

Paramyosin is a major target of the human IgA response against *Schistosoma japonicum*

MARIA GENEVIVE H.HERNANDEZ¹, JULIUS CLEMENCE R.HAFALLA¹, LUZ P.ACOSTA¹, FE F.ALIGUI¹, GEMILIANO D.ALIGUI¹, BERNADETTE L.RAMIREZ¹, DAVID W.DUNNE² & MARIO LUIS SANTIAGO¹

¹Schistosomiasis Study Group, Research Institute for Tropical Medicine, Alabang, Muntinlupa City, Philippines and ²Microbiology and Parasitology Division, Department of Pathology, Cambridge University, Cambridge, UK

SUMMARY

Human resistance and susceptibility to schistosomiasis is associated with age and specific antibody isotype responses against worm (SWAP) and egg (SEA) antigens. In a cross-sectional study of 176 individuals infected with Schistosoma japonicum in the Philippines, strikingly similar isotype response patterns against SWAP and SEA was observed when compared to other endemic areas. Interestingly, IgA titres to SWAP correlated with older age among S. japonicum-infected individuals (n = 176, P < 0.01), suggesting a role for this isotype in protective immunity. To identify the molecular targets of human IgA, 17 high-IgA/SWAP responders were identified from the said population. IgA antibodies from the majority (14/17) of these individuals recognized a band of 97 kDa (Sj97), comigrating in immunoblots with the myofibrillar protein paramyosin. The antigen was confirmed as paramyosin by expressed sequence tag (EST)-analysis of four clones obtained by screening an adult S. japonicum cDNA library with pooled IgA antisera and mouse antiparamyosin polyclonal antibodies. The identification of paramyosin as a major target of human IgA raises its potential as a vaccine candidate that targets mucosal immune responses. Since this antigen is exposed on the parasite surface only during the lung stages, we propose that human IgA contributes to parasite attrition during schistosome migration in the lungs.

Keywords paramyosin, *Schistosoma japonicum*, Philippines, IgA, lung stage

INTRODUCTION

Vaccine development remains to be an important long-term goal for the control of schistosomiasis. Extensive work has been carried out to identify schistosome molecules which confer significant partial protection in different animal models. Several vaccine candidates have been identified since (Bergquist 1995), but it is still unclear which vaccine formulation will be optimal for testing in humans, since it is becoming apparent that different antigens may be targets of different immunological responses. Characterizing which responses are protective in different stages of infection through human immune correlate studies may therefore aid in developing appropriate immunization strategies.

A comparison of human antibody isotype responses against worm (soluble worm antigen, SWAP) and egg (soluble egg antigen, SEA) antigens in different geographical regions showed strikingly similar immunoepidemiological patterns. In Kenya (Rihet *et al.* 1991, Dunne *et al.* 1992), Brazil (Webster *et al.* 1997a), the Gambia (Hagan *et al.* 1991), Zimbabwe (Ndhlovu *et al.* 1996) and the Philippines (Webster *et al.* 1997b), IgE and IgG4 antibodies against SWAP correlated with older age and/or resistance to reinfection, while IgM and IgG2 titres against SEA correlated with younger age and/or susceptibility. These data suggest that similar immune mechanisms govern protective immunity and susceptibility to the three major schistosome species in geographically diverse endemic populations. Hence, the targets of the 'protective' human IgE response may be considered as potential vaccine candidates (Li *et al.* 1995, Webster *et al.* 1996, Santiago *et al.* 1998).

While the role of IgE in age-acquired immunity has been well-documented in field studies, the role of another Th2-driven isotype, IgA, is more obscure. IgA is widely accepted as a noninflammatory isotype which may have protective functions in the gut, particularly by binding to bacteria or gut-dwelling parasites without complement activation

Correspondence: Mario Luis Santiago, Department of Microbiology, LHRB 620, University of Alabama at Birmingham, Birmingham, AL 35294, USA.

