WORM BURDEN AND HOST RESPONSIVENESS IN *WUCHERERIA BANCROFTI* INFECTION: USE OF ANTIGEN DETECTION TO REFINE EARLIER ASSESSMENTS FROM THE SOUTH PACIFIC

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Abstract. A population from the *Wuchereria bancrofti*–endemic island of Mauke was reevaluated retrospectively by use of the Og4C3 circulating antigen (CAg) enzyme-linked immunosorbent assay to assess active infection in relation to host responses by age and gender. Use of microfilaremia (Mf) alone misclassified ~ 50% of infected people, although CAg and Mf levels were positively correlated. Levels of CAg peaked between those aged 31–60 years; men aged > 60 years had a significantly higher CAg prevalence (> 90%) than women. Filaria-specific immunoglobulin (Ig) G4 reached maximum levels in both genders at age 51–60 years. By analysis of variance, both age and gender significantly influenced CAg and IgG4, with men having higher levels of both in the total population. Individuals positive for CAg had significantly lower lymphocyte proliferation responses to parasite antigen than did CAg-negative people, regardless of clinical status. This study reemphasizes the importance of CAg measurements for accurately assessing filarial prevalence and clinical status and demonstrates the relationship between active infection and immune responsiveness.

INTRODUCTION

With the recent introduction of sensitive and specific circulating antigen (CAg) assays, populations living in areas endemic for *Wuchereria bancrofti* infections have been assessed more accurately for both prevalence and intensity of infection. Before the availability of these assays, assessments based on microfilaria detection by nocturnal blood samples or by prevalence of clinically apparent disease[1–9] seriously underestimated the true prevalence of lymphatic filarial infection. Indeed, mathematical models based on microfilarial counts have suggested that prevalence may be underestimated by as much as 25–100%.[10] The available tests for CAg can identify infection as early as the first month of patency[11] and, in animal models, they correlate directly with adult worm burden.[12] Although a qualitative rapid-format card test version of the CAg assay has already been applied successfully as a survey tool in filaria-endemic areas,[13] the available enzyme-linked immunosorbent assay (ELISA)[14] provides quantitative as well as qualitative information. Such quantitative assays have now been used not only for cross-sectional[15,16] and longitudinal[17] analyses of community prevalence, but also for surveying populations in lymphatic filariasis–endemic regions after treatment.[18–20]

As the use of antifilarial drugs becomes more widespread in many endemic regions, the opportunities for studying drug-naive populations using these newer, more refined antigen detection techniques become fewer and fewer. Therefore, in the present study, we have taken advantage of the opportunity to reexamine a population residing on an island in the South Pacific, which had been studied before the introduction of antifilarial chemotherapy. Assays were performed in a retrospective fashion to determine the prevalence and intensity of the response to filarial infection by use of a quantitative measure of CAg as well as traditional parasitologic methods. These assessments have also permitted an examination of the influence of age and gender on susceptibility to infection, an evaluation of the effect of CAg levels and/or status on immunologic responsiveness, and a direct comparison with similar findings made in other previously studied populations.

MATERIALS AND METHODS

Study population. Three hundred sixty people representing 57% of the total population of the island of Mauke in the Cook Islands were randomly selected for study in 1974 (Table 1). This island was endemic for the subperiodic form of the filarial parasite *Wuchereria bancrofti* and had not undergone prior antifilarial treatment, as described in detail elsewhere.[1] Sera from each of the 360 subjects in the current study had been stored in aliquots at −70°C since collection.

1974 analyses. Microfilarial levels were quantified by filtration of 1 ml of heparinized blood through 5-μm polycarbonate filters (Nuclepore, Pleasanton, CA). Parasite and nonparasite antigen-specific lymphocyte proliferation assessment was performed on a subgroup of patients as described previously.[21] Antigens used in these assays included a saline extract of adult male and female worms from the related parasite *Brugia malayi* (BmA), tuberculin-purified protein derivative (Connaught Laboratories, Willowdale, Ontario, Canada), and streptokinase-streptodornase (Lederle Labs, Pearl River, NY). To represent the response to nonparasite antigens, the higher of the 2 responses (tuberculin-purified protein derivative and streptokinase-streptodornase) in each individual was used in the analyses. Stimulation indices (SI) were calculated by dividing the response (^{3}H-thymidine–incorporated counts per minute) of antigen-stimulated cultures by the response in unstimulated cultures (cells cultured in media alone).

