IMMUNO-EPIDEMIOLOGY OF BANCROFTIAN FILARIASIS : A 14-YEAR FOLLOW-UP STUDY IN ODISHA, INDIA

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Abstract. Forty asymptomatic, circulating filarial antigen negative (CFA^{-ve}) and ten asymptomatic, circulating filarial antigen positive (CFA^{+ve}) individuals were followed up longitudinally over a period of 14 years at intervals of 7 years in order to investigate the immunological, parasitological and clinical changes that took place in an endemic area due to natural process. The clinical status, microfilaremia, circulating filarial antigenemia and immunological responses to filarial antigens ($DSSd_1$ and Sd30) prepared from cattle filarial parasite *Setaria digitata*, were examined. The observations showed that 19 individuals had developed either antigenemia or filarial symptoms (acute filarial lymphangitis/hydrocele) from CFA^{-ve} group. Three individuals had cleared antigenemia and one had developed microfilaremia from CFA^{+ve} group after 7 years. Increased IgG and IgM and low IgG2 and IgG4 level responses along with high lymphocyte production were observed in CFA-negative individuals. This was in contrast to observations made in CFA^{+ve} subjects. The results of the present study indicated that the changes taking place in the immunological, clinical and CFA status of individuals residing in filaria endemic regions developed different clinical manifestation with course of time.

Keywords: bancroftian filariasis, circulating filarial antigen, endemic normal, India

INTRODUCTION

Bancroftian filariasis is a major public health problem affecting millions of people in tropical countries. A wide spectrum of clinical manifestations is observed in this disease, which is thought to be determined mostly by the host-parasite immune responses (Ottesen, 1992). Based on clinical presentation, presence of circulating microfilariae (Mf) and circulating filarial antigenemia (CFA, a metabolic product of lymphatic-dwelling adult worms), individuals living in bancroftian filarial endemic area can be classified into: 1) asymptomatic microfilaria carrier; 2) patients with history of one or more episodes of acute filariasis, such as adenolymphangitis (ADL); 3) patients with chronic disease such as hydrocele and elephantiasis; 4) asymptomatic and amicrofilaremic individuals with cryptic filarial infection (as demonstrated by the

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presence of CFA); and 5) endemic normal (EN) individuals without any symptom, microfilaremia or demonstrable CFA (Sahoo *et al*, 2002).

EN individuals are refractory from infection in an endemic area and are considered to be "putatively immune". The immunological correlation in this population has been investigated in order to understand the nature of protective immunity in human lymphatic filariasis (Ravindran *et al*, 2003). EN individuals carry high levels of IgG and IgM antibodies against the parasite antigens, which are believed to be protective (Freedmen *et al*, 1989). In order to study the natural history of the disease, several cross sectional studies have assessed the immunological reactivity in individuals from different filariasis endemic areas (Nutman *et al*, 1987; Ottesen, 1992; King *et al*, 1993; Nielsen *et al*, 2002), but very few longitudinal studies have been undertaken in this aspect in endemic populations because it is rare to have an opportunity to carry out the long term follow-up studies of persons living in an area endemic for filariasis, although it is most informative (Meyrowitsh *et al*, 1995).

In the present study a cohort of 50 individuals were followed up over a period of 14 years at regular intervals to investigate the immunological, parasitological and clinical changes taking place in an endemic area due to natural process.

MATERIALS AND METHODS

Study population

The study population included 50 individuals, aged 15 to 50 years (at initial survey period in 1994). All are residents of a *Wuchereria bancrofti* endemic area of Olosingh Village, Khurda District, Orissa, India (Bal and Das, 1996b; Mandal *et al*, 2000). At least 90% (n = 800) of the residents of two villages were thoroughly examined for clinically (including past and present history and filarial symptoms) and parasitologically (presence of circulating Mf by thick blood smear by taking 50 l of finger prick blood collected between 08:30 PM to 11:30 PM) along with their CFA status in 1994 (initial start period). The study population was selected among the examined individuals based on their CFA status and willingness to participate in the study. Informed consent was obtained from all participants.