Received: 22 January 1999

Accepted for publication: 21 July 1999

(Lamm 1997). Schistosomes, however, mainly reside in the bloodstream of the host, hence, a protective role for IgA, majority of which is usually transcytosed to the mucosal tissues, is more difficult to reconcile. Nonetheless, serum IgA responses against a candidate *Schistosoma mansoni* vaccine molecule, Sm28GST, exhibit enzyme inhibitory, antifecundity and antiembryonation effects (Auriault *et al.* 1990) and was significantly elevated among resistant individuals (Grzych *et al.* 1993). Studies done in Brazil (*S. mansoni*) and Zimbabwe (*Schistosoma haematobium*) also revealed a weak but statistically significant correlation between serum IgA responses to SWAP and age/resistance to reinfection (Ndhlovu *et al.* 1996, Webster *et al.* 1997a). A similar correlation between IgA titres to SWAP and age was also observed in the Philippines (Webster *et al.* 1997b), where evidence for age-acquired immunity was obtained (Olds *et al.* 1996, Olveda *et al.* 1996). These data suggest that IgA antibodies may also play a role in immunity to *S. japonicum* infection.

We therefore sought to identify the molecular targets of the human IgA response using serum from individuals who have high IgA titres to SWAP (high-IgA/SWAP responders). Interestingly, the majority of these individuals recognized antigen(s) in the 97 kDa range. Using a double immunoscreening approach, we subsequently show evidence that this antigen is the myofibrillar protein paramyosin.

MATERIALS AND METHODS

Antigen preparation

Adult *S. japonicum* worms were obtained by perfusion of New Zealand rabbits experimentally infected with cercariae from *Oncomelania quadrasi* snails from Mindoro Island, the Philippines. Soluble worm antigen (SWAP) was prepared by grinding liquid nitrogen-frozen adult worms and gentle shaking in buffer with Triton X-100 (Kurtis *et al.* 1997). Native paramyosin was biochemically purified from adult *S. japonicum* worms using a salt-extraction method (Ramirez *et al.* 1996). Adult worm tegument (AWT) and Denuded Worm Antigen (DWA) were also prepared as described (Santiago *et al.* 1998).

Polyclonal antisera

Polyclonal antibodies against native *S. japonicum* paramyosin were obtained from female ICR mice immunized with native paramyosin on days 0, 4 and 18 without adjuvant. The said protocol resulted in a significant reduction in worm burden (60–80%) following challenge with *S. japonicum* cercariae (Ramirez *et al.* 1996). Specificity was assessed

such that a 97 kDa band was uniquely recognized in immunoblot analyses with SWAP. Lots with the highest IgG titres in an indirect enzyme-linked immunosorbent assay (ELISA) were pooled and preadsorbed in *E. coli* lysate prior to the subsequent experiments.

Study population

Human antisera were obtained from two endemic communities in Leyte, the Philippines (Pastrana-Cinanoan and Lanawan), which were part of a government program on annual mass chemotherapy with praziquantel. Serum from these individuals were previously subjected to isotype-specific ELISAs (IgA, E, G1, G2, G3, G4, M) with SWAP and SEA, as described (Webster *et al.* 1997b).

Immunoblots

Approximately 30 µg SWAP was separated in 10% polyacrylamide gels in an SDS-PAGE then transferred to nitrocellulose. For IgA-specific blots 0.4-cm strips were blocked with 5% skim-milk/TBST for 2 h, then incubated with 1 : 100 antisera for 6 h in a flat rotary shaker below ambient temperature. Following three 10-min washes with TBST, the strips were incubated for 2 h with 1 : 1000 goat anti-human IgA conjugated to alkaline phosphatase (Sigma, St Louis, MO, USA). Blots were developed using the BCIP/NBT substrate system. For paramyosin blots, mouse anti-paramyosin was used at a dilution of 1 : 100 and incubated for 3 h, followed by incubation with 1 : 1000 goat antimouse antibodies (IgA, G and M) for 1 h prior to development with BCIP/NBT.

Immunoscreening

Approximately 5×10^5 clones from a λ -ZAP adult *S. japonicum* (Mindoro strain) cDNA expression library (Kurtis *et al.* 1997) were screened using pooled high-IgA/SWAP antisera, using conditions specified in the IgA-blots mentioned above. Clones not recognized in the primary screen were utilized as negative controls for the secondary and tertiary screens. Immunoreactive plaques from the tertiary screen, designated gA1 to gA8, were subsequently plated in individual NZY 100-mm plates then induced for expression with 10 mM IPTG-treated nitrocellulose. These plaque-lifts were subscreened with mouse anti-paramyosin as described in the immunoblots. pBluescript constructs from the IgA-reactive plaques were excised, transformed into XL-1 blue cells (Stratagene, La Jolla, CA, USA), and EST-sequenced using the T3 promoter as sequencing primer. DNA sequences were analysed using the BLAST software (NCBI, NIH, Bethesda, MD, USA).

