

# Infection and Immunity

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## Immunoglobulin E (IgE) Responses to Paramyosin Predict Resistance to Reinfection with *Schistosoma japonicum* and Are Attenuated by IgG4<sup>∇</sup>

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Schistosomiasis remains a public health concern in developing countries, and rapid reinfection fostered by continued exposure to contaminated water sources necessitates a vaccine to augment current mass treatment-based control strategies. We report isotype-specific (immunoglobulin A [IgA], IgE, IgG1, IgG4, and IgG) antibody responses to soluble worm antigen preparation and the recombinant vaccine candidates rSj97, rSj67, and rSj22 from a *Schistosoma japonicum*-infected cohort in Leyte, the Philippines, where schistosomiasis is endemic. Sera were collected from infected individuals 1 month posttreatment with praziquantel, and antibody responses were measured using a bead-based multiplex platform. Reinfection was monitored by stool sampling every 3 months, and data up to 1 year were included in the analysis ( $n = 553$ ). In repeated-measures models, individuals with detectible IgE responses to rSj97 had a 26% lower intensity of reinfection at 12 months posttreatment compared to nonresponders after adjusting for age, gender, village, exposure, pretreatment infection intensity, and clustering by household ( $P = 0.018$ ). In contrast, IgG4 responses to rSj97 as well as rSj67 and rSj22 were associated with markedly increased reinfection intensity. When stratified by IgG4 and IgE responder status, individuals with IgE but not IgG4 responses to rSj97 ( $n = 16$ ) had a 77% lower intensity of reinfection at 12 months compared to individuals with IgG4 responses but not IgE responses ( $n = 274$ ), even after adjusting for potential confounders ( $P = 0.016$ ). Together with our previously described protective cytokine responses, these data further support paramyosin as a leading vaccine candidate for human schistosomiasis japonica and underscore the importance of careful adjuvant selection to avoid the generation of blocking IgG4 antibody responses.

Schistosomiasis, caused by parasitic helminths of the genus *Schistosoma*, remains a major public health concern and currently infects 200 million individuals with 600 million individuals at risk of infection in 74 developing countries (19). National control strategies focusing on mass chemotherapy with praziquantel have significantly reduced severe liver and urinary tract pathology; however, rapid reinfection with consequent subtle morbidities such as anemia, malnutrition, and cognitive impairment persist despite years of annual treatment (30). Continued exposure to contaminated water sources mandates alternative control strategies such as vaccine-linked chemotherapy (3).

In vitro studies have demonstrated that schistosome larvae are susceptible to damage in the presence of sera from infected individuals together with leukocytes from uninfected donors, suggesting that parasite-specific antibody-dependent cellular cytotoxicity (ADCC) plays a key role in parasite elimination (8). Subsequent investigations identified the role of protective immunoglobulin E (IgE), IgA, and IgG antibody isotypes (11, 23, 29) and the participation of eosinophils and mast cells in

orchestrating this attack (7, 12). However, several studies have also demonstrated the presence of inhibitory antibodies which block schistosomular killing (22, 28). In particular, the antibody isotype IgG4 is a poor initiator of ADCC and blocks killing by competing with protective isotypes (29). These data suggest that schistosomes induce both protective and antagonistic antibody responses, which may alter the balance between parasite elimination and immune evasion (3).

Consonant with in vitro studies, several immunoepidemiologic surveys conducted in areas where schistosomiasis is endemic have demonstrated associations between antibody responses to crude parasite antigens and reinfection outcomes in humans. Protective IgE responses to worm (17, 24) and egg (43) antigens have been described across schistosome species, as well as IgA responses mediating antifecundity effects (23). These cohort studies have also described antiparasite IgG4 (16, 24, 32), IgM, and IgG2 (5, 22, 28) as isotypes associated with susceptibility.

Based on a model of antibody-mediated protection, the antigenic targets of both protective antibody isotypes and protective monoclonal antibodies have been identified in parasite extracts and genomic libraries. Numerous candidates have been identified (4), and a panel of the most promising of these was evaluated in immunoepidemiologic studies in Egypt (2), Brazil (35), and to a limited extent in China and the Philippines (1, 32). Despite this progress (3), only one vaccine candidate

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(*Schistosoma haematobium* glutathione S-transferase) has advanced to early-phase clinical trials (10).

We have previously described cytokine responses to crude antigen preparations (soluble worm antigen preparation [SWAP], SEA) and defined vaccine candidates (Sj97, Sj67, and Sj22) in a cohort of schistosomiasis-infected individuals between 7 and 30 years old and residing in Leyte, the Philippines (31). Here, we extend this work by measuring isotype-specific (IgA, IgE, IgG1, IgG4, and total IgG, henceforth referred to as IgG) antibody responses to these antigens in the same cohort and evaluating their association with resistance to reinfection over 12 months of posttreatment follow-up. We report that IgE responses to rSj97 are associated with resistance to reinfection and are attenuated by IgG4.

