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Filarial antibody responses in *Wuchereria bancrofti* transmission area are related to parasitological but not clinical status

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SUMMARY

In Wuchereria bancrofti *transmission areas, three groups of individuals have been identified, according to the presence or absence of microfilariae or adult worm derived molecules in the blood compartment. These groups likely reflect individuals with different permissivity/resistance to the complete development of* W. bancrofti *cycle. The profile of filarial-specific immunoglobulins was analysed in* W. bancrofti*-exposed individuals in French Polynesia, according to the presence or absence of microfilariae (Mf) and adult worms, measured by Og4C3 circulating antigen. Individuals harbouring adult worms, have higher filarial-specific IgG4 but lower IgG3 and IgE levels, than adult worm-free individuals, independently of the presence of Mf. Low filarial-specific IgG1 and IgG2 levels were associated with the presence of Mf but independent of the presence/absence of adult worms. The filarial antibody responses were associated with the parasitological status of individuals but not with clinical symptoms such as hydroceles or limb lymphangitis or elephantiasis. The reduction of filarial-specific immunoglobulin levels was higher after treatment with diethylcarbamazine than ivermectin, which likely reflects the better effect of the former on* W. bancrofti *adult worms. However, reduction of antibody levels was also observed in Mf-and adult worm-negative individuals. This could be due to the overall reduction of* W. bancrofti *transmission in the island where this study took place.*

Keywords *bancroftian filariasis, immunoglobulins,* W. bancrofti*, adult worm antigen, diethylcarbamazine, ivermectin*

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INTRODUCTION

Epidemiological data indicate that lymphatic filariasis affects 120 million individuals worldwide, 90% infected by *Wuchereria bancrofti* and 10% by *Brugia malayi* (Ottesen & Ramachandran 1995). These data include microfilaraemic individuals and individuals exhibiting overt clinical symptoms such as hydrocele, adenolymphangitis or elephantiasis. In the last decade, immunodiagnostic tools have been developed to monitor *W. bancrofti* adult worm burden, using the monoclonal antibodies AD 12 (Weil *et al*. 1987) or Og4C3 (More & Copeman 1990) which both specifically recognize *W. bancrofti* adult worm circulating filarial antigen(s) (CFA) (Chanteau *et al*. 1994, Weil *et al*. 1996). Cross sectional surveys, carried out in different geographical areas have shown that a large proportion (18–40%) of microfilaria (Mf)-negative individuals are CFA-positive, thus likely harbouring adult worms (Nicolas 1997, for a review). This means that the prevalence of adult worm parasitism is approximately twice the microfilarial prevalence. Therefore, in endemic areas, the individuals subjected to *W. bancrofti* transmission, as revealed by the presence of antifilarial antibodies in their blood, can be classified into three groups of individuals according to their parasitological status. These groups which likely reflect the different permissivity/resistance of individuals to the complete development of *W. bancrofti* cycle are: (i) Mf (and adult worm) carriers, contributing to the transmission of *W. bancrofti (*ii) Mf-negative but CFA-positive individuals, presumed to harbour adult worms only, and (iii) individuals who are Mf and CFA negative, and presumed to be able to prevent the development of adult worms from earlier L3 or L4 stages. These latter can be considered as 'resistant' to *W. bancrofti* development. In clinical filarial cases, CFA prevalence is also higher than Mf prevalence (Chanteau *et al*. 1994, Lammie *et al*. 1994, Nicolas *et al*. 1997).

Antibody responses in lymphatic filariasis have been mainly analysed in microfilaraemics in comparison to individuals suffering chronic lymphatic pathology. Persons permissive to successful parasite development

(microfilaraemics) were found to have lower levels of IgE and higher IgG4, whereas chronic pathology patients with low or no Mf, had higher IgE/IgG4 ratios (Hussain *et al*. 1981, Hitch *et al*. 1991, Kurniawan *et al*. 1993). The development of non-invasive diagnostic tools for monitoring the presence of adult worms allows the investigation of the immunological status of 'resistant' individuals but also of individuals harbouring only adult worms. The reasons why some individuals harbour adult worms in the absence of Mf are unknown. Possible reasons include Mf densities below the detection limit, lack of mating or fecundity of adult worms, or an efficient host immune response against Mf. Therefore we have investigated filarial antibody responses of amicrofilaraemics, according to their parasitological but also clinical status. In addition, we have followed the evolution of filarial antibody responses following chemotherapy with diethylcarbamazine (DEC) or ivermectin, as adult worms are more susceptible to the former than to the latter (Nicolas *et al*. 1997).