RESULTS

Immunoblot profile of antigens recognized by human IgA

The study population consisted of 176 individuals from two villages, whose antisera were subjected to isotype-specific ELISAs against worm and egg antigens. Previous data (Webster *et al.* 1997b) showed similar isotype titre versus age trends compared with Kenya, where resistance to reinfection data was obtained. In addition, a weak IgA/SWAP versus age correlation was observed. To investigate whether this weak correlation may be due to the recognition of a small proportion of SWAP antigens, high IgA/SWAP responders were selected by ranking absorbance readings against SWAP and obtaining serum from individuals with IgA titres above the 90th percentile (Figure 1). A total of 19 individuals were identified, but only 17 serum samples were available. High-IgA/SWAP responders ($n = 17$) had ages ranging from 7 to 55 years, had very similar IgA/SWAP titres (with the exception of patients 3 and 17) and had lower intensities of infection (70.6 ± 71.9 mean egg per gram, $n = 17$), though not statistically significant, than the rest of the population (170 ± 556 mean egg per gram, $n = 166$).

The profile of antigens recognized by human IgA among these individuals was relatively limited to some high-molecular weight antigens (Figure 2), with the majority (14/17) recognizing antigen(s) in the 97 kDa range (Sj97). Intriguingly, the patients who had intense IgA/Sj97 signals were

older (≥ 27 years older) than the patients who did not recognize or had faint reactivities to Sj97 (< 27 years older, with the exception of patients 4 and 7), suggesting a possible age-specific association. The same pattern could not be discerned for the other IgA-reactive antigens.

In IgA-blots with different worm preparations using pooled antisera, a 97 kDa antigen also emerged as the immunodominant antigen in DWA and SWAP, but not in AWT and SEA (Figure 3, lanes A1–4). The molecular weight of Sj97 and its subtegumental location led us to suspect that it corresponds to a known vaccine candidate, paramyosin. Parallel Western blots were therefore performed, and comigration of Sj97 with paramyosin was observed (Figure 3).

Molecular identification of Sj97 as paramyosin

To verify the molecular nature of Sj97 as paramyosin and identify other antigens recognized by the high-IgA/SWAP responders, we screened a λ -ZAP cDNA expression library with the pooled antisera. Eight cDNA clones, designated gA1 to gA8, were consistently recognized by human IgA up to the tertiary screen. Subscreening of these clones with mouse antiparamyosin antisera revealed six (gA1 to gA5 and gA7) as dually reactive. gA6 and gA8 did not react with these antisera, implying that these clones encode different antigens which do not cross-react with paramyosin (data not shown).

EST analyses were performed on the eight cDNA clones identified. The partial sequences from clones gA1, 2, 3 and 4 matched paramyosin sequences in the database (Becker *et al.* 1995, Accession no. SJU1185), with 100% homology from amino acid positions 124–277. On the other hand, EST-analyses from clones gA5 and gA7 suggest that they encode novel, identical antigens which cross-react with paramyosin. Clones gA6 and gA8 did not exhibit strong similarity to any known entries in the database, but was identical to MJ-2, one of the nine cDNA clones identified by high-IgE/SWAP responders (Santiago *et al.* 1998).

DISCUSSION

The role of IgA antibodies in immunity to gastrointestinal helminths is gaining wide acceptance, however, its contribution in immunity to blood-borne helminth parasites such as schistosomes remains to be clarified. Previous studies suggested that IgA antibodies may contribute in anti-fecundity and anti-embryonation immunity by neutralizing GST activity (Auriault *et al.* 1990, Grzych *et al.* 1993), however, it is still unclear whether these phenomena would account for the association between IgA reactivity to total worm antigens and age and/or resistance to reinfection