## MATERIALS AND METHODS

**Study population.** We enrolled individuals from three rice-farming villages (Macanip, Buri, and Pitogo) in Jaro, a town of Leyte, the Philippines, as described previously (31). Exclusion criteria included pregnancy, lactation, evidence of chronic illness, severe anemia, malnutrition, or hepatomegaly. Overall, a total of 616 eligible individuals between 7 and 30 years old consented to participation in the study. Active schistosome infection was assessed by three consecutive Kato-Katz stool examinations performed in duplicate and expressed as average eggs per gram of fecal matter (epg). All study participants were treated with a split dose of 60 mg praziquantel/kg of body weight at the start of the study period (October 2002 for Macanip; March 2003 for Buri and Pitogo). Phlebotomy was performed 1 month after treatment using Vacutainer serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). The serum was prepared by centrifugation, divided into single-use aliquots, stored at  $-80^{\circ}\text{C}$  on site, shipped to the Center for International Health Research (CIHR) on dry ice, and stored at  $-80^{\circ}\text{C}$  at CIHR until use. Subjects were assessed for reinfection every 3 months for 18 months with three Kato-Katz stool examinations performed in duplicate as described above. In addition, water contact was quantified as a proximate marker for infection exposure, as previously detailed (31). Data collected from this study were encoded using Filemaker 7.0 software (Filemaker Inc., Santa Clara, CA). This study was approved by the institutional review boards of Brown University and the Philippine Research Institute for Tropical Medicine.

***Schistosoma japonicum* antigens.** SWAP was prepared from *S. japonicum* adult worms courtesy of the Biomedical Research Institute (Rockville, MD). Worms were resuspended in phosphate-buffered saline, pH 7.4 (Gibco, Invitrogen, Carlsbad, CA), disrupted in ice using a Dounce homogenizer, and sonicated five times using 10-s bursts. The suspension then was centrifuged at  $60,000 \times g$  at  $4^{\circ}\text{C}$  for 1 h, and the supernatant was collected and filter sterilized through 0.22- $\mu\text{m}$  syringe filters (Millipore, Billerica, MA). Freshly prepared SWAP was used to couple microsphere beads as described below. The recombinant antigens rSj97, rSj67, and rSj22 were prepared as previously described (27, 31, 38). Briefly, the respective cDNA sequences were cloned and ligated into pET-32 Xa/LIC expression vectors (Novagen, EMD Biosciences, San Diego, CA) downstream of a thioredoxin fusion tag and transfected to *Escherichia coli* BL21(DE3) (Novagen). Recombinant protein expression was induced with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and purified by liquid chromatography. rSj97 was purified as a thioredoxin fusion protein from *E. coli* inclusion body extracts using successive anion-exchange, hydroxyapatite, and size-exclusion chromatography. rSj67 was purified from induced cell lysates using successive metal affinity, anion-exchange, and size-exclusion chromatography, and the thioredoxin tag was cleaved by protease digestion followed by a final polishing step to remove cleaved thioredoxin. Similarly, rSj22 was successively purified using metal affinity, anion-exchange, and hydrophobic-interaction chromatography, and the thioredoxin tag was cleaved by protease digestion followed by a final polishing step to remove cleaved thioredoxin. The thioredoxin fusion tag, rThio, was expressed by transfecting self-ligated pET32 Xa/LIC to BL21(DE3) and purified by sequential metal affinity chromatography, hydrophobic-interaction chromatography, and size-exclusion chromatography. Recombinant antigens were stored at  $-80^{\circ}\text{C}$ .

**Antibody assay.** Isotype-specific (IgA, IgE, IgG1, IgG4, and IgG) antibody responses to SWAP, rSj97, rSj67, rSj22, and rThio were assessed using a high-throughput bead-based platform (Bio-Plex; Bio-Rad, Hercules, CA). This method allowed the simultaneous determination of antibody responses of a particular isotype to the panel of antigens from a single serum sample. One

