Differential antibody response to parasite lipid antigens in lymphatic filariasis

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Antibody response to lipid antigens from microfilariae of cattle filarial parasite *Setaria digitata* was evaluated in human filarial sera of Orissa, India. Increased antibody (IgG and IgM) levels were observed in amicrofilaraemic groups of chronic patients and endemic normals compared to microfilaraemic carriers. Antibody levels were negligible in people living in non-filarial regions and in leprosy patients suggesting filariae specificity. Anti-lipid antibodies were also found to be considerably elevated in *Brugia malayi* (L_3)-infected mastomys without microfilaraemia than in those animals with microfilariae. These data indicate that high antibody levels to lipid antigens are associated with microfilariae negativity. IgG subclass analysis revealed the predominance of IgG_2 in asymptomatic microfilaraemics IgG_3 and IgG_1 in chronic patients and IgG_1 and IgG_2 in endemic normals. Diethylcarbamazine treatment in asymptomatic microfilaraemic subjects led to enhanced antibody response that sustained more than six months post treatment. The nature of antigenic determinant recognized by filarial antibodies was assessed by periodate treatment and indicated these antigens to be glycolipids. Periodate oxidation of lipids resulted in an overall decrease in antibody binding, the magnitude of which varied depending on antibody isotypes and filarial groups. The periodate-sensitive carbohydrate epitopes were recognized by both IgG and IgM in asymptomatic microfilaraemic carrier sera, but only by IgG in endemic normals and chronic filarial sera; IgM binding in the latter sera remained unaffected. Carbohydrate recognition by filarial antibodies seems to be dissimilar in different clinical groups of filariasis and might modulate the course of filarial infection in humans.

LYMPHATIC filariasis caused by the parasite *Wuchereria bancrofti* is a major disease in India and in other tropical regions of the world. Individuals are infected by mosquitoes that transmit infective third stage larvae (L_3). L_3 mature into the adult worms which live in the lymphatics in humans. The viviparous filarial nematodes produce a large number of microfilariae (MF), which are again taken up by the arthropod vectors and develop into infective L_3. The human immune system is thus challenged with three stages of filarial parasites – infective larvae, adult and MF. The disease exhibits diverse clinical symptoms in humans living in endemic regions. The three major distinct groups in endemic population are chronic filariasis patients with overt symptoms of lymphatic dysfunction, viz. elephantiasis and/or hydrocele, asymptomatic microfilaraemic carriers without clinical symptoms and the so-called endemic normal individuals who are apparently free from clinical symptoms and MF. It is hypothesized that such diverse symptoms in humans may be caused at least partly due to the types of immune responses the hosts elicit to parasite antigens. Extensive studies on anti-filarial immune response using protein antigens have been carried out in trying to understand the complex relationship between the immune response and progression or manifestation of the diseases. Lipids, an important group of antigens besides proteins, have not received enough attention in filarial immunological investigations. However, lipids have been incriminated as important antigens in a variety of infectious diseases. Immunological reactivity to glycolipid antigens has been widely reported in diseases such as cysticercosis[1], leishmaniasis[2] and schistosomiasis[3,4]. Recently, we have attempted to evaluate the antigenicity of lipids isolated from the cattle filarial parasite *Setaria digitata* as a replacement for the human parasite *W. bancrofti* in endemic individuals of Orissa, India. The presence of elevated antibodies to lipids from both MF and adult parasites in amicrofilaraemic individuals is reported[5]. Asymptomatic microfilaraemic carriers exhibited depressed antibody (IgG and IgM) levels compared to chronic filarial patients and endemic normals. The present paper describes IgG subclass profiles, the recognition of carbohydrate epitopes and the effect of diethylcarbamazine (DEC) therapy on anti-lipid antibody response in microfilaraemic subjects. Antibodies were also measured in *Brugia malayi*-infected *Mastomys coucha* with and without microfilaraemia.

