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Influence of infection with non-filarial helminths on the specificity of serological assays for antifilarial immunoglobulin G4

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Abstract

Serological assays based on the detection of immunoglobulin (Ig) G4 antibodies to crude filarial extracts are widely used for epidemiological and diagnostic purposes. We tested 195 samples collected in 1998 from an area of Brazil where filariasis is not endemic and 13 (6.7%) had levels of antifilarial IgG4 antibodies that were defined as positive. Both *Strongyloides* infection and the presence of *Strongyloides* antibody responses were associated with higher antifilarial antibody responses. None of the specimens had a positive response to the *Brugia malayi* recombinant antigen (Bm14). These data suggest that serodiagnostic assays based on the use of crude filarial antigens should be interpreted with caution because of the potential for cross-reactivity with *Strongyloides*.

Keywords: lymphatic filariasis, *Brugia malayi*, *Brugia pahangi*, *Strongyloides*, diagnosis, ELISA, Brazil

Introduction

Historically, the most widely used method for the diagnosis of lymphatic filarial infection has been the examination of blood films for the presence of microfilariae (mf). Although highly specific, thick films are not very sensitive and the nocturnal nature of microfilaraemia in much of the world limits the usefulness of blood film examination. The measurement of antifilarial antibody responses provides an alternative measure of filarial infection that can be used for diagnosis. Assays for antifilarial immunoglobulin (Ig) G4 antibodies have been proposed as sensitive and specific indicators of active filarial infection (Lal & Ottesen, 1988; Kwan-Lim *et al.*, 1990; Hitch *et al.*, 1991). In seroepidemiological studies antifilarial IgG4 has been used extensively to define filarial infection prevalence, especially in *Brugia*-endemic areas (Haarbrink *et al.*, 1995; Rahmah *et al.*, 1998; Terhell *et al.*, 2000). A number of laboratories, including those at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA interpret patients' positive antifilarial IgG4 responses to crude filarial extracts as evidence of active infection and may make treatment recommendations on the basis of a positive antifilarial IgG4 response.

We assessed antifilarial IgG4 responses to adult worm antigens in a population that was not exposed to lymphatic filariasis. Our results suggest that specific recombinant filarial antigens should be employed instead of crude filarial extracts to improve the specificity of serological assays for lymphatic filariasis. These results may be relevant to efforts to define improved serological assays for the monitoring of filariasis elimination programmes.

Materials and Methods

Study population

The current study was an extension of a hospital-based evaluation of parasitic infections in patients with eosinophilia and asthma. The patient population included 195 individuals from the Butantan community of São Paulo, Brazil, an area which is non-endemic for filariasis. The group comprised 75 women and 120 men with a mean age of 40.4 years (range 3–83 years). Venous blood samples were collected in 1998 at the University Hospital of São Paulo University and serum specimens were stored at −20 °C. Total peripheral eosinophil counts were performed with an automated counter (Coulter Electronics, Miami, FL, USA). Stools were examined to assess infection with intestinal parasites.

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Serological assays

Serological assays for filariasis and *Strongyloides* were run in the CDC laboratories in 1999 and 2000. Antibody levels to soluble *B. pahangi* adult worm antigens were determined by ELISA as previously described (Hitch *et al.*, 1991). Briefly, Immulon 2 HB microtitre plates (Dynex Tech Inc., Chantilly, VA, USA) were sensitized overnight with 2 μg/mL of antigen and blocked with 0.3% Tween 20 (Sigma Chemical Co., St Louis, MO, USA) in phosphate-buffered saline (PBS) (0.01 M, pH 7.2) for 1 h at 4 °C. Serum samples were diluted in PBS/0.05% Tween 20 and added to the sensitized plates in duplicate at a dilution of 1:50. Biotinylated mouse monoclonal antibody, specific for human IgG4 (Zymed Laboratories, Inc., South San Francisco, CA, USA), and streptavidin alkaline phosphatase (Gibco BRL/Life Technologies, Rockville, MD, USA) were added in sequence with washing steps in between. Colour development was monitored after the addition of *p*-nitrophenyl phosphate (Sigma Chemical Co.) dissolved in 10% diethanolamine–3 mM MgCl, pH 9.8; the optical density (OD) of the reaction product was read at 405 nM with an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Serum samples from non-exposed North Americans were run in parallel as negative controls. Patient specimens were defined as positive if the antibody level was greater than the mean plus 2 SD of the response of the negative controls (6.0 arbitrary units [AU]).