Current analyses. Analysis of CAg utilized the Og4C3 ELISA from TropBio Pty Ltd (Townsville, Australia) following the manufacturer's instructions. Antigen units were extrapolated from a standard curve provided with the assay kit; values ≤ 32 units were considered to be antigen negative. The BmA-specific immunoglobulin (Ig) G4 levels were determined by ELISA as described previously.[22]

Statistical analyses. The Mann-Whitney *U*-test was used

Age group (yr)	Sex M/F	No. Mf positive (%)		No. CAg positive (%)	
		M	F	M	F
1–10	43/56	7 (16)	10 (18)	15 (35)	17 (30)
11–20	29/48	5 (17)	10 (21)	12 (41)	17 (35)
21–30	17/22	7 (41)	5 (23)	10 (59)	11 (50)
31–40	25/23	14 (56)	6 (26)	18 (72)	14 (61)
41–50	14/16	7 (50)	6 (38)	9 (64)	9 (56)
51–60	15/18	10 (67)	10 (56)	11 (73)	12 (67)
>60	16/18	9 (56)	5 (28)	15 (94)*	8 (44)

* Indicates significant difference between male (M) and female (F) subjects ($P = 0.0031$; Fisher's exact test).

for comparisons between males and females within a specific age group. To evaluate the effects of age and gender on CAg and IgG4 levels within the study population as a whole, an analysis of variance (ANOVA) model on logarithmically transformed data was used. The Tukey-Kramer test was used to make pairwise comparisons between age groups for CAg and IgG4 levels without the influence of gender. To determine the relationship between CAg, IgG4, and microfilarial levels, Spearman rank correlations were performed. Fisher's exact test was used to test differences in response rates.

RESULTS

Antigenemia and microfilaremia: age-specific prevalence and comparative diagnostic effectiveness. The age-specific prevalence of both microfilaremia (Mf) and CAg positivity is shown in Table 1. Dividing the study population into smaller groups of 5-year age intervals did not alter the conclusions; thus, the larger 10-year divisions were used for analysis. Microfilarial levels generally plateaued for men and women aged > 20 years, and geometric mean (GM) levels of Mf+ patients were lowest in males aged 1–10 years and in males and females aged 11–20 years (data not shown).

Although the majority (67 of 99) of children ≤ 10 years were CAg negative (CAg⁻), positive CAg levels were still observed in many (32%). Nine of 18 (50%) 3- to 4-year-olds in this study were CAg positive (CAg+), although only one was Mf positive (Mf+), indicating the advantage of this test for detecting infection in young children. The differences observed between the 2 detection methods (Mf and CAg) continued through all age groups, with a mean difference between the 2 curves of 19.6% (Figure 1A). The difference between the 2 methods is more striking, however, when considered as the percentage of infected people actually misclassified when Mf detection was used as the indicator of infection. For example, 17% of children aged 1–10 years were Mf+, whereas 32.3% were CAg+. Because all microfilaremic children were CAg+, these findings indicate that almost 50% of infected children were not diagnosed using Mf positivity alone. The percentage of such "misclassified" people in the population decreased gradually with age except for an unexpected increase to nearly 40% in subjects aged > 60 years (Figure 1B).

Age and sex influence on antigenemia. The percentage of antigen-positive subjects increased steadily with age for males and females (Table 1), reaching a peak in the 31–40-
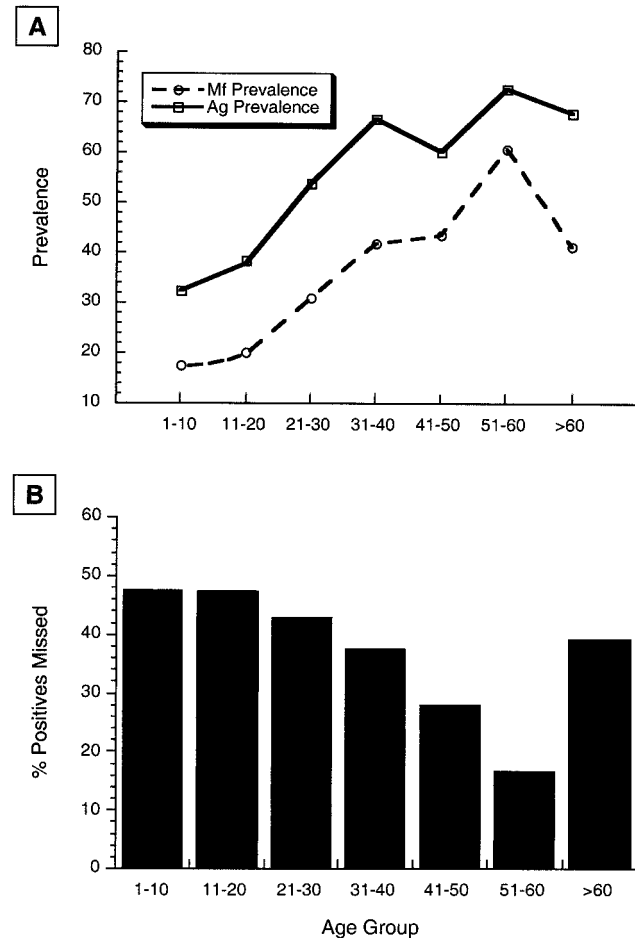

