NATURAL HUMAN IMMUNOGLOBULIN G SUBCLASS RESPONSES TO PLASMODIUM FALCIPARUM SERINE REPEAT ANTIGEN IN UGANDA

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Abstract. Serum samples from Ugandan residents of a malaria-hyperendemic region were tested by enzyme-linked immunosorbent assay for reactivity against recombinant constructs of the 47 (SE47')- and 50 (SE50A)-kDa fragments of Plasmodium falciparum serine repeat antigen (SERA). Immunoglobulin (Ig) G3 and IgG1 were the predominant subclass responses to SE47' and SE50A, respectively. The geometric mean optical density (OD) for IgG3 anti-SE47' was significantly lower in children < 15 years compared with adults (P < 0.0001). By contrast, the geometric mean IgG1 anti-SE50A was slightly higher in children compared with adults (P < 0.01). The proportion of high responders (ODs > 0.5) to SE47' was significantly lower in children compared with adults (P < 0.001), whereas the proportion of high responders to SE50A was comparable in children and adults (P = 0.07). This first detailed study of SERA in a malaria-hyperendemic region suggests that natural human IgG3 anti-SE47' might be associated with immunity to malaria.

INTRODUCTION

Plasmodium falciparum serine repeat antigen (SERA) is a promising malaria vaccine candidate antigen1 that accumulates in the parasitophorous vacuole at the late trophozoite and schizont stage and appears at the merozoite surface.2–4 It contains 989 amino acids, including a 35-serine-residues repeat,5 and has a limited homology to the active site of serine proteases.6,7 It is processed into 3 fragments—47, 56, and 18 kDa—coincident with merozoite release.8 The amino acid sequence of SERA is well conserved among Plasmodium strains and isolates except for the N-terminal domain of 47 kDa, which is diverse to some extent at the serine repeat and its proximal regions.9 According to the genome sequence of chromosome 2, there are 8 genes that encode a family of SERA-like proteins, designated SERA1, SERA2, SERA3, SERA4, SERA5, SERA6, SERA7, and SERA8.10 The present work is concerned with the gene product of SERA4.

Recombinant amino terminal fragment of SERA4 expressed in yeast induces protective immune responses against P. falciparum infection in Aotus monkeys.11–13 We reported the construction of a synthetic gene sequence with Escherichia coli codons that encodes the N-terminal domain of SERA of Honduras 1 strain, SE47' (amino acids 17–382), and the middle domain containing the cysteine protease-like domain SE50A (amino acids 568–802); we also reported immunization of rats with SE47'-induced antibodies that inhibited parasite growth in vitro.14 The SE47' protein also induced protective antibodies in Saimiri monkeys.15 Antibody titers against the recombinant SE47' in 2 of the SE47'-immunized monkeys were boosted by P. falciparum challenge infection. The boosting of antibody titers after challenge infection correlated with the degree of protection in the monkeys. Affinity-purified immunoglobulin (Ig) G specific to SE47' inhibited parasite proliferation by agglutinating merozoites released from rupturing schizonts and by complement-dependent lysis of blood stage schizonts by the classical pathway.16,17

The foregoing observations in nonhuman model systems provide strong evidence that SERA4 might be a useful malaria asexual stage vaccine antigen. However, limited data are available on human immune responses to SERA4 by residents of malaria endemic regions.18 We therefore investigated the natural human humoral responses to SERA4 by residents of Atopi Parish, Apac District, in northern Uganda. This is a region characterized by intense perennial hyperendemic malaria transmission.19 In this article, we describe IgG subclass responses to 2 recombinant constructs representing the amino terminal and central domain of SERA4 (SE47' and SE50A, respectively) in residents of Atopi Parish. We report that there is a remarkable differential IgG subclass responses to the amino terminal and central parts of SERA4, with IgG3 and IgG1 being the predominant subclass responses against SE47' and SE50A, respectively, and that higher levels of IgG3 anti-SE47' were associated with lack of fever and lower parasitemia in children aged < 15 years.

