



Contents lists available at [SciVerse ScienceDirect](#)

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



A 22 year follow-up study on lymphatic filariasis in Tanzania: Analysis of immunological responsiveness in relation to long-term infection pattern

Paul Bloch^{a,b,*}, Nina O. Nielsen^c, Dan W. Meyrowitsch^d, Mwelecele N. Malecela^e, Paul E. Simonsen^a

^a DBL – Centre for Health Research and Development, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark

^b Steno Health Promotion Center, Steno Diabetes Center, Gentofte, Denmark

^c Department of Epidemiology Research, Statens Serum Institut, Copenhagen, Denmark

^d Department of Public Health, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

^e National Institute for Medical Research, Dar es Salaam, Tanzania

ARTICLE INFO

Article history:

Received 28 February 2011

Received in revised form

12 September 2011

Accepted 15 September 2011

Available online xxx

Keywords:

Lymphatic filariasis

Immunology

Epidemiology

Cellular responsiveness

Antibodies

Cytokines

Tanzania

ABSTRACT

Seventy-one individuals who had been examined for *Wuchereria bancrofti* microfilaraemia in 1975, some of whom had been offered mass treatment with diethylcarbamazine (DEC) in subsequent years, were re-identified in 1996 and examined for microfilaraemia, circulating filarial antigenemia and cellular and humoral immunoresponsiveness to crude antigen homogenates prepared from *Brugia pahangi* parasite material. 85.9% of the study individuals had the same infection status in 1975 and 1996, suggesting strong predisposition to infection over extended periods of time. IL-4, IL-5 and IFN γ responses were associated with being infection negative in 1996 whereas IL-10 responses were associated with being infection positive. Similarly, specific IgG3 and IgE were strongly associated with being infection negative in 1996 whereas specific IgG4, and thus high IgG4/IgE ratios, were strongly associated with being infection positive. Intermediary levels of mainly IL-5, IFN γ and PBMC stimulation indices were observed for study individuals who changed from being infection positive in 1975 to infection negative in 1996, or vice versa, suggesting a transition in cellular immunoresponsiveness associated with changing infection status. The findings suggest that some people are more disposed to infection with bancroftian filariasis than others and that this is largely unaffected by treatment with DEC. The findings also suggest that specific cellular and antibody responses are more related to current than past infection status, and that IL-4, IL-5, IFN γ , specific IgG3 and IgE are associated with parasite clearance, whereas IL-10 and specific IgG4 are associated with parasite protection.

© 2011 Elsevier B.V. All rights reserved.

1. Background

Limited documentation is available about the long-term dynamics of lymphatic filariasis infection and antifilarial immunity. Cross-sectional studies have shown that infection prevalence, as determined by detection of circulating filarial antigens (CFA) and microfilariae (MF), increases steadily from ages less than 5 years up to 20 years and remains high but fluctuating thereafter (Lammie et al., 1994; Simonsen et al., 1996, 2002; Steel et al., 2001). Moreover, cross-sectional studies have shown that the prevalence of infection in childhood is correlated with the prevalence of infection in adulthood (Witt and Ottesen, 2001). Whereas, the levels of specific antibodies against filarial infection in humans are generally

higher in adults than children, the pattern of humoral responsiveness is rather similar and largely depends on infection status (De Almeida et al., 1996; Jaoko et al., 2006, 2007; Simonsen et al., 1996; Witt and Ottesen, 2001). Although responses in amicrofilaraemic children are stronger than in amicrofilaraemic adults, the pattern of cellular responsiveness in children and adults is also quite similar; antigen-negative or amicrofilaraemic individuals in endemic populations generally have higher proliferative responses to filarial antigens than antigen-positive or microfilaraemic individuals (Ottesen et al., 1977; Hitch et al., 1991; Nielsen et al., 2002a). Whereas these data are based on cross-sectional studies and apply to populations, documentation about the long-term dynamics of immune responses in relation to infection is limited due to scarcity of longitudinal cohort studies.

The present study aimed at adding to the understanding of the epidemiology and immunology of lymphatic filariasis by taking advantage of a unique opportunity to carry out a 22 year follow-up of individuals living in an area of north-eastern Tanzania, which is highly endemic for lymphatic filariasis caused by *Wuchereria bancrofti*. The study population was examined for microfilaraemia and

* Corresponding author at: Steno Health Promotion Center, Niels Steensens Vej 8, DK-2820 Gentofte, Denmark.

E-mail addresses: pabc@steno.dk (P. Bloch), nij@ssi.dk (N.O. Nielsen), D.Meyrowitsch@sund.ku.dk (D.W. Meyrowitsch), mwelentuli@gmail.com (M.N. Malecela), pesi@life.ku.dk (P.E. Simonsen).

clinical manifestations for the first time in 1975 (McMahon et al., 1981). In 1996 – 22 years later – 71 individuals from the same population were re-identified and invited to participate in a follow-up study during which they were examined for clinical manifestations, CFA and MF based infection status, and cellular and humoral immunoresponsiveness.

Cellular responses were analysed with emphasis on cellular proliferation of peripheral blood mononuclear cells (PBMC) and secretion of the cytokines IL-4, IL-5, IL-10 and IFN γ following stimulation with crude *Brugia pahangi* microfilarial and adult worm antigens. Furthermore, humoral responses were analysed by measuring the occurrence of plasma antibodies (IgG1, IgG2, IgG3, IgG4 and IgE) against *B. pahangi* microfilarial and adult worm antigens. The immunological parameters were analysed in relation to the infection status of the study individuals in 1975 and 1996.

2. Methods

2.1. Study population

The study was carried out in four coastal villages – Kwale, Machui, Moa and Tawalani – in Tanga Region, north-eastern Tanzania. Cross-sectional surveys for lymphatic filariasis were first carried out in these villages in 1975 (McMahon et al., 1981), during which individuals were examined for MF at daytime following administration of a low dose of diethylcarbamazine (DEC), – the so-called DEC provocative day test. One hundred microliters of finger-prick blood were examined with the counting chamber technique (McMahon et al., 1979a). This was immediately followed by application of various control measures (vector control in Kwale and Moa, DEC mass treatment in Machui and Tawalani), and follow-up surveys were carried out during the next couple of years to evaluate the effect of the measures (Kolstrup et al., 1981). Cross-sectional surveys were again carried out in Kwale, Machui and Tawalani in 1991 (Meyrowitsch et al., 1995a), and were followed by DEC mass treatment in Kwale and Tawalani in 1992 (Meyrowitsch et al., 1996). In 1996, attempts were made to re-identify as many as possible of the individuals originally examined in 1975. The procedure for re-identification made use of available records from the earlier surveys (i.e. name, age and place of living) and detailed communication with community elders on the identity and whereabouts of the previously examined individuals, and, subsequently, questioning of the identified individuals about their participation in the 1975 survey.

A total of 71 individuals aged 34–74 years (in 1996) were re-identified and gave their consent to participate in this study. They were examined clinically for chronic manifestations of lymphatic filariasis. Five persons had a hydrocele (one Grade 1 and four Grade 2) and two had elephantiasis (Grade 1). In addition, two persons had both a hydrocele (Grade 2) and elephantiasis (Grade 2). The grading of clinical manifestations followed procedures described by Casley-Smith et al. (1993). Six of the study individuals reported to have had a hydrocele removed by operation in the past. Two of these were among those individuals who had a (re-developed) hydrocele detected in this study.

Analyses of stool and urine for helminth eggs (by Kato smear and nucleopore filtration techniques, respectively) indicated that infections with hookworm (95.5%), *Trichuris trichiura* (38.8%) and *Ascaris lumbricoides* (7.5%) were common in the study population. These infections were not significantly associated with any of the study groups.

2.2. Examination for MF and preparation of plasma and PBMC

Night blood samples of 30 ml were collected from the study individuals by venipuncture from 2100 h to 2400 h. One milliliter

of blood was removed for MF examination using the nucleopore filtration technique (McMahon et al., 1979a). From the remaining blood, peripheral blood mononuclear cells (PBMC) and plasma were isolated from each sample in Lymphoprep tubes (Nycomed Pharma, Norway) by following procedures from the manufacturer. PBMC were washed in RPMI 1640 containing 25 mM Hepes, 2 mM L-glutamine, 50 mg/l gentamycin and 5% fetal calf serum, and were then diluted in the same medium containing 15% normal human serum (NHS; Statens Serum Institute, Copenhagen, Denmark) instead of calf serum to a concentration of 5×10^6 cells/ml prior to cryopreservation in liquid nitrogen using an automated controlled-gradient cryopreservation device (Hviid et al., 1993). Sodium azide (NaN $_3$) was added to the plasma samples to a concentration of 15 mM prior to freezing of the samples at -20°C . PBMC and plasma samples were transported to Denmark for further analyses.