FIGURE 1. The circulating antigen (CAg) assay identifies more infected people than does microfilaria (Mf) detection. **(A)** Prevalence for each age group determined by Mf detection **(dashed line)** and CAg detection **(solid line). (B)** Percentage of infected individuals misclassified for each age group when Mf positivity is used alone as the diagnostic tool. The percentage missed was calculated as $100 - [(\% \, Mf^+/\% \, CAg^+) \times 100]$.

year age group. After a slight decrease at 41–50 years, the prevalence in men and women increased; however, in the > 60-year age group, the genders diverged significantly ($P = 0.0031$ by Fisher's exact test), with men having a higher rate (94%) of antigen positivity than women (44%).

Geometric mean levels of CAg for the age groups are shown in Figure 2. Absolute CAg levels were similar in the < 20-year-old age group for both genders but thereafter were generally higher in men than in women. Only for people aged > 60 years, however, were the differences statistically significant ($P = 0.0134$). Notably, when the antigen levels were plotted on different scales by gender (Figure 2, inset), the mean antigen levels had very similar age-specific distributions, although absolute values differed. When the subjects were segregated into prereproductive (1–15 years), reproductive (16–40 years), and postreproductive (41–60 years) age groupings with respect to CAg levels, a significant predominance of males over females was seen during the reproductive years ($P = 0.0343$; data not shown). An ANOVA of the entire study population to examine the effects of age and gender (as independent variables) on CAg
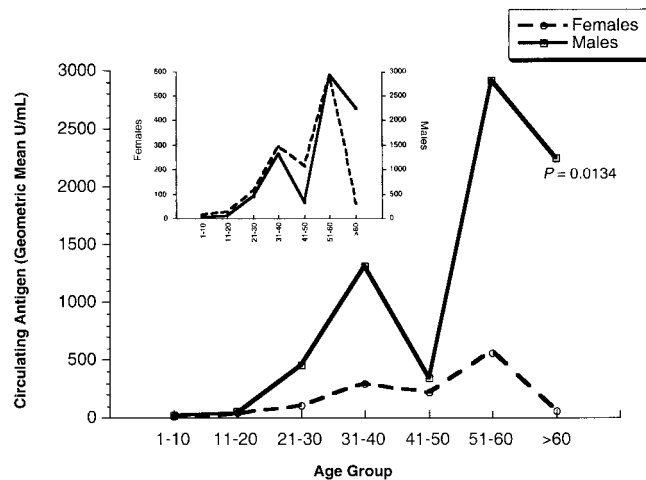
FIGURE 2. Quantitative levels of circulating antigen (CAg) as a function of age and gender. Geometric mean CAg levels (U/mL) in males **(solid line)** and females **(dashed line)** for each age group. Inset shows the same data plotted with different y-axes.

FIGURE 3. Relationship between immunoglobulin (Ig) G4 and age in males and females. Geometric mean IgG4 levels (ng/mL) in males **(solid line),** females **(dashed line),** and all individuals **(dotted line)** for each age group. The Mann-Whitney U-test was used to compare groups.

levels showed that both played a significant role (for age, $P = 0.0001$; for gender, $P = 0.0227$). The adjusted GM for CAg in male and female subjects in this analysis were 327 and 115 antigen units, respectively.

Specific IgG4 antibody in relation to age, gender, Mf, and antigenemia. When BmA-specific IgG4 levels were examined, the peak responses for males and females were found between 51 and 60 years (GM = 60,500 and 52,200 ng/mL, respectively; Figure 3). Although a similar pattern of responses was seen for both genders, ANOVA indicated that IgG4 levels for the entire study population were influenced significantly by both age ($P = 0.0001$) and gender ($P = 0.0023$; adjusted GM, males = 33,500 ng/mL, females = 19,300 ng/mL).