The study was carried out in villages of Khurda district, Orissa, a region where *W. bancrofti* infection is endemic. Sera were collected from individuals who are life-long residents of this region as described previously[6,7]. Each participant was examined for symptoms of lymphatic filariasis and microfilaraemia was detected by microscopic examination of Giemsa-stained 50 µl blood smears obtained by finger prick between 20.30 and 23.30 h. The sera were then classified according to the symptoms and microfilaraemia of each donor: Chronic filarial patients (CP), with elephantiasis and/or hydrocele; asymptomatic carriers (AS), microfilaraemics but with no clinical symptoms; and endemic normals (EN), permanent residents of the study area but with no apparent symptoms or microfilaraemia. The absence of microfilaraemia was confirmed three times over a period of two weeks. Sera from normal people belonging to non-filarial regions (such as Koraput and Karanjia in Orissa) were used as non-endemic normal (NEN) controls for serological comparison. Sera from patients infected with *M. leprea* (lepromatous leprosy, LL) were kind gifts from Dr U. Sengupta, JALMA (ICMR), Agra. Pooled sera were made whenever

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needed by adding equal volumes of sera from subjects belonging to different categories of lymphatic filariasis.

The adult *Setaria* worms (both sexes) were obtained from a local slaughterhouse. MF were released from the uterine canal of gravid female worms and subsequently purified by gel-filtration (Sephadex G-10)[8]. The lipid fraction of MF was prepared following a previous procedure[5]. Briefly, MF were homogenized and sonicated in PBS and then centrifuged. The aqueous-insoluble pellet[9] was extracted in organic solvent (chloroform/methanol 2 : 1). The lipidic fraction contained 20 µg of neutral carbohydrates per mg of lipids[10] as determined by the method of Dubois *et al.*[11] and proteins were not detected by Folin–phenol assay.

Antibody levels to MF lipids were assessed by ELISA following the published procedure[12] and as described in our earlier study[5]. The plates were coated with 100 µl of antigen in 70% ethanol (15 µg/ml lipids), kept at 37°C overnight to dry, blocked with 2% BSA in washing buffer (2 M NaCl, 0.04 M $MgSO_4$). Each serum diluted (1 : 100) in washing buffer containing 10% foetal calf serum (FCS) was tested in triplicate, the plates were incubated at 37°C for 3 h, washed (in washing buffer, 3 ×), and the plates were incubated with HRP-conjugated rabbit anti-human IgG or IgM antibodies (1 : 1000, washing buffer + 10% FCS). Colour was developed with O-phenylene-diamine plus H_2O_2 and absorbance measured by an automatic ELISA reader (Bio-Rad, Richmond, USA). For IgG subclass determination, the method described earlier was performed with the following modification: after incubation with 1 : 100 diluted sera, the plates were washed and mouse monoclonal anti-human immunoglobulin IgG1 (clone HP-6001, 1 : 2000), IgG_2 (clone HP-6014, 1 : 5000), IgG_3 (clone HP-6050, 1 : 5000) and IgG_4 (clone HP-6025, 1 : 5000) (Sigma Chemical Co, USA) diluted in washing buffer added and incubated for overnight at 4°C. The plates were then washed and anti-mouse Igs conjugated with peroxidase (1 : 4000 fold in washing buffer) was added and incubated at 37°C for 2 h. The bound enzyme was then quantitated as described earlier. Statistical significance (P values) was calculated by Student's t test.

To assess the role of carbohydrate epitopes, antigens were coated onto the plates as usual. Sodium-meta periodate (0.01 M in 0.01 M Na-acetate buffer, pH 4.5) was added to the wells (100 µl/well) incubated for 20 h in dark at 4°C, washed, and then ELISA was followed. Individual serum of filarial groups was checked in triplicate with and without treatment.

Asymptomatic microfilaraemic individuals ($n = 7$) who wished to participate were subsequently enrolled in a treatment programme with DEC at the WHO recommended dosage of 6 mg/kg body wt for 12 days. Three endemic normals also participated in the programme. Subjects were visited daily during the treatment to assure their compliance with the regimen. Blood samples were collected pre- and post-treatment each month up to one year.