2.3. Antigens and mitogens

Antigens for cellular stimulation and antibody measurements consisted of crude homogenates of *B. pahangi* microfilariae or adult worms. Adult *B. pahangi* antigen (BPAD) was prepared by homogenisation of the parasite material at 0°C in a glass homogeniser together with 2 ml phosphate buffered saline (PBS, pH 7.4) containing 1% (w/v) CHAPS (Sigma, USA) and protease inhibitors (PMSF: 174 $\mu\text{g/ml}$, TPCK: 50 $\mu\text{g/ml}$ and TLCK: 25 $\mu\text{g/ml}$; all from Sigma). The homogenate was ultrasound sonicated on ice for 3 min using a Soniprep 150 MSE (Branson, USA). Following overnight incubation at 4°C the homogenate was homogenated and sonicated again and centrifuged at 13,000 rpm for 15 min at 4°C . The supernatant was then passed through a 0.45 μm Sartorius Minisart filter (Goettingen, Germany), dialyzed against PBS for 2 h in a Slide-a-lyzer (Pierce, USA), and filter sterilised using a 0.2 μm Dynagard filter (Microgon, USA). The protein content of the filtrate was measured by the Bio-Rad Protein Assay (Bio-Rad, USA). The crude homogenate was aliquoted into small vials and frozen at -80°C until use.

Microfilarial *B. pahangi* antigen (BPMF) was prepared by centrifuging the larvae for 10–15 min at 3000 rpm. The supernatant was removed and replaced with 1 ml of distilled water in order to lyse contaminating erythrocytes and incubated for 5 min. The suspension was filtered through a 5 μm Nucleopore filter with a 2.5 cm diameter to remove erythrocyte debris. The eluate was checked for microfilariae under a microscope using 40x magnifications and refiltered if some were found. The filter was soaked in 2 ml PBS containing protease inhibitors (as described above) and left on a shaker for 2 h. After removing the filter, the microfilarial suspension was ultrasound sonicated on ice for 4 min. Two ml PBS containing protease inhibitors and 2% Chaps detergent (as described above) was added and the suspension was left incubating overnight at 4°C . The dissolved larvae were centrifuged at 13,000 rpm for 10 min. The supernatant was then passed through a 0.45 μm Sartorius Minisart filter and dialyzed for 2 h against PBS using a Slide-a-lyzer. The suspension was not sterile filtered. The protein content was measured by the Bio-Rad Protein Assay (Bio-Rad, USA). The crude homogenate was aliquoted into small vials and frozen at -80°C until use.

In addition, purified protein derivative of *Mycobacterium tuberculosis* (PPD, Statens Serum Institute, Copenhagen, Denmark) and tetanus toxoid (TT, Statens Serum Institute, Copenhagen, Denmark) were used as antigens in cell cultures, and phytohemagglutinin (PHA) was used as mitogen.

2.4. Circulating filarial antigen

Plasma samples were examined for circulating filarial antigens (CFA) using the TropBio enzyme-linked immunosorbent assay (ELISA) kit (TropBio, JCU, Tropical Biotechnology, Townsville,

Australia) as described by the manufacturer. Samples were tested in duplicate and the mean optical density (OD) values were calculated. Samples with a mean OD value $\geq$ standard 2 (32 antigen units) were considered positive for CFA.

2.5. Cellular proliferation

PBMC were cultured in RPMI 1640 containing 25 mM Hepes, 2 mM L-glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin, and 15% NHS at a concentration of 0.67×10^6 cells/ml for 7 days together with BPAD (11.8 μ g/ml) or BPMF (1 μ g/ml), PPD (11.8 μ g/ml), TT (6.9 μ g/ml) or mitogen (11.8 μ g/ml). Control cultures, in which only medium was added, were included. The cultures were prepared in triplicate in round-bottomed microtiter plates (Nunc, Denmark) which were placed in an incubator with a humidified atmosphere and 5% CO₂ at 37 °C. On day six, 20 μ l [³H] thymidine (Dupont de Nemours, Belgium) was added to each well and 24 h later the cells were harvested onto a glass fiber filter using a Matrix 96 Filtermate Harvester (Packard Instruments, USA). The quantity of [³H] thymidine incorporated in the cellular genome was measured by a scintillation counter (Packard Instruments) and the results expressed as stimulation indices (SIs): mean counts per minute (cpm) of stimulated cultures/mean cpm of unstimulated cultures.

2.6. Cytokines in cell culture supernatants

For measuring the levels of IL-4, IL-5, IL-10 and IFN γ secreted from stimulated and non-stimulated cells, cultures of PBMC were prepared as described above, except that [³H] thymidine was not added. Supernatants of 80–100 μ l were removed from each cell culture well after optimum periods of culturing (3 days for IL-4, 5 days for IL-5, 3 days for IL-10 and 7 days for IFN γ) and frozen at –20 °C until analysis. Following pooling of the triplicate samples the cytokine levels in the supernatants were measured by sandwich ELISA using commercially available kits (Genzyme, USA, for IL-4, IL-10 and IFN γ ; Pharmingen, USA, for IL-5) and following procedures from the manufacturer. One day before collecting supernatants, PMA (50 ng/ml) and ionomycin (750 ng/ml) were added to cell cultures prepared for IL-4 measurement. For each cytokine level obtained from a (pooled) antigen stimulated cell culture the level obtained from a (pooled) non-stimulated cell culture was subtracted. Negative values resulting from this subtraction were given the value of zero and analysed as such.

2.7. Specific antibodies

Specific IgG1, IgG2, IgG3, IgG4 and IgE in plasma samples were measured against *B. pahangi* microfilarial (BPMF) and adult worm (BPAD) antigen homogenates using indirect ELISA, as previously described (Bloch et al., 1993; Nielsen et al., 2002b). BPMF and BPAD antigen homogenates were applied in optimal concentrations of 3.5 μ g/ml and 5 μ g/ml, respectively. Plasma was used in optimal dilutions of 1:2000 for IgG1, 1:1000 for IgG2 1:500 for IgG3, 1:1000

for IgG4 and 1:3 for IgE. Horse-radish peroxidase (HRP) conjugated monoclonal mouse antibodies against each of the four human IgG subclasses (CLB, Amsterdam) were diluted 1:2000. For measuring specific IgE antibodies in the plasma samples IgG antibodies were preabsorbed prior to use in a Bio-Rad Spin Column to which a Protein G Sepharose Fast Flow gel (Pharmacia LKB, Uppsala, Sweden) was added. HRP conjugated monoclonal rabbit antibody against human IgE (Dako, Denmark) was diluted 1:1000. A pool of positive control plasma samples were included on all plates to adjust for plate to plate variation. All sera were tested in triplicate and for each plasma sample the results were expressed as the mean absorbance value.

2.8. Data analysis

For analytical purposes, all study individuals were grouped according to their infection status in 1975 and 1996. Those individuals who were MF positive in 1975 and CFA positive in 1996 were labelled “MF+/CFA+”. Likewise, those individuals who were MF positive in 1975 and CFA negative in 1996 were labelled “MF+/CFA–”, those who were MF negative in 1975 and CFA positive in 1996 were labelled “MF–/CFA+”, and those who were MF negative in 1975 and CFA negative in 1996 were labelled “MF–/CFA–”.

MF intensities were calculated as geometric mean (GM) intensities using the formula, $GM = \text{antilog}[\Sigma(\log(x))/n]$, where x is the number of MF/ml for all MF-positive study individuals. Differences in microfilariae intensities between groups were compared using *t*-test for independent samples on the logtransformed values. Age differences were also compared using *t*-test for independent samples. Differences in MF and CFA prevalence rates between groups were compared using Pearson's Chi-Square test. Mean concentrations of cytokines (pg/ml) and stimulation indices (SIs) were calculated and compared statistically between groups using the Mann–Whitney two sample *U*-test and the Kruskal–Wallis *k*-sample test. Absorbance values and ratios for antibody measurements were also compared statistically between groups using these two non-parametric tests. Probability values (*p*-values) less than 0.05 were considered statistically significant.

