

Human antibody responses to *Wuchereria bancrofti* infective larvae

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SUMMARY

Human IgG antibody responses to *Wuchereria bancrofti* third stage infective larvae (L3) surface and somatic antigens were studied by indirect immunofluorescence (IFA) and immunoblot with endemic Egyptian sera ($n = 115$) with the aim of identifying targets of protective immunity. Human sera variably recognized 14 major bands in L3 by immunoblot. The statistical significance of group differences in antibody prevalence was assessed by the chi-squared test. Children and young adults (aged 10–20 years) tended to have antibodies to more L3 somatic antigens than older adults, with significant differences for bands at 66, 60 and 5 kDa. Infected subjects had more consistent antibody responses to antigens at 55, 50 and 6 kDa than endemic normal subjects with negative serum filarial antigen tests, who are presumed to be uninfected. A 5 kDa antigen was preferentially recognized by the latter group. Antibodies to L3 surface antigens were equally prevalent in uninfected children (75%) and adults (90%) but less prevalent in people with microfilaremia (38%) than in amicrofilaremic subjects with or without filarial antigenemia (81%) ($P < 0.001$). IFA-positive sera showed significantly enhanced recognition of antigens at 66, 40 and 14 kDa in immunoblots relative to IFA-negative sera. Additional studies are needed to further characterize antigens identified in this study and to establish whether they are indeed targets of protective immunity in humans.

Keywords filariasis, *Wuchereria bancrofti*, antibody, infective larvae, human

INTRODUCTION

Lymphatic filariasis (caused by the mosquito borne, lymphatic dwelling nematodes *Wuchereria bancrofti* and *Brugia malayi*) is a major public health problem in tropical and subtropical regions (Ottesen *et al.* 1997). Recent reports have estimated that at least 120 million people are infected with these parasites in 73 countries and that over 40 million people have overt clinical disease (Michael *et al.* 1996). Bancroftian filariasis accounts for approximately 90% of this disease burden. Chronic filariasis results from lymphatic insufficiency or blockage and presents in different ways, depending on the location of involved lymphatic vessels. Lymphoedema and elephantiasis of the legs are the most visible forms of the disease, but hydrocoeles are more common than limb oedema in most endemic areas.

W. bancrofti and *B. malayi* are transmitted by mosquitoes, which release infective stage filarial larvae (L3) when they feed on human blood. L3 enter the skin and migrate in subcutaneous tissues until they enter lymphatic vessels. Larvae mature to become adult worms over a period of months, and adult worms live in lymphatic vessels for years; they are not cleared by the immune system, and living adult worms do not normally stimulate strong local inflammatory responses. In contrast to adult worms, the L3 is believed to be an important target for immunity in filariasis. Animals immunized with irradiated (and developmentally arrested) L3 develop significant immunity to challenge infections (Yates & Higashi 1985, Chusattayanond & Denham 1986, Weil *et al.* 1992). The antigenic targets and mechanisms of immunity in animal filariasis are areas of active study. Vaccinated animals produce strong antibody responses to L3 surface antigens and also to certain internal or somatic antigens (Yates & Higashi 1985, Lucius *et al.* 1991, Weil *et al.* 1992), and recent studies have shown that immunization with defined L3 antigens or passive transfer of antibodies to L3 surface antigens can confer significant immunity to filariasis in some animal models (Parab *et al.* 1988, Oikawa *et al.* 1992, Li *et al.* 1993, Hartmann *et al.* 1997).

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Much less is known about protective immunity to filariasis in humans, and it is difficult to study this subject directly. However, epidemiological data suggest that humans develop at least partial immunity after years of exposure to the parasite (Day *et al.* 1991b). Infection prevalence rates in endemic areas tend to peak in early adulthood and decline in later years (Grenfell & Michael 1992). Filarial infection intensity (measured by microfilaria counts or filarial antigen levels) also tends to decrease with age (Weil *et al.* 1999), and recent studies have shown that incidence rates for filarial infection are higher in children and young adults than in older adults (Vanamail *et al.* 1989, Weil *et al.* 1999).

