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Immune responses to recombinant *Brugia malayi* pepsin inhibitor homolog (Bm-33) in patients with human lymphatic filariae

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Abstract

Immune responses to recombinant *Brugia malayi* pepsin inhibitor homolog (rBm-33) were investigated in patients with human lymphatic filariasis (microfilaremics (MF) and chronic pathology (CP)) along with endemic normals (EN). Flow cytometric analysis (24 h) revealed CD4⁺ T cell activation in patients (MF and CP) compared to normals (EN), with increased expression of CD69 and diminished levels of CD62L and CD127. This was associated with an elevated expression of CD154 but not CD28 and CTLA4 in CP patients. However, Bm-33-induced cytokine expression profile (IL-1 β , IL-12, IL-8, IFN- γ , IL-10 and TGF- β) did not exhibit any significant difference between normals and patients at the same time point. Although CD4⁺ T cell activation was observed initially in filarial patients (24 h), lymphoproliferation studies (96 h) suggested diminished proliferation compared to normals, indicating functional inactivation in the former upon prolonged antigen exposure. This indicates that rBm-33 induces an early T cell activation in MF and CP patients followed by a decreased lymphoproliferation that might contribute to immune suppression in these individuals.

Introduction

Human lymphatic filariasis, caused by lymph-dwelling nematode parasites, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, is a chronic endemic disease of tropical and subtropical countries of the world. A major contributing factor for this chronicity is the

parasite's ability to survive in the hostile immune niche of the host. This is made feasible by the remarkable ability of the parasite to modulate host immune responses. As parasite proteins are important cogs in this phenomenon, the characterisation of parasite recombinant proteins is an important study to understand these complex immune modulation mechanisms.

Previous studies from our laboratory have investigated the role of recombinant WbSXP-1 in inducing the modulation of T cell responses in microfilaremic patients (Sasisekhar et al. 2005). However, considering the complexity of parasite life cycle and the myriad proteins involved in regulating host immune regulatory networks, there is a need to identify and functionally characterise new parasite recombinant proteins. *B. malayi* pepsin inhibitor homolog (Bm-33) identified previously (Dissanayake et al. 1993) is an aspartyl protease inhibitor (Aspin) believed to play an important role in immune evasion (Maizels et al. 2001). One important nematode Aspin that has been studied extensively is PI-3 (pepsin inhibitor) from *Ascaris suum* that inhibited pepsin, indicating its role in protecting the parasite from the digestive enzymes of the host gastrointestinal tract. In addition, PI-3 was also shown to inhibit cathepsin E and antigen processing by T cells, suggesting an immunomodulatory function (Kageyama 1998). In this regard, rBm-33 was characterised previously in our laboratory by serology and immunolocalisation that demonstrated Bm-33 to be an immunodominant, hypodermal antigen, inducing elevated IgG4 isotype reactivity in microfilaremic patients (Krushna et al. 2009).

In order to continue this further, recombinant Bm-33-induced cellular immune responses were investigated in filarial patients (microfilaremic (MF) and chronic pathology (CP)) and normals (endemic normals, EN) to evaluate its role in immune modulation. The expression of activation markers, CD69, CD62L and CD127, and co-stimulatory molecules, CD154, CD28 and CTLA-4, was assessed by whole blood flow cytometry on CD4⁺ T cells. Subsequently, real-time PCR analysis was carried out with peripheral blood mononuclear cells (PBMC) of the same patients to monitor the cytokine expression profile. The expression of pro-inflammatory cytokines like IL-1 β , IL-8, IL-12 and IFN- γ was assessed along with suppressor cytokines like IL-10 and TGF- β . Finally, lymphoproliferation studies were done using thymidine uptake assay to evaluate T cell proliferation. The results suggest T cell activation at the end of 24 h in MF and CP patients compared to EN, as shown by the increased expression of CD69 and CD154 as well as the decreased expression of CD62L and CD127. However, this early T cell activation was not sustained since Bm-33 stimulation resulted in a suppressed lymphoproliferation at later time points in filarial patients.

Materials and methods

Materials

RPMI 1640, lymphocyte separation medium (Pancoll) and foetal calf serum were obtained from PAN BIOTECH, GmbH. HEPES was obtained from USB, Amersham Life Sciences (Cleveland, OH, USA). NaHCO₃ and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Gentamicin was obtained from Ranbaxy Pharmaceuticals (New Delhi, India).

