Identification of a Novel Antigen of *Schistosoma mansoni* Shared with *Plasmodium falciparum* and Evaluation of Different Cross-Reactive Antibody Subclasses Induced by Human Schistosomiasis and Malaria

Christine Pierrot,[1] Shona Wilson,[2] Hélène Lallet,[1] Sophia Lafitte,[1] Frances M. Jones,[2] Wassim Daher,[1] Monique Capron,[1] David W. Dunne,[2]* and Jamal Khalife[1]*

Unité Inserm 547, IFR 17, Institut Pasteur de Lille, 1 rue du Prof. Calmette, 59019 Lille, France,[1] and Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom[2]

Plasmodium falciparum and *Schistosoma mansoni* are often found in human coinfections, and cross-reactive antibodies to different components of the two parasites have been detected. In this work, we identified a cross-reactive *S. mansoni* gene product, referred to as SmLRR, that seems to belong to the leucine-rich repeat protein family. Comparative analysis of SmLRR revealed 57% similarity with a putative gene product encoded in the *P. falciparum* genome. Antibodies to SmLRR were found in experimental infections and in both *S. mansoni*- and *P. falciparum*-infected individuals. Correlative analysis of human anti-SmLRR responses in Kenya and Uganda suggested that malaria and schistosomiasis drive the immunoglobulin G3 (IgG3) and IgG4 isotypes, respectively, against SmLRR, suggesting that there is differential regulation of cross-reactive isotypes depending on the infection. In addition, the levels of anti-SmLRR IgG4, but not the levels of IgG3, correlated positively with the intensity of *S. mansoni* infection.

Epidemiological studies of malaria and schistosomiasis indicate that mixed infections are common in people living in areas where these diseases are endemic. For many parasites, protection has been associated with either Th1- or Th2-type cytokine production and the dependent immune effector mechanisms. For malaria, based on studies with mouse models, workers have concluded that cellular and humoral responses are required to control and clear malaria parasites (for reviews see references 21, 22, and 30). For schistosomiasis, efforts have been made to obtain insight into the immune responses involved in protection. For mice, a permissive host, there is a considerable amount of direct evidence indicating that the protection can be mediated by a Th1-type response (for a review see reference 28). In rats, a semipermissive host, Th0/Th2- and Th2-type responses are involved in resistance to infection and reinfection, respectively (for a review see reference 16). For humans, although Th1-type responses can be detected, immunoepidemiological studies in different areas where the disease is endemic have firmly correlated Th2 responses with resistance to reinfection after chemotherapy (5, 10).

Studies of the influence of coinfection with *Schistosoma mansoni* on the immune response to experimental malaria have suggested that the modulation of immunological responses is due to cross-regulation of the Th1 and Th2 responses, which are known to be induced by malaria and schistosome infections, respectively (11, 34). This is consistent with the observation that malaria-specific immunoglobulin E (IgE) responses, which are not induced following a single infection with *Plasmodium chabaudi*, are induced by a single infection during schistosomiasis coinfection (12). More recently, examination of humoral responses to *Plasmodium falciparum* and to *S. mansoni* egg and worm antigens in individuals exposed to malaria and schistosomiasis indicated that there is a strong correlation between malaria- and schistosome-specific IgG3 responses (25). Unexpectedly, this association seems to result from the presence of shared components of the two parasites that bind cross-reactive antibodies rather than from mediation by immunological cross-regulation induced by either parasite. Although the cross-reactivity was confirmed to occur in individuals living in areas where each disease is monoendemic (25), results obtained with murine models, in which the environment was tightly controlled, showed that sera from mice infected solely with *S. mansoni* did not react with *P. chabaudi* antigen and vice versa (11). From these observations, it is not possible to exclude the possibility that mice cannot mount a cross-reactive humoral response due to their genetic background, the infection dose, the inoculation route, and/or to the species or strain of *Plasmodium* used. Investigating the rat host as a model for studying experimental malaria (1), we observed that like sera from *P. falciparum*-infected humans, sera from rats infected with *Plasmodium berghei* cross-reacted with *S. mansoni* antigens (unpublished data). In the present study, we molecularly characterized a cross-reactive *S. mansoni* gene product, designated SmLRR, and evaluated its reactivity with sera from individuals living in areas where the diseases are endemic who were exposed to malaria and/or schistosomiasis.

* Corresponding author. Mailing address for Jamal Khalife: Unité Inserm 547, IFR 17, Institut Pasteur de Lille, 1 rue du Prof. Calmette, 59019 Lille, France. Phone: (33) 320877968. Fax: (33) 320877888. E-mail: jamal.khalife@pasteur-lille.fr. Mailing address for David W. Dunne: Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom. Phone: (44) 1223333336. Fax: (44) 1223333741. E-mail: dd@mole.bio.cam.ac.uk.

MATERIALS AND METHODS

Animals, parasites, and antigen preparations. The experiments were performed in accordance with local animal ethics committee regulations by using

8-week-old Fischer F344 male rats purchased from HARLAN (Holland). Sera were collected 5 to 7 weeks after inoculation of *P. berghei* or after infection with *S. mansoni* via the retroorbital venous plexus. The Puerto Rican strain of *S. mansoni* used was maintained in *Biomphalaria glabrata* snails and golden hamsters. *P. berghei* ANKA was maintained in Fischer rats as described previously, and parasite extracts were prepared as described by Adam et al. (1).