MATERIALS AND METHODS

Study site and study population. The study site, Atopi Parish, is located 5 km west of Apac Town, 300 km north of Kampala, at an elevation of 3,450 feet (1,035 m) above sea level, in Maruzi County, Apac District, in northern Uganda. Maruzi County is bordered by 2 large water bodies, the Victoria Nile to the west and Lake Kwanza to the southeast. These water bodies feed seasonal and papyrus swamps that interrupt the normal savanna type of vegetation throughout the county. Aroca, the major perennial swamp, not only runs north of Atopi Parish, but one seasonal branch passes through the parish and divides into 2 smaller branches. The Aroca swamp therefore constitutes an important breeding site for the mosquito vectors Anopheles gambiae and An. funestus, which transmit malaria in this region.20

There is a long dry season from November to February and 2 short rainy seasons from March to June and from August to October. The population of Atopi consists predominantly of the Lango tribe, a Nilotic ethnic group whose major occupation is peasant agriculture. The crops cultivated consist of millet, beans, cassava, cotton, and sorghum. According to the 1991 census, the male-to-female ratio was 1.0:1.1, and subjects aged < 5, 5–9, 10–14, 15–19, and > 20

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years constituted 19.7, 17.2, 12.6, 11.6, and 39.4%, respectively, of the population. These demographic data were confirmed by a census we carried out in December 1995 that established that Atopi Parish had 4,007 inhabitants living in 739 households.

**Study design.** During a cross-sectional survey conducted in 1995, blood samples from children and adults were obtained after informed consent (and approval by the Uganda National Council for Science and Technology) by finger-pricks and venipuncture, respectively, and collected in microtainers or Vacutainers containing ethylenediamine-tetraacetic acid. Thin and thick blood films were made from each sample, stained with Giemsa, and examined for malaria parasites under high-power microscopy. Parasite density, defined as the number of parasites per microliter of blood, was estimated by counting the number of parasites in 100 high-power fields and multiplying the mean number of parasites per field by 500. Serum samples were separated into fresh serum vials and transported on ice to Kampala, where they were stored at −20°C.

**Antigens and immunochemicals.** Recombinant SE47 and SE50A were expressed and purified as described previously. Alkaline phosphatase-conjugated murine monoclonal antibodies to IgG1, IgG2, IgG3, and IgG4 antibodies were purchased from Zymed Laboratories (South San Francisco, CA) and from The Binding Site (Birmingham, UK). p-nitrophenyl phosphate substrate tablets and other chemicals were purchased from Sigma Chemical (St. Louis, MO).

**Enzyme-linked immunosorbent assay (ELISA).** The IgG subclass antibodies reacting with SE47 and SE50A were detected by ELISA. Immunon 4 microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight at 4°C with 100 µL of antigen diluted to a concentration of 1 µg/mL in carbonate buffer. Plates were washed 3 times in phosphate-buffered saline (PBS) containing 0.5% v/v Tween 20 (PBS-T) and blocked for 1 hr with blocking buffer. Secondary antibodies were diluted 1:1,000 in PBS-T or 1:1,600 in PBS-T. After the final wash, the wells were filled with the substrate p-nitrophenyl phosphate and the reactions were read at 405 nm after 30 min. Serum samples from 30 Europeans who had never been exposed to malaria were tested by ELISA for reactivity with SE47 and SE50A. All subsequent analyses were performed in duplicate, and incubated for 1 hr at 37°C. The plates were then washed 3 times in PBS-T; they were blocked for 1 hr with blocking buffer consisting of 1% nonfat dry milk in PBS-T. Serum samples were diluted 1:50 in blocking buffer, added to the plates in duplicate, and incubated for 1 hr at 37°C. The plates were then washed 3 times in PBS-T; they were then incubated for 1 hr at 37°C with 100 µL of alkaline phosphatase–conjugated mouse monoclonal antibodies against human IgG1, IgG2, IgG3, or IgG4 appropriately diluted in blocking buffer. Secondary antibodies were diluted 1:1,000 or 1:1,600 in PBS-T. After the final wash, the wells were filled with the substrate p-nitrophenyl phosphate and the reactions were read at 405 nm after 30 min. Serum samples from 30 Europeans who had never been exposed to malaria were used as negative controls to provide a cutoff value for positive antibody reactions.