The study population was classified into two groups: Group I (n = 40) with CFA^{-ve} and Group II (n = 10) with CFA^{+ve}. Enrolled individuals were re-examined clinically, parasitologically and for CFA status after 7 years (2001) and 14 years (2008), and were divided consequently into to various sub-groups based on their physical examination results. The mass drug administration (MDA) program has been instituted in this area since 2004, at least 10 years after the initiation of this study. The history of drug consumption by the study population has been recorded through questionnaires at the start and after every round of MDA.

Antigen preparation

Parasite antigens, $DSSd_1$ and Sd30 (Fr-III), were isolated from cattle filarial parasite *Setaria digitata*. $DSSd_1$, a water insoluble, detergent soluble, surface-associated glycoprotein was purified from filarial parasites as described by Bal *et al* (2003). This glycoprotein is 210 kDa based on SDS-PAGE and contains 45 g of carbohydrate per mg of protein. The allergenic 30 kDa (as determined by SDS-PAGE) Fr - III was purified from *S. digitata* as described by earlier (Beuria and Das,

1992). These two antigens were used to determine the filarial-specific antibody level.

Immunological assessments

Serum IgG, IgM, IgG2 and IgG4 responses to parasite antigens were determined by ELISA following the procedure maintained earlier (Beuria *et al*, 2000). $DSSd_1$ was used to assess IgG and IgM levels (Bal and Das, 1996a) and Fr-III for active filarial infection by measuring IgG4 and IgG2 levels. The OD value was converted to antibody unit (OD of test sample / OD of +ve control sample x100) and mean ± SD value is presented. Circulating filarial antigen (marker of active filarial infection) was detected using Og4C3 ELISA kit (finger prick compatible; Trop Bio, Townsville, Australia). Antigen units of 100 were used as a cut-off value for identification of antigenemia as per the manufacturer's instruction.

Cellular proliferation

Cell proliferation response to parasite antigen ($DSSd_1$) was measured in peripheral blood mononuclear cells (PBMC) isolated from 5 ml of heparinized blood (Steel *et al*, 1996). A total of 15 samples from various groups were collected during the 3rd survey (2008) because of non availability of enrolled individuals. In brief, 10^5 PBMC were cultured in the presence and/or absence of antigen for 5 days. After which [^{3}H] thymidine was added 1 Ci [^{3}H] / well, which incorporate in to the DNA of proliferating cells. The radioactive incorporation was measured (counts per min; cpm) after 18 hours of thymidine addition using a LS-6500 Beta Liquid Scintillation counter (Beckman Coulter, Basel, Switzerland). Proliferative activity is expressed as stimulation index (SI) by the formula: mean cpm of stimulated cell cultures/mean cpm of un-stimulated (control medium only) cell cultures.

Statistical analysis

Wilcoxon signed-rank test was used for comparison of paired data to determine significance of difference between survey points and within different subgroups. A p-value < 0.05 is considered statistically significant.

RESULTS

Disease status

During the initial survey (1994) all subjects in Group I ($n = 40$) were asymptomatic, amicrofilaremic and CFA^{-ve}, but during the 2nd survey (2001) 30% ($n = 12$) had developed filarial symptoms [8: acute filarial lymphangitis (AFL); 4: hydrocele] without microfilaremia (Fig 1). Seven (17%) individuals were CFA^{+ve} without any clinical symptoms and microfilaremia and 5/8 in AFL category and 3/4 in hydrocele category were CFA^{-ve}. These 19 individuals continued to manifest the same filarial status during the 3rd round of survey (2008). No changes were observed among the remaining 21 subjects as regards their filarial status throughout the whole study period.

On the other hand during 2nd round of survey in Group II (asymptomatic, amicrofilaremic but CFA^{+ve} during enrollment in 1994), 3 (30%) individuals were converted to CFA^{-ve}, 1 (10%) became microfilaremic and 6 (60%) showed no change in their filarial status. During the 3rd round of survey only the microfilaremic individual had developed filarial symptom (AFL) after clearing the microfilaria, while the remaining subjects continued with the same status as in 2001.