cDNA library, immunoscreening, and DNA sequencing. A cDNA library derived from mRNA of *S. mansoni* adult worms, constructed in λZAP II, was immunoscreened with a pool of sera from *P. berghei*-infected rats. This pool was checked for reactivity with *S. mansoni* antigen extract and was used at a dilution of 1:100. Positive clones were detected using anti-rat IgG antibodies labeled with peroxidase (Sigma). This procedure was repeated until the clones were 100% pure. Sequencing reactions were carried out using a BigDye terminator cycle sequencing kit, and sequences were analyzed with an ABI sequencer (PE Applied Biosystems). The sequence obtained was compared with the plasmoDB database (http://plasmodb.org), the Pfam database (http://www.sanger.ac.uk), and the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Recombinant SmLRR expression and antiserum production. SmLRR full-length cDNA was obtained by reverse transcription-PCR performed with *S. mansoni* total RNA using the following specific synthetic primers: forward primer 5′-GGATCCATGAGTGTGGAAGTGGAAATTCAATCTCC-3′ and reverse primer 5′-AAGCTTTCATCCTTTGCATTGGATGAAGTAACAG-3′. To orient the cloning, we inserted restriction sites in the 5′ (BamHI) and 3′ (HindIII) primer sequences. Recombinant SmLRR protein was expressed using the pQE30 vector (QIAGEN). To obtain the recombinant protein, SmLRR was prepared as follows. An overnight culture of bacteria containing pQE30 SmLRR was diluted in culture medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin. At an optical density at 600 nm of 0.6, expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were grown for 4 h at 37°C. Cells were then harvested and lysed by sonication on ice in lysis buffer (25 mM Tris [pH 8.0], 100 mM NaCl, 1 mM 2-mercaptoethanol, 0.1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, an EDTA-free inhibitor cocktail (Boehringer Mannheim), and 100 μg/ml lysozyme. After centrifugation, the supernatant was incubated with Ni-nitrilotriacetic acid agarose (QIAGEN) resin for 1 h at 4°C to allow binding. The resin was washed 10 times in sonication buffer containing 20 mM imidazole, and bound protein was eluted with elution buffer (25 mM Tris [pH 8.0], 100 mM NaCl, 10% glycerol, 250 mM imidazole). Supernatants were filtered and dialyzed against phosphate-buffered saline (PBS) containing 5% glycerol, and protein purity was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The purified proteins (>95%) were stored at −20°C until they were used.

Rats were immunized by injection of 100 μg of recombinant SmLRR protein in the presence of complete Freund's adjuvant and were boosted in the presence of incomplete Freund's adjuvant 4 weeks later. Serum was collected 2 weeks later, and reactivity with SmLRR was tested.

Reverse transcription-PCR, Western blotting, and immunofluorescence experiments. Total RNA was extracted from different *S. mansoni* stages (adult worms, cercariae, and eggs) using RNAzol (Qbiogene). A reverse transcriptase reaction with 2 μg of total RNA was performed with Superscript II (Gibco BRL, Bethesda, MD) according to the manufacturer's recommendations. PCRs were performed with *Taq* Gold DNA polymerase (Applied Biosystems) with the following primers that amplified a 150-bp PCR product in the SmLRR cDNA: forward primer 5′-TTAGATGATCGTCCTGTTTTTC-3′ and reverse primer 5′-GCTTACTGGATAACCATTTGACA-3′. As negative control, we used RNA prepared from rat spleen cells. Amplification reactions were performed under the following conditions: one cycle of 94°C for 10 min, 35 cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 7 min. *S. mansoni* adult worm antigen preparations were fractionated on 6 to 20% SDS–polyacrylamide gradient gels and transferred to nitrocellulose membranes. Filters were probed with rat anti-SmLRR antiserum (1/50). Detection was carried out as previously described (18). For immunolocalization experiments, adult worms were fixed in Immunohistofix (ULB, Belgium), and sections were prepared as previously described (33). Slides were saturated for 30 min in 3% milk in PBS and then washed in PBS. This step was followed by 2 h of incubation at room temperature with anti-SmLRR mouse antibodies diluted 1:100 in PBS–3% milk. After three washes in PBS–0.05% Tween, the slides were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) at a 1:500 dilution. After several washes in PBS, the slides were mounted using the Fluoromount G reagent (Southern Biotechnology Associated). Green fluorescence was detected using a UV light microscope (Leitz DM RB; Leica) with a 488-nm filter.

Rat antibody assays with SmLRR. Plates (Nunc MaxiSorp) were coated for 3 h at 37°C with 100 μl/well of purified SmLRR or *P. berghei* or *S. mansoni* antigens (5 μg/ml) in PBS. After three washes in PBS containing 0.1% Tween 20 and incubation for 1 h at room temperature with PBS containing 0.5% gelatin, 100 μl/well of *P. berghei* or *S. mansoni* rat sera diluted in PBS–0.1% Tween 20 was added and incubated overnight at 4°C. After three washes, the plates were incubated for 90 min at 37°C with 100 μl/well of peroxidase-labeled anti-rat IgG (1:10,000). After five final washes, 1 mg/ml substrate (o-phenylenediamine; Sigma) in 0.1 M sodium phosphate buffer (pH 5.5) containing 0.03% H_2O_2 was added and incubated for 30 min at 37°C. After addition of 100 μl/well of 2 M HCl, the optical density at 492 nm was determined. Statistical comparisons of animal groups were carried out by using the Mann-Whitney U test. Differences between experimental groups were considered significant if the *P* value was <0.05.