Study population

We studied a total of 25 individuals (asymptomatic amicrofilaremic individuals (EN, $n=10$), symptomatic individuals with chronic lymphedema and/or lymphatic obstruction and no microfilaria in their blood (CP, $n=10$) and asymptomatic individuals with microfilaraemia (MF, $n=5$) from Chennai, India, an area endemic for *Wuchereria bancrofti* infections. Standardised histories were obtained and physical examinations were done on all the

participant residents during epidemiological surveys. Parasitological examination of all individuals was done by the detection of microfilariae in blood smears taken from endemic individuals after 10 P.M. Patients were recruited through the National Filariasis Control Units under the Department of Public Health and Preventive Medicine (Chennai, India) after informed consent was obtained with protocols approved by the institutional review board of Anna University (Table 1). All the individuals were screened for the presence of circulating filarial antigens by Og4C3 mAb ELISA, a marker of *W. bancrofti* infection and adult worm burden (Chanteau et al. 1994; TropBio, Townsville, Queensland, Australia).

A probable limitation in this regard is the low number of MF patients used in the present study. This may be attributed to the success of mass drug administration programme adopted by the Government of Tamil Nadu under the Global Filariasis Elimination Programme of WHO, which has effectively controlled the filariasis and hence MF cases by administering DEC in these endemic areas. The obtained positive cases were recruited in the study after screening nearly 300 volunteers. These MF-positive asymptomatic individuals had not received any treatment for their filarial infection prior to blood collection.

Antigens and mitogens

Recombinant *B. malayi* pepsin inhibitor homolog (rBm-33) was expressed and purified as described previously (Krushna et al. 2009). Briefly, Bm-33 cDNA (645 bp) was cloned in pRSET-A and the recombinant plasmid was transformed into BL21(DE3) for large-scale expression of the recombinant protein. rBm-33 (33 kDa) protein expression was induced using 1 mM IPTG, and the protein was purified by immobilised metal affinity chromatography. Assessment of endotoxin contamination was done using a *Limulus* amoebocyte lysate assay, which showed <1 pg of LPS/10 µg of protein. Apart from recombinant filarial antigen, phytohemagglutinin (PHA; Sigma Chemical Co.) was used as a positive control for whole blood and PBMC stimulation at a concentration of 10 µg/ml.

Antigen-driven and polyclonally stimulated proliferation of human PBMC

Peripheral mononuclear cells from normal healthy volunteers (EN) and infected patients (MF, CP) were isolated from heparinized venous blood by density gradient centrifugation with Ficoll-Hypaque (Pancoll, PAN BIOTECH, GmbH). Cells were washed in RPMI 1640 medium (PAN BIOTECH, GmbH, HEPES (25 mM) and 80 mg gentamicin per litre of RPMI 1640) for 10 min at 1,200 rpm and resuspended in medium supplemented with 10% foetal calf serum (PAN BIOTECH, GmbH). Finally, PBMCs (0.2×10^6 /well) were cultured in 96-well round-bottom tissue culture plates (Becton Dickinson, Franklin, NJ, USA) at 37°C in a humidified 5% CO₂ incubator and stimulated with 10 µg/ml of PHA. A dose-response study was carried out with PBMCs from normal healthy volunteers to determine the optimum proliferative dose of recombinant Bm-33, which was found to be 10 µg/ml. Antigen-stimulated PBMCs were cultured for 96 h and PBMCs stimulated with PHA were cultured for 48 h. Antigen-specific proliferation was quantified as [³H] thymidine (Amersham-Life Science, UK) incorporation during the last 20 h of incubation. All experiments were performed in triplicates. The viability of the cells in the presence of recombinant Bm-33 was monitored by Trypan Blue exclusion. Recombinant Bm-33-induced lymphoproliferation with endemic normal PBMC, in the presence and absence of Polymixin-B sulphate, indicated no role for contaminating LPS in the proliferative responses (data not shown).