Relatively few studies have examined human antibody responses to filarial L3. One study reported that sera from people in South India who were exposed to bancroftian filariasis but who were amicrofilaremic and clinically normal (and thus considered to be 'putatively immune individuals') often contained antibodies to a 43 kDa *B. malayi* L3 antigen by immunoblot that was not recognized by most sera from people with microfilaremia (Freedman *et al.* 1989). Immunoblot studies of anti-L3 antibodies with filariasis sera from India and Papua-New Guinea did not identify antigens preferentially recognized by exposed but uninfected individuals (Day *et al.* 1991a, Zhang *et al.* 1999). However, Day *et al.* (1991a) found that antibodies to L3 surface antigens (detected by indirect immunofluorescence, IFA) were significantly more prevalent in sera from adults and uncommon in sera from children. Other studies have shown that sera with antibodies to L3 surface antigens promote leucocyte adherence to filarial L3 *in vitro* with parasite killing (Higashi & Chowdhury 1970, Sim *et al.* 1982, Chandrashekar *et al.* 1990).

The purpose of the present study was to obtain additional information on human antibody responses to *W. bancrofti* L3. The study was performed with well-characterized sera from a *W. bancrofti*-endemic area in Egypt and designed to specifically answer the following questions: (1) are particular L3 antigens preferentially recognized by antibodies in sera from exposed but uninfected subjects? (2) Do antibodies to filarial L3 surface or somatic antigens in sera from adults (who are believed to be relatively immune to new infections) differ from those in sera from children (who are believed to be relatively susceptible to new infections)? (3) Do sera with antibodies to L3 surface antigens (as assessed by IFA) have characteristic antigen binding patterns by immunoblot?

MATERIALS AND METHODS

Clinical specimens and classification of human subjects

Venous blood samples were collected during a

population-based study of bancroftian filariasis in Kafr Tahoria, a village 35 km north east of Cairo, Egypt (Faris *et al.* 1993). Informed consent was obtained from all research subjects (or from their parents or guardians in the case of children). The study population (aged >10 years) had a microfilaria prevalence rate of 28% and a filarial antigen prevalence rate of 39% at that time, but very little clinical disease (5%, mostly small hydrocoeles).

Plasma specimens for the present study were selected from the Kafr Tahoria collection to represent various groups defined on the basis of infection status and age as noted below. Microfilaremia was detected by 5 μ M membrane filtration of 1 ml blood samples collected at night (Dennis *et al.* 1976). *W. bancrofti* antigens were detected in plasma by direct-sandwich ELISA as previously described (Ramzy *et al.* 1994). Subjects aged 10–20 years were considered to be 'young' while older subjects were 'old'. Both sexes were equally represented. Amicrofilaremic subjects with no evidence of clinical filariasis were considered to be 'endemic normals', and this group was further subdivided on the basis of antigen test results (Weil *et al.* 1996). Nonendemic control sera were from adult residents of Cairo, Egypt (nonendemic for filariasis) who had no history of living in endemic areas.

Production of *W. bancrofti* infective larvae

Larvae of *Culex pipiens*, the principal mosquito vector for filariasis in Egypt, were collected from abandoned wells in Kafr Tahoria village and maintained in an insectary until adult mosquitoes emerged. Adult female mosquitoes were allowed to feed at night on volunteers with microfilaria counts of 400–700 per ml. Mosquitoes were dissected in saline solution 12–14 days after feeding to collect L3, which were counted and stored frozen at -70°C until they were needed for immunological studies.

Immunoblot analysis of antibody responses to *W. bancrofti* infective larvae

Soluble *W. bancrofti* L3 somatic antigens were prepared by sonication of 1000 L3 per ml on ice with an ultrasonic homogenizer (Cole-Parmer Instrument Co., Chicago, IL, USA; six cycles of 30 s each at 20 kHz with 30 s cooling between cycles). The sonicated L3 homogenate was boiled for 3 min in SDS-PAGE sample buffer and centrifuged for 3 min at 16 000 g. The supernatant was separated by SDS-PAGE under denaturing conditions using 5–20% gradient gels with 500 μ l of parasite extract per trough, as previously described (Weil *et al.* 1992), and fractionated proteins were electrophoretically transferred to nitrocellulose membranes as previously described (Towbin *et al.* 1979). After blotting, nitrocellulose sheets were cut into 0.4 cm strips,