3. Results

3.1. Characterisation of study individuals

Of the 71 study individuals 47 (66.2%) were positive for CFA and 38 (53.5%) had MF (Table 1). At the time of the present study (1996) the mean age of males and females was 52.5 and 49.3 years, respectively. The MF density for the microfilaremic males was 140 MF/ml and for females was 68 MF/ml. When grouped according to CFA status in 1996 the mean ages of CFA positive and negative study individuals were 51.8 and 47.8 years, respectively. None of the above-mentioned differences were statistically significant. Of the 26 males 22 (84.6%) were CFA positive and 18 (69.2%) were MF positive. Of the 45 females 25 (55.6%) were CFA positive and 20 (44.4%) were MF positive. Both of these differences between males and

Table 1

Characteristics related to age, sex and infection status of the 71 study individuals totally and by CFA status in 1996.

	All study individuals (n = 71)	CFA positive individuals only (n = 47)	CFA negative individuals only (n = 24)
Mean age in years ^a	50.5 (34–74)	51.8 (36–74)	47.8 (34–65)
Males	26 (36.6%)	22 (46.8%)	4 (16.7%)
Circulating filarial antigen (CFA) positives	47 (66.2%)	47 (100.0%)	0
Microfilariae positives	38 (53.5%)	38 (80.9%)	0
Microfilariae intensity ^b	92 (2–4708)	194 (2–4708)	0

^a The age range is presented in brackets.

^b Presented as the geometric mean intensity (MF/ml) for MF positive study individuals; the range is presented in brackets.

Table 2

Age, sex, CFA prevalence, and MF prevalence and intensity of the 71 study individuals by infection status in 1975 and 1996.

	MF positive in 1975, CFA positive in 1996 (n = 41)	MF positive in 1975, CFA negative in 1996 (n = 4)	MF negative in 1975, CFA positive in 1996 (n = 6)	MF negative in 1975, CFA negative in 1996 (n = 20)
Mean age in years	52.2	53.3	49.2	46.7
Males	22 (53.7%)	0	0	4 (20.0%)
Circulating filarial antigen (CFA) positives (1996)	41 (100.0%)	0	6 (100.0%)	0
Microfilariae positives (1996)	36 (87.8%)	0	2 (33.3%)	0
Microfilariae intensity (1996) ^a	226 (2–4708)	0	15 (2–120)	0

^a Presented as geometric mean intensities (MF/ml) for MF positive study individuals; ranges are presented in brackets.

females were statistically significant (Chi-square, $p < 0.05$). Within the group of CFA positive individuals, there were 22 (46.8%) males as compared to 4 (16.7%) males within the group of CFA negative individuals. This difference was statistically significant (Chi-square, $p < 0.05$). Among the CFA positive individuals, 38 (80.9%) were also MF positive (in 1996). Among the CFA negatives none were MF positive.

The majority of the study individuals remained with the same infection status in the two surveys (Table 2). Those who were MF positive in 1975 were ten times more likely to present with microfilaraemia in 1996 as compared to those who were MF negative in 1975 (RR = 10.40; CI95%: 2.72–39.70, $p < 0.0001$). Those who were MF positive in 1975 were four times more likely to present as CFA positive in 1996 (RR = 3.94; CI95%: 1.95–8.01, $p < 0.0001$) as compared to those who were MF negative in 1975. Hence, a positive MF status in 1975 was a strong and statistically significant predictor for presenting as MF positive and CFA positive in 1996.

3.2. Cytokine responses and stimulation indices

Cytokine levels (IL-4, IL-5, IL-10 and IFN γ) in cell culture supernatant from individuals belonging to the four infection categories are shown in Fig. 1. For IL-4, statistically significant differences between infection categories were obtained when stimulating with BPMF (Kruskal–Wallis, $p < 0.01$) but not BPAD. For BPMF, cytokine levels were significantly higher among “MF–/CFA–” compared with “MF+/CFA+” (Mann–Whitney, $p < 0.01$). For IL-5, statistically significant differences between infection categories were observed when stimulating with BPAD (Kruskal–Wallis, $p < 0.01$), but not BPMF. IL-5 levels (for BPAD stimulation) were significantly higher for “MF–/CFA–” compared with “MF+/CFA+” (Mann–Whitney, $p = 0.001$) but not between any other infection categories. For IL-10, statistically significant differences between infection categories were only observed when stimulating with BPMF (Kruskal–Wallis, $p < 0.05$). IL-10 levels (for BPMF

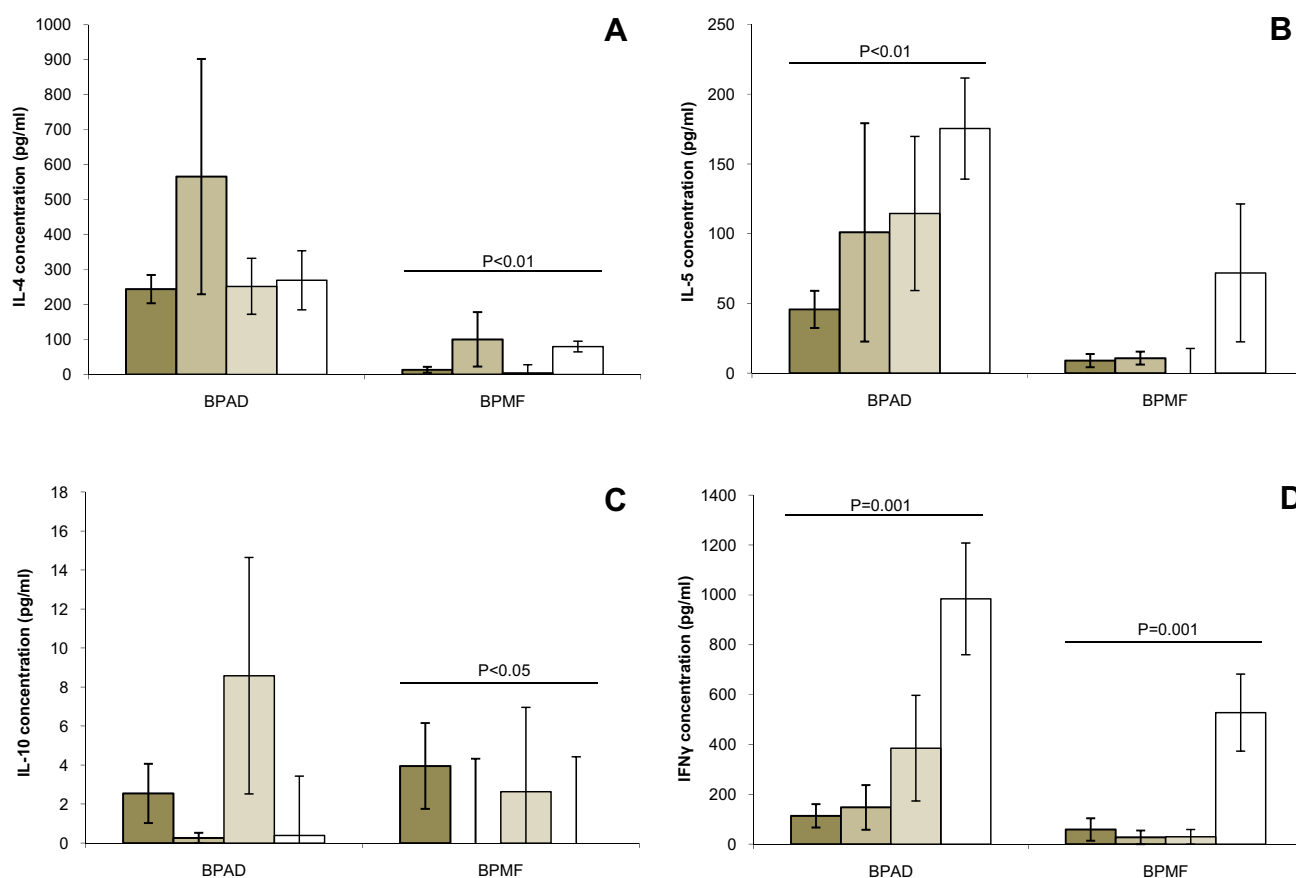


Fig. 1. Levels of IL-4 (A), IL-5 (B), IL-10 (C) and IFN γ (D) from peripheral blood mononuclear cells (BPMC) stimulated with two filarial antigen homogenates (BPAD and BPMF) for individuals who were positive in 1975 and 1996 (“MF+/CFA+”; dark columns), positive in 1975 but negative in 1996 (“MF+/CFA–”; medium dark columns), negative in 1975 but positive in 1996 (“MF–/CFA+”; light columns), and negative in 1975 and 1996 (“MF–/CFA–”; open columns). Mean values are presented as columns and standard errors are presented as vertical lines. No columns are shown where mean values were zero or negative.