The infection of *M. coucha* is initiated by injection of the infective larvae (L_3) of *B. malayi* harvested from infected *Aedes aegypti* (Liverpool strain) mosquitoes. Infection is monitored by means of microfilarial counts in the peripheral blood. L_3 develop into adult stage parasites and the infected animals become microfilaraemic after 90–100 days and the MF in circulation last for several months[13]. Sera were collected after six months of L_3 infection and checked in ELISA for anti-lipid antibodies using peroxidase conjugated goat anti-mouse Igs. Sera from uninfected mastomys were used as controls in the ELISA. These sera contained negligible anti-lipid antibodies.

A representating pattern of IgG levels to MF lipid in human filarial sera is shown in Figure 1. The mean IgG response in the AS group ($n = 35$) was significantly ($P < 0.01$) reduced in comparison to that of either EN or CP groups. IgG response in EN was also found to be higher ($P < 0.01$) compared to CP. There was a significant difference in antibody levels between the microfilaraemic and amicrofilaraemic groups. Anti-lipid antibodies were significantly low in sera collected from non-filarial endemic areas of Orissa such as Koraput and Karanjia in contrast to sera from filarial endemic areas. Sera from leprosy patients (LL) were also tested and found to be antibody negative. A simillar result was also noticed for IgM antibodies to MF lipid (data not shown).

IgG subclass response of anti-lipid antibodies was evaluated in filarial sera (Figure 2). IgG_2 was found to be the predominant subclass in asymptomatic microfilaraemic sera. EN sera had both IgG_1 and IgG_2 as the predominant subclass while during chronic phase of infection IgG_3 and IgG_1 were found to be increased substantially.