Human study cohorts, plasma collection, and human antibody isotype assays. Plasma samples from two human cohorts, Kenyan schoolchildren and a Ugandan community cohort, were analyzed for anti-schistosome, anti-malaria, and anti-SmRRL IgG subclass responses. The first study group consisted of Kenyan children who were 5 to 17 years old and were attending Yumbuni Primary School in Makueni District, which was within 15 km of the area examined in previously described studies of malaria/schistosome antibody and clinical interactions (23, 25). At the time of this study, approximately 20% of the schoolchildren in the area were blood smear positive; 99% of these infections were *P. falciparum* infections, and 1% were *Plasmodium malariae* infections. For cohort selection, five separate stools were collected from each child and examined for *S. mansoni* eggs by using two Kato-Katz thick smears (15) per stool. Thick and thin blood smears were examined for the presence of malaria parasitemia and for species identification. Only *P. falciparum* was found in the study cohort selected. The children were divided into two groups. The group A children were malaria and schistosomiasis negative, had no detectable malaria parasitemia or schistosome eggs, and had low levels of anti-*P. falciparum* schizont antigen (Pfs) IgG3 and anti-soluble egg antigen (SEA) IgG4 (*n* = 20; age range, 5 to 16 years); the group B children were malaria positive and schistosomiasis negative, had detectable *P. falciparum* parasitemia and anti-Pfs IgG3, and had no detectable schistosome eggs and low levels of anti-SEA IgG4 (*n* = 20; age range, 8 to 17 years). It is important to note that there was no previous treatment for schistosomiasis in this area, which eliminated the likelihood of the presence of preexisting antischistosome antibodies from previous infections. The *Plasmodium* parasitemia range in group B was 9 to 334 parasites/200 white blood cells.

The Ugandan study cohort was from the village of Booma in the Butiaba area, which has been described elsewhere (25). This cohort consisted of 277 individuals. Blood samples were obtained from 219 subjects before treatment for schistosomiasis. The pretreatment prevalence of malaria and the pretreatment prevalence of *S. mansoni* in different age groups were as follows: for subjects who were 7 to 9 years old (*n* = 34), 50% and 81%, respectively; for subjects who were 10 to 12 years old (*n* = 38), 46% and 100%, respectively; for subjects who were 13 to 16 years old (*n* = 35), 42% and 100%, respectively; for subjects who were 17 to 22 years old (*n* = 32), 38% and 92%, respectively; for subjects who were 23 to 30 years old (*n* = 47), 29% and 85%, respectively; for subjects who were 31 to 38 years old (*n* = 45), 25% and 88%, respectively; and for subjects who were 39 to 50 years old (*n* = 46), 18% and 94%, respectively.

The methods used for preparation of *S. mansoni* soluble worm antigen (SWA) and crude *P. falciparum* schizont antigen have been described previously (7, 25). Specific levels of IgG1, IgG3, and IgG4 against SEA, SWA, and Pfs were determined by enzyme-linked immunosorbent assays (ELISA), as described previously (25). Human isotype assays against SmLRR were carried out as described above. Sera from the two groups were randomly distributed in the wells of triplicate microtiter plates to avoid the effects of interplate variability, and all preparations were assayed at the same time. Sera were diluted 1/200 for detection of IgG1 and IgG4 and 1/100 for detection of IgG3.

The Mann-Whitney U test for nonparametric data was used for statistical comparisons of the antibody isotype responses of the Kenyan noninfected (group A) and malaria-infected (group B) children to each antigen preparation. Correlations between the antibody isotype responses of the malaria-infected children (group B) to the different antigen preparations were analyzed by Spearman's rank correlation. All analyses were carried out using SPSS-10. A *P* value of <0.05 was considered statistically significant.

Human plasma depletion. *P. falciparum* extracts prepared as previously described (25) were coated on Sepharose beads according to the manufacturer's instructions (Amersham Biosciences). Plasma samples known to have IgG1 and IgG3 reactions with SmLRR were diluted 1:100 and preincubated with 25 μl or 100 μl of *P. falciparum* or bovine serum albumin (BSA)-Sepharose beads. After overnight incubation at 4°C, samples were centrifuged for 15 min at 2,000 × *g*
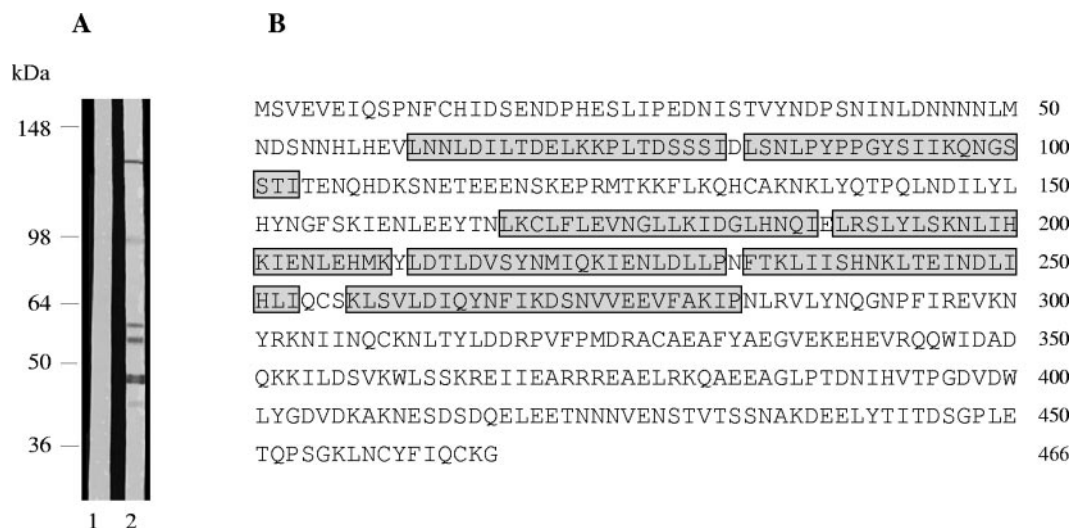
FIG. 1. (A) Western blot analysis of common antigenicity in *S. mansoni* and *P. berghei*. Soluble *S. mansoni* antigens were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a pool of noninfected control rat sera (lane 1) or a pool of sera collected from rats infected with *P. berghei* (lane 2). (B) Predicted amino acid sequence of the SmLRR cDNA. Leucine-rich repeats are enclosed in boxes.

and tested against recombinant SmLRR by performing an ELISA. As a control, we used Sepharose beads coated with BSA.