MATERIALS AND METHODS

Study site and populations

This study was conducted in Tahaa island, French Polynesia, where a large scale chemotherapy trial was conducted to evaluate the effect of annual treatments of ivermectin or DEC, either alone or in combination, on microfilaraemia and adult worm burden (Moulia-Pelat *et al*. 1995, Nicolas *et al*. 1997). In February 1994 and February 1995, this island of approximately 4000 inhabitants, was divided into four geographical areas and all inhabitants aged > two years were treated with a single annual dose of one of the four treatments: DEC at 6 mg/kg (DEC6), ivermectin at 400 μg/kg (IVR), ivermectin at 400 μg/kg plus DEC at 6 mg/kg or at 3 mg/kg. The population had been left untreated since 1982 and transmission was ongoing as assessed by entomological indices and to high prevalence of antifilarial antibodies which were detected in 78% of the population (data not shown).

Venous blood was collected from adult individuals (> 20 years) to determine their microfilaraemia by membrane filtration of 1 ml blood (Moulia-Pelat *et al*. 1995) and CFA levels measured using the Og4C3 antigen assay (JCU Tropical Biotechnologies Ltd) as described in Nicolas *et al*. (1997). Prior to treatment, 22% of adult individuals had microfilariae (Moulia-Pelat *et al*. 1995) and 46% had detectable CFA (Nicolas *et al*. 1997).

Blood was collected prior to the first treatment (month 0) and the second treatment (month 12) and one year after the second treatment (month 24). Individuals were examined clinically for the presence of adenolymphangitis, scrotal hydrocele or elephantiasis. Antifilarial immunoglobulin responses were analysed on sera kept frozen from a subset of individuals treated with either DEC or ivermectin, choosen randomly among asymptomatic individuals (Table 1). All clinically affected individuals, treated with either DEC or ivermectin alone, were enrolled in the study. The subgroups of individuals who were be treated by DEC or ivermectin were matched for Mf and CFA level (data not shown).

Adult worm antigens

A protein extract was prepared from *B. malayi* adult worms sonicated in PBS and the protein concentration was determined, after centrifugation, by the BCA Protein Assay (Pierce).

Enzyme-linked immunosorbent assays with human sera

The levels of filarial-specific IgG isotypes were determined in endemic individuals as follows. Microtitre plates (Luxlon@ M29 LSE, CEB) were coated with 0·15 μg/well of extract in 0·06 M carbonate buffer pH 9·6. Plates were incubated for two h at 37°C then stored overnight at 4°C, and blocked before use in ELISA by a one-h incubation in 0·1% Tween 20 and 10% goat serum in PBS. After washing with 0·1% Tween 20 in PBS, two-fold dilutions of patients sera

Table 1 Characteristics of the parasitological status of the groups of asymptomatic individuals through the monitoring of microfilariae (Mf) and adult worm circulating filarial antigen (CFA)

Group status	I Mf+CFA+	II Mf−CF+	III Mf−CFA−
n	49	53	56
No. female/no. male	26/23	26/27	33/23
Median age, years (range)	48·6 (25–75)	42·7 (21–74)	43·6 (21–75)
Mf/ml* (range)	1070 (221–7650)	0	0
CFA*, Og4C3 (units (range)	5577 (1406–21844)	705 (135–3141)	0

* Data are geometric mean levels.