hundred micrograms each of rSj97, rSj67, rSj22, rThio, and freshly prepared SWAP was covalently bound to  $1.25 \times 10^7$  microspheres (beads) per the manufacturer's instructions (Luminex, Austin, TX). Each antigen was coupled to beads with a unique dye signature, allowing automated antigen-specific discrimination of fluorescence values in the multiplex assay. The bead regions were pooled after confirming that fluorescence values did not differ between single-bead analysis and multiplex analysis. Pooled beads were divided into aliquots for single use, lyophilized, and stored at  $-80^{\circ}\text{C}$ . Pooled plasma obtained from the Macanip cohort was used to optimize the antibody assays, with sample and secondary antibody dilutions and volumes selected based on a dose-sensitive fluorescence response. For the IgE and IgA assays, beads were preincubated with 2.8 mg/ml nonspecific human IgG (Sigma, St. Louis, MO) prior to sample incubation in order to mask the antibody binding sites of rSj97. Individual patient sera ( $n = 601$ ) and North American controls from an area of nonendemicity (termed "nonendemic controls") ( $n = 15$ ) were diluted in assay buffer (phosphate-buffered saline, 1 mg/ml bovine serum albumin, 0.05% Tween 20, 0.05% sodium azide) at optimized concentrations (1:20 for IgE and 1:100 for IgA, IgG, IgG1, and IgG4) and incubated with beads for 30 min at room temperature (RT) with shaking at optimized volumes (50  $\mu\text{l}$  for IgE and 25  $\mu\text{l}$  for IgA, IgG, IgG1, and IgG4). After several washes in assay buffer, the beads were incubated with 50  $\mu\text{l}$  of isotype-specific detecting antibodies (Pharmingen, San Diego, CA) at optimized dilutions (1:100 for IgA, IgE, and IgG1 and 1:5,000 for IgG4 and IgG) and incubated for 30 min at RT with shaking. After being washed, the beads were incubated in 50  $\mu\text{l}$  of streptavidin-phycoerythrin (1:500 dilution; Pharmingen) for 10 min at RT with shaking, washed, and resuspended in assay buffer, and then the fluorescence was quantified using the BioPlex analyzer (model 100 for IgE, IgA, and IgG1 assays and model 200 for IgG4 and IgG assays; Bio-Rad). All liquid handling was performed by a high-speed pipetting robot (Megaflex; Tecan, Research Triangle Park, NC). Cytokine responses to Sj97 were determined on peripheral blood mononuclear cells obtained 4 weeks posttreatment using a multiplexed bead assay as described previously (31).

**Statistical analysis.** Individuals were classified as antibody responders or nonresponders using a plate-specific cutoff (mean plus 2 standard deviations) calculated from nonendemic controls included in each assay plate ( $n = 15$ ). Raw fluorescence values were used for SWAP, rSj67, and rSj22 classification; for rSj97, thioredoxin fluorescence values were subtracted from raw rSj97 values prior to classification due to the presence of the thioredoxin fusion tag on rSj97. This dichotomous classification allowed for the examination of reinfection trends over time between responders and nonresponders, as well as the interaction between isotype-specific responses (i.e., combined IgE-IgG4 levels). Egg counts were log transformed [ $\ln(\text{value} + 1)$ ] to approximate normal distribution. Only successfully treated individuals with available antibody measurements and who contributed at least one stool sample during any of the reinfection time points were included in the analysis ( $n = 553$ ). In the bivariate analysis, contingency tables were constructed to detect differences between antigen- and isotype-specific antibody responses and the potentially confounding categorical variables of gender, age group, water contact (two-level nominal divided at the 2.5 percentile as described previously [31]), and village, while Student's *t* test was used for continuous baseline intensity measurements. To assess the relationship between antibody responses and resistance to reinfection, a repeated-measures regression model with random intercepts was utilized, with the interaction term of binary antibody response variable by time point as the primary predictor. Included in the model are the following potential confounders: age group (children 7 to 11 years old, adolescents 12 to 21 years old, and adults 22 to 30 years old), gender, village of residence, water contact, and baseline intensity of infection. In addition, the analysis was adjusted for the nonindependence of responses from individuals of the same household (clustering). Results are presented as the least squares (LS) mean intensity of reinfection between antibody responders and nonresponders across the four follow-up time points (3, 6, 9, and 12 months posttreatment).

Potentially opposing effects of IgE and IgG4 responses to rSj97 were tested using a similar repeated-measures model with the interaction of interest between the time point and the combined IgE and IgG4 responder status with four levels (IgE only, IgG4 only, IgE and IgG4, and neither). Results for this analysis are presented as the LS mean intensity of reinfection among the categories of the combined IgE and IgG4 responder variable at 12 months posttreatment after adjusting for potential confounders. Finally, simple logistic regression was used to assess the relationship between antibody responses to rSj97 and  $\ln$ -transformed [ $\ln(\text{cytokine} + 1)$ ] values. Measured cytokines included gamma interferon, interleukin-4 (IL-4), IL-5, IL-10, IL-12, and IL-13 from Sj97-stimulated peripheral blood mononuclear cells collected 4 weeks posttreatment. *P* values of  $<0.05$  were considered significant. Longitudinal analysis with clustering was performed in SAS version 8.1, while bivariate analysis for epidemiological pa-

TABLE 1. Baseline cohort characteristics (*n* = 553)

| Characteristic              | No. (%) of subjects |
|-----------------------------|---------------------|
| Village                     |                     |
| Macanip.....                | 378 (68)            |
| Buri.....                   | 80 (15)             |
| Pitogo.....                 | 95 (17)             |
| Gender                      |                     |
| Male.....                   | 348 (63)            |
| Female.....                 | 215 (37)            |
| Age                         |                     |
| Child (7–11 yr).....        | 204 (37)            |
| Adolescent (12–21 yr).....  | 257 (46)            |
| Adult (22–30 yr).....       | 92 (17)             |
| Infection intensity         |                     |
| Light (1–99 epɡ).....       | 402 (73)            |
| Moderate (100–399 epɡ)..... | 121 (22)            |
| Heavy (>400 epɡ).....       | 30 (5)              |

Parameters and cytokine levels were performed in JMP version 5.1.1 (both from the SAS Institute, Cary, NC).