RESULTS

Isolation and identification of cross-reactive clones. To examine the patterns of *S. mansoni* antigens recognized by anti-*P. berghei* antibodies, Western blot experiments were performed with a pool of sera collected from rats infected with *P. berghei*. As shown in Fig. 1A, immunoblot analysis showed that IgG antibodies produced during a *P. berghei* infection recognized several *S. mansoni* bands that included protein and nonprotein antigens, supporting the results of a previous human study (23). In order to identify the proteins of *S. mansoni* involved in the cross-reactivity, we screened a cDNA library of *S. mansoni*. After several rounds of plaque purification, only one clone still reacted strongly with *P. berghei* antibodies. This clone, referred to as SmLRR, contained an 1,398-bp open reading frame (ORF), a 12-bp 5′ end, and a 929-bp 3′ noncoding region with a poly(A) tract and a possible polyadenylation signal (AATAAAATAAA) 20 bp upstream of the poly(A) tract (GenBank accession no. AY623659). The encoded protein comprises 466 amino acids and has a predicted molecular mass of 53.8 kDa (Fig. 1B).

Comparison of SmLRR sequence with databases. The SmLRR ORF was compared by using the BLAST program to the plasmoDB database (all open reading frames encoding >50 amino acids), the GenBank database, and the Pfam protein family data bank. With the plasmoDB database, we observed 47% identity and up to 66% similarity of the SmLRR ORF with an ORF present in *P. berghei* (positions 136 to 192 and 12 to 68, respectively) (Fig. 2A). Moreover, the SmLRR ORF was found to be similar to a hypothetical ORF, gene 222, found on chromosome 13 of *P. falciparum*, and the amino acid sequence exhibited 35% identity and up to 57% similarity (Fig. 2B). When the sequence was compared with the GenBank database, we observed 38% identity of the Nt side (positions 129 to 270) of SmLRR with the protein phosphatase PP1 regulatory subunit sds22 (positions 17 to 154) (suppressor of the dis 2 mutant) (accession no. P22194).

Expression of SmLRR in *S. mansoni*. Figure 3A shows the mRNA expression by adult worms, cercariae, and eggs. Next, we analyzed whether the SmLRR protein is present in *S. mansoni*. Western blots of parasite extracts were probed with antibodies raised against SmLRR. The SmLRR antibodies detected a single protein with a molecular mass of about 64 kDa in homogenates of adult worms, cercariae, and eggs (Fig. 3B). The difference between the size of the protein measured and the size calculated (53.8 kDa) was due to abnormal migration of the native protein. This was confirmed by the fact that the recombinant protein produced in *Escherichia coli* migrated at 64 kDa (Fig. 3C). Next, immunofluorescence experiments were performed to localize the native SmLRR in adult worms, a stage that persists for a long time in the host liver. Immunofluorescence studies with anti-SmLRR antibodies revealed punctate labeling in parenchyma cells below the muscle layers. Strong reactivity was also detected in the tubercles of the tegument and in the tegument around the tubercles (not shown). Sections were also incubated with control sera, and there was no staining in these sections.

Antibody response to SmLRR in experimental infections. As shown in Fig. 4, infection by *S. mansoni* induced production of antibodies directed against SmLRR. When samples from rats infected with *P. berghei* (which had never been exposed to *S. mansoni*) were tested, we observed the presence of antibodies which reacted with SmLRR, suggesting that this antigen could be involved in humoral cross-reactivity between *S. mansoni* and *P. berghei*.

Antibody responses to SmLRR in *P. falciparum*-infected individuals. Kenyan schoolchildren were selected because they were blood smear positive for *P. falciparum* and had detectable IgG3 anti-Pfs responses (group B). As previously reported for similar groups of children (23), these children were found to have significantly higher IgG1 and IgG3 responses against SWA than non-malaria-infected children (control group A)

A

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SmLRR  NKLYQTPQLNDILYLHYNGFSKIENLEEYTNLKCLFLEVNGLLKIDGLHNQIELRSL  192
       N LY T :LN::LYLH  GF  I: L  : NLKCLFL  N : KI: L : : L::L
P.b.   NNLYDTDELNEVLYLHMKGFHNIDGLSTFKNLKCLFLNNNCIKKIENLKDLVNLKAL  68
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B

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SmLRR  LKQHCAKNK-LYQTPQLNDILYLHYNGFSKIENLEEYTNLKCLFLEVNGLLKIDGLHNQI  187
       :K    KN:  Y   :LND:LYL:   ::KIE L:   NLK L:L  N L KI:GL   I
P.f.   VKNIIEKNEGYYSIIELNDVLYLNNKLYTKIECLQNLHNLKTLYLNNNALEKIEGLECCI  69

SmLRR  ELRSLYLSKNLIHKIENLEHMKYLDTLDVSYNMIQKIENLDLLPNFTKLIISHNKL----  243
       L :LYL: N I :IENL ::  L  L::  N I  IENL: L     L :S:N L
P.f.   NLIALYLNCNQIKRIENLNNLIRLRILNLEDNNIFVIENLECLDCLEDLNLSNNCLGSKG  129

SmLRR  TEINDLIHLIQCSKLSVLDIQYNFIKDSNVVEEVFAKIPNLRVLYNQGNPFIREVKNYRK  303
       :    ::H  :  K: :L::   N I : :::: :   : NL :LY  NP : :   NYRK
P.f.   SPNTSILH--KNKKIYILNLSNNQINE-DILDNLLM-LENLSILYLMNNPILLKYNNYRK  185

SmLRR  NIINQCKNLTYLDDRPVFPMDRACAEAFYAEGVEKEHEVRQQWIDADQKKIL--DSVKWL  361
       I : K:LT:LD :P:   :R C EAF: G  KE EV Q: I:  QKK:   :S:: :
P.f.   LFIYKLKSLTFLDYKPIKTDERRCVEAFFEGGPLKEQEVMQK-IER-QKKLQHRNSIECI  243