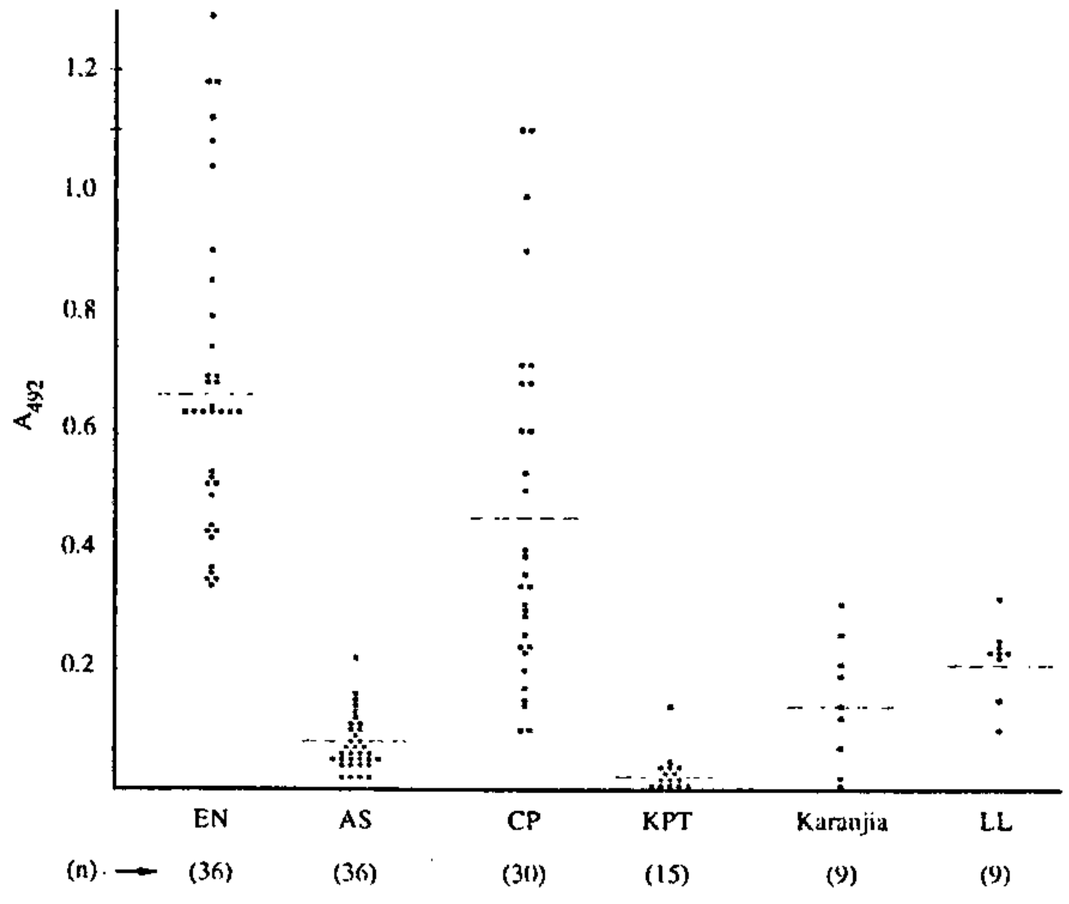

Figure 1. IgG levels (A_{492}) to microfilariae lipid of *Setaria digitata* in filarial (*W. bancrofti* exposed) and non-filarial sera (1 : 100-fold dilution). Dotted lines represent the mean value of each group. n refers to the number of subjects in each group. EN, endemic normals; AS, asymptomatic microfilaraemics; CP, chronic filarial patients; KPT, Koraput; LL, lepromatous leprosy.

Of specific interest IgG_4 was low in AS as well as in other groups.

The effect of periodate treatment of lipid antigens on the antibody reactivities in different filarial sera was evaluated (Table 1). Prior to periodate treatment antibody levels in asymptomatic microfilaraemic individuals were reduced compared to those of either EN or CP. The periodate oxidation of lipid resulted in decreased IgG binding in all filarial sera. However, there was no significant changes in IgM binding in CP, and EN sera in contrast to IgM in AS sera which exhibited reduction following periodate oxidation.

To determine anti-lipid antibodies in animals with and without MF, mastomys sera from both microfilaraemic (MF^+) and amicrofilaraemic (MF^-) states ($n = 7$ in each group) were collected after 6 months of L_3 infection and tested against the lipid antigens (Figure 3). Microfilaria negative mastomys had significantly ($P < 0.01$) increased levels of anti-lipid antibodies compared to microfilaraemic animals.

The kinetics of anti-lipid antibody responses after DEC treatment in seven asymptomatic microfilaraemic individuals was measured (Figure 4). The subjects before treatment exhibited very low levels of antibody. The antibody levels increased three to five folds after 1 month of treatment and sustained up to 6 months. The level decreased around one year post-treatment, however four individuals out of seven continued to exhibit high antibody response. All the subjects became amicrofilaraemic around one month and remained such till one year post treatment. Three EN individuals after taking DEC did not exhibit any increase in anti-lipid antibody response.

Antibodies against parasite glycolipids have been detected in patients with hydatid disease[14], leishmaniasis and trypanosomiasis[15]. Earlier antibody levels of glycolipids from *Litmosoides carinii* MF were measured in experimental filarial infection *M. coucha*-infected with *B. malayi*[16]. The present paper supports our previous observation of reduced IgG and IgM levels to lipid fraction of *S. digitata* in asymptomatic microfilaraemic individuals living in a *W. bancrofti* endemic region of Orissa[6]. In contrast, high levels of antibodies were detected in amicrofilaraemic CP and EN groups. Antibody levels were reduced in people living in non-filarial region (Koraput and Karanjia) of Orissa and also in leprosy patients suggesting the filarial specificity of the antibody response. It is of interest to note that mastomys with circulating microfilaria elicited considerably reduced antibody response. On the contrary, mastomys without MF exhibited high antibody level to lipid antigen. These data of both human and mastomys sera appear to reflect some degree of association between high antibody response to lipids and the amicrofilaraemic state. Elevated antibody response to filarial lipid antigens will probably identify persons who are MF negative in the endemic region. Further, chemotherapy (DEC-treatment) of asymptomatic microfilaraemic patients leading to amicrofilariaemia resulted in enhanced antibody production to lipid antigens. Clearance of MF may have led to the release of antigens and stimulation of higher antibody response to lipids in the treated subjects who became amicrofilaraemic. We have earlier demonstrated elevated antibody response to a protein antigen in a larger number of microfilaraemic subjects treated with DEC[9]. The protein antigen is recently demonstrated to be on the surface of filarial parasites[7]. The enhanced anti-lipid antibodies suggest MF negativity. Increased levels of these antibodies could be the potential correlates