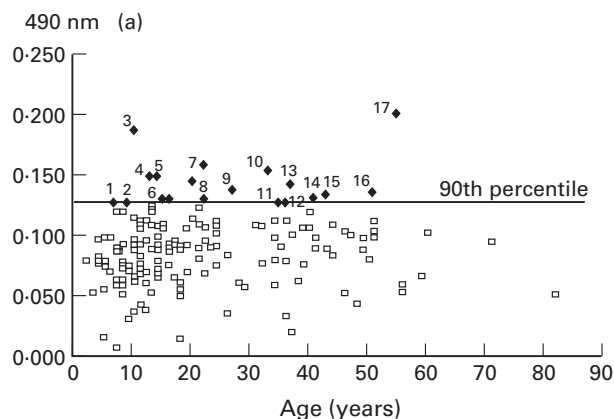


Figure 1 Identification of high-IgA/SWAP responders. Serum samples from 176 individuals in the Philippines were subjected to an IgA-specific ELISA with SWAP. Individuals whose absorbance readings $\geq$ 90th percentile cut-off value were classified as 'high-IgA/SWAP responders' (diamonds). Nineteen individuals were identified, but sera from only 17 individuals were available. These individuals, depicted as patients 1–17, have a broad age distribution (age 7–55 years). Except for two outliers (patients 3 and 17), the IgA/SWAP titres of these individuals do not vary considerably from each other.