SmLRR  SSKREII  368
       S  :II
P.f.   ISITKII  250
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FIG. 2. (A) Alignment of the SmLRR deduced amino acid sequence (positions 136 to 192) (*Sm50*) and the sequence of *P. berghei* antigen (Pb_283d11q1c; positions 12 to 68) from the plasmoDB database (*P.b.*). (B) Alignment of the SmLRR deduced amino acid sequence (positions 129 to 368) and the sequence of *P. falciparum* antigen (chr13gene222; positions 10 to 250) from the plasmoDB database (*P.f.*).

had ($P < 0.001$ for both types of responses), who had lower anti-Pfs IgG3 responses. Despite the absence of *S. mansoni* infection in both groups of children, the children in malaria-infected group B exhibited significantly greater IgG1 and IgG3 responses against SmLRR than control group A children exhibited ($P < 0.001$ for both types of responses) (Fig. 5). This contrasted with the pattern of IgG4 responses to the same antigen. The children in malaria-infected group B showed significantly higher anti-Pfs IgG4 responses than the noninfected group A children showed ($P < 0.001$). The anti-SWA IgG4 responses in both groups were low and not statistically different, as were the anti-SmLRR IgG4 levels.

The anti-Pfs IgG3 responses of the malaria-infected group B children were positively correlated with both the anti-SWA IgG3 ($r = 0.543$, $P = 0.013$) and anti-SmLRR IgG3 ($r = 0.632$, $P = 0.003$) responses, and the anti-SWA and anti-SmLRR IgG3 responses were strongly correlated with each other ($r = 0.557$, $P = 0.011$). The anti-SWA and anti-SmLRR IgG1 responses were also very significantly correlated ($r = 0.574$, $P = 0.008$); however, although there were positive relationships between the anti-Pfs IgG1 responses and both the anti-SWA IgG1 ($r = 0.421$, $P = 0.064$) and anti-SmLRR ($r = 0.381$, $P = 0.098$) responses, these relationships were not statistically sig-

nificant. In the absence of any schistosomiasis in this study cohort, there were no correlations between the IgG4 responses (for Pfs and SWA, $r = 0.221$ and not significant; for Pfs and SmLRR, $r = 0.141$ and not significant; and for SWA and SmLRR, $r = 0.229$ and not significant).

To confirm that the reactivity of IgG antibodies of the malaria-infected children with SmLRR was effectively related to *P. falciparum* infection, serum samples from cross-responders were adsorbed on *P. falciparum* extracts immobilized on Sepharose beads. As shown in Fig. 6, in depletion experiments there were 40% to 85% decreases in IgG binding to SmLRR according to the quantity of Pf-Sepharose beads used. Depletion of the same serum samples on BSA-Sepharose beads did not reveal any relevant decreases (<18%).

Antibody responses to SmLRR in an area where *S. mansoni* and malaria are coendemic. To evaluate the evolution of humoral immune responses to SmLRR with age and schistosome coinfection, we looked for interactions between the IgG isotype antibody responses in individuals from a community cohort study in an area of Uganda where schistosomiasis and malaria are coendemic. Correlations between IgG isotype responses to SEA, SWA, and Pfs antigens and recombinant SmLRR antigen are shown in Table 1. Only the IgG1 response
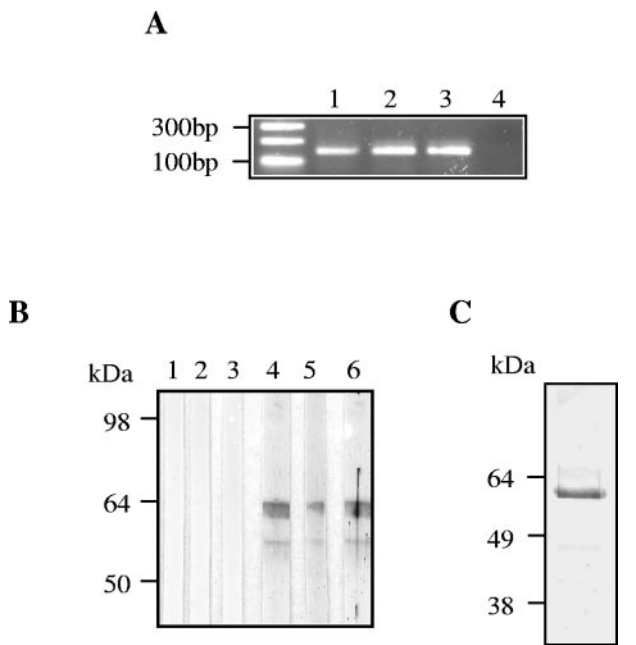
FIG. 3. Expression of SmLRR in *S. mansoni*. (A) Reverse transcription-PCR analysis of total RNA extracted from adult worms (lane 1), cercariae (lane 2), and eggs (lane 3) and control RNA obtained from rat spleen cells (lane 4). (B) Western blot analysis of immunoreactivity of rat anti-SmLRR antiserum with *S. mansoni* antigen. Soluble adult worm (lanes 1 and 4), cercaria (lanes 2 and 5), and egg (lanes 3 and 6) antigens were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with preimmune serum (1/50) (lanes 1 to 3) or with rat anti-SmLRR antiserum (1/50) (lanes 4 to 6). (C) Coomassie blue-stained gel showing the position of recombinant SmLRR protein.
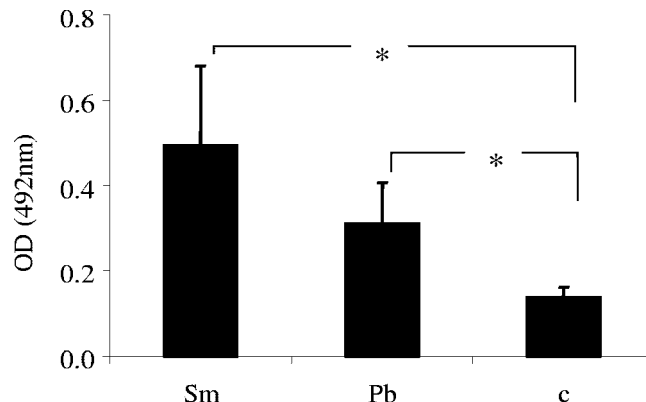