Cultures for whole blood flow cytometry

Heparinized whole blood (6 ml) was diluted with equal volume of RPMI 1640 in a 50-ml sterile centrifuge tube. Of this diluted blood, 4 ml was seeded into six-well tissue culture plate (Becton Dickinson) and cultured for 24 h in a humidified atmosphere of 5% CO₂ at

37°C in the presence and absence of stimulation with 10 µg/ml of PHA and recombinant Bm-33. After 24 h, cells were scraped with cell scraper (Axygen, USA) and washed with 1:10 diluted BD FACS lysing solution (BD Biosciences, San Jose, CA, USA) in 1X PBS. Subsequently, the cells were fixed in 4% paraformaldehyde and permeabilised in PBS/0.1% Saponin (J T Baker) for the intracellular detection of co-stimulatory molecules and cytokines. Staining was done with previously titrated volumes of FITC and PE-conjugated antibodies and incubated at 4°C for 1 h. The cells were subsequently washed and fluorescence was measured on a FACS Calibur (BD Biosciences) using 50,000 gated lymphocytes. Analysis was performed using Flow JO software, and the data for the stimulated conditions were expressed as per cent fold change (%CD4⁺ T cells expressing a particular marker upon stimulation/%CD4⁺ T cells expressing a particular marker under unstimulated condition).

Reagents for flow cytometry

The antibodies used in the study were, FITC-labelled antihuman CD4, CD14; Per CP-labelled antihuman CD4; and PE-labelled antihuman CD3, CD69, CD62L, CD127, CD154, CD28 and CTLA-4. All these antibodies were obtained from BD Biosciences.

Cultures for real-time PCR

PBMC (1×10⁶ cells/ml) were cultured in cRPMI 1640 medium supplemented with 10% foetal calf serum in 24-well tissue culture plates (Becton Dickinson) for 24 h in the presence or absence of stimulation with 10 µg/ml of PHA and r Bm-33. The cultures were harvested by centrifugation, following which the supernatants were stored at –20°C and the pellet was used for RNA extraction.

RNA extraction

PBMC were lysed using the reagents of a commercial kit (QIAshredder; Qiagen). Total RNA was extracted according to the manufacturer's protocol (RNeasy mini kit, Qiagen) and RNA was dissolved in 20 µl of RNase-free water.

cDNA synthesis

Reverse transcription of RNA was performed in a final volume of 20 µl containing 0.25 mM mix of the four deoxynucleotide triphosphates (dATP, dGTP, dTTP and dCTP) (New England Biolabs, MA, USA), 1X reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 8 mM DTT, 20 U RNase inhibitor (RNasin, Gibco BRL) and 200 U of MMLV-reverse transcriptase (New England Biolabs) and followed by incubation of the tubes at 37°C for 60 min. Moloney murine leukaemia virus reverse transcriptase is an RNA-dependent DNA polymerase which has RNase H activity. The reverse transcription reaction was stopped by heating the tubes at 90°C for 5 min. The cDNAs were snap-chilled in ice for 5–10 min and stored at –20°C until use.

Real-time PCR

Real-time quantitative RT-PCR was performed in an ABI 7700 sequence detection system (Applied Biosystems, Fullerton, CA, USA) using TaqMan Assays-on-Demand reagents for IL-1β, IL-12, IL-8, IFN-γ, IL-10, TGF-β and endogenous 18S ribosomal RNA control. Quantification of gene expression was performed using the comparative CT method (Sequence Detector User Bulletin 2, Applied Biosystems) and reported as the fold change relative to the housekeeping gene. To calculate the fold change, CT of the house keeping gene (18s rRNA) was subtracted from the CT of the target gene to yield the ΔCT. Change in the expression of the normalised target gene as a result of antigenic exposure was expressed

as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ of stimulated $-\Delta CT$ of unstimulated. Along with fold change, basal level expression of the same genes was also assessed.

Statistical analysis

Statistical analysis were carried out using Graph Pad Prism software. Comparison among the clinical groups was performed using the Mann–Whitney *U* test. A *p* value of 0.05 was considered statistically significant.

Results

Recombinant Bm-33-induced CD4⁺ T cell activation

Bm-33-induced CD4⁺ T cell activation was evaluated for the expression of T cell activation markers, CD69, CD62L (L-selectin) and CD127. Recombinant Bm-33-induced CD69 expression exhibited a significant ($p < 0.05$) upregulation in filarial patients (CP and MF) compared to EN (CP and MF $>$ EN; Fig. 1a), whilst CD62L expression was significantly ($p < 0.05$) decreased in both MF and CP patients compared to EN (MF and CP $<$ EN; Fig. 1b). A similar trend was observed with respect to CD127 expression where filarial patients, particularly MF individuals, showed a significant decrease compared to both CP and EN (Fig. 1c).