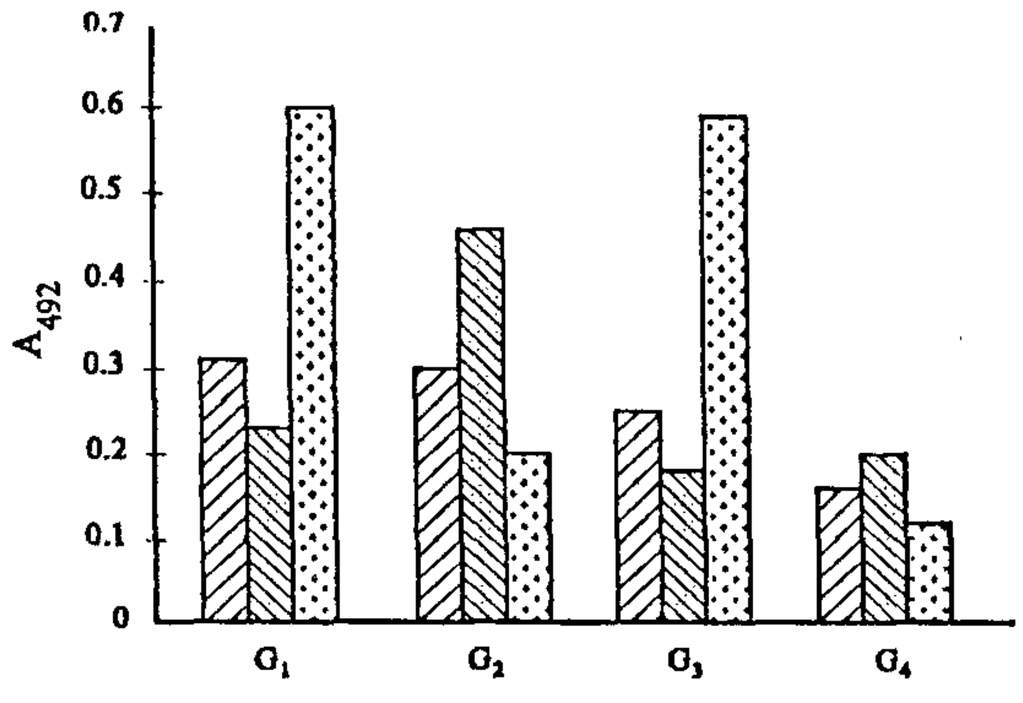

Figure 2. IgG subclass antibodies to MF lipid antigens in filariasis. A serum pool was made from 20 individuals in each filarial group. Endemic normals (▨), asymptomatic microfilaraemics (▧), chronic filarial patients (⁘).

Table 1. Effect of periodate treatment of microfilarial lipid antigens on serum antibody binding ($n = 30$ in each group). Each serum was checked in triplicate and the data shown below are the mean OD 492 values ± S.D.

	IgG		IgM	
Group	Pre treatment	Post treatment	Pre treatment	Post treatment
Endemic normals	0.65 ± 0.12	0.40 ± 0.10*	0.95 ± 0.60	0.69 ± 0.42
Asymptomatic microfilaraemic carriers	0.15 ± 0.06	0.06 ± 0.03*	0.65 ± 0.31	0.30 ± 0.12*
Chronic filarial patients	0.45 ± 0.22	0.25 ± 0.12*	0.83 ± 0.36	0.74 ± 0.40

*$P < 0.01$ compared to pretreated values.