Antibody response

Serum IgG, IgM, IgG4 and IgG2 responses to filarial antigens were assessed in all the study population. Initially (1994), there is a significant difference in

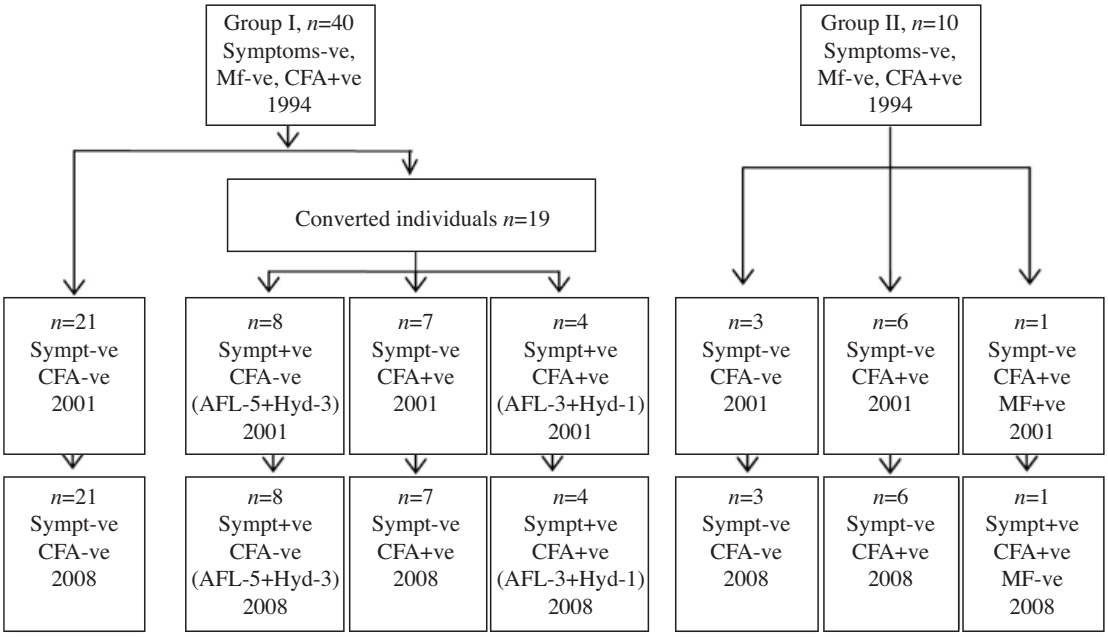

Fig 1–Details of study population. In Group I cohort, 19 individuals developed either clinical filarial symptoms (AFL/Hyd) or antigenemia or both and 21 remained unchanged after 7 years from start of the project. In Group II cohort, 4 individuals had converted their CFA status or developed microfilaremia, 6 remained unchanged after 7 years. No further changes were observed at the end of the 14 years survey except for one microfilaremic individual. Sympt, clinical filarial symptoms; CFA, circulating filarial antigen; Mf, microfilariae; AFL, acute filarial lymphangitis; Hyd, hydrocele.

the antibody levels between Group I and II subjects, with Group I in comparison with Group II having higher levels of IgG (49 ± 13 *vs* 26 ± 4) and IgM (48 ± 11 *vs* 31 ± 3), and decreased levels of IgG4 (25 ± 10 *vs* 58 ± 12) and IgG2 (27 ± 8 *vs* 56 ± 11). A significant change in antibody isotypes levels were observed among individuals who showed conversion in their CFA status, either negative to positive or vice-versa (Fig 2C and D) during the study period, but no changes were observed in antibody levels in those who maintained their initial CFA status (either CFA^{-ve} or CFA^{+ve}) throughout the study period (Fig 2A and B).