diluted in the same diluant (ELISA diluent) were added in duplicate and incubated 1·5 h at 37°C. Plates were washed before addition of murine monoclonal antihuman IgG Fc (clone HP 6017, Sigma) diluted 1/1000. After two h incubation at room temperature (≈ 25°C), all wells were washed and then incubated with a 1/2000 HRP-conjugated rabbit antimouse reagent (Biosys) for two h at room temperature. After washing, o-phenylenediamine substrate (OPDA, Sigma) was added to the wells and readings at 492 nm were taken after 30 min. The antifilarial IgG level of each serum was determined by comparison with a standard curve established from a pool of serum from ten microfilaraemic Polynesian individuals. A negative control serum, made of a serum pool of five nonendemic European individuals, was also added to each plate. The sera collected from a given individual at three times of sampling were analysed on the same plate to avoid interplate variation. The protocol for determination of antifilarial isotypes was similar, using the following isotype-specific mouse monoclonal antibodies (Sigma): IgG1, clone HP-6001; IgG2, clone HP-6002; IgG3, clone HP-6047 and IgG4, clone HP-6023, all used at a dilution of 1/2000 except IgG1 which was used at 1/1000. Specific antifilarial IgE levels were determined using a similar protocol, with monoclonal antihuman IgE antibody (Sigma) used at a dilution of 1/5000.

Effect of drug treatments on antifilarial antibody levels

Residual antibody level after one (month 12) or two annual treatments (month 24) was calculated as R (%) = (level after treatment/level prior treatment) × 100.

Statistical analysis

Data were subjected to statistical analyses by the nonparametric Spearman Rank Correlation test. A value $P < 0{\cdot}05$ was taken to be significant. Filaria-specific immunoglobulin levels were transformed to log values before statistical testing. The percentage of Mf or CFA reduction between the groups were compared with Chi-square test. A multivariate unconditional logistic regression model was developed using the logarithms of microfilaraemia, antigen level and antibody levels to predict the likelihood of clinical status or infection. A reduced model was obtained by deleting nonsignificant variables from the model.

RESULTS

Filarial parasitism and IgG isotypes in asymptomatic individuals prior treatment

The three groups of asymptomatic individuals analysed prior treatment are described in Table 1. Microfilaraemic individuals (group I) had a mean of 1070 Mf/ml and 5577 CFA (Og4C3 antigen) u/ml, while mean CFA level in amicrofilaraemic adult worm carriers (group II) was only 705 CFA units/mL, i.e. 7·8 times less than in microfilaraemics. The three groups were matched for age and sex ratio. Filarial antibody levels prior to treatment are illustrated in Figure 1(a). Mean filarial-specific IgG1 and IgG2 levels were significantly lower (7–10 times, respectively, $P < 0{\cdot}02$) in microfilaraemics than in amicrofilaraemic individuals, whether or not CFA positive. The mean filarial-specific IgG3 level was 4–8 higher in unparasitized individuals than in parasitized individuals, although the differences were not significant. Mean IgG4 level was significantly higher in individuals harbouring adult worms only than in the other two groups ($P < 0{\cdot}02$), and was higher in microfilaraemics than in Mf-and adult worm-negative individuals (although the difference was not significant). In contrast, the mean IgE level was significantly ($P < 0{\cdot}01$) lower (≈ 5 times) in parasitized individuals (Mf-and/or CFA-positive) than in Mf-and adult worm-negative individuals. The ratio of IgG4/IgE was very similar in both groups of parasitized individuals and significantly higher (4 times, $P < 0{\cdot}01$) than in Mf-and adult worm-negative individuals. The subgroups of individuals treated by DEC or ivermectin were matched for isotype levels (data not shown).

CFA level in microfilaraemics was correlated with Mf density ($r = 0{\cdot}59$), and there was a slight association between CFA level and IgG1 ($r = 0{\cdot}236$, $n = 102$, $P = 0{\cdot}55$) or IgG2 ($r = 0{\cdot}210$, $n = 102$, $P = 0{\cdot}51$). No correlation were found between Mf levels and isotype levels.