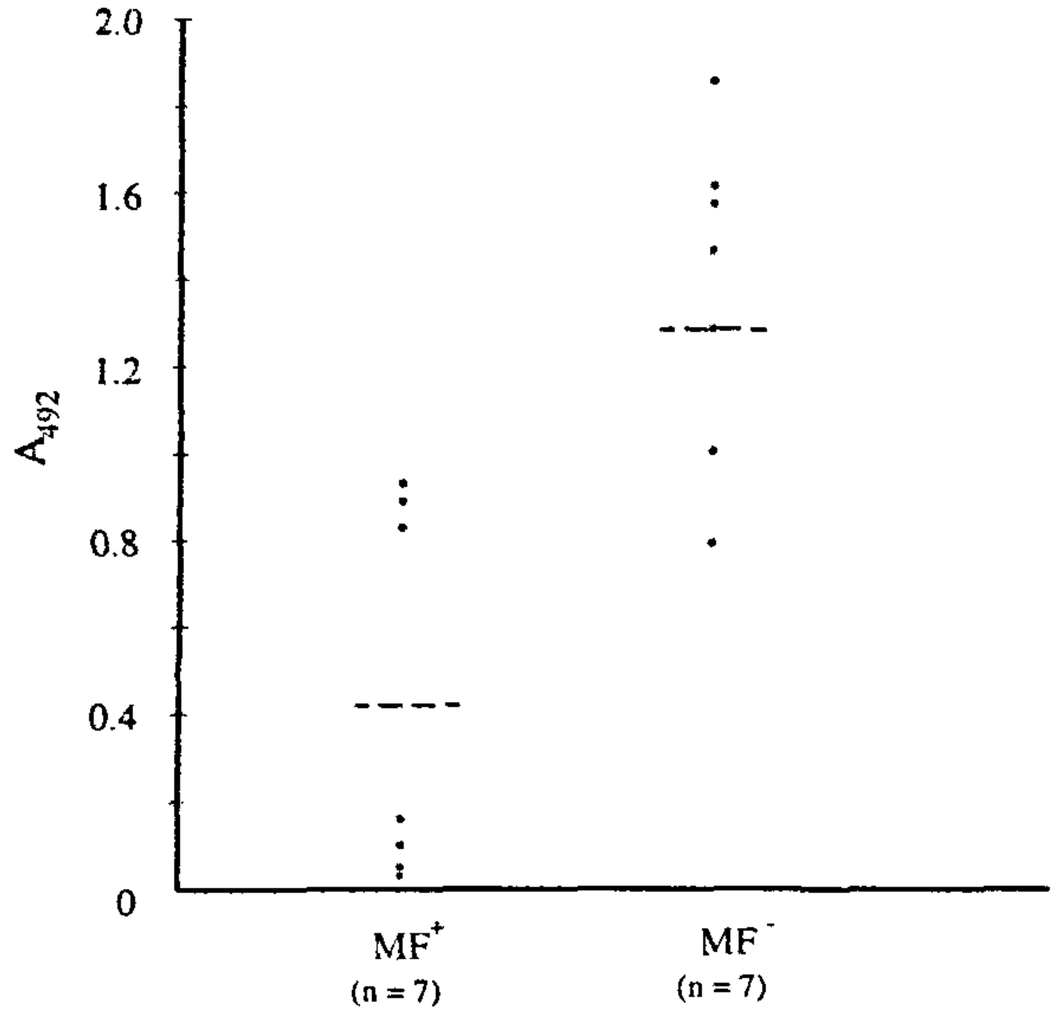

Figure 3. Antibody levels to lipid antigens in *B. malayi*-infected mastomys with microfilariae (MF^+) and without (MF^-) microfilaraemia ($n = 7$ in each group). Sera were collected six months after infection. Each dot represents antibody levels (A_{492}) of each animal and horizontal lines represent mean value of each group.