PHA-induced expression of T cell activation markers showed a significant ($p < 0.05$) change only in CD69 expression where an enhanced expression was observed in EN compared to MF patients (EN $>$ MF).

Besides this, basal level expression of CD62L was low ($p < 0.05$) in CP compared to EN patients (CP $<$ EN). No difference in expression was observed with respect to other molecules that were examined.

Thus, recombinant Bm-33-induced enhanced CD69 expression and lowered CD62L and CD127 expression in filarial patients compared to normals suggest T cell activation in the former. This is further confirmed by the positive control PHA that augments CD69 expression in EN individuals compared to filarial patients.

Recombinant Bm-33-induced co-stimulatory molecule expression in CD4⁺ T cells

rBm-33 induced elevated CD154 expression in CP patients compared to EN ($p < 0.05$, CP $>$ EN; Fig. 2a). Apart from this, neither CD28 (Fig. 2b) nor CTLA-4 (Fig. 2c) showed any significant difference in their expression upon Bm-33 stimulation in endemic population.

With respect to mitogen stimulation, PHA-induced expression of all the above co-stimulatory molecules (CD154, CD28, CTLA-4) did not exhibit any significant difference between the groups. Basal level CD154 expression was elevated in EN compared to MF and CP patients (EN $>$ MF and CP, $p < 0.05$). Apart from this, neither CD28 nor CTLA-4 showed any significant difference in their expression profiles.

Thus, recombinant Bm-33 induced enhanced CD154 expression in CP compared to EN.

Recombinant Bm-33-induced modulation of cytokine gene expression

rBm33-induced expression of pro-inflammatory cytokines—IL-1 β , IL-8, IL-12 and IFN- γ —was assessed in PBMCs of the endemic population (Fig. 3a–d). No significant difference was observed in the antigen-induced (recombinant Bm-33 and PHA) expression of IL-1 β , IL-8 and IL-12. Although recombinant Bm-33-induced IFN- γ expression did not exhibit any

significant difference between patients (MF and CP) and endemic normals, MF individuals showed a significant increase ($p < 0.05$) compared to CP (MF > CP; Fig. 3d).

PHA-induced IFN- γ expression showed significant augmentation in EN individuals compared to MF and CP patients.

Basal level expression of all the above cytokines were ($p < 0.05$) elevated in CP patients compared to MF and EN (CP > MF > EN).

Assessment of anti-inflammatory cytokines revealed no change in IL-10 expression in both rBm-33- and PHA-stimulated cultures (Fig. 4a). As expected, basal level IL-10 expression was ($p < 0.05$) elevated in MF compared to CP and EN (MF > CP > EN).

TGF- β expression among the groups did not exhibit any significant difference upon rBm-33 stimulation. But PHA stimulation increased the expression levels in EN compared to CP patients (EN > CP; Fig. 4b). Besides this, basal level expression of TGF- β was ($p < 0.05$) elevated in CP compared to MF and EN (CP > MF > EN).

Thus, rBm-33 stimulation did not exhibit any modulation in cytokine gene expression in filarial patients and EN.

Lymphoproliferative responses induced by recombinant Bm-33

Recombinant Bm-33 induced a significant suppression of lymphoproliferative responses (SI) in MF patients compared to CP and EN ($p < 0.05$, MF < CP < EN) at the 96-h time point (Fig. 5). However, PHA-induced proliferation did not exhibit any significant difference between the groups at 48 h.

Discussion

Human filarial parasite's prolonged survival in the hostile immune niche of the host necessitates the modulation of host immune responses by parasite proteins. Thus, identification and characterisation of filarial parasite proteins should provide important insights into their role in immune modulation and host pathogenesis. Earlier studies at our laboratory have shown Bm-33 as an immunodominant filarial protein expressed in the adult as well as the larval stages of the parasite life cycle and stimulating IgG4 antibodies production in individuals with patent infection (Krushna et al. 2009). The present study describes host immune responses, particularly cellular responses to rBm-33, in filarial patients and putatively immune individuals.