blocked in 0.01 M phosphate buffered saline (pH 7.4) with 0.05% Tween 20 (PBS/T) and 5% non fat dry milk for 30 min, and incubated with human sera (1 : 250 for IgG blots and 1 : 100 for IgG subclass blots) in PBS/T with 5% foetal calf serum for 2 h at 37°C. After washing, the strips were incubated in alkaline phosphatase conjugated antihuman IgG antibodies (Promega, Madison, WI, USA) in PBS/T with 5% FCS for 2 h at 37°C. Biotin labelled IgG subclass-specific monoclonal antibodies (purchased from Hybridoma Reagent Laboratory, Baltimore, MD, USA) and alkaline phosphatase-conjugated avidin were used as detection reagents for antibody subclass studies. After washing, nitrocellulose strips were developed with the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma Chemical Co., St Louis, MO, USA) in 100 mM Tris-HCL with 100 mM NaCl and 5 mM MgCl₂ for 15 min at 37°C. Positive and negative human serum pools and two immune rabbit sera (rabbit antibody to a PBS extract of *B. malayi* adult worms and rabbit antibody to a PBS extract of *W. bancrofti* L3) were included in each immunoblot run as quality controls. Mono-specific mouse antibodies to recombinant *B. malayi* paramyosin (97 kDa) (Li *et al.* 1991), myosin (Li *et al.* 1995), and tubulin (50–55 kDa) were used as reference sera to identify these bands in immunoblots.

Indirect immunofluorescence (IFA)

Antibodies to *W. bancrofti* L3 surface antigens were detected essentially as previously described (Weil *et al.* 1992). Briefly, larvae were incubated overnight at 4°C with human serum diluted 1 : 10 in filtered PBS. After washing, larvae were incubated in fluorescein-conjugated goat antihuman IgG (Organon Teknika-Cappel, West Chester, PA, USA) diluted in PBS for 2 h at 37°C. Larvae were washed and examined with the high dry objective (total magnification ×400) of an epifluorescence microscope. The intensity of fluorescence was rated on a scale of 0–3+; linear fluorescence had to be present on a majority of L3 for a test to be scored positive.

Statistical analysis

Data were analysed on a microcomputer with EpiInfo software (version 6.0 (Dean *et al.* 1994). The chi-squared test was used to assess the significance of group differences in antibody prevalence.

RESULTS

Detection of IgG antibodies to *W. bancrofti* L3 by immunoblot

Endemic sera recognized 14 major bands at 200, 97, 66, 60, 55, 50, 45, 40, 35, 30, 25, 14, 6 and 5 kDa in the *W. bancrofti*

L3 somatic antigen extract (Figure 1). Sera from groups of subjects classified according to age and infection status differentially recognized certain antigen bands, but considerable individual variation was observed within each group (Table 1). Nonendemic control sera did not react with any of these antigens.

Table 2 summarizes the results of the statistical analysis of differences in antigen recognition by group. In general, IgG antibodies in sera from 'young' subjects (<21 years of age) showed stronger and more frequent recognition of *W. bancrofti* L3 somatic antigens than sera from older subjects. This was especially true for antigen bands at 66, 60 and 5 kDa (chi-squared = 4.95, 6.24 and 6.56, respectively, $P < 0.05$). Among endemic normal subjects, sera from antigen-positive cases showed stronger antibody reactivity with L3 antigens than sera from antigen-negative sera. Antigens at 97, 55, 50 and 6 kDa were more frequently recognized by antigen-positive than by antigen-negative endemic normal sera (chi-squared = 4.81, 4.49, 9.64 and 4.27, respectively, $P < 0.05$). Sera from infected subjects (MF carriers or antigen-positive endemic normals) were more reactive with antigen bands at 55 and 50 kDa than sera antigen-negative endemic normals (Table 2). It is likely (but not formally proven) that these bands correspond to parasite tubulin, because antigen bands at the same molecular weight