RESULTS

**Cohort description.** The cohort consisted of 616 *S. japonicum*-infected individuals between 7 and 30 years old who were treated with praziquantel at baseline. Forty study subjects were initially excluded due to treatment failure (*n* = 20) or missing stool data to confirm treatment status (*n* = 20). An additional 23 participants were censored due to missing reinfection data; thus, a total of 553 individuals contributed to the analysis. Study participants were followed for reinfection every 3 months for 18 months; however, analysis was limited to reinfection data extending to 12 months posttreatment due to difficulty in predicting heterogeneity in reinfection from a single posttreatment antibody measurement. This design is in agreement with the known boosting effect of treatment on antibody responses with the subsequent decay of antibody levels (6). Transmission occurs year-round in the study area, with 318 individuals (57.5%) and 431 individuals (77.9%) cumulatively reinfected at 6 and 12 months posttreatment, respectively. Table 1 summarizes the cohort characteristics at baseline.

**Antibody responses.** We utilized a high-throughput bead-based platform to determine isotype-specific antibody responses to several vaccine candidates in serum collected 1 month posttreatment. The antibody isotypes included in this report (IgA, IgE, IgG1, IgG4, and IgG) were selected based on relevance to schistosome infection in the context of the antigens tested.

The prevalence of antigen- and isotype-specific antibody responders in the cohort is presented in Table 2. The percentage of IgA responders was very low for all antigens tested, while there was substantial heterogeneity in IgE and IgG1 responses to all antigens. In contrast, IgG4 and IgG responses were common to all antigens, with the large majority of the cohort being classified as responders for IgG4 and IgG to SWAP.

In previous reports, we have identified gender, age, village of residence, water contact, and baseline intensity as predictors of

TABLE 2. Prevalence of isotype-specific antibody responders to SWAP, rSj97, rSj67, and rSj22<sup>a</sup>

| Ig        | % of antibody responders to: |       |       |       |
|-----------|------------------------------|-------|-------|-------|
|           | SWAP                         | rSj97 | rSj67 | rSj22 |
| IgA       | 2.7                          | 11.9  | 2.7   | 8.0   |
| IgE       | 42.7                         | 39.8  | 42.7  | 38.2  |
| IgG1      | 86.4                         | 31.1  | 26.7  | 55.5  |
| IgG4      | 96.3                         | 86.4  | 89.3  | 91.1  |
| Total IgG | 92.6                         | 73.6  | 68.9  | 64.6  |

<sup>a</sup> Responders were classified based on plate-specific cutoffs established by mean results for nonendemic controls (*n* = 15) plus 2 standard deviations.

reinfection in this cohort. If related to antibody levels, these factors may confound our main analysis of interest between antibody levels and reinfection. Therefore, we examined their relationship with antibody levels in a bivariate analysis. These analyses reveal that males have an increased prevalence of antibody responders compared to females (IgE to SWAP, rSj67, and rSj22; IgG1 to SWAP and rSj97; IgG4 and IgG to all antigens; *P* < 0.05 for all). Antibody prevalence differed between villages (IgA, IgG1, and IgG to all antigens; IgE to SWAP, rSj97, and rSj22; and IgG4 to rSj97; *P* < 0.05 for all). Significant differences were also detected between several antibody responses and age groups. There were more antibody responders in adults than in children and adolescents for IgA to rSj97, rSj67, and rSj22, for IgG1 to rSj97, and for IgE to SWAP, rSj67, and rSj22. In addition, adolescents were more likely to be IgG4 responders to rSj67 and rSj22 than children and adults, while children were less likely to be responders to SWAP-specific IgG, IgG4, and IgE and for IgA to rSj22 than adults and adolescents (*P* < 0.05 for all). Consistent with praziquantel-mediated antigen challenge, antibody responders had significantly higher baseline intensities than nonresponders to all antigens and isotypes tested (*P* < 0.05 for all). Lastly, water contact levels were significantly lower among IgG1 and IgG responders to SWAP than among nonresponders (*P* < 0.05).

**Antibody responses and resistance to reinfection.** We used a repeated-measures linear regression model to evaluate the relationship between antibody responses measured 1 month posttreatment with longitudinal reinfection data measured at 3, 6, 9, and 12 months posttreatment. The predictor of interest was the interaction of the time point by antibody response, which allowed for the assessment of differences in reinfection trends over time between responders and nonresponders. As previously demonstrated (23), reinfection was significantly associated with gender (males more than females), age group (children more than adults), village of residence, baseline intensity of infection, and water contact (*P* < 0.05 for all), and these variables were included in our model as potential confounders.