Suppression of T cell function in response to parasite antigens *in vitro* is a hallmark of helminth infections (Nutman et al. 1987; Yazdanbakhsh et al. 1993). However, in the present study, rBm-33 stimulation augmented CD4⁺ T cell activation in MF and CP patients by the virtue of elevated CD69 expression and diminished CD62L expression in MF and CP patients compared to EN at 24 h. In addition, a profound suppression of CD127 expression levels in MF compared to CP at the same time point indicates rBm-33-induced CD4⁺ T cell activation in the asymptomatic patients. Similar observations were reported earlier with respect to the expression of these activation markers (Sprent and Surh 2002; Sallusto et al. 2004; Bradley et al. 2005) that suggests an early T cell activation. Furthermore, rBm-33 stimulation did not result in increased co-stimulatory molecule expression in MF patients as there was no significant modulation in the expression of CD154, CD28 and CTLA-4 between individuals with patent infection and normals.

Although cytokines are important mediators in regulating antigen-specific immune responses, none of cytokines (IL-1 β , IL-12, IL-8, IFN- γ , IL-10 and TGF- β) assessed in the

present study showed any significant difference in their expression profiles between patients and putatively immune individuals. This might, however, necessitate a closer look by monitoring the time kinetics of expression.

Finally, a profound suppression of lymphoproliferation (at 96 h) in MF patients compared to CP and EN upon stimulation with rBm-33 suggests the short-lived nature of Bm-33-induced early CD4⁺ T cell activation in this cohort. In this regard, studies with another protease inhibitor Bm Serpin (Bm SPN-2) showed similar observations pertaining to strong and short-lived immune responses (Zang et al. 2000). This relatively unsustained T cell activation in MF patients might be attributed to the activation of other regulatory cascades like apoptosis by Bm-33 after 24 h. In this regard, activation of previously activated T lymphocytes under certain circumstances has been implicated in apoptosis (Akbar and Salmon 1997). This phenomenon, termed as activation-induced cell death, is critically important for the maintenance of T cell homeostasis and self-tolerance during disease progression (Akbar and Salmon 1997; Revillard et al. 1998; Parijis and Abbas 1998). Studies in several experimental models suggested the role of CD4⁺ T cell apoptosis in proliferative defect observed in filarial patients (Connor et al. 2003; Jenson et al. 2002). Thus, rBm-33 may induce apoptosis in MF patients, and this is being pursued.

In CP patients also, rBm-33 induced an early CD4⁺ T cell activation characterised by elevated CD69 and diminished CD62L expression levels compared to EN. In addition, rBm-33 stimulation, enhanced CD154 expression in CP, but not in MF. This increase could be a direct consequence of rBm-33-induced CD4⁺ T cell activation that was observed in this group of patients. Enhanced CD154 levels, leading to CD40/CD154 ligation, may influence the production of Th-1 cytokines like TNF- α , IL-1, IL-12, etc. (Raman et al. 1999; Wagner et al. 1994; Shu et al. 1995). However, in our study, rBm-33 did not induce any of these cytokines at 24 h of PBMC culture. Besides this, suppressed rBm-33-induced lymphoproliferation in CP compared to EN supports previous reports with parasite recombinant protein like WbSXP-1 (Sasisekhar et al. 2005) that induces proliferative suppression in patients (MF and CP).

Apart from antigen (rBm-33)-induced modulation, the present study also provides interesting insights into basic immune mechanisms operating in the endemic population by determining the basal expression profiles of some molecules. For instance, elevated CD62L expression in EN compared to patients indicates the presence of activated phenotype in CP patients. In addition to this, elevated CD154 and CD28 levels in EN compared to patients (MF and CP) highlights the immune competence of the normals compared to patients. Besides this, elevated expression levels of pro-inflammatory cytokines (IFN- γ , IL-1 β , IL-12, and IL-8) in patients (CP and MF) were observed. These may be produced by antigen-exposed T cells themselves or by macrophages. With respect to suppressor cytokines like IL-10 and TGF- β , spontaneous levels of elevated IL-10 in MF patients have been demonstrated earlier (Mahanty et al. 1996) and have been implicated in immune suppression. In addition, elevated TGF- β expression in CP compared to MF and EN at the basal level supports the previous observations (Babu et al. 2006).

Thus, rBm-33-induced immune response substantiates its immunodominant nature as it induced CD4⁺ T cell activation in MF patients. In addition to this, the present study supports our previous findings pertaining to T cell epitope analysis (Krushna et al. 2009). Besides this, unsustained CD4⁺ T cell activation in filarial patients suggests that rBm-33 contributes to the pathogenesis of filarial infections.

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Fig 1.