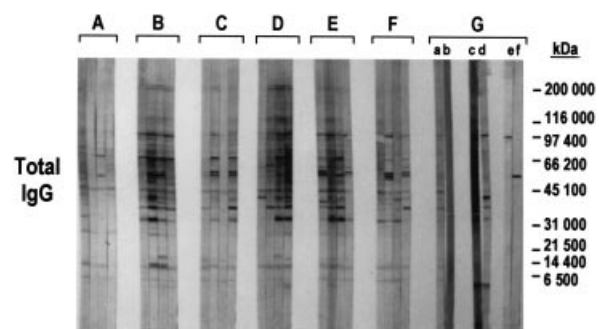


Figure 1 Representative immunoblot patterns showing IgG antibody reactivity with *W. bancrofti* L3 somatic antigens obtained with sera from human subjects classified by age and infection status. Blots developed with sera from 'young' subjects (<21 years of age) are shown in (A,B), and (C–F) show blots developed with sera from 'old' subjects (>20 years of age). Immunoblots in (A) and (D) were developed with sera from antigen-negative endemic normals; (B,E), antigen-positive endemic normals; (C,F), asymptomatic microfilaremia. Four representative blots are shown for each group, and each shows the IgG antibody binding pattern obtained with a single serum sample. Control blots shown in (G) were developed with an antibody-positive pool prepared with (a) endemic human sera; (b) a pool of nonendemic human sera; (c) rabbit antibody to *B. malayi* adult worm extract; (d) rabbit antibody to *W. bancrofti* L3 extract; (e) mouse monoclonal antibody to 97 kDa filarial paramyosin; and (f) mouse antibody to recombinant *B. malayi* tubulin (50–55 kDa).

Table 1 Summary of IgG immunoblot results shows antibody reactivity (%) by infection status and age for major *Wuchereria bancrofti* L3 somatic antigens

Serum type	No. tested	Molecular weight (kDa)													
		200	97	66	60	55	50	45	40	35	30	25	14	6	5
Antigen-negative endemic-normals															
'Young' ¹	14	29	21	71	79	29	0	93	43	14	79	29	79	86	57
'Old'	14	50	36	43	29	0	14	10	43	43	29	21	79	50	21
Total	28	39	26	57	54	14	7	96	43	29	57	25	79	68	39
Antigen-positive endemic normals															
Young	8	50	63	75	89	25	38	100	75	63	63	38	88	88	50
Old	14	43	64	36	43	57	57	100	43	50	71	64	64	100	7
Total	22	45	64	50	59	45	50	100	55	55	68	55	73	95	27
Asymptomatic microfilaremia															
Young	14	50	36	43	43	64	50	100	29	29	71	21	64	100	7
Old	14	43	50	21	36	50	57	100	43	50	57	29	71	86	0
Total	28	46	43	32	39	57	54	100	36	39	64	25	68	93	4

¹ 'Young' subjects were ≤ 20 years of age; 'old' > 20 years of age.

were also labelled with monospecific antibodies to *B. malayi* tubulin. A 5-kDa antigen was the only antigen that was more frequently recognized by antibodies in sera from antigen-negative endemic normals (40%) than by sera from microfilaria carriers (4%, $P = 0.001$).

Detection of IgG subclass antibodies to *W. bancrofti* L3 by immunoblot

IgG subclass reactivity to somatic *W. bancrofti* L3 antigens was studied with sera from 30 endemic individuals (Figure 2). These sera were randomly selected from the larger serum

panel to represent microfilaria carriers and antigen-positive and antigen-negative endemic normal groups. Five 'young' and five 'old' subjects were tested for each group. IgG₁ antibodies in sera from each group tended to bind strongly to antigens in the middle molecular weight range (31–97 kDa) and to low molecular weight antigens at 5, 6, and 14 kDa (Figure 2a). Very little IgG₂ antibody reactivity was observed (Figure 2b). IgG₃ antibody binding was almost completely limited to low molecular weight antigens (≤ 35 kDa), with the strongest binding to antigens at 5 and 6 kDa.