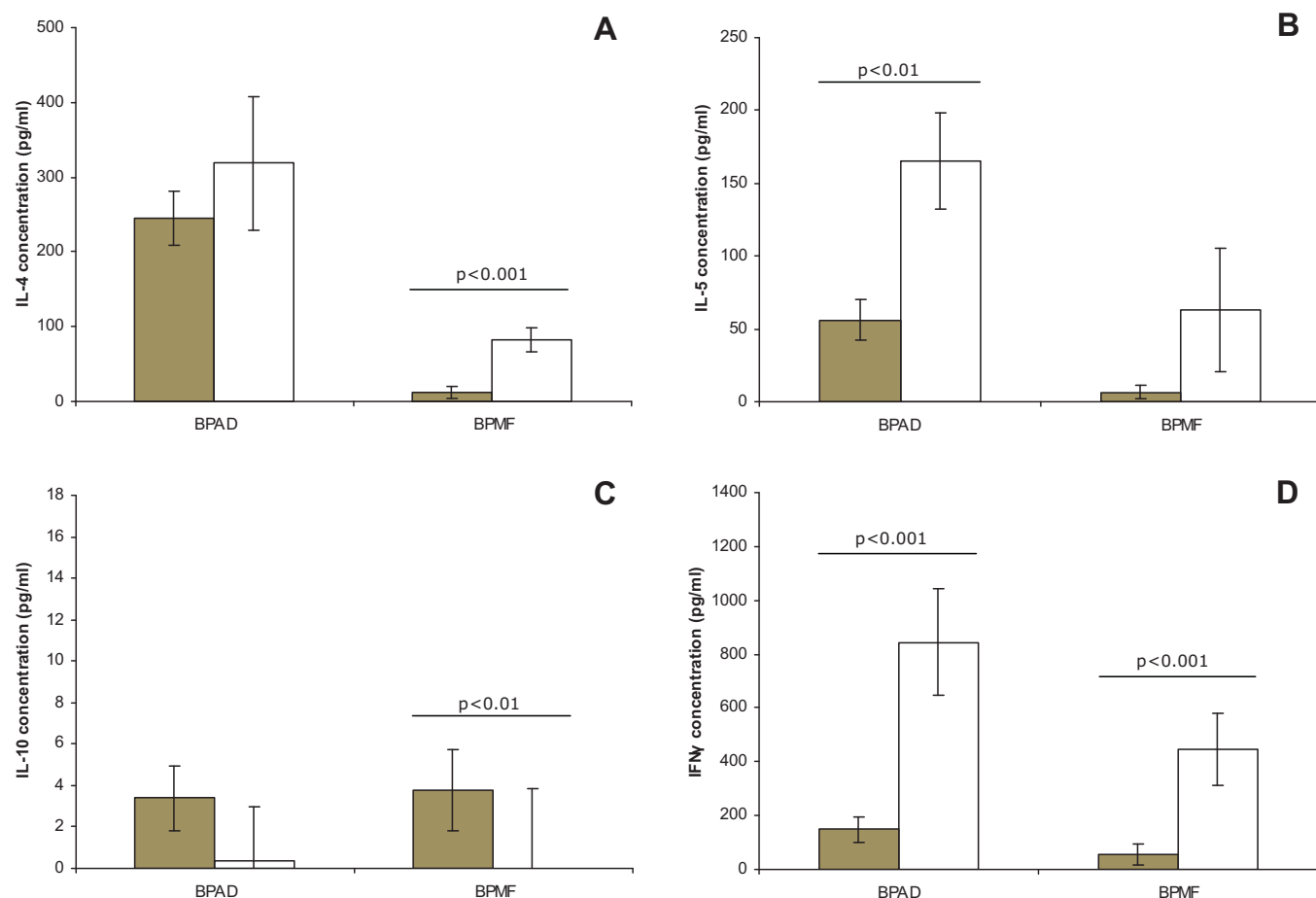


Fig. 2. Levels of IL-4 (A), IL-5 (B), IL-10 (C) and IFN γ (D) from peripheral blood mononuclear cells (PBMC) stimulated with two filarial antigen homogenates (BPAD and BPMF) for individuals who were circulating filarial antigen positive (dark columns) and negative (open columns) in 1996. Mean values are presented as columns and standard errors are presented as vertical lines. No columns are shown where mean values were zero or negative.

stimulation) were significantly higher for “MF+/CFA+” compared with “MF–/CFA–” (Mann–Whitney, $p = 0.01$). Finally, for IFN γ , statistically significant differences between infection categories were observed when stimulating with BPAD and BPMF (Kruskal–Wallis, $p = 0.001$ for both antigens). IFN γ levels were significantly higher for “MF–/CFA–” compared with “MF+/CFA+” (Mann–Whitney, $p < 0.001$ for both of these antigens) but not between any other infection categories.

When the study individuals were grouped according to their CFA status in 1996 (Fig. 2), CFA-positive individuals generally responded with lower levels of IL-4, IL-5 and IFN γ , and higher levels of IL-10, as compared with CFA-negative individuals. For IL-4, this difference was statistically significant when stimulating with BPMF (Mann–Whitney, $p < 0.001$), but not BPAD. For IL-5, this difference was statistically significant when stimulating with BPAD (Mann–Whitney, $p < 0.01$), but not BPMF. For IL-10, the difference was statistically significant only for BPMF (Mann–Whitney, $p < 0.01$), and for IFN γ , the difference was statistically significant for both BPAD and BPMF (Mann–Whitney, $p < 0.001$ for both antigens).

When the study individuals were grouped according to their MF status in 1996, the IL-4, IL-5, IL-10 and IFN γ responses followed the same pattern as seen when grouped according to CFA status, except that none of the differences observed between IL-10 levels were statistically significant (data not shown).

Stimulation indices were considerably higher in response to BPAD as compared to BPMF (Fig. 3). For both antigens the differences in stimulation indices between infection categories were statistically significant (Kruskal–Wallis, $p = 0.001$ for

BPAD, and $p < 0.01$ for BPMF). The index was significantly higher for “MF–/CFA–” compared with “MF+/CFA+” (Mann–Whitney, $p < 0.001$ for BPAD and BPMF). For BPMF, the index was also higher for “MF–/CFA–” compared with “MF–/CFA+” (Mann–Whitney, $p < 0.01$ for BPMF). For BPAD the index was higher for “MF–/CFA+” compared with “MF+/CFA+” (Mann–Whitney, $p < 0.05$). Other differences were not statistically significant. When grouping the study individuals according to their CFA status in 1996, CFA-negative individuals had higher indices compared with CFA-positive individuals. These differences were statistically significant for both antigens (Mann–Whitney, $p < 0.01$ for BPAD and $p = 0.001$ for BPMF). Similar observations were made when grouping the study individuals according to their MF status in 1996 (data not shown).