To determine whether age groups differed from one another for both CAg and IgG4 levels, pairwise comparisons were made by the Tukey-Kramer test for multiple comparisons and defining age groups as significantly different when $P \leq 0.10$. Not unexpectedly, children ≤ 10 years had CAg levels that were significantly lower ($P < 0.023$) than those of all other age groups except for individuals in the 11–20-year group, and these children had lower levels of IgG4 than those seen in all other age categories ($P < 0.001$; Figures 2 and 3). In addition, children 11–20 years of age had significantly lower levels of CAg than did individuals 31–40 and 51–60 years old ($P < 0.009$). The IgG4 levels were also lower than those in the 51–60-year category (where the highest level of BmA-specific IgG4 was seen; $P = 0.080$).

Spearman rank analysis was used to demonstrate a positive correlation between levels of CAg and both Mf number and IgG4 levels (rho = 0.727 and 0.659, respectively; $P < 0.0001$). When those individuals who were CAg$^-$ (≤ 32 antigen units) were removed from this analysis, a positive correlation remained between CAg and Mf levels (rho = 0.694), but not between CAg and IgG4 (rho = 0.268). There did not appear to be a strong relationship between Mf and IgG4 levels (rho = 0.401).

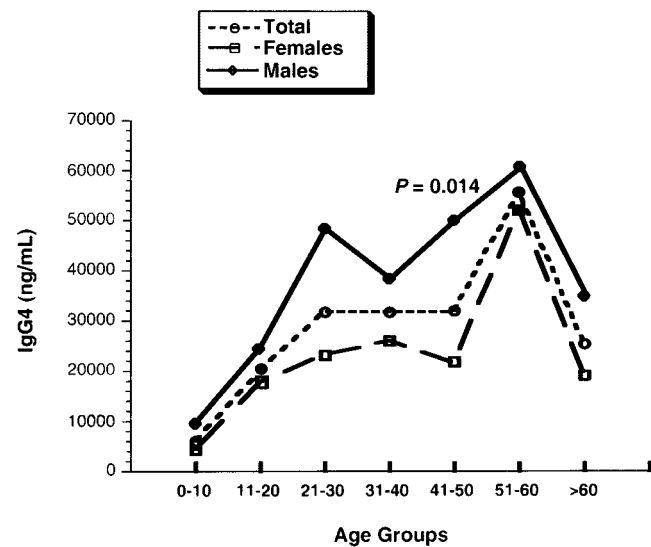**Relationship between clinical status and microfilarial or CAg positivity.** Of the 360 individuals included in this study, 313 had detailed clinical examinations as part of the original study.[1] Thirty-two of these 313 had objective clinical manifestations (elephantiasis, hydrocele, or both) of lymphatic filariasis (Table 2). Among those with disease associated with *W. bancrofti* infection (mostly hydroceles), 17 (53%) were Mf$^+$, and all but one of these were also CAg$^+$. Those with elephantiasis alone were less likely to be Mf$^+$ (2 of 7; 29%) than those with hydrocele alone (12 of 17; 71%), and only 1 of 5 of the Mf$^-$ patients with elephantiasis (and no hydrocele) was CAg$^+$. There was, however, no difference in the absolute levels of CAg between those with clinical disease and those with subclinical or asymptomatic infections (data not shown), and the direct relationship between Mf positivity and CAg positivity remained intact regardless of clinical status.

Effect of CAg on lymphocyte proliferation responses. Lymphocyte proliferation had also been performed in 1974 on a subgroup of infected and apparently uninfected adults and children. Individuals for this immunological study were selected at that time on the basis of their clinical status (i.e., "endemic normal" control, elephantiasis, presence of hydroceles, and history of filarial fevers) and Mf levels (high or

TABLE 2
Relationship between clinical status and circulating antigen*

Clinical status	Mf$^+$ CAg$^+$	Mf$^+$ CAg$^-$	Mf$^-$ CAg$^+$	Mf$^-$ CAg$^-$	Total
Asymptomatic	70	6†	61	138	281
Clinical manifestations					
Elephantiasis	2	0	1	4	7
Hydrocele	11	0	1	5	17
Elephantiasis/hydrocele	3	1	3	1	8
Total	86	7	66	148	313