IgG₄ antibodies were more reactive with L3 antigens than

Table 2 Statistical analysis of differences in antibody reactivity by group to *Wuchereria bancrofti* L3 antigens, as assessed by immunoblot

Groups compared	Molecular weight (kDa)									
	97	66	60	55	50	40	14	6	5	
'Young' versus 'old' subjects	–	0.03	0.01	–	–	–	–	–	0.01	
Antigen-positive endemic normals versus antigen-negative endemic normals	0.03 ¹	–	–	0.03	< 0.01	–	–	0.03	–	
Infected (MF and/or antigenemia) versus antigen-negative endemic normals	–	–	–	< 0.01	< 0.01	–	–	0.01	–	
Antigen-negative endemic normals versus infected (MF and/or antigenemia)	–	–	–	–	–	–	–	–	0.01	
Microfilaremic versus endemic normals	–	–	–	0.02	0.02	–	–	–	< 0.01	
Endemic normals versus microfilaremic	–	–	–	–	–	–	–	–	0.01	
IFA positive cases to L3 versus IFA negative	–	0.04	–	–	–	0.04	0.03	–	–	

¹ Group differences in antibody binding to L3 antigens were assessed by chi-squared. Numbers given as P -values uncorrected for multiple comparisons.

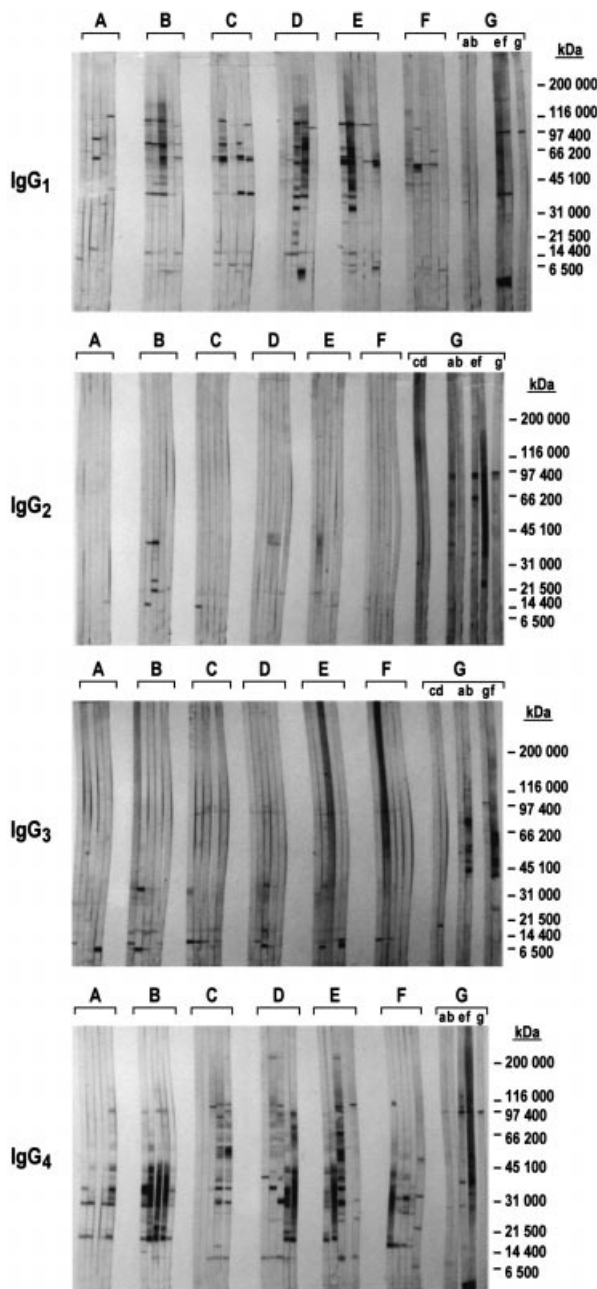


Figure 2 Representative immunoblots obtained with *W. bancrofti* L3 antigens and human sera developed with secondary antibodies to human IgG₁, IgG₂, IgG₃, and IgG₄ are shown. Immunoblots were obtained with sera from (A) antigen-negative endemic normal 'young' subjects, (B) antigen-positive endemic normal 'young' subjects, (C) asymptomatic microfilaremic 'young' subjects, (D) antigen-negative endemic normal 'old' subjects, (E) antigen-positive endemic normal 'old' subjects, (F) asymptomatic microfilaremic 'old' subjects and (G) reference sera. Control positive and negative sera in (G) are: (a,c) IgG positive endemic serum pool, (b,d) nonendemic serum pool. Ga and Gb were developed antihuman IgG while Gc and Gd were developed with antihuman IgG₂ or IgG₃, respectively; Ge was developed with rabbit antibody to *W. bancrofti* L3 antigen; Gf rabbit antibody to *B. malayi* adult worm antigen; Gg mouse monoclonal antibody to filarial paramyosin.