3.3. Antibody responses

The antibody subclasses IgG1, IgG2, IgG3, IgG4 and IgE were measured against BPAD and BPMF. Moreover, the ratio between IgG4 and IgE was calculated for each study individual. When grouping the study individuals according to infection status in 1975 and 1996 (Fig. 4), significant differences between the infection categories were observed for IgG4 responses and for IgG4/IgE ratios (Kruskal–Wallis, $p < 0.001$ for both BPAD and BPMF). IgG4 absorbance values were higher for “MF+/CFA+” compared with “MF+/CFA–” (Mann–Whitney, $p < 0.01$ for BPAD and $p < 0.05$ for BPMF) and “MF–/CFA–” (Mann–Whitney, $p = 0.001$ for BPAD and $p < 0.001$ for BPMF). Moreover, IgG4 absorbance values were higher for “MF–/CFA+” compared with “MF+/CFA–” (Mann–Whitney,

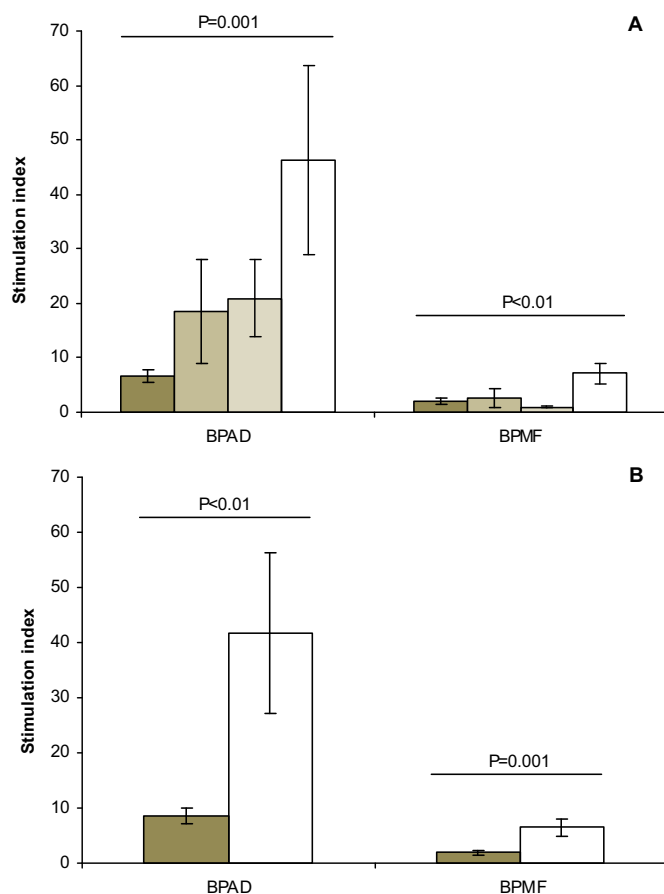


Fig. 3. Stimulation indices from responses of peripheral blood mononuclear cells (PBMC) stimulated with two filarial antigen homogenates (BPAD and BPMF) for individuals who were positive in 1975 and 1996 ("MF+/CFA+"; dark columns), positive in 1975 but negative in 1996 ("MF+/CFA-"; medium dark columns), negative in 1975 but positive in 1996 ("MF-/CFA+"; light columns), and negative in 1975 and 1996 ("MF-/CFA-"; open columns) in (A), and from circulating filarial antigen positive (dark columns) and negative (open columns) individuals in (B). Mean values are presented as columns and standard errors are presented as vertical lines.

$p=0.01$ for BPAD and $p<0.05$ for BPMF) and "MF-/CFA-" (Mann–Whitney, $p<0.05$ for both BPAD and BPMF). Likewise, IgG4/IgE ratios were higher for "MF+/CFA+" compared with "MF+/CFA-" (Mann–Whitney, $p<0.01$ for BPAD) and "MF-/CFA-" (Mann–Whitney, $p<0.001$ for both antigens), and they were higher for "MF-/CFA+" compared with "MF+/CFA-" (Mann–Whitney, $p<0.05$ for BPAD) and "MF-/CFA-" (Mann–Whitney, $p<0.01$ for BPAD and $p=0.001$ for BPMF). For antibody subclasses IgG1, IgG2, IgG3 and IgE differences in absorbance values were not statistically significant when using k-sample (Kruskal–Wallis) tests. However, statistically significant differences between some of the individual infection categories were observed. Thus, IgG1 absorbance values for "MF+/CFA+" were significantly higher than for "MF+/CFA-" (Mann–Whitney, $p<0.05$ for both antigens). The same tendency was observed for IgG2 but the differences were not statistically significant. For IgG3 responses, the absorbance values for "MF+/CFA+" were significantly lower than for "MF-/CFA-" (Mann–Whitney, $p<0.05$ for both antigens). Likewise, for IgE responses, the absorbance values for "MF+/CFA+" were significantly lower than for "MF-/CFA-" (Mann–Whitney, $p<0.05$ for BPAD and $p<0.01$ for BPMF).

When grouping the study individuals according to CFA status in 1996, CFA-negative individuals responded with significantly higher IgG3 and IgE absorbance values (Mann–Whitney, $p<0.05$ for both antibody subclasses and for both antigens) and

significantly lower IgG4 absorbance values (Mann–Whitney, $p<0.001$ for both antigens) than CFA-positive individuals (Fig. 5). Moreover, the IgG4/IgE ratios were significantly higher for CFA positive individuals compared with CFA-negative individuals (Mann–Whitney, $p<0.001$ for both antigens). No statistically significant differences in IgG1 and IgG2 responses were observed between CFA-positive and CFA-negative individuals. Similar observations were made when grouping the study individuals according to their MF status in 1996 (data not shown).

4. Discussion

The present study took advantage of a unique opportunity to make a 22 years follow-up examination of 71 individuals living in four villages in a highly endemic area for *W. bancrofti* infections in north-eastern Tanzania. The villagers were examined for microfilaraemia in 1975 (McMahon et al., 1981). Individuals from two of the villages (Machui and Tawalani) were offered mass treatment with DEC shortly thereafter (Kolstrup et al., 1981). In 1992, individuals from two of the villages (Kwale and Tawalani) were again offered mass treatment with DEC (Meyrowitsch et al., 1996).

The DEC provocative day test was used to diagnose study individuals in the surveys carried out in 1975. In a study of the sensitivity of this test, McMahon et al. (1979b) demonstrated it to be as sensitive for determining infection status as night blood screening. However, diagnosing infection based on MF detection is less sensitive than measuring CFA (Steel et al., 2001) and, as a result, the number of study individuals found MF negative in 1975, and thus belonging to infection categories "MF-/CFA-" or "MF-/CFA+", is somewhat overestimated. Unfortunately, plasma or serum samples for more accurate diagnosis were not available from the surveys carried out in 1975.

In the present study, re-identified individuals were examined for MF, CFA, and humoral and cellular immunoresponsiveness, and the relationship between past and present infection status and current immunoresponsiveness was analysed. Only few chronic manifestations of lymphatic filariasis were observed in the study individuals and statistical differences and conclusions were not affected by these individuals. Significantly more males than females were CFA positive and MF positive, which is a common observation in surveys for lymphatic filariasis. Detailed epidemiological information about infection and disease within the study communities can be found elsewhere (McMahon et al., 1981; Meyrowitsch et al., 1995a).

Interestingly, 61 (85.9%) of the study individuals had the same infection status in 1975 and 1996. Most of these individuals, namely 41 (67.2%), were infection positive in both past and present despite many of them receiving treatment with DEC in 1975 and 1992. Only 10 individuals had a different infection status, namely four who changed from being infection positive to negative and six who changed from being infection negative to positive. This finding is in accordance with those of several other studies (Lammie et al., 1994; Meyrowitsch et al., 1995b, 2004; Steel and Ottesen, 2001; Witt and Ottesen, 2001) and suggests either strong predisposition to infection or that some individuals are more exposed to infection than others. A genetic marker associated with *W. bancrofti* infection has recently been identified (Meyrowitsch et al., 2010).