FIG. 4. ELISA analysis of immunoreactivity of *S. mansoni*- or *P. berghei*-infected rat sera with recombinant SmLRR. Sm, sera of *S. mansoni*-infected rats ($n = 9$); Pb, sera of *P. berghei*-infected rats ($n = 7$); c, sera of control rats ($n = 5$) (dilution, 1/100). The data are the mean ± standard error of the mean for each group. An asterisk indicates that the P value is <0.05. OD (492nm), optical density at 492 nm.

to SmLRR was significantly associated with anti-Pfs IgG1. The IgG3 responses to all antigens were significantly associated with each other. Anti-Pfs IgG4 responses were significantly associated with anti-SEA, anti-SWA, and anti-SmLRR IgG4 responses. Based on *S. mansoni* egg counts (Table 2), *S. mansoni* infection intensity was found to be associated significantly with anti-SEA, anti-SWA, anti-Pfs, and anti-SmLRR IgG4. This suggested that at the highest levels of infection *S. mansoni* induced a strong anti-SmLRR IgG4 response and very likely a stronger cross-reactive response to Pfs.

DISCUSSION

Since in regions where schistosomiasis is endemic other common parasitic infections, particularly malaria, are often also endemic, it was important to address the question of immunological cross-reactivity between parasites and to identify the molecular basis of this phenomenon. While analyzing the immune responses in experimental rat malaria, we observed that *P. berghei* infection induced antibodies that bound to *S. mansoni*. This suggests that the rats, like infected humans, produced cross-reactive antibodies which recognized common epitopes for the two parasites. In an attempt to identify the proteins expressing the cross-reactive epitope(s), we screened an *S. mansoni* cDNA library using a pool of sera taken from rats exposed to *P. berghei*. This led isolation of one clone designated SmLRR. The deduced amino acid sequence

exhibits significant identity with *P. berghei* and *P. falciparum* putative proteins that have yet to be experimentally identified. However, it is very likely that a portion of the antibodies that react with *S. mansoni* could be induced by this *P. berghei* antigen. To analyze the product of the SmLRR gene expressed by *S. mansoni*, we produced the recombinant protein and raised specific antisera. Immune rat sera against the recombinant protein reacted with a 64-kDa protein under reducing conditions in a Western blot analysis. Comparative analysis of the deduced amino acid sequence using the GenBank and Pfam databases revealed the presence of seven leucine-rich repeats. Leucine-rich repeats are short sequence motifs that are present in a number of proteins, such as Toll-like receptors, insulin-like growth factor, and receptor-like protein kinase (for a review see reference 19). The function of the protein encoded by the SmLRR gene, which is present in different stages of *S. mansoni*, is not known yet. Nevertheless, some inferences about the biology of SmLRR in *S. mansoni* can be made based on the immunofluorescence experiments. Interestingly, the SmLRR gene product was in the tegument tubercles of adult worms, suggesting that SmLRR functions at least in part at the host-parasite interface and thereby induces cellular and humoral responses.