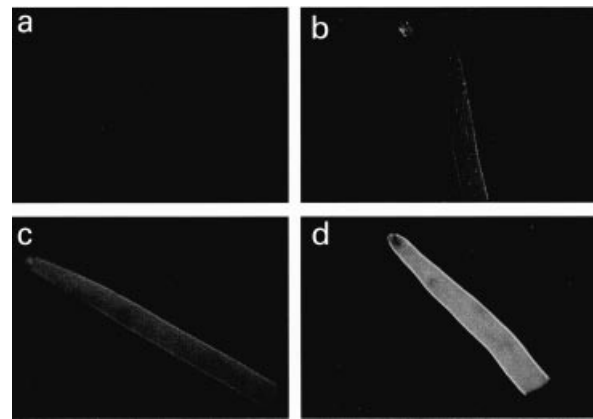


Figure 3 Showing binding of human IgG antibodies to *W. bancrofti* L3 surface antigens as assessed by indirect immunofluorescence. (a) Shows a negative IFA obtained with pooled normal human serum; (b) 1+ positive reaction; (c) 2+; (d) 3+. Photographs were taken at a magnification of $\times 400$.

the other IgG subclasses (Figure 2d). IgG₄ antibodies labelled over 90% of the antigen bands from total IgG immunoblots that were performed with the same sera. In general, IgG₄ antibodies had stronger reactivity with antigens under 45 kDa than IgG₁. However, this was not true for the very low molecular weight antigens at 5 and 6 kDa, which were not labelled by IgG₄ antibodies.

Antibodies to L3 surface antigens by IFA

Surface fluorescence was rated on a scale of 0–3+ (Figure 3). No staining was observed with 15 nonendemic control human sera. Results obtained with endemic sera are shown in Table 3. Sera from MF carriers had significantly weaker reactivity to L3 surface antigens than sera from amicrofilaremic subjects, regardless of antigen status (chi-squared = 30.56, $P < 0.001$). IFA reactivity did not differ between 'young' and 'old' subjects. IFA-positive sera were significantly more reactive with L3 antigens at 66, 40 and 14 kDa than IFA-negative sera (chi-squared = 2.94, 2.84 and 3.74, respectively, $P < 0.05$).

DISCUSSION

Although third-stage larvae initiate filarial infections in humans and are likely to be a primary target of protective immune responses, relatively few studies have examined human antibody responses to filarial L3. Several features distinguish the present study from previous efforts. First, the current study employed *W. bancrofti* parasite material to study antibodies in sera from a *W. bancrofti* endemic area, while some prior studies have substituted L3 from other

Table 3 IgG antibody reactivity of human sera to L₃ surfact antigens by infection status and age as detected by indirect immunofluorescence

Serum type	No. tested	Positive		Reaction intensity			
		No.	%	Neg	1+	2+	3+
Antigen-negative endemic normals							
'Young' ¹	20	15	75	5	3	5	7
'Old'	20	18	90	2	2	6	10
Antigen-positive endemic normals							
Young	10	9	90	1	0	1	8
Old	10	6	60	4	1	5	0
Asymptomatic microfilaremic							
Young	20	7	35	13	0	4	3
Old	22	9	41	13	0	5	4

¹ 'Young' subjects were ≤ 20 years of age; 'old' > 20 years.

filial species. The current study also tested a larger number of sera than most prior studies; this improved the statistical power of the study to correctly identify group differences in antibody reactivity. Finally, most prior studies have not tested sera for the presence of filarial antigens. Antigen testing in the current study allowed us to identify an infected subset within the group of asymptomatic and amicrofilaremic subjects that have been considered to be uninfected or 'putatively immune' in previous studies.

Immunoblot studies showed that the human sera tested in this study contained antibodies to 14 major antigens in *W. bancrofti* L3. Three of these antigens appeared to be paramyosin, myosin and tubulin, based on results obtained with monospecific antibodies. Additional work will be needed to further characterize the other L3 antigens labelled by human antibodies.