Associations between antibody responses and susceptibility to reinfection were common in this cohort. IgG4 responders consistently had increased reinfection intensities over time across all antigens compared to nonresponders (Fig. 1). This association with increased reinfection reached significance for both rSj67 (*P* = 0.0055) and rSj22 (*P* = 0.0383), while a similar trend was shown for SWAP (*P* = 0.1315) and rSj97 (*P* =

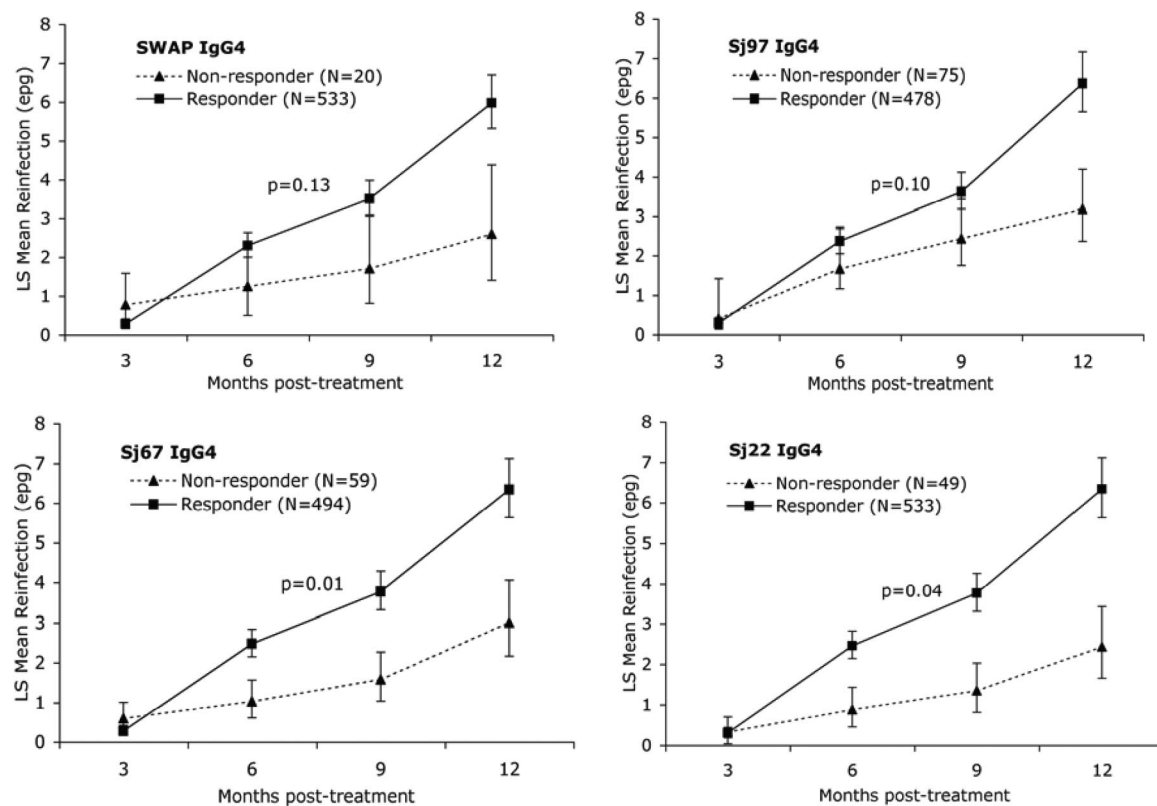


FIG. 1. Intensity of reinfection over time for IgG4 responses to SWAP and the recombinant antigens Sj97, Sj67, and Sj22. LS means represent the mean reinfection egg burden after adjusting for potential confounders and clustering by household in a repeated-measures model.  $P$  values are for time by antibody response interaction. Error bars represent standard errors.

0.0969). Strikingly, divergence in reinfection intensities between IgG4 responders and nonresponders was observed as early as 6 months posttreatment. Similarly, IgG responses to rSj22 were significantly associated with susceptibility ( $P = 0.0185$ ), particularly at 6 months posttreatment. Increased reinfection intensities over time were also observed for IgA responses to two antigens, reaching significance for rSj22 ( $P = 0.0486$ ) and marginal significance for SWAP ( $P = 0.0778$ ). However, the wide standard errors due to low positivity of IgA responders limit interpretation (data not shown). No significant associations were detected for IgG1 responses and reinfection.

In contrast to the multiple associations with susceptibility to reinfection, resistance to reinfection was solely predicted by IgE responses to rSj97 ( $P = 0.0186$ ). Reinfection intensities between IgE responders and nonresponders were similar up to 9 months; however, responders showed a 26% decreased intensity of reinfection compared to nonresponders at 12 months posttreatment (Fig. 2). IgE responses for rSj22, rSj67, and SWAP were not associated with resistance.