Development of filarial symptoms was not correlated with the antibody responses. Those individuals who developed filarial symptoms without changing their CFA status (either $Symp^{-ve}$ /CFA^{-ve} to $Symp^{+ve}$/CFA^{-ve}, or $Symp^{-ve}$ /CFA^{+ve} to $Symp^{+ve}$/CFA^{+ve}) did not show any changes in their antibody levels (Fig 2E and F). However, antibody levels changed when the individuals developed symptoms with changes in CFA status (Fig 2G), whereas antibody levels did not change in the Group II individual who developed microfilariae and symptoms during the study period (Fig 2F). Surprisingly, individuals who had cleared the circulating

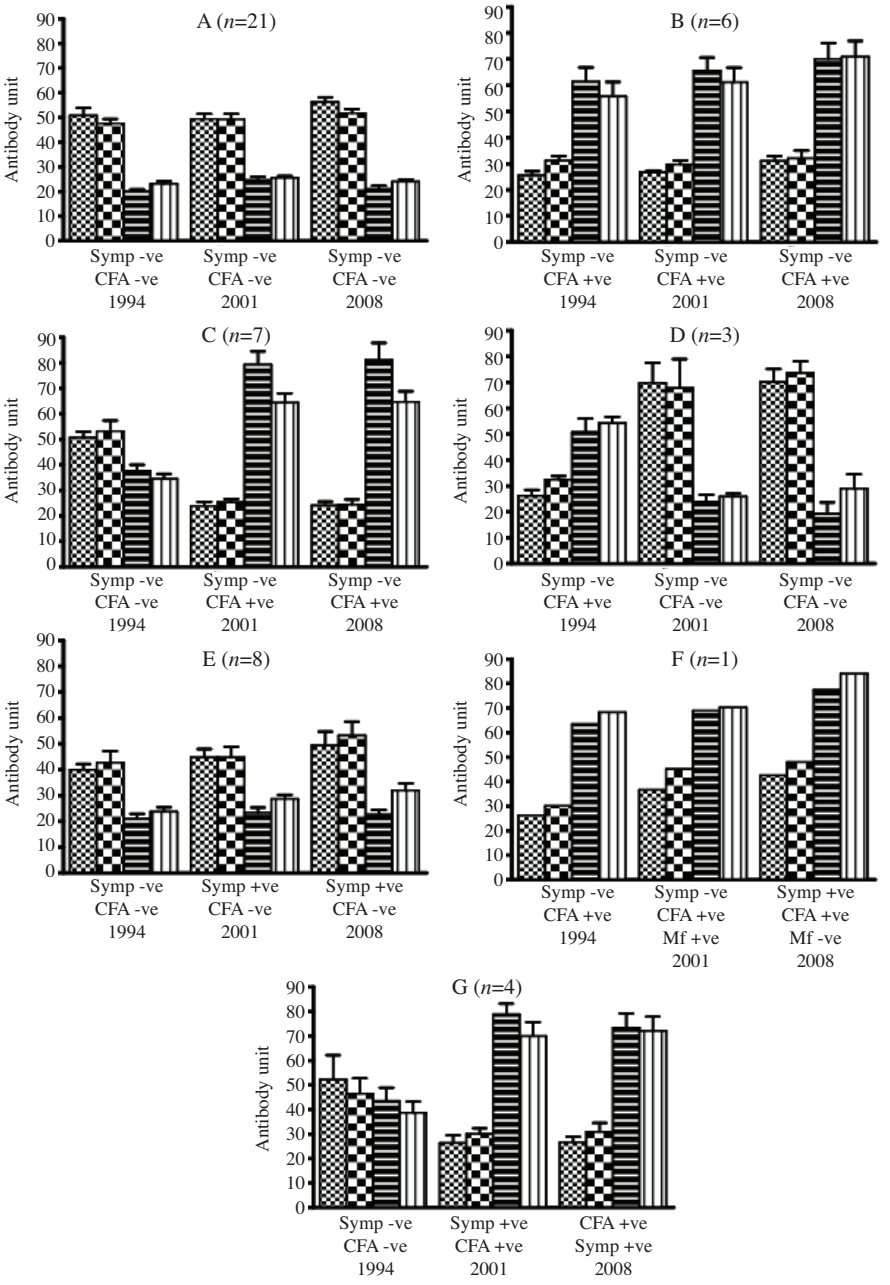

Fig 2–Antibody levels in different groups of study population at three survey points. Protocols for determination of antibody levels are described in Materials and Methods. IgG (▩), IgM (▩), IgG4 (▤) and IgG2 (▥). Fig A: CFA^{-ve} and symptom $^{-ve}$ individuals throughout the study period; B : CFA^{+ve} and $symptom^{-ve}$ individuals throughout the study period; C: $CFA^{-/+/+ ve}$ and symptom $^{-ve}$ group; D: $CFA^{+/-/-ve}$ and symptom $^{-ve}$ group; E: Symptom $^{-/+/+ ve}$ and CFA^{-ve} throughout the study period; F: $CFA^{+/+/+ ve}$, symptom $^{-/-/+ ve}$ and MF $^{-/+/- ve}$ individual; and G : $CFA^{-/+/+ ve}$ and symptom $^{-/+/+ ve}$ individuals. CFA, circulating filarial antigen; Mf, microfilariae; Sympt, clinical filarial symptoms.