Filarial parasitism and IgG isotypes in clinical groups

Mf and CFA levels and antifilarial isotypes were determined in 36 individuals suffering scrotal hydrocele (HYD), lymphangitis (LYM) or elephantiasis (ELE) (Table 2). Most of the men with hydrocele were parasitized by Mf and exhibit high CFA levels. All microfilaraemics were CFA positive and in addition there were five individuals (three elephantiasis and two lymphangitis) who were Mf negative and CFA positive. In contrast, most individuals suffering lymphangitis or elephantiasis were unparasitized or poorly parasitized with low levels of Mf and/or adult worm antigen. The distribution of antifilarial antibodies (Table 2) shows two different patterns between individuals with hydroceles and individuals with lymphangitis or elephantiasis. Individuals with hydroceles had significantly lower levels of IgG1, IgG2, IgG3 and IgE and higher levels of IgG4 and IgG4/IgE ratio than individuals with lymphangitis or elephantiasis. Individuals with hydroceles and lymphangitis/elephantiasis differ mainly by their parasitological status and when

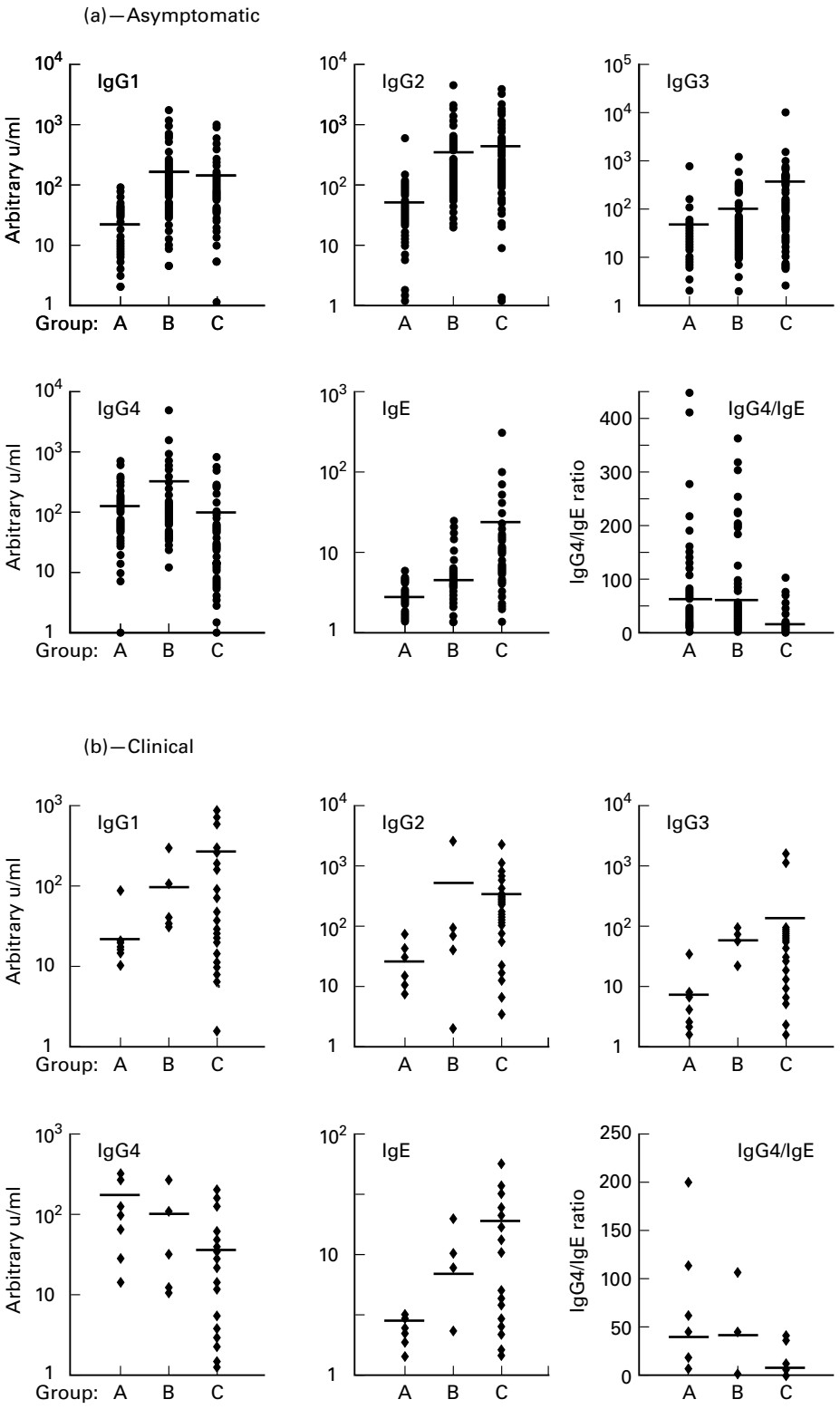

Table 2 Characteristics of the parasitological status and antifilarial antibody responses of clinical individuals

Clinical symptom	HYD	LYM	ELE
n	6	13	17
No. female/no. male	0/6	1/12	10/7
Median age, years (range)	59·7 (33–78)	57·9 (30–79)	61·5 (30–82)
Mf positive (%)	83·3	15·4	5·8
Mf/ml*	187	2·4	1·4
CFA positive (%)	83·3	30·8	23·5
CFA* Og4C3 units	991	8	6
Antifilarial isotype levels*			
IgG1	17·7†,‡	63·5†	60·4‡
IgG2	28·2†,‡	89·5†	117·5‡
IgG3	8·8†,‡	20·3†	27·0‡
IgG4	72·2†	28·6	16·2‡
IgE	1·9†,‡	6·3†	4·6‡
IgG4/IgE	38·0†,‡	4·5†	3·5‡

* Data are geometric mean levels. †, ‡: $P < 0{\cdot}05$; HYD: hydrocele, LYM: limb lymphangitis, ELE: elephantiasis.

individuals were classified according to Mf and CFA levels instead of clinical symptoms, the antifilarial antibody response showed a similar pattern in symptomatic and in asymptomatic individuals (Figure 1b). Furthermore, a logistic regression model showed that levels of isotypes do not allow the prediction of whether a given individual has clinical symptoms or not. The lowest mean filarial-specific IgG1, IgG3 and IgE levels were observed in microfilaraemics and the highest in Mf-and adult worm-negative