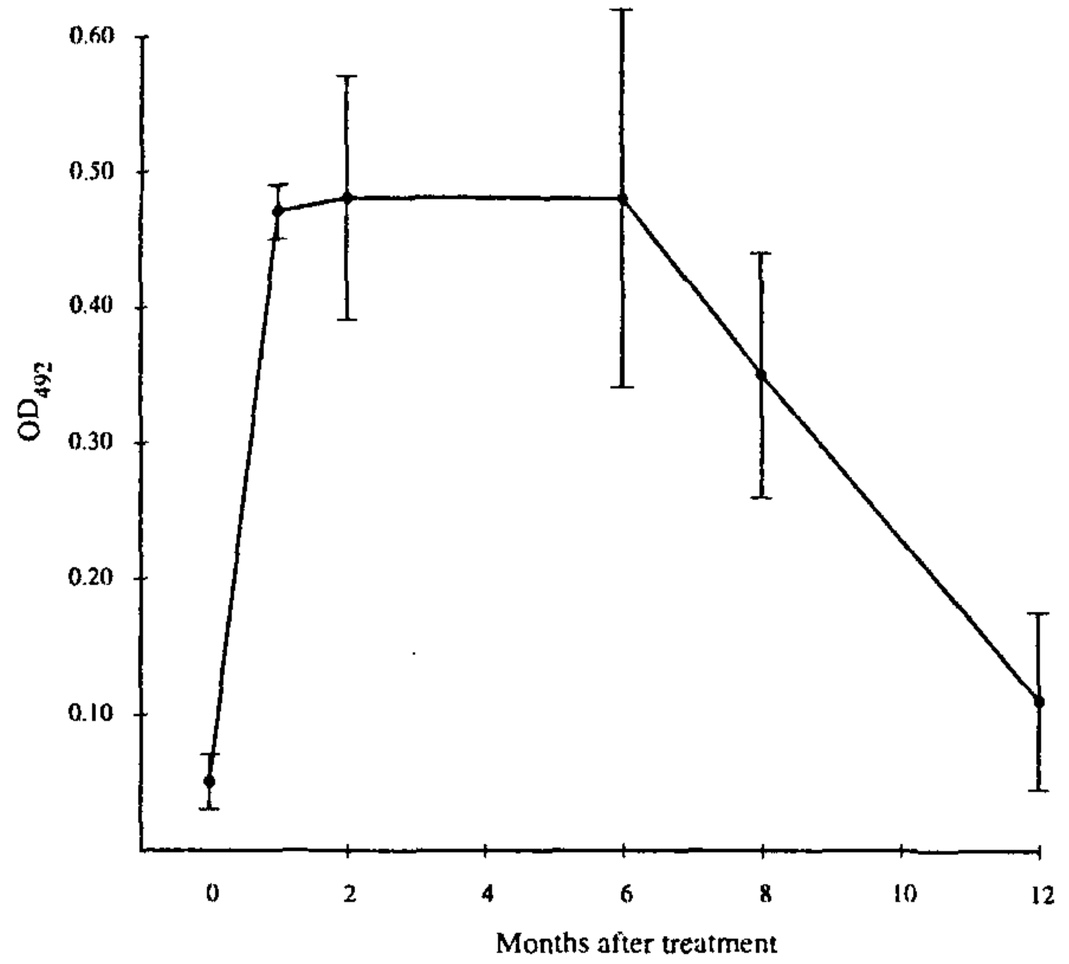

Figure 4. Effect of diethylcarbamazine treatment on antifilarial IgG levels to MF lipid antigens in seven asymptomatic microfilaraemic individuals. Each point represents IgG level (mean ± SD) of all seven subjects as monitored at different time intervals.

of the amicrofilaraemic state. Immunolongitudinal study using lipid antigens will be useful to assess the role of anti-lipid antibodies in controlling microfilaraemia in people living in the endemic regions.

The periodate oxidation data tend to indicate these antigens to be glycolipids. The binding of antibodies in asymptomatic microfilaraemic sera to MF lipids was sensitive to periodate oxidation, but in other groups it is dependent on antibody isotypes. IgM reactivity in microfilaraemic individuals but not in amicrofilaraemic EN and CP groups was affected by periodate oxidation of MF lipids. IgG reactivities, however, in all groups were decreased. The extent of antibody recognition to carbohydrate epitopes appears to be dissimilar in different clinical groups of filariasis. It is of interest to speculate that the antibody recognition of epitopes is different during the progression of lymphatic filariasis, and might modulate the status of the disease (microfilaraemics/clinical symptoms).

The expression of IgG subclass to parasite lipids differed in filarial sera. IgG_2 was predominant in AS, while CP showed high IgG_1 and IgG_3, the two cytophilic antibodies. EN normal sera exhibited IgG_1 and IgG_2 responses. Interestingly the IgG_4 subclass, otherwise known to be selectively elevated in asymptomatic microfilaraemics[17,18], to the parasite lipids was decreased in all filarial sera including asymptomatic microfilaraemics. The dominance of IgG_2 in asymptomatic microfilaraemics suggests the involvement of carbohydrate epitopes as these antigens generally elicit IgG_2 response in humans[19,20]. The immunodominance of carbohydrate epitopes is also known to induce a suppressed IgG_4 response[21]. The selective recognition of carbohydrate epitopes of lipid antigens in asymptomatic microfilaraemics sera as corroborated from periodate oxidation studies is in agreement with subclass recognition.