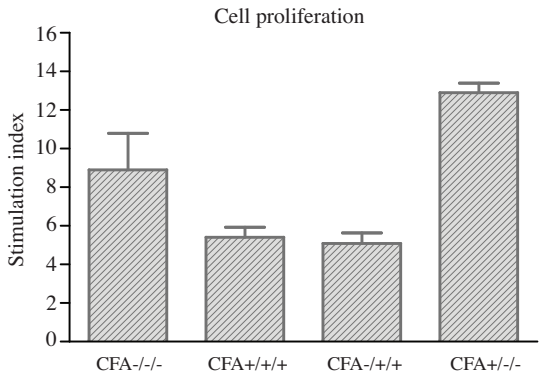

Fig 3–Lymphocyte proliferation response. Protocols for measuring lymphocyte proliferation, expressed as stimulation index, in response to filarial antigen $DSSd_1$ are described in Materials and Methods. Mean ± SD values are shown for asymptomatic $CFA^{-/-/-}$ ($n = 5$), $CFA^{+/+/+}$ ($n = 4$), $CFA^{-/+/+}$ ($n = 4$) and $CFA^{+/-/-}$ ($n = 2$) individuals.

filarial antigenemia without developing any symptoms exhibited compared with EN subjects statistically higher levels of IgG (70 ± 8 *vs* 56 ± 9) and IgM (73 ± 9 *vs* 52 ± 8) ($p < 0.05$) (Fig 2A and D).

Cellular response

Elevated levels of lymphocyte proliferation were observed in individuals with CFA^{-ve} throughout the study period ($CFA^{-/-/-}$) (SI = 8.9 ± 1.9) compared with those with consistent CFA^{+ve} ($CFA^{+/+/+}$) (SI = 5.4 ±0.5). Significantly higher levels of cellular proliferation were observed also in individuals who had converted from CFA^{+ve} to CFA^{-ve} (SI = 12.9 ± 0.5) than the un-converted $CFA^{+/+/+}$ individuals (SI = 5.4 ±0.5, Fig 3). No significant differences in SI values were found in those who acquired antigenimia ($CFA^{-/+/+}$) (conversion from initial CFA^{-ve} to CFA^{+ve} in subsequent two surveys) compared to ($CFA^{-/-/-}$) individuals (SI = 8.9 ± 1.9). $CFA^{+/+/+}$ individuals and those converted from initial CFA^{-ve} to CFA^{+ve} in subsequent two surveys ($CFA^{-/+/+}$; SI = 5.09 ± 0 .54) had similar proliferation activity.