* CAg = circulating antigen; CAg$^-$ = CAg negative; CAg$^+$ = CAg positive; Mf = microfilaria; Mf$^-$ = Mf negative; Mf$^+$ = microfilaria positive.
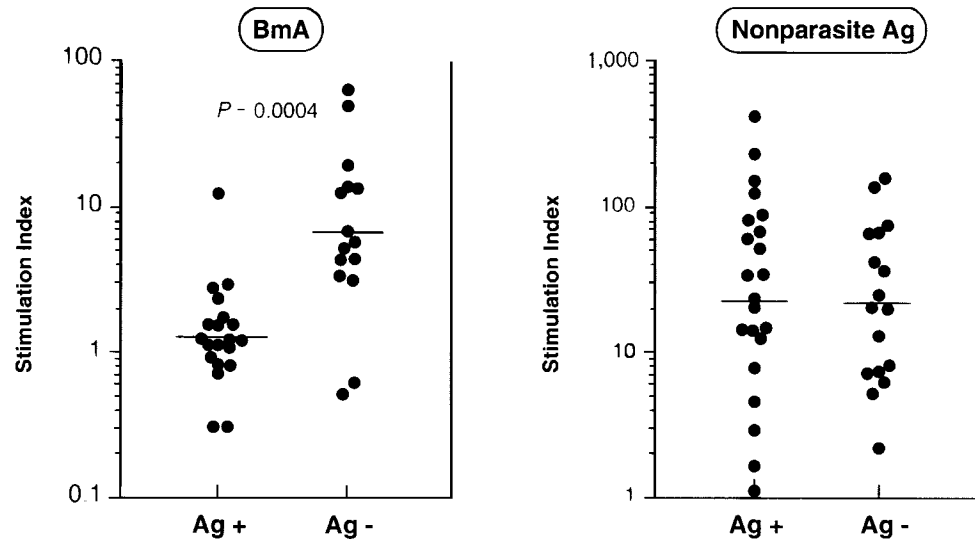† Five of 6 individuals had very low density microfilaremia (<5 Mf/mL).

FIGURE 4. Lymphocyte proliferation stimulation indices (SI) to adult parasite antigen (BmA; **left panel**) and nonparasite antigen **(right panel)** in circulating antigen–positive and–negative individuals. Each data point represents one patient; horizontal bars are geometric means. The Mann-Whitney *U*-test was used to compare groups.

low density of Mf).[21] For the present study, a comparison of the responses to parasite and nonparasite antigens was made for individuals now determined to be CAg+ or CAg− (Figure 4). The CAg+ subjects had a significantly lower proliferative response to BmA than did CAg− people (GM SI = 1.27 and 6.42, respectively; $P = 0.0004$); however, there was no difference in response to nonparasite antigen (GM SI = 23.07 and 21.58, respectively). Of note, 2 individuals originally classified as adult endemic normals in 1974 were found to be CAg+ and had SI values to BmA that were < 1.0. A negative correlation was found between proliferation responses to BmA and CAg levels (rho = −0.605). When CAg− individuals were removed from this analysis, however, the relationship lost its statistical significance, indicating that it is the presence of viable adult worms themselves rather than their absolute number that may be the determinant in immune responsiveness. Indeed, individuals who were CAg+ were less likely to proliferate to BmA (SI < 2) than were CAg− subjects ($P = 0.0001$; Fisher's exact test).

DISCUSSION

During the past decade, our understanding of clinical disease in bancroftian filariasis has changed dramatically. By lymphoscintigraphy[23,24] and ultrasound,[25] it has been demonstrated that subclinical lymphatic dysfunction and pathology occur even in asymptomatic Mf+ individuals, a group once thought to be free of pathology. With the widespread availability of CAg assays, which reflect adult worm burden,[12] it can now be demonstrated that most earlier studies likely underestimated the prevalence of filariasis in endemic communities.

In the present study, the prevalence of infection in the population was ~20% higher when determined by CAg positivity than when determined by Mf, with as many as 50% of infected individuals in some age groups being misclassified. This difference was most apparent in children and adolescents, groups now recognized[17] to be much more susceptible to infection than previously thought. For filariasis elimination programs, this finding has particular significance, because the general ineffectiveness of diethylcarbamazine to reverse lymphatic damage in treated adults[26,27] emphasizes the importance of targeting individuals infected early (especially young children) to prevent the subsequent development of clinical disease.

Although CAg prevalence was higher in this study than Mf prevalence, the distribution pattern over the age groups appeared similar, and there was a positive correlation between the 2 detection methods. Although there were individuals with very low Mf levels who may have been missed by use of the available CAg assay,[20,28] this assay is nonetheless a far more accurate tool for assessing infection status than is microfilaremia or any other method of rapid assessment.