Signs of progression in the move from being infection positive to negative or vice versa were observed for some of the measured cytokine responses. This was most notable for IL-5 and IFN γ where mean response levels of "MF+/CFA-" and "MF-/CFA+" were intermediary between low response levels "MF+/CFA+" and high response levels of "MF-/CFA-". Such intermediary levels of cellular responsiveness were even more pronounced and statistically significant for cellular stimulation indices (SIs), especially when

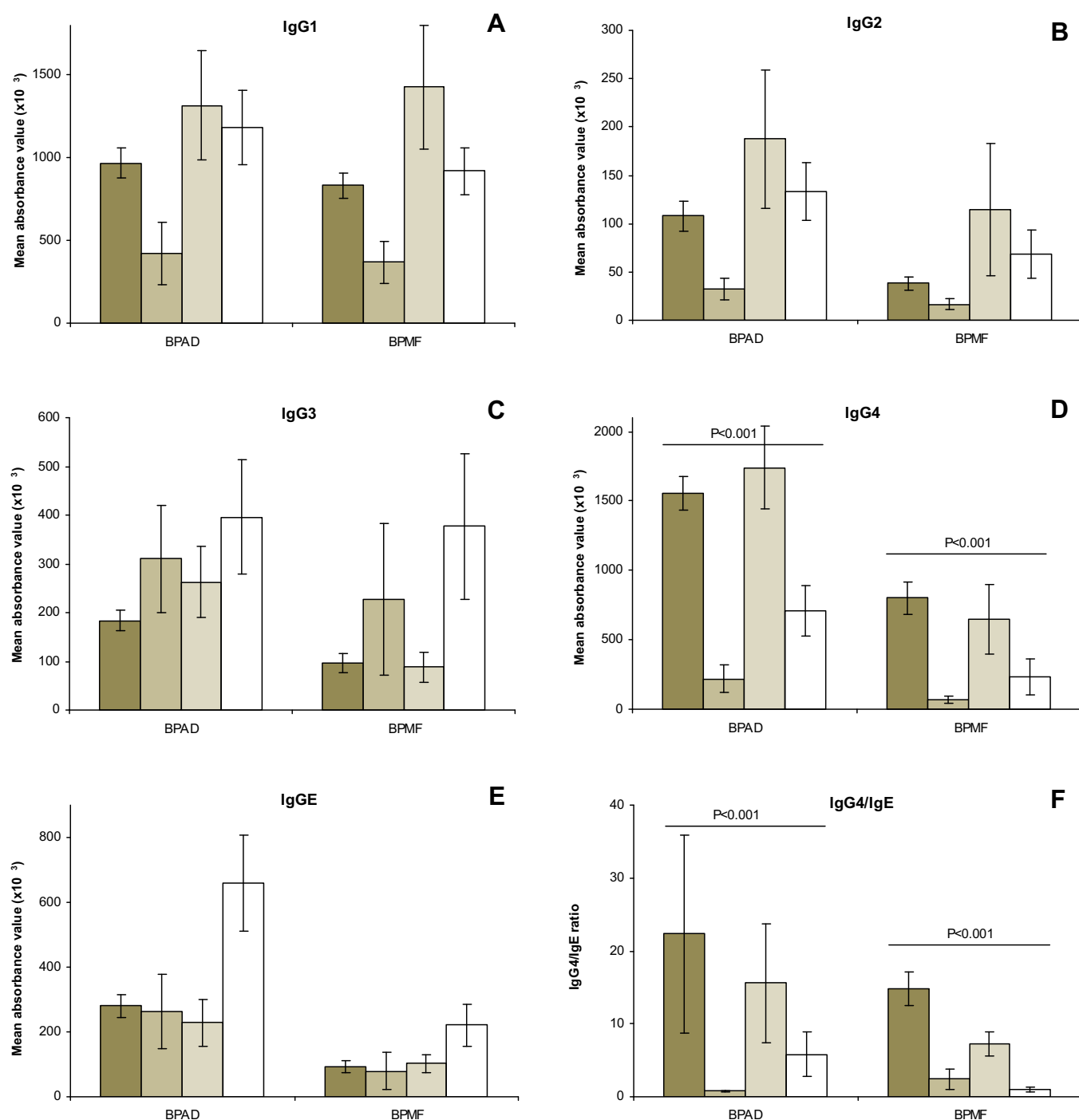


Fig. 4. Absorbance values of IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), and IgE (E), and IgG4/IgE ratios (F), specific to two antigen homogenates (BPAD and BPMF) and measured by ELISA in plasma samples for individuals who were positive in 1975 and 1996 ("MF+/CFA+"; dark columns), positive in 1975 but negative in 1996 ("MF+/CFA-"; medium dark columns), negative in 1975 but positive in 1996 ("MF-/CFA+"; light columns), and negative in 1975 and 1996 ("MF-/CFA-"; open columns). Mean values are presented as columns and standard errors are presented as vertical lines.

stimulated with BPAD. Although study groups are small, this suggests that a transition in immunoresponsiveness takes place when changing infection status, or, more likely, that a maturation of the immunological responsiveness affects the survival of the parasite in its human host. Long-term persistence of cellular hypo-responsiveness to filarial antigens after clearance of microfilaraemia has been shown in other studies (Gopinath et al., 1999; Steel and Ottesen, 2001). Possible mechanisms behind immunological maturation in lymphatic filariasis have previously been discussed (Nielsen et al., 2002a,b).

Substantial individual variation in response levels within the categories was observed. Although based on relatively weak responses, this was most notable for IL-10 where only BPMF stimulated responses that were significantly different for the four infection categories. Interestingly, the patterns of IL-10 responses were opposite the patterns observed for IL-4, IL-5 and IFN γ . Thus, the category of "MF+/CFA+" individuals responded with significantly higher levels of IL-10 and with significantly lower levels of IL-4, IL-5 and IFN γ as compared with the category of "MF-/CFA-" individuals.

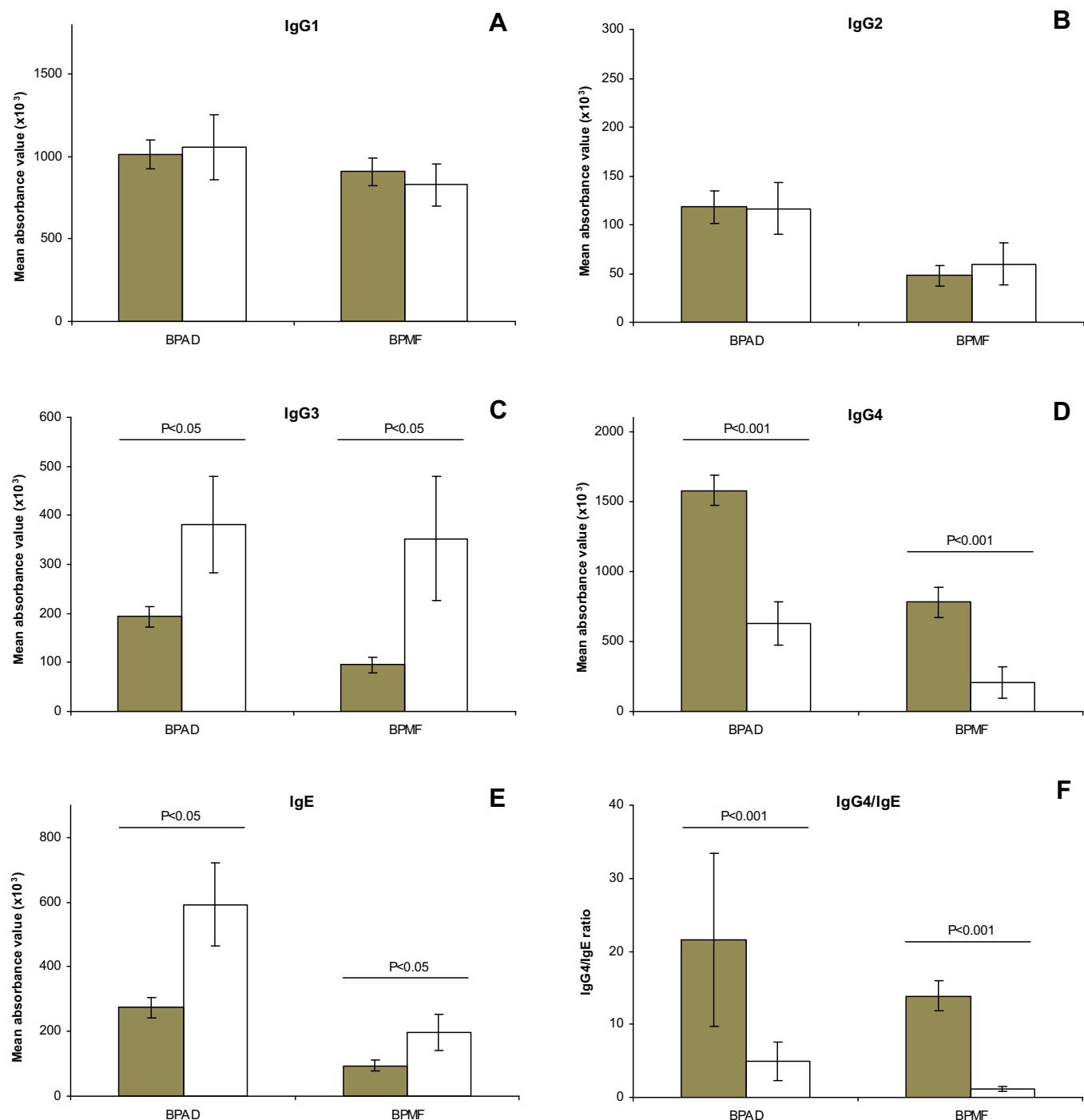


Fig. 5. Absorbance values of IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), and IgE (E), and IgG4/IgE ratios (F), specific to two antigen homogenates (BPAD and BPMF) and measured by ELISA in plasma samples for individuals who were circulating filarial antigen positive (dark columns) and negative (open columns) in 1996. Mean values are presented as columns and standard errors are presented as vertical lines.