individuals, although a significant difference ($P < 0{\cdot}01$) was only found for IgG1 levels. Mean filarial-specific IgG2 level was much lower in microfilaraemics than in amicrofilaraemics, whether or not parasitized by adult worms (15–22 times less), and a predictive model showed that there was a linear regression between Mf density and IgG2 level ($\text{Mf} = 587 - 0{\cdot}34\ \text{IgG2}$, $P < 0{\cdot}008$).

As the antibody responses showed a similar pattern in clinical and in asymptomatic individuals, clinical and asymptomatic individuals were combined and the effects of filaricidal treatments on parasitism and isotype levels was assessed according to the parasitological status independently.

Effect of the filaricidal drugs on MF and CFA levels

As observed in previous studies (Moulia-Pelat *et al*. 1995, Nicolas *et al*. 1997), both DEC and ivermectin strongly reduced microfilaraemia after one (data not shown) or two annual treatments, although the difference was not significant (Table 3). By contrast, DEC had a significantly better effect than ivermectin on adult worm burden reduction, monitored by CFA levels, whether the individuals were microfilaraemic ($P < 0{\cdot}02$) or amicrofilaraemic ($P < 0{\cdot}001$) (Table 3).

Effect of filaricidal drug treatment on antifilarial antibody levels

Antifilarial antibody levels were measured in all individuals after one (month 12) and two annual (month 24) treatments with DEC or ivermectin. A multivariate analysis was carried out to measure the effect of parasitological status or drug on evolution of antibody levels. Globally all antibody levels were gradually reduced after one treatment (not shown) and two treatments (Figure 2). The highest reductions of antibody levels were observed with IgG1 and IgG4, while IgE levels remained rather stable, even after two treatments (Figure 2). However, for a given isotype, there was no significant effect of the parasitological status in reduction of the level. Reduction of levels was observed in Mf-and CFA-negative individuals as well as in parasitized individuals (Figure 2a). In contrast, there was a higher reduction of all antibody levels in individuals treated with DEC than in those treated with ivermectin (Figure 2b), and this trend was significant for IgG2 and IgG4 levels.