Because IgG4 and IgE responses to rSj97 were associated with susceptibility and resistance, respectively, we evaluated the relationship between reinfection and time by combined response group interaction. Individual rSj97 responses were stratified as “none,” “IgE only,” “IgG4 only,” or “IgE and IgG4.” Reinfection trends over time significantly differed among categories, with the most pronounced deviation observed at 12 months posttreatment (interaction of time point

by antibody strata;  $P = 0.0230$ ) (Fig. 3). Individuals classified as IgE responders but not IgG4 responders to rSj97 (IgE only, 16 total) had a 77% lower intensity of reinfection than individuals classified as IgG4 but not IgE responders (IgG4 only, 274 total;  $P = 0.0161$ ). Interestingly, individuals with both IgE and IgG4 responses to rSj97 (IgE and IgG4, 204 total) showed a trend of increased intensity of reinfection compared to individuals expressing IgE alone ( $P = 0.0776$ ), suggesting that the presence of IgG4 attenuated the protective effect of IgE.

**Cytokine and antibody analysis.** In a previous report, we described cytokine responses of antigen-stimulated peripheral blood mononuclear cells from this cohort collected at 4 weeks posttreatment, coincident with serum collection for antibody measurement (31). Therefore, we sought to assess the cross-sectional relationship between antibody responses and cytokine levels to rSj97 by logistic regression. Surprisingly, IgG4 and IgE responses to rSj97 were associated with both Th1 and Th2 cytokine production. Specifically, IgG4 responses to rSj97 were associated with increased IL-5, IL-13, and IL-12 to Sj97 ( $P$  value of  $<0.05$  for all), while IgE responses to rSj97 were associated with increased IL-12, IFN- $\gamma$ , IL-13, and IL-10 responses to Sj97 ( $P < 0.05$  for all).

## DISCUSSION

The search for a schistosome vaccine in the praziquantel era remains of paramount public health importance due to rapid reinfection and prevalent subtle morbidities, particularly in

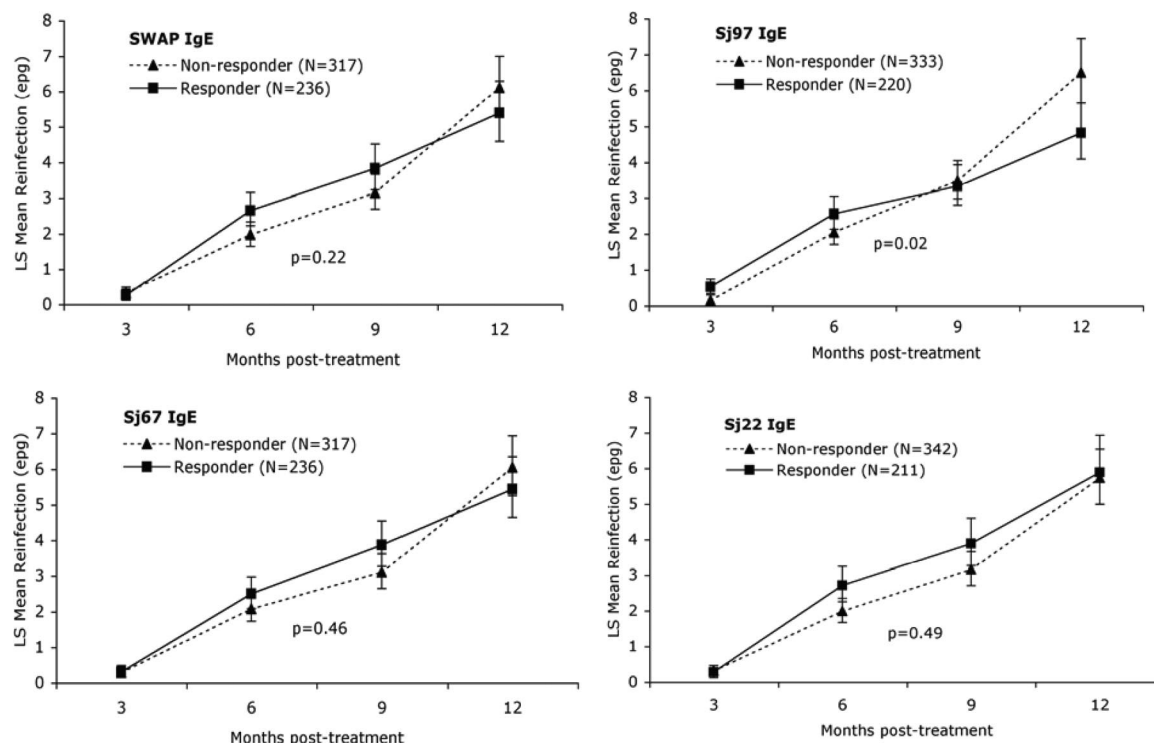


FIG. 2. Intensity of reinfection over time for IgE responses to SWAP, Sj97, Sj67, and Sj22. LS means represent the mean reinfection egg burden after adjusting for potential confounders and clustering by household in a repeated-measures model.  $P$  values are for time by antibody response interaction. Error bars represent standard errors.