In view of the similarity of the SmLRR, *P. berghei*, and *P. falciparum* gene products, it was pertinent to evaluate the antibody response against SmLRR in malaria and schistosomiasis. In this context, we confirmed that *S. mansoni* or *P. berghei* experimental infections produced antibodies that reacted with recombinant SmLRR. Moreover, our data for Kenyan schoolchildren infected with *P. falciparum* are consistent with the hypothesis that similar cross-reactive antibodies could occur in human infections. Indeed, the association of *P. falciparum* infection with increased levels of anti-SWA activity and the positive correlations between anti-SWA and anti-Pfs responses support the previous suggestion that malaria induces human IgG3 that cross-reacts with *S. mansoni* antigens (25). This suggestion was also supported by the depletion experiments, whose results suggested that a high proportion of the anti-SmLRR IgG observed is *P. falciparum* specific. In addition, the present data show that in the absence of schistosomiasis, the
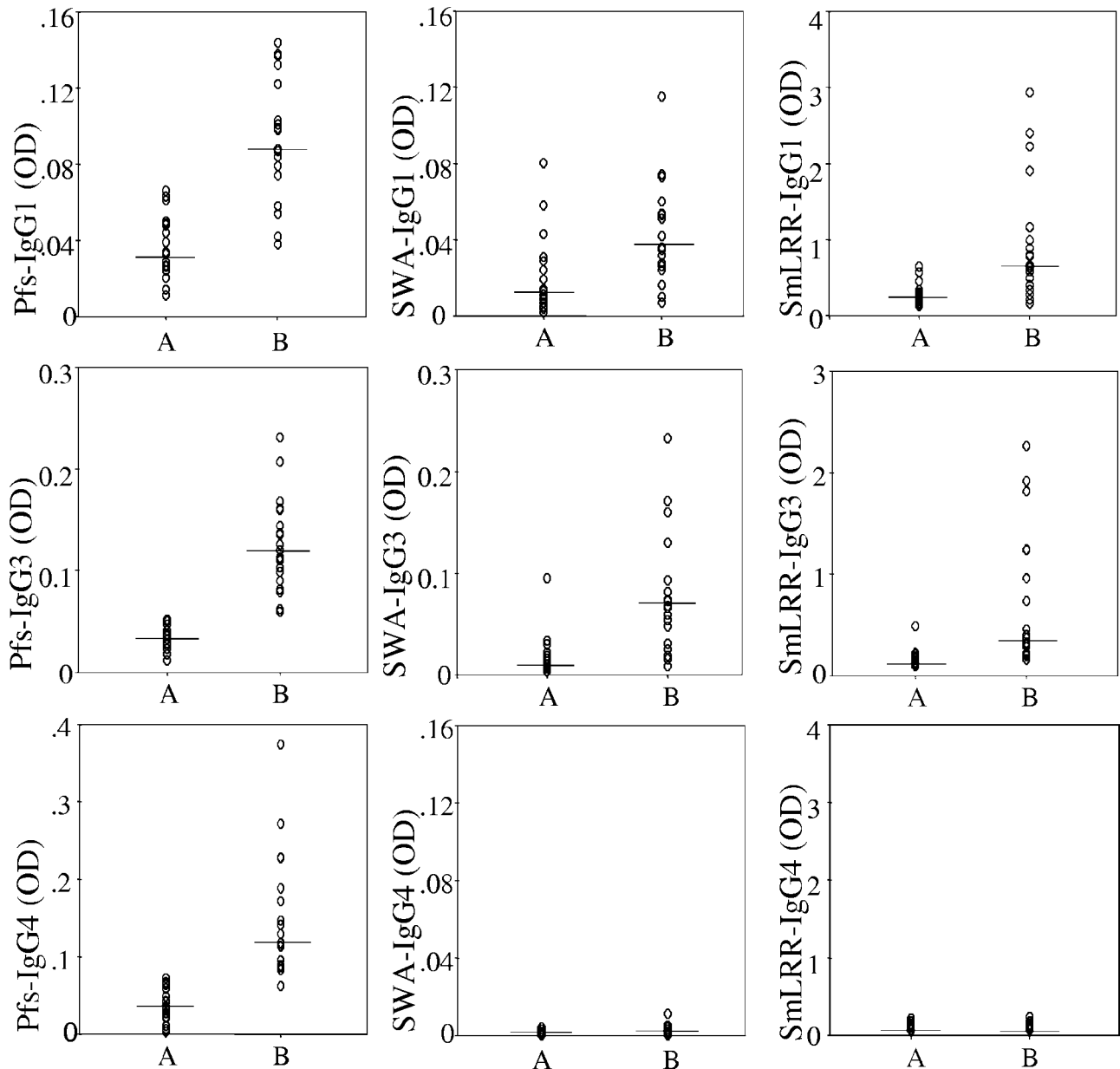
FIG. 5. ELISA analysis of the antibody responses of *P. falciparum*-infected children to *S. mansoni* SmLRR. Plasma samples were obtained from the following two groups of Kenyan schoolchildren: children who were malaria and schistosomiasis negative and had low levels of anti-Pfs IgG3 and anti-SEA IgG4 ($n = 20$) (A) and children who were malaria positive and schistosomiasis negative and had detectable levels of anti-Pfs IgG3 but low levels of anti-SEA IgG4 ($n = 20$) (B). These samples were assayed for IgG1, IgG3, and IgG4 reactivity with Pfs, *S. mansoni*, SWA, and recombinant SmLRR. The results are expressed as the optical density at 490 nm (OD) for each individual (○) and as the median for each group (horizontal line).

levels of anti-malaria IgG3 have a similar significant positive relationship with IgG3 responses to SmLRR. In contrast, although malaria infection induced a significant anti-Pfs IgG4 response, no anti-SWA or anti-SmLRR cross-reactive IgG4 was detected in the schoolchildren in this study, and there was no relationship between the IgG4 responses to malaria and schistosome antigens. Thus, the pattern of *P. falciparum* iso-type-specific correlations with SmLRR was very similar to the pattern seen with native *S. mansoni* worm antigen preparations, suggesting that this may at least in part contribute to the

previously reported cross-reactivity between these two commonly coinfecting parasites (23, 25). Interestingly, in the same area of Kenya, it has been shown that schistosomiasis-associated hepatosplenic morbidity (32) is greatest when school-age children are exposed to both malaria and schistosomiasis rather than to either infection alone (2).

In an area of Uganda where malaria and schistosomiasis caused by *S. mansoni* are highly endemic, anti-SmLRR IgG1, IgG3, and IgG4 responses were analyzed using a cross-sectional community cohort whose ages ranged from 7 to 50 years.
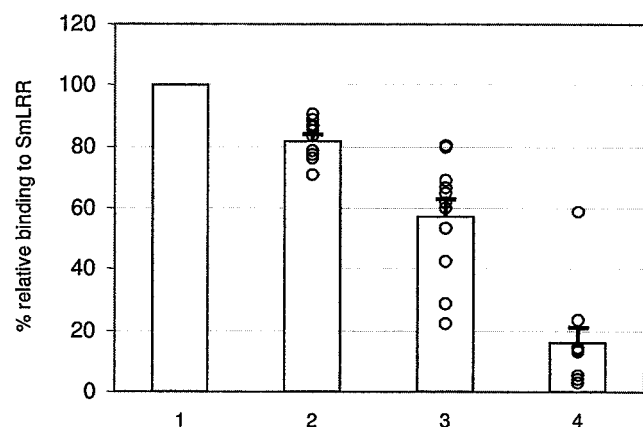
FIG. 6. Analysis of antibody responses of *P. falciparum*-infected children to *S. mansoni* SmLRR. Plasma samples from children who were malaria positive and schistosomiasis negative and had detectable levels of anti-Pfs IgG3 but low levels of anti-SEA IgG4 were assayed for IgG reactivity with recombinant SmLRR before depletion (bar 1) and after depletion on 100 μl of BSA-Sepharose beads (bar 2) or on 25 μl (bar 3) or 100 μl (bar 4) of *P. falciparum*-Sepharose beads. The results are expressed as relative binding to SmLRR, where the binding before depletion was defined as 100%. The circles indicate values for individual plasma samples, the bars indicate means, and the error bars indicate standard errors of the means ($n = 11$).