Figure 1 Levels of antibody responses to a *B. malayi* adult worm extract in asymptomatic individuals (a) and individuals with clinical symptoms (b), prior treatment, according to parasitological status. Groups: A: microfilaraemics (Mf and CFA positive); B: Mf negative and CFA positive individuals; C: Mf and CFA negative individuals. Horizontal bars indicate geometric means.

Table 3 Effect of two annual treatments with either ivermectin (IVR) or DEC on *W. bancrofti* burden

Parasitic status	Mf+CFA+		Mf-CFA+	
Treatment	IVR	DEC	IVR	DEC
% residual microfilaraemia	3·8	1·5	–	–
% residual CFA level	50·3*	35·1*	78·3**	13·1**

* $P < 0{\cdot}02$, ** $P < 0{\cdot}001$.

DISCUSSION

So far, the immune responses triggered by filariae in human populations have been mainly compared between microfilaraemics, clinically symptomatic individuals and 'putatively immune' individuals (Kurniawan *et al*. 1993). The recent development of immunodiagnostic tools for monitoring the presence of adult worms in humans, through the detection of circulating antigens, has led to a better characterization of so-called 'putatively immune' individuals in *W. bancrofti* endemic populations. Such tools are still lacking for *B. malayi* infection but one candidate parasite molecule has been identified (Langy *et al*. 1998). Therefore three groups of individuals can be defined in *W. bancrofti* transmission areas, such as Polynesia. Beside microfilaraemic individuals (who harbour adult worms as well), a large proportion of individuals harbour adult worms (CFA positive) but no Mf. Finally a large proportion of individuals have neither adult worms nor Mf. These latter are subjected to *W. bancrofti* L3 transmission, as they have antifilarial antibodies and are living in a transmission area for several years, and are likely able to prevent the development of adult worms from L3 or L4 larvae. They can be considered as 'resistant' to the development of *W. bancrofti*. Amicrofilaraemic individuals, previously considered as 'putatively immune' must be divided into two groups of individuals, harbouring adult worms or not, hence with different immune responses to filarial parasites. The characterization of these three groups of individuals should help in determining the reasons why different individuals are able to block the development of *W. bancrofti* at different stages of its cycle in humans.

This study shows that the three groups have in fact three different patterns of antifilarial antibody responses. Individuals harbouring adult worms (independantly of the presence of Mf) have high IgG4 and low IgE levels, hence a high IgG4/IgE ratio, which confirms data observed in other endemic populations in Haïti (Dimock *et al*. 1996) and in Tanzania (Simonsen *et al*. 1996). In addition, low levels of IgG3 are associated with the presence of adult worms and low levels of IgG1 and IgG2 with Mf infection. The antibody responses of individuals who harbour only adult worms differ from microfilaraemics in IgG1, IgG2 and IgG4 levels, and from unparasitized individuals in IgG3, IgG4 and IgE (and ratio IgG4/IgE). These data, at the humoral level, confirm data obtained in Haïti showing that elevated antifilarial IgG4 level was associated with the presence of adult worm and microfilaraemia was associated with decreased antifilarial IgG2 (Dimock *et al*. 1996). Our data strengthen data on cellular responses showing that a Th1-like response dominates in unparasitized individuals and a Th2-like in individuals parasitized by adult worms and/or Mf (Dimock *et al*. 1996, Steel *et al*. 1996).