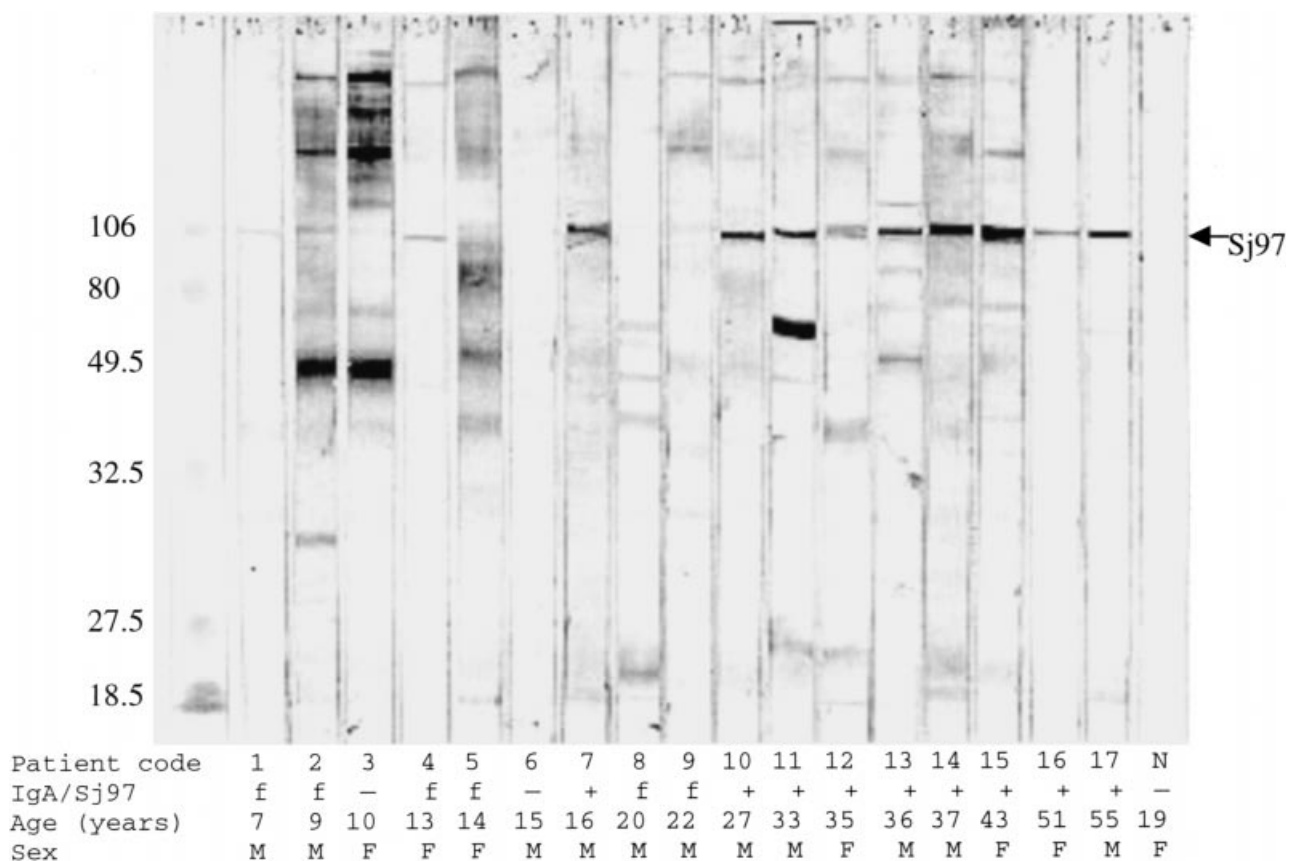


Figure 2 Immunoblot profile of antigens recognized by the human IgA response. Serum samples from high-IgA/SWAP responders were utilized in an IgA-specific immunoblot with SWAP. As shown, multiple antigens were recognized by human IgA among these individuals, including an immunodominant 97 kDa band (Sj97) recognized by the majority of older, high-IgA/SWAP responders. IgA/Sj97 signals were qualitatively classified either as intense (+), faint (f) or none (—). Patient codes, with ages in ascending order, correspond to those of Figure 1. The sex of each individual is also shown. Molecular weight markers are depicted on the far left.

observed in at least three endemic populations (Ndhlovu *et al.* 1996, Webster *et al.* 1997a, Webster *et al.* 1997b). The dearth of knowledge on the precise molecular target(s) of the IgA response in these populations is therefore a critical gap in understanding the mechanisms underlying the protective role of IgA in human resistance models.

An effort has therefore been undertaken to identify the molecular targets of human IgA. In this study, we utilized an isotype-screening approach, similar to our previous studies which identified Sj22·6 (Santiago *et al.* 1998) and Sj21·7 (Hafalla *et al.* 1999) as targets of human IgE. Our approach focused on the identification of 17 high-IgA/SWAP responders, whose antisera were pooled to screen a λ -ZAP cDNA expression library. By subscreening with antiparamyosin and confirmation by DNA sequencing, we unambiguously identified the vaccine candidate paramyosin (Ramirez *et al.* 1996, Yang *et al.* 1997) as a major target of the human IgA response.

Since paramyosin immunolocalizes to the subtegument and postacetabular glands (Matsumoto *et al.* 1988, Nara *et al.* 1994), our findings raise the issue on how this antigen can be an effective target of IgA antibodies. More recently, paramyosin was shown to be exposed on the parasite surface only in lung-stage schistosomulae (Goibert *et al.* 1997). These findings suggest that IgA antibodies may exert a protective effect in the lungs when schistosomes migrate in this compartment. Hence, the stimulation of paramyosin-specific mucosal responses may confer significant damage to these lung-stage parasites. In support of this hypothesis, oral immunization of mice with recombinant paramyosin resulted in tegumental damage to perfused *S. japonicum* worms (Yang *et al.* 1997). While IgA antibodies were not detected in serum in this experiment, it is feasible that such antibodies localize to the lungs, or oral immunization may not be an optimal route for eliciting lung-phase immune responses (Kremer *et al.* 1998).

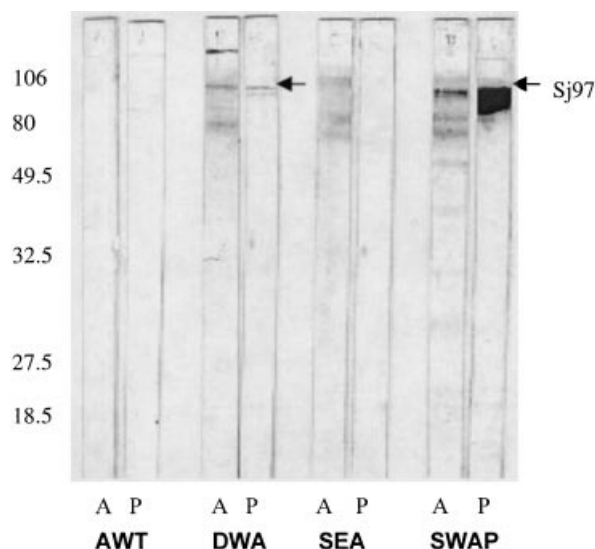


Figure 3 Co-migration of Sj97 with paramyosin. Different parasite preparations were probed with (A) pooled high-IgA/SWAP responder antisera (IgA blot) and (P) mouse antiparamyosin polyclonal antibodies. AWT, adult worm tegument; DWA, denuded worm antigen; SEA, soluble egg antigen; SWAP, soluble worm antigen preparation. Pooled antiserum was prepared by mixing equal amounts of antisera from the 17 high-IgA/SWAP responders. Molecular weight markers are depicted on the far left.