high-transmission areas (3, 30). Despite the difficulty of developing vaccines for a complex immunomodulating parasite such as schistosomes, mathematical models (14, 41) suggest that schistosome vaccines do not require sterile immunity to reap long-term benefits when combined with chemotherapy (3). In

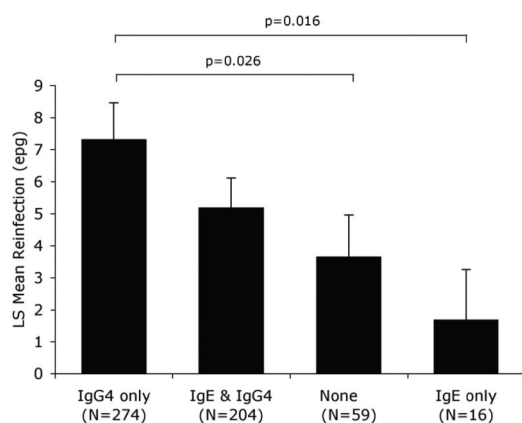


FIG. 3. IgE responses to rSj97 (paramyosin) predict resistance to *S. japonicum* reinfection at 12 months posttreatment and are attenuated by IgG4. LS means represent the mean reinfection egg burden after adjusting for potential confounders and clustering by household in a repeated-measures model using the combined Sj97 IgE and IgG4 response variable (a  $P$  value of 0.023 for time by combined IgE-IgG4 variable interaction). Confounders in this model include age, gender, village of residence, exposure, and baseline intensity.  $P$  values represent pair-wise comparisons between IgG4 only and the indicated groups. Error bars represent standard errors.

response to this need, several vaccine candidates have been identified, yet only one has reached early-phase clinical trials (3, 10), and none has been approved for clinical use to date.

One approach to vaccine development involves characterization of protective immune responses in humans. However, well-designed, longitudinal immunoepidemiological studies for schistosomiasis japonica that adjust for relevant confounders and repeated measures remain limited. In this study, we enrolled a large cohort of infected individuals living in villages where *S. japonicum* is endemic, treated them with praziquantel, and followed them for the acquisition of reinfection. We then measured antibody responses to several vaccine candidates after treatment but prior to reinfection, which strengthens the causal interpretation of any detected antibody-reinfection relationships. Additionally, the use of longitudinal reinfection data enabled us to assess intensities of reinfection over time between antibody responders and nonresponders. In order to more accurately assess this relationship, the model was adjusted for potential confounders of age, gender, village of residence, water contact, baseline intensity, and clustering by household.

Our analysis of antibody responses to adult worm antigens (SWAP) revealed marginal associations with susceptibility to reinfection with IgG4 and IgG; however, we failed to detect any protective associations for SWAP. This is in partial agreement with work by Hagan et al. demonstrating that worm-specific IgG4 was associated with susceptibility, while IgE responses predicted resistance to *S. haematobium* reinfection in The Gambia (24). In contrast, a study of schoolchildren in

Kenya by Dunne et al. showed that IgE responses to SWAP were associated with resistance to *Schistosoma mansoni* reinfection, after adjusting for age (17). In combined IgE and IgG4 analysis, Li et al. observed that excess IgE over IgG4 to SWAP is associated with *S. japonicum* resistance in Dongting Lake, China (32). Similarly, a study of *S. mansoni* in Brazil showed that increased levels of antischistosomal IgE over IgG4 predicted resistance to reinfection after adjusting for age and water contact (16).

In the present study, resistance to reinfection was solely predicted by IgE responses to paramyosin (rSj97), with decreased reinfection intensities among responders particularly at 12 months posttreatment. However, similar to SWAP responses, IgG4 responses to rSj97 were marginally associated with susceptibility (Fig. 1). In an analysis of combined IgE and IgG4 responses to rSj97, maximal resistance was observed in individuals with IgE but without IgG4 responses, while individuals without IgE but with IgG4 responses had the highest reinfection intensities. Interestingly, the presence of both IgE and IgG4 responses showed a trend of compromising the resistance associated with IgE alone, suggesting that IgE responses are attenuated by IgG4. IgG4 is a poor inducer of ADCC, and it is hypothesized to compete with paramyosin-specific IgE bound to eosinophils and to inhibit this protective immune mechanism. Together, these results highlight the importance of minimizing anti-paramyosin IgG4 responses to generate maximal resistance to schistosomiasis japonica reinfection.