In summary, we have suggested the importance of carbohydrate determinant in lipid antigens of filarial parasites for antibody binding in filarial serum. Reduced levels of these antibodies in mastomys with microfilariae in contrast to amicrofilaraemic animals support the results found in humans living in endemic regions. Reduced antibody levels in microfilaraemic individuals was enhanced by DEC treatment.

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Isolation of endophytic fungi from leaves of neem (*Azadirachta indica* A. Juss.)

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Occurrence of asymptomatic fungal endophytes in green and senescent leaves of neem is reported. Only five endophytes were isolated from the leaves. Of these, four were sterile forms and one was *Fusarium avenaceum*. The frequency of occurrence of endophytes was significantly higher in the basal leaflets than in the apical or middle leaflets and in the main vein of the leaflet than in the lamina tissue. The frequency of colonization of green leaves by endophytes increased during the rainy season although no new endophyte species could be recovered. The restricted number of endophytic fungal genera and the absence of common endophytic fungi in the neem leaves could be due to the antifungal metabolites present in the leaves. The results suggest that occurrence of foliar endophytes in tropical trees is influenced by environment, and type and chemistry of the host tissue.

ENDOPHYTIC fungi colonize living plant tissues without producing any apparent disease symptoms or obvious negative effects[1]. The endophytes are usually ascomycete and mitosporic fungi. Endophytes are being intensively studied since some of them may be sources of novel biochemicals of pharmaceutical importance[2,3]. The investigation of this relatively unexplored group of fungi may also reveal new forms[4]. Most work on fungal endophytes however, has been centred on plants from temperate regions and relatively few tropical plants have been studied for the presence of endophytes[5]. The tropical plants that have been studied for the presence of endophyte include members of Araceae, Bromeliaceae and Orchidaceae from French Guyana[6], Piperaceae and Crassulaceae from Brazil[7], Musaceae[8] and Poaceae[9] from Hong Kong and various palms[10–12]. In India, only two species of mangrove trees[13], some trees of tropical montane evergreen forest and dry deciduous forest[14], halophytes[15] and an angiosperm parasite[16] have been screened for the presence of endophytes. We report here the presence of endophytes in the leaves of neem, a tropical tree native to India.

The neem belongs to the family Meliaceae and is of considerable economic importance. The beneficial uses of the products of neem tree have been known to the people of India for centuries, but more recently their economic properties have been recognized by the developed countries[17]. Neem products are broad-spectrum pesticides and act on several insect species including phytophagous insects[18]. The medicinal importance of different parts of neem are well known to the practitioners of Indian Ayurveda and Unani systems of medicine[18].

Mature green leaves and senescent (yellow) leaves of neem were collected from five medium-sized trees growing in Chennai, South India, which is at sea level and experiences a dissymmetric tropical climate[19]. To determine the influence of seasons on the occurrence of endophytes, green leaves were collected on a monthly basis for twenty-four months (May 1996 to April 1998) and screened for the presence of endophytes.

Each leaf was thoroughly washed in running water before sampling. Lamina segments (approximately 0.5 cm^2) cut from the middle portion of the leaflets of green and senescent leaves were dipped in 70% ethanol for 5 sec immersed in 4% NaOCl for 1 min and rinsed in sterile water for 10 sec (ref. 20) and plated on potato dextrose agar medium amended with streptopenicillin (150 mg l^{-1}). To determine the distribution of endophytes in a single leaflet, the middle portion with the main vein, the leaf blade devoid of the main vein and the rachis were sampled.

The sterilized segments of the leaf were placed on 15 ml of the medium contained in a petri dish (9 cm diameter) and incubated in a light chamber for 21 days at 26°C (refs 21, 22). The source of light was three 4-feet Philips daylight fluorescent lamps. The leaf segments received approximately 2200 lux of light through the petri dish lid as measured by a Lutron LX-101 (Germany) lux meter. At each sampling period, twenty compound leaves were collected from each tree and three hundred segments were screened for the presence of endophytes.

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