DISCUSSION

The main objective of this study was to evaluate the parasitological, clinical and immunological changes that occur among individuals living in an area endemic for bancroftian filariasis. To the best of our knowledge this is the first study of this kind. We observed that almost 60% of EN populations with CFA^{-ve} had developed circulating filarial antigenemia (27%) and filarial symptoms (30%) after 7 years. The development of circulating filarial antigenemia was associated with decreased levels of IgG and IgM and increased levels of IgG4 and IgG2 antibody responses to specific filarial antigens (indicating acquisition of infection/antigenemia. Earlier studies have reported that increased levels of IgG4 and IgG2 isotypes are indicators for active filarial infection (Kwan-Lim *et al*, 1990; Bal *et al*, 2003; Frances *et al*, 2008). On the other hand, no changes to antibody responses were observed among subjects who had developed symptoms without detectable antigenemia. This is because immunological responses are inversely associated with filarial antigenemia and not with the development of filarial symptoms (Bal *et al*, 2003).

The persistence of antigenemia among 60% of CFA^{+ve} individuals throughout the study period was associated with increased levels of IgG4 and IgG2 and decreased levels of IgG and IgM antibody responses. Low levels of lymphocyte proliferation were also observed among this group of individuals. However, 30% of individuals who had cleared their antigenemia showed increased levels of IgG and IgM as well as lymphocyte pro-

liferation throughout the study period. Similar findings have also been reported in experimental animals, where clearance of microfilaremia is accompanied by increased production of specific antifilarial IgG and IgM antibodies (Weil *et al*, 1982; Fletcher *et al*, 1986; Mandal *et al*, 2009).

The overall findings of this study indicates that increased production of IgG and IgM antibodies and lymphocytes to specific filarial antigens might play a major role in clearance of antigenemia among cryptic individuals (CFA^{+ve}, asymptomatic and amicrofilaremic) residing in endemic area. These findings may predict that once the CFA^{+ve} individuals clear their antigenemia but with high levels of IgG and IgM and elevated levels of lymphocyte production will never be re-infected or develop filarial symptoms even after being continuously exposed to infection. This hypothesis needs to be validated by undertaking long term studies in larger samples in other filarial endemic areas.

Interestingly one cryptic individual, who had developed microfilaremia but without any symptoms after 7 years, was found to be amicrofilaremic with clinical symptoms and antigenemia after 14 years. As the area under study is covered by MDA under National Filaria Control Programme (NFCP), the disappearance of Mf might be due to the effect of the annual single dose of diethyalcarbamazine (DEC) used in the program. The elevated IgG4 and IgG2 levels are directly correlated with parasitemia not clinical manifestation (Mohanty *et al*, 2007), in agreement with our findings. This finding indicates that microfilaria plays an important role in the development of filarial symptoms in cryptic individuals. This hypothesis also needs validation in larger samples in other filarial endemic areas.

The low level of IgG4 and IgG2 along with increased level of IgG and IgM and elevated lymphocyte production to the filarial antigen ($DSSd_1$) were observed in 52% of putatively immune individuals ($Symp^{-ve}$ and CFA^{-ve}) during the entire study period. At the same time the long lasting elevated parasite antigen-specific T cell and B cell responses have been observed in individuals who have never been infected (Sym^{-ve}, CFA^{-ve}) even though continuously exposed to filarial infection. The level of antibody responses and lymphocyte proliferations in $CFA^{+/-ve}$ converted individuals are significantly higher compared to putatively immune individuals ($CFA^{-/-/-ve}$), which might be due to the development of protection in the converted individuals ($CFA^{+/-/-ve}$).

In summary, our study has revealed that persistence of elevated levels of cellular as well as humoral responses to filarial-specific antigen ($DSSd_1$) for long periods of time leads to infection- and symptoms-free conditions among the endemic population. The findings also establish the consequence of a change in status either parasitological or of CFA leads to development of symptoms in the endemic area. The present study also established the correlation between up-regulation of hypo-responsiveness in cryptic individuals and clearance of antigenemia. However, how much up-regulation of humoral and cellular responses is required to clear infection in cryptic individual is not clear and needs in-depth longitudinal studies in each group of filarial patients in a cohort fashion.

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