Protective IgE responses to native paramyosin have been previously demonstrated in *S. mansoni* by the use of retrospective infection data (19). However, this study did not detect protective IgE responses following treatment and reinfection and lacked adjustment for important potential confounders. Other cohort studies of paramyosin have implicated IgG responses with resistance in *S. mansoni* (15) and IgG4 with susceptibility in both *S. mansoni* (2) and *S. japonicum* (1). The relatively higher prevalence of IgA responders to this antigen confirms that it is a major IgA target in parasite extracts as previously demonstrated (25). However, these responses were associated with susceptibility as previously described by others (2). Interestingly, our results echo protective IgE responses observed for other schistosomiasis vaccine candidates, notably the 22-kDa tegumental antigen Sm22 and the 28-kDa glutathione *S*-transferase Sm28GST (10, 18).

The 22-kDa tegumental antigen was initially identified as the major IgE target of *S. mansoni*-infected sera (17), and this was subsequently confirmed in *S. japonicum* (37) and *S. hematobium* (20). Indeed, we detected a high prevalence of IgE responses to rSj22 in this cohort (38%). However, these responses were not associated with resistance. This is in contrast to previous work in *S. mansoni* observing lower reinfection rates among IgE responders to this antigen (40) but in agreement with studies in *S. japonicum* (1, 32) which did not detect such associations. In this cohort, IgG4 responses to rSj22 and to rSj67 both predicted susceptibility to reinfection.

The results from this antibody analysis concur with our previous cytokine study in identifying paramyosin as a promising vaccine candidate for schistosomiasis japonica. In brief, our cytokine report demonstrated that type 2-biased cytokine responses to paramyosin predicted resistance to reinfection (31).

In cross-sectional analysis comparing posttreatment cytokine and antibody levels, we observed that both Th1 and Th2 cytokine responses were associated with IgE anti-rSj97 responses as well as SWAP responses (data not shown). This contrasts with results from a study of schoolchildren in Uganda, where pretreatment SWAP-stimulated IL-5 levels were associated with posttreatment IgE to SWAP after adjusting for age, infection intensity, and pretreatment antibody levels (39). We attribute this discordance to differences in the timing of cytokine measurement, and it is a limitation of our study that pretreatment cytokine analyses were not performed. Alternatively, the disparity may reflect intrinsic differences between human immune responses generated by *S. mansoni* and those by *S. japonicum* (36).

Several additional study limitations merit discussion. First, our primary conclusion that resistance to reinfection is associated with the balance of IgE versus IgG4 responses to paramyosin may be attributed to residual confounding by differential exposure or baseline intensity among the antibody strata despite adjustment in the model. We believe residual confounding is unlikely as water contact levels and baseline intensities did not differ among the four antibody strata (data not shown). Second, we did not purify IgE from serum prior to assessing the antigen-specific IgE antibody levels, making our IgE results reflective of competitive binding occurring in vitro with other isotypes for the same antigen. We believe that this approach is appropriate as it more accurately reflects the competition that occurs in vivo at the host-parasite interface between IgG4 and IgE, and this view is supported by the results of our stratified analyses. It would be of interest in further studies to assess if both IgE and IgG4 target the same epitope by measuring isotype-specific antibody responses to paramyosin fragments. Lastly, our multiplex platform simultaneously determined antibody responses to SWAP and the recombinant antigens, each of which is present in SWAP. Again, this could have led to competition for antigen binding. During optimization, however, we conducted separate antibody measurements for each antigen and found that values did not differ when multiplexed, suggesting that the simultaneous measurement did not compromise results, in agreement with our previously published work (13).

In conclusion, we have characterized both antibody and cytokine responses to several *S. japonicum* vaccine candidates, and our results strongly support paramyosin as a vaccine for schistosomiasis japonica. In spite of these data, we acknowledge that immunoepidemiological surveys are limited to observing protective immune responses in the context of natural infection and are susceptible to both bias and confounding (3). Further studies should therefore be devoted to preclinical and clinical evaluation of the vaccine efficacy of paramyosin, taking into consideration various factors such as adjuvants, antigen load, immunization schedule, and route. Both IgE and IgG4 are produced in the presence of IL-4; however, IgE production is preferentially favored by low levels of IFN- $\gamma$  and IL-10 (9, 21, 26). Accordingly, vaccination studies must be directed toward developing such a cytokine profile in order to ensure the generation of protective immune responses. A caveat of inducing antigen-specific IgE responses is the risk of allergic reactions in previously exposed and sensitized individuals. Evidence from experimental models (33, 42) and epidemiologic

surveys (34), however, suggest that a state of allergic hyporeactivity is observed in the context of schistosome infection.

In support of early-phase clinical studies, we have developed a good manufacturing practice-ready, pilot-scale process to produce recombinant full-length *S. japonicum* paramyosin, rSj97 (27), and are currently conducting efficacy and safety studies in rodents and large-animal models.

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