The *Strongyloides* ELISA was performed with Chaffee extract antigen of *S. stercoralis* at a concentration of 0.03 μg/mL (generously provided by M. Wilson, CDC). Serum samples were assayed at a dilution of 1:50 and bound antibodies were detected using biotinylated mouse monoclonal antibody specific for IgG (Zymed Laboratories, Inc.). A known, negative serum sample was included as a negative control on each plate. For the purposes of this study, a patient serum sample was considered to have a positive *Strongyloides* antibody response if the OD was greater than the mean plus 2 SD of the OD of the negative control (13.8 AU).

Antibody responses to the *B. malayi* recombinant antigen (Bm14) have been used as serological markers of filarial infection (Chandrashekar *et al.*, 1994; Ramzy *et al.*, 1995; Weil *et al.*, 1999). The Bm14 ELISA was performed with a 2 μg/mL concentration of the Bm14-GST (*Schistosoma japonicum* glutathione-S-transferase) fusion protein (generously provided by Dr Gary Weil, Washington University School of Medicine, St Louis, MO, USA). For this assay, serum samples were assayed at a dilution of 1:100 and bound antibodies were detected with biotinylated mouse monoclonal antibody specific for IgG4 (Zymed Laboratories, Inc.). Serum samples from non-exposed North Americans were run in parallel as controls. Antibody responses to the GST

fusion partner were not assessed in parallel. Previous studies have demonstrated that IgG4 antibodies to GST are typically absent in human serum specimens.

A rapid-format card test for detection of *Wuchereria bancrofti* antigen (AMRAD ICT, New South Wales, Australia) was used to assess filarial antigen status. The assay was performed according to the manufacturer's instructions.

Results

Antifilarial antibody responses for specimens collected from 195 patients were measured by ELISA. Thirteen patients (6.7%) had antifilarial IgG4 responses that were defined as positive (Table 1) (range 6.5–1000 AU) using the cut-off based on North American samples (mean + 2 SD = 6.0 AU). Using a more conservative cut-off (mean + 3 SD = 7.8 AU), nine of the 13 had positive antifilarial IgG4 responses. Patients with a positive antifilarial IgG4 response had a higher mean eosinophil count than those with negative responses, but this difference was not statistically significant (Table 1). None of the samples were *W. bancrofti* antigen-positive using the ICT test for circulating filarial antigen.

To evaluate the potential contribution of other helminths to antifilarial IgG4 responses, antifilarial antibody responses were analysed, based on results of stool examinations for intestinal parasites. Thirty-eight patients with parasitological evidence of *Strongyloides* infection had significantly higher antifilarial IgG4 responses than uninfected persons ($P < 0.002$; data not shown). There was no difference in antifilarial IgG4 reactivity based on infection status with *Ascaris* or *Trichuris*. Too few patients were infected with hookworm or *Schistosoma mansoni* to evaluate their contribution to antifilarial antibody responses.

The relationship between *Strongyloides* infection and antifilarial IgG was further analysed by ELISA using *S. stercoralis* antigen (Table 2). Patients considered to have *Strongyloides*-specific antibodies were older and had a significantly higher mean eosinophil count than antibody-negative persons (Table 2). Patients with positive *Strongyloides* antibody responses had higher antifilarial antibody responses and, conversely, there was a tendency, albeit not statistically significant, for persons with positive antifilarial IgG4 responses to have higher *Strongyloides* antibody levels ($P = 0.08$; data not shown).

IgG4 antibody responses to the Bm14 recombinant antigen provide an alternative to the use of crude filarial antigens for diagnostic purposes (Ramzy *et al.*, 1995). All serum specimens were uniformly negative with this assay, independent of *Strongyloides* infection status or antibody responses to parasite antigens.

Discussion

Although intestinal helminth infections have been recognized as a potential source of IgG antibodies that cross-react with filarial antigens, IgG4 antibodies have generally been considered to be far more specific (Lal & Ottesen, 1988; Eberhard & Lammie, 1991). Despite having lived in filarial non-endemic areas of Brazil only and having no evidence of circulating filarial antigen, 13 of 195 patients had positive antifilarial IgG4 responses to crude filarial antigen, two in the range expected of serum samples from microfilaraemic persons. Based on these findings, we investigated helminth infections as a possible source of cross-reactive antibodies and evaluated an alternative assay using Bm14 recombinant antigen.