The current study yielded some unexpected results to the questions posed in the Introduction. For example, since epidemiological studies have suggested that people acquire a degree of immunity to filariasis after years of exposure to the parasite, we expected to find increased antibody reactivity in sera from older subjects. The opposite result was obtained by immunoblot (stronger reactivity was observed with sera from 'young' subjects), and no age difference was observed by IFA. These results can be interpreted several ways. First, it may be that both 'young' and 'old' subjects in our study had equivalent levels of partial immunity to L3. Lower levels of protective antibodies might have been observed in children if we had studied children under 10 years of age. A second possibility is that antibodies may not be involved in protective immunity to filarial L3. We doubt this explanation, because antibodies are clearly involved in immune clearance of microfilariae (Weil *et al.* 1982, Canlas *et al.* 1984), antibodies promote adherence and killing of L3 by human leucocytes *in vitro* (Higashi &

Chowdhury 1970, Sim *et al.* 1982, Chandrashekar *et al.* 1990), and antibodies have been shown to be protective in some studies of immunity to filariasis in animals (Abraham *et al.* 1988, Parab *et al.* 1988, Oikawa *et al.* 1992). A third potential explanation for our results comes from the relative dominance of IgG₄ antibodies (albeit with fairly strong IgG₁ responses), in sera from each of the groups studied. The prominence of IgG₄ antibody responses in filariasis has been described previously (Ottesen *et al.* 1985). The current results extend this observation to L3 antigens of *W. bancrofti* and are consistent with those recently reported from a study of brugian filariasis sera (Kurniawan-Atmadja *et al.* 1998).

IgG₄ antibodies are not likely to be protective against filarial worms; indeed, they may function as blocking antibodies to shield parasites from protective immune responses (Hussain & Ottesen 1988). The strong contribution of IgG₄ to total IgG reactivity in immunoblots may have concealed group differences in antibody reactivity that correlated with immune protection. This interpretation would be consistent with results of seroepidemiology studies of immunity to reinfection after treatment in schistosomiasis (Hagan *et al.* 1991, Dunne *et al.* 1992). Besides the prominence of IgG₄ and the relative absence of IgG₂ antibodies to filarial antigens in immunoblots, there were intriguing qualitative differences in antigen recognition by subclass that are difficult to explain at present. Additional studies are needed to further investigate the possible protective role of antibody isotypes other than IgG₄ and to explore the issue of isotype balance in human filariasis.

Based on the assumption that immunity to filariasis is mediated (at least in part) by antibodies to L3, we expected to find that antibodies in sera from antigen-negative endemic normals (considered to be 'putatively immune' by some authors whereas we consider these people to be 'relatively

immune') would preferentially bind particular L3 antigens, which subsequently could be considered to be potential targets of protective immune responses. Unfortunately, no such smoking gun was observed in this study, although the lukewarm preferential recognition of a 5-kDa antigen by endemic normal sera is a clue that merits further investigation. Apart from this antigen, our results are consistent with the negative results reported by Day *et al.* (1991a) and Bailey *et al.* (1995) who found that sera from infected subjects were at least as reactive with L3 antigens as sera from uninfected subjects.

Previous studies of antibody reactivity to filarial L3 failed to find a correlation between IFA and immunoblot results (Day *et al.* 1991a, Zhang *et al.* 1999). To explain this result, Day *et al.* suggested that the critically important surface antigens may not be solubilized by detergents and reducing agents in SDS-PAGE sample buffer. Curiously, IFA-positive sera did preferentially bind to several L3 antigens (at 66, 40 and 14 kDa) by immunoblot in the current study. Additional studies are needed to further characterize these antigens and to establish whether they are exposed on the L3 surface.

It should be noted that the serum set we studied was cross-sectional, with only one time point sampled per subject. This limitation also applies to all previous studies of anti-L3 antibodies. Future studies should focus on the issue of changes in anti-L3 antibody reactivity over time with special attention paid to sera from those who acquire or clear infections.

In conclusion, although our study has not clearly identified targets of protective immunity to *W. bancrofti* L3 or answered the question of whether antibodies to L3 are protective in human filariasis, it has provided new information on potential targets of immunity and indicated clear directions for future research.

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