Expression of activation markers on CD4⁺ T cells (**a** CD69, **b** CD62L, **c** CD127) in EN, MF and CP at basal level (*i*) and upon stimulation with PHA (*ii*) and Bm-33 (*iii*) by flow cytometry in whole blood cultures (4 ml) of EN, MF and CP individuals stimulated for 24 h at 37°C in 5% CO₂. Each *dot* represents the expression levels (*i*) and fold change (*ii*, *iii*) for each sample and *horizontal bars* indicate geometric mean. Fold change (basal level) = % CD4⁺ T cells expressing a particular marker. Fold change under stimulated condition = % CD4⁺ T cells expressing a particular marker/% CD4⁺ T cells expressing a particular marker under unstimulated condition

Fig 2.

Expression of co-stimulatory molecules on CD⁺ T cells (**a** CD154, **b** CD28, **c** CTLA-4) in EN, MF and CP at basal level (*i*) and upon stimulation with PHA (*ii*) and Bm-33 (*iii*) by flow cytometry in whole blood cultures (4 ml) of EN, MF and CP individuals stimulated for 24 h at 37°C in 5% CO₂. Each *dot* represents expression levels (*i*) and fold change (*ii*, *iii*) for each sample and *horizontal bars* indicate geometric mean. Fold change (basal level) = % CD4⁺ T cells expressing a particular marker. Fold change under stimulated condition = % CD4⁺ T cells expressing a particular marker/% CD4⁺ T cells expressing a particular marker under unstimulated condition

Fig 3.

Expression of Th-1 cytokines IL-1 β (a), IL-8 (b), IL-12 (c) and IFN- γ (d) at basal level (i) and upon stimulation with PHA (ii) and Bm-33 (iii) in PBMC cultures (1 $\times$ 10⁶/ml, 24 h) of EN, MF and CP by real-time PCR. Antigen-induced fold change was plotted on Log 10 scale for the values on the Y-axis upon PHA (ii) and Bm-33 (iii) stimulation. Each dot represents expression levels (i) and fold change (ii, iii) for each sample and horizontal bars indicate geometric mean. Fold change (Δ CT) = CT of the housekeeping gene (18s rRNA) – CT of the target gene. Change in the expression of the normalised target gene as a result of antigenic exposure ($2^{-\Delta\Delta$ CT), where $\Delta\Delta$ CT = Δ CT of stimulated – Δ CT of unstimulated

Fig 4.

Expression of suppressor cytokines IL-10 (a) and TGF-β (b) at basal level (i) and upon stimulation with PHA (ii) and Bm-33 (iii) in PBMC cultures ($1 \times 10^6/\text{ml}$, 24 h) of EN, MF and CP by real-time PCR. Antigen-induced fold change was plotted on Log 10 scale for the values on the Y-axis upon PHA (ii) and Bm-33 (iii) stimulation. Each dot represents expression levels (i) and fold change (ii, iii) for each sample and horizontal bars indicate geometric mean. Fold change (ΔCT) = CT of the housekeeping gene (18s rRNA) – CT of the target gene. Change in the expression of the normalised target gene as a result of antigenic exposure ($2^{-\Delta\Delta\text{CT}}$), where $\Delta\Delta\text{CT} = \Delta\text{CT}$ of stimulated – ΔCT of unstimulated

Fig 5.

Lymphocyte proliferative responses to recombinant Bm-33 in endemic population. Lymphocyte proliferation assay was carried out with 0.2×10^6 PBMCs from EN, MF and CP individuals stimulated with recombinant Bm-33 (96 h) along with PHA (48 h). The results are expressed as Log 10 values of stimulation index. Individual data are represented by *symbols* for each category; *vertical bars* indicate the geometric mean of the values and *horizontal bars* indicate mean $\pm$ standard deviation. Bm-33: EN vs MF, $p=0.007$; EN vs CP, $p=0.007$; MF vs CP, $p=0.03$

Table 1

Demographic details of filarial patients

	No. of male/female	Median age	Range (years)	Clinical manifestations	Treatment	MF load (mf/ml)	CFA Og4C3
EN	6/4	25	23–32	–	–	None	None
MF	3/2	37	12–40	–	–	60–2000	2000–60,000
CP	5/5	28	25–50	Grades II–IV lymph edema	DEC/heat therapy/compression therapy	None	None

GM geometric mean, *DEC* diethylcarbamazine, *Og4C3* *Onchocerca gibsoni* antigen, *EN* endemic normal, *MF* microfilaremics, *CP* chronic pathology, *AU* antigenic units