Strongyloides infection appears to be a source of IgG4 antibodies that cross-react with filarial antigens in assays using filarial adult worm extracts. Persons infected with *Strongyloides* had significantly higher antifilarial IgG4 responses than persons without evidence of current *Strongyloides* infection ($P < 0.002$). Furthermore, persons with a positive antibody response to *Strongyloides* had higher antifilarial IgG4 responses ($P < 0.001$). These observations emphasize that positive results obtained with antifilarial antibody assays, even those based on detection of IgG4, should be interpreted with caution, as first noted by Rocha *et al.* (1995). We suspect that our results can be extended to laboratories using assays run with crude antigens derived from other filarial species (e.g. *B. malayi* or *Dirofilaria immitis*) because of the correlation in antibody responses that we observed using different antigenic extracts. We recommend that diagnostic laboratories rule out *Strongyloides* infection when antifilarial antibody responses are detected using assays based on crude filarial antigens and there is no other parasitological evidence supporting a diagnosis of lymphatic filariasis. It is important to point out that our observations also do not rule out the possibility that other helminth infections contribute to antifilarial IgG4 responses as well because of the relatively small number of persons in our study that were infected with other parasites.

To avoid issues of cross-reactivity in the serodiagnosis of filariasis, an assay based on detection of IgG4 antibodies to the Bm14 antigen offers an attractive alternative (Ramzy *et al.*, 1995; Weil *et al.*, 1999). The reported sensitivity of this assay is quite good; approximately 90% of microfilaraemic persons have IgG4 anti-

Table 1. Comparison of Brazilian patients with positive and negative antifilarial immunoglobulin G4 responses determined by ELISA

Antifilarial IgG4 response	*n*	Median age (range) (years)[a]	Mean eosinophil count (cells/mm^3)[a]	No. infected with *Strongyloides*	No. infected with *Ascaris*
Positive	13	42 (10–72)	700	5 (38%)	0 (0%)
Negative	182	40 (3–83)	471	33 (18%)	19 (10%)

[a]None of the differences between patients with positive and negative antifilarial IgG4 responses were statistically significant.

Table 2. Comparison of Brazilian patients with positive and negative *Strongyloides* antibody responses determined by ELISA using *Strongyloides stercoralis* antigen

Strongyloides antibody response	*n*	Median age (range) (years)	Mean eosinophil count (cells/mm^3)	No. infected with *Strongyloides*	No. with antifilarial IgG4	Geometric mean antifilarial IgG4
Positive	58	50 (24–80)	760	37 (64%)	8	1.36 AU
Negative	137	36 (3–83)	370	1 (0.7%)	5	0.52 AU
P		<0.001	<0.001	<0.001	0.022	<0.001

body responses to this antigen (Chandrashekar *et al.*, 1994; Ramzy *et al.*, 1995), comparable to what has been reported in studies of antifilarial IgG4 using crude filarial antigen (Marley *et al.*, 1995). In addition, although the absence of cross-reactivity with *Ascaris* was noted in previous studies of Bm14, samples from persons infected with *Strongyloides* were not tested. In our study, there were no positive Bm14 responses among those that tested positive with crude filarial antigen; thus, the Bm14 assay is more specific than the crude antigen ELISA and this specificity extends to *Strongyloides* infection.

With the advent of new tools for diagnosis and treatment, the World Health Assembly has called for the elimination of lymphatic filariasis as a public health problem. To effectively eliminate the infection, reliable and practical tools are needed not only for diagnosis, but also for epidemiological surveys and programme monitoring. Although assays for circulating *W. bancrofti* antigen appear to be specific and sensitive measures for diagnosis and extremely useful for prevalence surveys, recent studies have demonstrated that circulating antigens persist in microfilaria-negative persons for up to three years after treatment (Schuetz *et al.*, 2000). This represents a potential limitation of the use of antigen assays for the purpose of monitoring filariasis elimination programmes, at least in early stages of the programme, and suggests a potential role for antibody assays. Given its increased specificity relative to immunoassays based on the use of crude filarial antigens, future studies should explore the use of assays based on the Bm14 recombinant antigen for monitoring of filariasis elimination programmes.

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Correction

Savioli *et al.* Schistosomiasis and soil-transmitted helminth infections: forging control efforts. *Transactions*, 2002, **96**, 577–579.

Paragraph two of the section on Chemotherapy-based morbidity control (p. 577) is modified to correct the cost of a single-dose treatment for soil-transmitted helminth infections with any of the 4 anthelminthics on the WHO list of essential drugs from less than U$0.30 to less than U$0.03:

Treatment of a child with praziquantel currently costs approximately US$0.20. A single-dose treatment for soil-transmitted helminth infections with any of the 4 anthelminthics on the WHO list of essential drugs (albendazole, levamisole, mebendazole and pyrantel) costs less than US$0.03. The WHO has calculated that the cost of intervention, including delivery, where schistosomiasis and soil-transmitted helminth infections are both endemic is typically less than US$1 and can be as low as US$0.30 per child per year, while treatment of soil-transmitted helminth infections alone costs as little as US$0.10 per child per year (Montresor *et al.*, 2002c; WHO, in preparation, a).