Our findings support the notion that it is the presence of live adult worms, and not just Mf, that alters cellular and humoral responsiveness. Lymphocyte proliferation results demonstrated that the CAg+ individuals, not just those with Mf, had little or no proliferative response to parasite antigen, although their responses to nonparasite antigen were equivalent to those of CAg− subjects. Diminished antigen-specific cytokine responsiveness has previously been described in CAg+ individuals.[29] It was also apparent that the presence or absence of adult worms is an important determinant of IgG4 levels, because CAg+ subjects had higher IgG4 levels than did CAg− subjects; however, a direct correlation between CAg and IgG4 levels was only seen when CAg− individuals were included in the analysis, indicating that IgG4 is more likely to be dependent on the presence or absence of parasites rather than on the number of worms present.

Age was shown to have a significant influence on CAg levels in this study, and in addition, CAg levels correlated significantly with Mf density, as observed previously by others.[20] Quantitative levels of CAg, but not CAg prevalence, Mf prevalence, or Mf intensity, showed a strikingly different distribution pattern across the population's age profile. Two

distinct peaks were observed in this study population, the first at 31–40 years of age and the second at 51–60 years. Such a profile perhaps indicates that adult worm burden in the population was fluctuating with age. In a study of patients with schistosomiasis,[30] similar fluctuations have been attributed to changes in levels of certain hormones, particularly dehydroepiandrosterone, shown to affect certain immune functions and leading to a decrease in infection intensity when dehydroepiandrosterone levels are highest.

Gender differences in response to helminth infections have been well documented in both animals and humans. For example, female rats are highly resistant to *Brugia pahangi* infection,[31] and female jirds generally have no *B. pahangi* Mf, even though they may have adult worms detected at autopsy,[32] indicating a resistance to at least one stage of the parasite. Suggestions made to explain this differential resistance to infection between males and females include influences of macrophages[33] and testosterone (which already has been shown to cause increased susceptibility to diseases such as cysticercosis[34] and schistosomiasis[35]).

The differences observed between the genders in the present investigation have been demonstrated in other studies of bancroftian filariasis as well, primarily during the postpubertal reproductive years.[2,36,37] Studies have demonstrated not only a greater prevalence of Mf [2,3,38–43] and disease[42,44] in male subjects, but also higher antigen[39] and antibody[37,39] levels. Except for children, males in the current study also tended toward not only a higher prevalence of both Mf and CAg but also higher levels of Mf, CAg, and IgG4. The major gender differences in antigen level (a reflection of adult worm burden) in this study were during the reproductive years (16–40 years) and in the > 60-year-old group.

Some have suggested a possible pregnancy-related mechanism for the lower disease rates found in women of reproductive age,[36] but others have found no such connection.[45] Indeed, aside from the obvious hormonal differences, there have been no conclusive data showing pregnancy to be a major factor. Cultural factors causing males to be more exposed to microfilaria at an earlier age[46] or even variations in clothing may account for gender differences in disease prevalence, but on the island of Mauke, no specific explanation for this difference could be found either in 1974 or in a follow-up study in 1992.[47]

The differences seen in the oldest age groups, especially in CAg prevalence and levels, were particularly striking and might be due to host physiological changes. Indeed, the observation that the women in this study population actually showed an increase in CAg levels between ages 51 and 60 (although still lower than men) mirrored the findings of Dutta and others,[37] who showed an increase in Mf density in women at menopause. Although one study found equivalent rates of Mf positivity in men and women aged > 75 years,[40] another study found that in *Onchocerca volvulus*–infected men aged > 60 years, there was a higher Mf density than in women.[41] In the present study, the differences in CAg very likely reflect a higher worm burden in men. It has been suggested that differences in worm burden between the genders also reflect the rate of transmission and therefore reinfection—the higher the rate of transmission, the more profound the differences between the genders.[48] It is possible, therefore, that males and females may have differing cumulative levels of immunity to reinfection, which in turn may account for the variations seen in older individuals. Whatever the reasons underlying the gender differences (physiological changes, immunologic differences, exposure, transmission levels), it is clear that they should be taken into consideration when assessing infection and disease in a population.

The CAg ELISA has provided additional insights into the analysis of infection and responses to it in a filarial-endemic region. Although Mf and clinical pathology are useful diagnostic tools, CAg assays appear to be more sensitive for quantifying infection prevalence in a population. This makes CAg an especially valuable tool for longitudinal studies of disease prevalence and reinfection potential following community-wide treatment programs focused on eliminating lymphatic filariasis as a public health problem throughout the world.[49]

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