Disregarding the infection status of the past and only addressing the infection status of the present revealed a distinct pattern of cytokine responsiveness in which IL-4, IL-5 and IFN γ responses are associated with being infection negative, and where IL-10 responses are associated with being infection positive. The same pattern is observed for the stimulation indices (SIs) where the responsiveness to both antigen homogenates is significantly higher for infection negative compared with infection positive individuals. Similar observations were made by Steel and Ottesen (2001) and by Metenou et al. (2009). However, for IL-4 in particular, other studies have found that cytokine levels either did not differ significantly

between infection positive and negative individuals (De Almeida et al., 1996; Dimock et al., 1996) or were higher in infection positive as compared to infection negative individuals (Yazdanbakhsh et al., 1993). Although the immune response in lymphatic filariasis may be dominated by the downregulation of antigen-specific proliferative responses and IFN γ -producing cells (associated with a Th1 type of response) and the upregulation of IL-4 driven IgG4 antibody responses (associated with a Th2 type of response) some studies suggest that applying this Th1/Th2 paradigm to human infection is too simplistic (Doetze et al., 1997; Sartono et al., 1997; Lawrence and Devaney, 2001). Studies in different inbred strains of mice have

shown that L3 stage larvae and, possibly, adult parasites, are strong inducers of IL-4 and IgG4 responses whereas MF induce IFN γ and IL-5 responses (Lawrence and Devaney, 2001). Immune responses determined by stimulating PBMC with parasite antigens may thus reflect a complex balance between different stages of filarial parasites present in the human host. This may also explain the lack of consistency in IL-4 responses observed in infected individuals in different studies. Our findings suggest that IL-4, IL-5 and IFN γ could play a role in parasite clearance and/or that IL-10 could be involved in parasite protection.

A very high immunological responsiveness to filarial antigens was observed among individuals belonging to the “MF–/CFA–” category despite being infection free, possibly for decades. This was observed for individual cytokine responses, mainly IL-5, IL-10 and IFN γ , but also, and more pronouncedly, for the stimulation indices (SIs), where the cellular responsiveness to both antigen homogenates was significantly higher for the “MF–/CFA–” category of individuals as compared with the other infection categories. This emphasises the capacity of the immune system to respond to parasite antigens for extensive periods of time, possibly maintained by memory cells and regular boosting by infective third stage larvae.

Two different types of antigen homogenates (BPAD and BPFM) were applied to analyse for antigen-induced differences in responsiveness. BPAD included adult female worms with microfilariae in their uteri. In general, the two homogenates resulted in different cytokine response levels but only limited differences in response patterns between human infection groups. Clearly, antigenic epitopes within the applied crude homogenates both share similar features and differ in antigenicity. These findings suggest that strong immune responses are induced by different antigens in different stages of parasites, each of which may contribute to parasite clearance or susceptibility. This should be considered in the interpretation of findings from studies in which only one antigen preparation, or a recombinant antigen such as BM14 (Lammie et al., 2004), are used.

The most pronounced differences in specific antibody responses between infection categories were observed for IgG4, where “MF+/CFA+” and “MF–/CFA+” responded with significantly higher absorbance values than “MF+/CFA–” and “MF–/CFA–”. The opposite tendency was observed for specific IgG3 and IgE, where individuals from the “MF–/CFA–” category responded with significantly higher absorbance values than individuals from the “MF+/CFA+” category. Similar, and statistically significant, differences between infection positive and negative individuals were observed when only grouping the study individuals according to CFA status in 1996. This dichotomy between IgG4 on the one hand and IgG3 and IgE on the other resulted in pronounced IgG4/IgE and IgG4/IgG3 ratios (the latter not shown), high for infection positive individuals and low for infection negative individuals, which has also been observed and discussed in other studies as a sign of the involvement of specific IgG3 and IgE in parasite clearance and of specific IgG4 in parasite protection (Maizels et al., 1995; Nielsen et al., 2002b; Jaoko et al., 2006).

As a result of some underestimation of the number of infection positive individuals when diagnosed only on the basis of MF examinations (as done in 1975) it can be speculated that most, if not all, of those who were found MF negative in 1975 and CFA positive in 1996 (“MF–/CFA+”) were actually infection positive in 1975 and rightfully should belong to the “MF+/CFA+” category. This is further supported by the epidemiological findings from a 26-year follow up study carried out within the same population (Meyrowitsch et al., 2004), confirming that infection status does not change much over decades even when DEC treatment is provided several times in between. Due to the low number of individuals in the “MF–/CFA+” category, the inclusion of these individuals into

the “MF+/CFA+” category did not significantly change the findings of this study. The findings thus suggest that some people are more disposed to infection with bancroftian filariasis than others and that the susceptibility is largely unaffected by DEC treatment. The findings also suggest that specific cellular and antibody responses are more related to current than past infection status, and that IL-4, IL-5, IFN γ , specific IgG3 and IgE could be involved in parasite clearance, whereas IL-10 and specific IgG4 could be involved in parasite protection.

Funding

The research was funded by the Council for Development Research under the Danish International Development Agency (Danida), and DBL – Centre for Health Research and Development in Denmark.

Ethical approval

Research and ethical clearance was granted by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania. Individuals were only included in the study after oral informed consent had been obtained. The study conformed to all aspects of the Helsinki Declaration.

Acknowledgements

The study participants are gratefully acknowledged for their collaboration, and the technical staff at Bombo Research Station (the late Joyce Kivugo as well as Charles Guzo, Zaina Maumba and Mwanaidi Bagabuje) and DBL – Centre for Health Research and Development (Mette Lund and Pernille Strøm) is thanked for valuable technical assistance in the laboratories in Tanzania and Denmark, respectively. Lars Hviid and Thor Theander, Center for Medical Parasitology, Faculty of Health Sciences, University of Copenhagen, Denmark are thanked for providing valuable technical advice and allowing the use of their facilities. The paper is published with the permission of the Director of the National Institute for Medical Research, Tanzania.

References

- Bloch, P., Simonsen, P.E., Vennervald, B.J., 1993. The antibody response to *Dracunculus medinensis* in an endemic human population of northern Ghana. *J. Helminthol.* 67, 48, 37.
- Casley-Smith, J.R., Jamal, S., Casley-Smith, J., 1993. Reduction of filaritic lymphoedema and elephantiasis by 5,6 benzo-a-pyrone (coumarin), and the effects of diethylcarbamazine (DEC). *Ann. Trop. Med. Parasitol.* 87, 247–258.
- De Almeida, A.B., Silva, M.C.M., Maciel, M.A., Freedman, D.O., 1996. The presence or absence of active infection, not clinical status, is most closely associated with cytokine responses in lymphatic filariasis. *J. Infect. Dis.* 173, 1453–1459.
- Dimock, K.A., Eberhard, M.L., Lammie, P.J., 1996. Th-1 like antifilarial responses predominate in antigen-negative persons. *Infect. Immun.* 64, 2962–2967.
- Doetze, A., Erttmann, K.D., Gallin, M.Y., Fleischer, B., Hoerauf, A., 1997. Production of both IFN-gamma and IL-5 by *Onchocerca volvulus* S1 antigen-specific CD4+ T cells from putatively immune individuals. *Int. Immunol.* 9, 721–729.
- Gopinath, R., Hanna, L.E., Kumaraswami, V., Pillai, S.V.P., Kavitha, V., Vijayasekaran, V., Rajasekharan, A., Nutman, T.B., 1999. Long-term persistence of cellular hyporesponsiveness to filarial antigens after clearance of microfilaremia. *Am. J. Trop. Med. Hyg.* 60, 848–853.
- Hitch, W.L., Lammie, P.J., Walker, E.M., Hightower, A.W., Eberhard, M.L., 1991. Antifilarial cellular responses detected in Haitian paediatric population by use of microblastogenesis assay. *J. Infect. Dis.* 164, 811–813.
- Hviid, L., Albeck, G., Hansen, B., Theander, T.G., Talbot, A., 1993. A new portable device for automatic controlled-gradient cryopreservation of blood mononuclear cells. *J. Immunol. Method* 157, 135–142.
- Jaoko, W.G., Simonsen, P.E., Meyrowitsch, D.W., Estambale, B.B.A., Malecela-Lazaro, M.N., Michael, E., 2006. Filarial-specific antibody response in East African bancroftian filariasis: effects of host infection, clinical disease, and filarial endemicity. *Am. J. Trop. Med. Hyg.* 75, 97–107.
- Jaoko, W.G., Michael, E., Meyrowitsch, D.W., Estambale, B.B.A., Malecela, M.N., Simonsen, P.E., 2007. Immunoepidemiology of *Wuchereria bancrofti* infection:

- parasite transmission intensity, filaria-specific antibodies, and host immunity in two East African communities. *Infect. Immun.* 75, 5661–5662.
- Kolstrup, N., McMahon, J.E., Magayuka, S.A., Mosha, F.W., Bushrod, F.M., Bryan, J.H., 1981. Control measures against bancroftian filariasis in coastal villages in Tanzania. *Ann. Trop. Med. Parasitol.* 75, 433–451.
- Lammie, P.J., Hightower, A.W., Eberhard, M.L., 1994. Age-specific prevalence of antigenemia in a *Wuchereria bancrofti*-exposed population. *Am. J. Trop. Med. Hyg.* 5, 348–355.
- Lammie, P.J., Weil, G., Noordin, R., Kaliraj, P., Steel, C., Goodman, D., Lakshmikanthan, V.B., Ottesen, E., 2004. Recombinant antigen-based antibody assay for the diagnosis and surveillance of lymphatic filariasis—a multicenter trial. *Filaria J.* 3, 9.
- Lawrence, R.A., Devaney, E., 2001. Lymphatic filariasis: parallels between the immunology of infection in humans and mice. *Parasite Immunol.* 23, 353–361.
- Maizels, R.M., Sarton, E., Kurniawan, A., Partono, F., Selkirk, M.E., Yazdanbakhsh, M., 1995. T-cell activation and the balance of antibody isotypes in human lymphatic filariasis. *Parasitol. Today* 11, 50–56.
- McMahon, J.E., Marshall, T.F.C., Vaughan, J.P., Abaru, D.E., 1979a. Bancroftian filariasis: a comparison of microfilariae counting techniques using counting chamber, standard slide and membrane (nucleopore) filtration. *Ann. Trop. Med. Parasitol.* 73, 457–464.
- McMahon, J.E., Marshall, T.F.C., Vaughan, J.P., Kolstrup, N., 1979b. Tanzania Filariasis Project: a provocative day test with diethylcarbamazine for the detection of *Wuchereria bancrofti* in the blood. *Bull. WHO* 57, 759–765.
- McMahon, J.E., Magayuka, S.A., Kolstrup, N., Mosha, F.W., Bushrod, F.M., Abaru, D.E., Bryan, J.H., 1981. Studies on the transmission and prevalence of bancroftian filariasis in four coastal villages of Tanzania. *Ann. Trop. Med. Parasitol.* 75, 415–431.
- Metenou, S., Dembele, B., Konate, S., Dolo, H., Coulibaly, S.Y., Coulibaly, Y.I., Diallo, A.A., Soumaoro, L., Coulibaly, M.E., Sanogo, D., Doumbia, S.S., Wagner, M., Traore, S.F., Klion, A., Mahanty, S., Nutman, T.B., 2009. Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filarial/malaria-coinfected population. *J. Immunol.* 183, 916–924.
- Meyrowitsch, D.W., Simonsen, P.E., Makunde, W.H., 1995a. Bancroftian filariasis: analysis of infection and disease in five endemic communities of north-eastern Tanzania. *Ann. Trop. Med. Parasitol.* 89, 653–663.
- Meyrowitsch, D.W., Simonsen, P.E., Makunde, W.H., 1995b. A 16-year follow-up study on bancroftian filariasis in three communities of north-eastern Tanzania. *Ann. Trop. Med. Parasitol.* 89, 665–675.
- Meyrowitsch, D.W., Simonsen, P.E., Makunde, W.H., 1996. Mass diethylcarbamazine chemotherapy for control of bancroftian filariasis: comparative efficacy of standard treatment and two semi-annual single-dose treatments. *Trans. R. Soc. Trop. Med. Hyg.* 90, 68–73.
- Meyrowitsch, D.W., Simonsen, P.E., Magesa, S.M., 2004. A 26-year follow-up of bancroftian filariasis in two communities in north-eastern Tanzania. *Ann. Trop. Med. Parasitol.* 98, 155–169.
- Meyrowitsch, D.W., Simonsen, P.E., Garred, P., Dalgaard, M., Magesa, S.M., Alifrangis, M., 2010. Association between mannose-binding lectin polymorphisms and *Wuchereria bancrofti* infection in two communities in north-eastern Tanzania. *Am. J. Trop. Med. Hyg.* 82, 115–120.
- Nielsen, N., Bloch, P., Simonsen, P.E., 2002a. Filariasis specific immune responses in relation to lymphoedema grade and infection status I. Cellular responses. *Trans. R. Soc. Trop. Med. Hyg.* 96, 446–452.
- Nielsen, N., Bloch, P., Simonsen, P.E., 2002b. Filariasis specific immune responses in relation to lymphoedema grade and infection status II. Humoral responses. *Trans. R. Soc. Trop. Med. Hyg.* 96, 453–458.
- Ottesen, E.A., Weller, P.F., Heck, L., 1977. Specific cellular immune unresponsiveness in human filariasis. *Immunology* 33, 413–421.
- Sarton, E., Kruize, Y.C., Kurniawan, A., Maizels, R.M., Yazdanbakhsh, M., 1997. Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age. *J. Infect. Dis.* 175, 1276–1280.
- Simonsen, P.E., Lemnge, M.M., Msangeni, H.A., Jakobsen, P.H., Bygbjerg, I.C., 1996. Bancroftian filariasis: the patterns of filarial-specific immunoglobulin G1 (IgG1) IgG4, and circulating antigens in an endemic community of northeastern Tanzania. *Am. J. Trop. Med. Hyg.* 55, 69–75.
- Simonsen, P.E., Meyrowitsch, D.W., Jaoko, W.G., Malecela, M.N., Mukoko, D., Pedersen, E.M., Ouma, J.H., Rwegoshora, R.T., Masese, N., Magnussen, P., Estambale, B.B.A., Michael, E., 2002. Bancroftian filariasis infection, disease, and specific antibody response patterns in a high and a low endemicity community in East Africa. *Am. J. Trop. Med. Hyg.* 66, 550–559.
- Steel, C., Ottesen, E.A., 2001. Evolution of immunologic responsiveness of persons living in an area of endemic bancroftian filariasis: a 17-year follow-up. *J. Infect. Dis.* 184, 73–79.
- Steel, C., Ottesen, E.A., Weller, P.F., Nutman, T.B., 2001. Worm burden and host responsiveness in *Wuchereria bancrofti* infection: use of antigen detection to refine earlier assessments from the South Pacific. *Am. J. Trop. Med. Hyg.* 65, 498–503.
- Witt, C., Ottesen, E.A., 2001. Lymphatic filariasis: an infection of childhood. *Trop. Med. Int. Health* 6, 582–606.
- Yazdanbakhsh, M., Sarton, E., Kruize, Y.C.M., Kurniawan, A., van der Pouw-Kraan, T., van der Meide, P.H., Selkirk, M.E., Partono, F., Hintzen, R.Q., van Lier, R.A.W., Maizels, R.M., 1993. Elevated levels of T cell activation antigen CD27 and increased interleukin-4 production in human lymphatic filariasis. *Eur. J. Immunol.* 23, 3312–3317.