The responses observed were not associated with the presence of blood smear-detectable parasitemia at the time of sampling. In Kenyan studies we found that anti-Pfs IgG3 responses, which increased with age, were greater in blood smear parasitemia-positive schoolchildren than in blood smear-negative children; however, this was not true for parasitemia-positive and -negative Kenyan adults, nor was it true in Kenyan community studies (Wilson, unpublished results). Thus, the association of increased anti-Pfs IgG3 responses with parasitemia that we observed appeared to be a childhood phenomenon. However, in this Ugandan community, there were significant positive correlations between the intensity of *S. mansoni* infection and IgG4 responses to *S. mansoni* antigens, including SmLRR, and anti-SmLRR IgG4 responses were strongly positively correlated with anti-Pfs IgG4 responses. The IgG4 response is a major human response to chronic helminthiasis (6, 8, 14, 27) and is often the only IgG subclass response that correlates with the intensity of schistosome (20, 24) and other helminth (20) infections. As seen previously, the IgG3 responses to malaria and schistosome antigens, including

TABLE 1. Pretreatment correlations between IgG subclass responses to different antigen preparations for the Ugandan community cohort ($n = 219$; Spearman's rank correlation)

Antigen	Association between specific IgG1 responses		Association between specific IgG3 responses		Association between specific IgG4 responses	
	SmLRR	Pfs	SmLRR	Pfs	SmLRR	Pfs
SWA	0.158[a]	0.044	0.331[a,b]	0.427[a,b]	0.059	0.373[a,b]
SEA	0.084	0.001	0.385[a,b]	0.495[a,b]	0.155[a]	0.464[a,b]
Pfs	0.225[a,b]		0.399[a,b]		0.245[a,b]	

[a] $P < 0.05$.
[b] Significance remains after Bonferroni's correction.

TABLE 2. Pretreatment antigen-specific IgG subclass correlations with *S. mansoni* egg counts for the Ugandan community cohort (Spearman's rank correlation)

Antigen	Association between specific IgG1 response and *S. mansoni* egg counts	Association between specific IgG3 response and *S. mansoni* egg counts	Association between specific IgG4 response and *S. mansoni* egg counts
SWA	0.476[a,b]	0.145[a]	0.395[a,b]
SEA	0.381[a,b]	0.030	0.525[a,b]
Pfs	−0.128	−0.022	0.222[a,b]
SmLRR	−0.008	0.088	0.213[a,b]

[a] $P < 0.05$.
[b] Significance remains after Bonferroni's correction.

SmLRR, positively correlated with each other but were not related to the intensity of *S. mansoni* infection. Taken together, these data suggest that malaria infection may drive the IgG3 cross-reactive response to SmLRR and that chronic, high-intensity *S. mansoni* infection can induce a typical, worm infection-associated anti-SmLRR IgG4 response, which then correlates with anti-Pfs IgG4 responses. The presence in the two parasites of shared epitopes with induction of two different cross-reactive isotypes may be due at least in part to differential immune regulation related to each parasite and/or to the nature of cross-reactive proteins per se. Regarding the functional role of antibodies, previous studies have shown that schistosomiasis- and malaria-specific IgG1 and IgG3 antibodies from infected individuals can mediate antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular inhibition, respectively (3, 17, 26). However, *S. mansoni* IgG4 blocked in vitro killing of schistosomula by human eosinophils (17), and noncytophilic anti-Pfs IgG4 antibodies have been reported to interfere with *P. falciparum* growth inhibition mediated by cytophilic IgG3 in vitro (9). The underlying mechanism of childhood hepatosplenic morbidity that is associated with coexposure to malaria and schistosomiasis caused by *S. mansoni* has not been identified yet. It is not associated with the ultrasonography-detectable periportal fibrosis that causes classical hepatosplenic schistosomiasis in a small proportion of older individuals. Nonetheless, enhanced inflammatory responses to components of either parasite or both parasites are still possible etiologies for this childhood disease. With the murine *S. mansoni* model, it has been shown that severe hepatic reactions to parasite eggs can be mediated by Th1 (13) or interleukin-17 (29) inflammatory responses, in addition to well-characterized Th2-mediated fibrobic mechanisms (13). IgG3 has the longest hinge region of all human IgG subclasses, which confers the highest effective complement activator activity and also the highest affinity for effector cell Fc receptors (4, 31). Thus, the up-regulation of IgG3 responses greatly increases the potential for host tissue damage as a collateral effect of reactions to the persistence in the liver of parasites and parasite antigens, as in chronic schistosomiasis caused by *S. mansoni*. It is not clear what proportion of the antigenic cross-reactivity observed in human responses to schistosomiasis and malaria is accounted for by SmLRR since, although this protein was the only cross-reactive protein selected by expression library screening, nonpeptide parasite components are additional potential sources of cross-reactivity. However, the

clear demonstration that highly reactive effector antibody isotypes, such as human IgG3, may be up-regulated by cross-reactivity between major human parasites that are coendemic provides evidence which supports the hypothesis that immune interactions between different parasites has significant potential to exacerbate human disease.

In conclusion, we observed that SmLRR expressed in different stages of *S. mansoni* contains humoral epitopes that cross-react with both *P. berghei* and *P. falciparum*. In human infections the cross-reactive responses seem to be predominantly an IgG3 isotype response to malaria and an IgG4 response to schistosomiasis. The SmLRR and the *P. falciparum* protein(s) expressing the cross-reactive epitopes could constitute the basis for future studies to evaluate their roles in both infections.

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