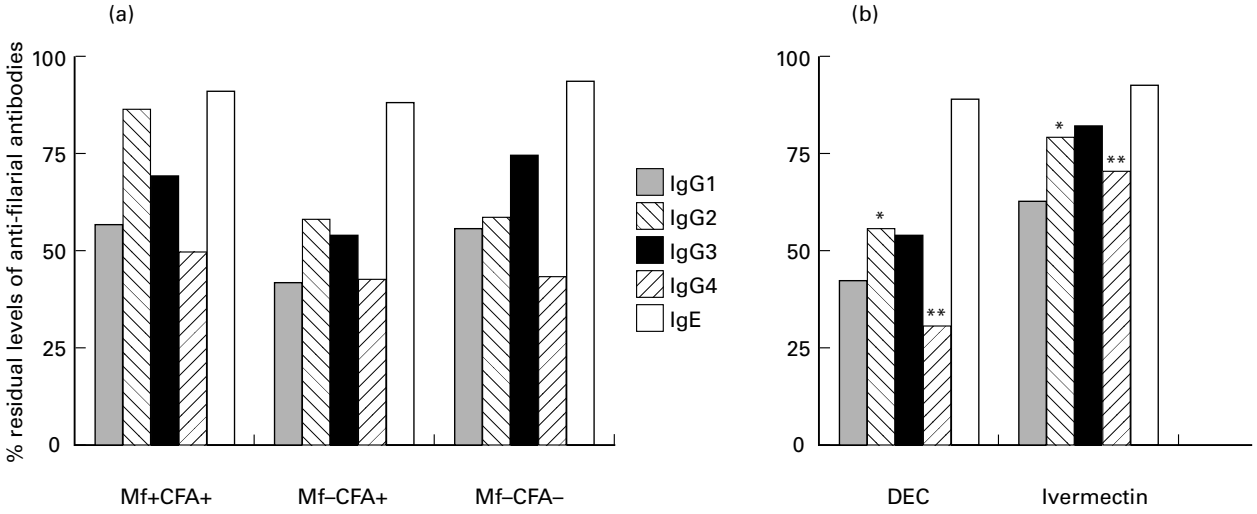

Figure 2 Effect of two annual treatments with filaricidal drugs on the residual levels of antifilarial antibodies (%) according to (a) their initial parasitological status (the individuals treated with DEC or ivermectin are combined) or (b) the filaricidal drug used (the three parasitic groups are combined). Data are geometric means of residual levels: *$P < 0{\cdot}05$; **$P < 0{\cdot}001$.

Individuals with filariasis clinical symptoms have been often compared with microfilaraemics. In fact they constitute an heterogenous group, according to the symptoms observed and their degree of parasitism, whatever the parasite stage. As observed in other endemic areas (Lammie *et al*. 1993, Addiss *et al*. 1995), individuals suffering scrotal hydroceles are often microfilaraemics and individuals suffering acute lymphangitis, and elephantiasis, are mainly amicrofilaraemics. The reasons why individuals develop hydroceles rather than lymphangitis remains unknown, in particular whether molecules produced by the different parasite stages may lead to different symptoms. In our study, although the number of clinically symptomatic individuals enrolled was low, it appears that antibody responses was associated with the parasitological status only and not with clinical symptoms. Previous studies performed on patients with clinical symptoms (hydroceles, lymphoedema or elephantiasis) have also shown that the antifilarial IgG2 responses was lower in Mf-positive individuals (Lammie *et al*. 1993) and that CFA but not Mf was associated with filarial-specific IgG4. In addition, another study in Brazil has shown that parasitism, but not clinical status, was also most closely associated with cytokine responses (de Almeida *et al*. 1996).

The evolution of antibody levels was followed after treatment with DEC or ivermectin. IgE responses were found to be relatively stable or showed a moderate decline after therapy in all individuals. However, reduction of IgG isotypes was observed in all individuals and was higher with DEC than with ivermectin. This reflects the fact that DEC has a better adulticidal effect than ivermectin, in addition to the microfilaricidal effect of both drugs. Surprisingly, the reduction of levels following treatment with DEC or ivermectin was similar in the three groups of individuals, including Mf-and CFA-negative individuals. This indicates that the humoral responses observed in these latter individuals, who have no detectable adult worms, could reflect responses against infective larvae, inoculated by mosquito bites, but putatively to L4 or early adult worms as well. In the present situation, the whole population of the island has been treated with DEC, ivermectin or a combination of both, which led to reduction of filarial burden in this population (Moulia-Pelat *et al*. 1995; Nicolas *et al*. 1997). Therefore, the global decrease of humoral responses in individuals enrolled in this study likely reflects the reduction of *W. bancrofti* transmission on this island.

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