IgA antibodies are potent triggers for eosinophil degranulation (Abu-Ghazaleh *et al.* 1989) and there is evidence that this occurs in the lungs in allergic asthma (Terada *et al.* 1996, Nahm & Park 1997, Peebles *et al.* 1998). In one experiment, IgA2 conferred the highest levels of in-vitro eosinophil-mediated killing of DNP-coated schistosomulae compared with other isotypes (Dunne *et al.* 1993). Combined with previous observations of pulmonary eosinophilia in mice multiply immunized with irradiated cercariae and treated with praziquantel (Menson *et al.* 1989, Piper *et al.* 1991), and the fact that IL-5, a central regulator of eosinophil biology and promoter of IgA biosynthesis, are elevated among resistant individuals (Roberts *et al.* 1993, Medhat *et al.* 1998), we speculate that paramyosin-specific IgA antibodies trigger an eosinophil-mediated response against lung-stage parasites. Further studies on the pulmonary mucosal immune responses of infected individuals will be needed to verify this hypothesis.

Compared to the strong IgE/SWAP-age correlation observed in different endemic populations, the IgA/SWAP-age correlation was usually weaker (Webster *et al.* 1997b, Ndhlovu *et al.* 1996), suggesting that only a few antigens in SWAP may be contributing to this trend. This was supported by the relatively limited set of antigens recognized by human IgA (Figure 2) compared to IgE

(Santiago *et al.* 1998). In this study, the age distribution of high-IgA/SWAP responders was broad, but the IgA/Sj97 signals were more intense among older individuals, suggesting an age-specific correlation. This was confirmed in our recent findings in the same population, where we observed a strong positive correlation ($\rho = 0.393$, $n = 145$, $P < 0.00001$) between serum IgA responses to native paramyosin and age in isotype-specific ELISAs and Spearman's rank correlation analyses (Santiago *et al.* in preparation). Furthermore, there was a strong positive correlation between IgA/pmy and IgA/SWAP titres ($\rho = 0.315$, $n = 145$, $P < 0.0001$), suggesting that paramyosin may be the critical component of SWAP in the IgA/SWAP versus age correlation. This was supported by the fact that no other antigen aside from Sj97 showed a qualitative age-specific correlation in the IgA-blot.

Intriguingly, the antigens recognized by human IgE and IgA antibodies appear to be mutually exclusive, since (1) immunodominant antigens recognized in the IgE-blot (Santiago *et al.* 1998) were not found in the IgA-blot; and (2) cDNA clones which were identified by human IgE were not recognized by human IgA and vice versa, except for a single clone designated as MJ-2 (Santiago *et al.* 1998) or gA6/gA8 (this study). Although a 97-kDa band was also identified in IgE-blot (Santiago *et al.* 1998), this does not appear to be paramyosin, since the corresponding clones (gA1 to gA4) were not recognized by IgE antibodies from high-IgE/SWAP responders which reacted to Sj22-6/Sj21-7 (Hernandez *et al.* unpublished results). This discrepancy between the molecular targets of IgE and IgA antibodies in the context of *S. japonicum* infection suggest that these isotypes may act at distinct levels of the parasite life cycle and/or host compartments, and hence play independent roles in host immunity against schistosomes. In particular, human IgA antibodies may be directed specifically to antigens expressed at mucosal tissues, such as the case for paramyosin in the lungs. A more detailed analysis of the expression patterns of these IgE/IgA targets may shed more insight to this phenomenon.

In conclusion, we unambiguously show through a double-immunoscreening approach that *S. japonicum* paramyosin is a major target of IgA antibodies from high-IgA/SWAP responders. Since mucosal immune responses are primarily geared towards the generation of specific IgA antibodies, paramyosin may have potential as a mucosal vaccine against human schistosomiasis which is targeted towards lung-stage parasites.

ACKNOWLEDGEMENTS

The authors thank Dr Remigio M. Olveda and the Schistosomiasis Study Group (RITM), Dr G. Richard Olds and

Dr Peter M. Wiest (Case Western University), the Schistosomiasis Control Program and the people of Leyte, the Philippines, for their support. We also thank Dr Frederick Oduol (New York University) for critically reviewing this manuscript. This work was supported in part by the National Institutes of Health Tropical Medicine and Research Center (US NIH TMRC) Grant P50 AI 30601.

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