360, 10 have shown antigen on Day 60 as well. The antigenic analysis on Day 720 showed the presence of antigen in all the 8 microfilaraemic subjects.

Filarial antigen in urine was detected in 41% of mf carriers before treatment. The detection of antigen in urine did not correlate with either mf count or antigen levels in blood. The detection of antigen in urine and blood of the same individuals using monoclonal antibody E 34 (Zheng et al., 1987) and anti BmA antibody (Prasad et al., 1987) showed no correlation between levels of microfilaraemia and urine levels of parasite antigen. The lack of correlation presumably reflect differential loss in kidney function of different patients as a result of chronic filariasis infection. However, urine antigen levels did correlate with increased dose of DEC administration in this study. After administration of 6 mg/kg body wt. dose on Day 3, 89% of the carriers showed antigen, most of them with reciprocal antigen titre of 1:64. All the carriers (100%) showed antigen in urine during the course of treatment. Rivas-Alcala et al. (1981) have observed microfilariae in urine of 20% of patients with onchocerciasis before treatment. However, during the first week of treatment microfilaruria raised to 100%. Screening of all the 27 urine samples in this study on Days 360 and 720 showed antigen in urine in 8/12 and 3/8 mf carriers. The detection of antigen in urine may be useful as a marker to determine whether or not the patient has taken the drug in control programmes during treatment.

Of all the parameters studied, the detection of mf ES antigen was found to be promising as an indicator of infection, despite mf were not detected by blood film examination. The data presented in Table 1 shows reappearance of mf on Days 360 and 720 in filarial cases who were administered DEC. Though all the 12 cases were amicrofilaraemic on Day 60, 10 cases showed antigenaemia,

Table 1. Microfilaraemia and mf ES antigen level in patients who showed reappearance of microfilariae on Days 360 and 720

Case No.	Pre-treatment		Post-treatment					
	Day 0		Day 60		Day 360		Day 720	
	Mf*	ES Ag	Mf*	ES Ag	Mf*	ES Ag	Mf*	ES Ag
6	6	300	-ve	300	2	300	1	300
30	2	2400	-ve	300	1	4800	-ve	-ve
34	10	600	-ve	300	2	1200	-ve	-ve
44	22	1200	-ve	600	2	300	2	1200
14	8	600	-ve	300	1	300	1	1200
37	26	600	-ve	300	4	300	-ve	-ve
35	17	600	-ve	300	2	300	-ve	-ve
3	4	600	-ve	300	6	300	-ve	-ve
15	1	-ve	-ve	-ve	1	300	-ve	-ve
17	9	300	-ve	-ve	1	1200	1	600
19	2	600	-ve	600	4	4800	5	1200
20	8	1200	-ve	1200	2	600	1	300
10	24	1200	-ve	300	-ve	-ve	1	600
12	10	600	absent		-ve	300	1	300

*Mf count/20 mm³

indicating the presence of infection. Furthermore, the presence of mf ES antigen on Days 0 (92%), 360 (100%) and 720 (100%) correlated well with microfilaraemia. Henceforth the detection of mf ES antigen by inhibition ELISA may be used as a parameter for the detection of active infection in filarial control programmes.

Acknowledgments

This work was supported by grants in part from ICMR, New Delhi, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Indo-US S & T initiative programme. We thank Dr. Sushila Nayar, Director for her keen interest and encouragement. Thanks are also due to MS. Meena Jotwani for her technical assistance. Special thanks to Dr. M. S. Bharti and staff of filaria research-cum-training centre, Wardha, for their help in contacting carriers.

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