**Statistical analysis.** Data were entered into Epi Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and checked for consistency before statistical analysis by Stata, version 6.0 (Stata, College Station, TX). Descriptive analysis was carried out to determine the frequency of categorical variables such as parasite rates, spleen rates, history of chloroquine consumption, bed net use, and presence or absence of fever. The cutoff value for positive ELISA optical densities (ODs) was calculated as the arithmetic mean OD plus 2 standard deviations of the European control sera. This cutoff value was 0.150–0.250; values above this were regarded as antibody positive. Because the OD and parasite density data were skewed, logarithmic transformation was carried out to normalize the data. The geometric mean ODs and parasite density in blood samples from children (aged <15 years old) and adults (aged ≥15 years old) were computed. Statistical significance for differences in geometric means was tested by the unpaired Student’s t-test. We also arbitrarily defined high and low responders as those patients whose serum samples reacted with the recombinant antigens with an OD value of >0.50 and <0.50, respectively. The proportion of high responders among children and adults was compared by the chi-square test.

**Malarriometric data.** During a cross-sectional study carried out in December 1995, 1,244 people were surveyed. All were examined for *P. falciparum* parasitemia and parasite counts, and the majority (97–99%) were assessed for body temperature and spleen size and questioned about their history of fever within the last 3 or 15 days, chloroquine consumption, and bed net use. Bed net use and chloroquine consumption were very low, at 10.5% (130 of 1,234) and 11.3% (139 of 1,234), respectively. Fever (temperature ≥37.5°C) was recorded in 64 (5.2%) patients, and the fever rate was significantly reduced in people aged ≥15 years (39% in adults versus 60.6% in children). The prevalence of parasitemia for all ages was 62%; however, the prevalence for the age group 1–9 years was 84%. The predominant species was *P. falciparum* (100%), with *P. malariae* in the minority (1.5%), and *P. ovale* was not seen. The geometric mean parasite density started to decline at the age of 6 years. The splenomegaly rate in subjects >12 years old was also lower than that in subjects aged <9 years (19.8% versus 63.1%). The overall data indicate that malaria transmission in Atopi Parish is hyperendemic, with a considerable degree of age-related acquired immunity. These malarriometric data have been reported previously.

**Differential IgG subclass responses to SE47 and SE50A.** Three hundred seventy-four serum samples were tested by ELISA for reactivity with SE47 and SE50A. Sera were tested for each of the 4 IgG subclasses. There was a remarkable differential IgG subclass response to the 2 recombinant constructs, with IgG3 and IgG1 antibodies predominating the humoral response to SE47 and SE50A, respectively (Figure 1). The geometric mean OD for IgG3 anti-SE47 and IgG1 anti-SE50A was significantly lower in children aged <15 years (0.34; 95% confidence interval [CI], 0.29–0.39) compared with adults aged ≥15 years (0.63; 95% CI, 0.51–0.80; P < 0.0001). In contrast, the geometric mean OD for IgG1 anti-SE50A was slightly higher in children compared with adults (0.65; 95% CI, 0.59–0.71; versus 0.54; 95% CI, 0.49–0.60; P = 0.01). Interestingly, whereas mean IgG3 anti-SE47 ODs increased with age (Figure 1A), mean IgG1 anti-SE50A ODs decreased in older people (15–30 years old; Figure 1B). There was virtually no IgG2 and IgG4 antibody responses to both SE47 and SE50A. All subsequent analyses of human IgG subclass responses to SERA4 therefore focused on IgG3 anti-SE47 and IgG1 anti-SE50A.

**Immunogenicity of SE47 and SE50A.** In order to provide possible insights about the immunogenicity of SE47 and
RESULTS

Relationship between antibodies against SE47 and SE50A and fever. It was of interest to determine if any relationship existed between levels of IgG3 anti-SE47 and IgG1 anti-SE50A in individual subjects on the one hand, and the presence or absence of fever in the subjects on the day of the blood sampling during the cross-sectional study on the other. The scatter of the individual OD values of IgG3 anti-SE47 and IgG1 anti-SE50A in 31 nonfebrile children (mean age, 6.1 years) and 9 febrile children (mean age, 6.3 years) with axillary temperatures of $\geq 37.5^\circ C$ is shown in Figure 2. All the children with fever were low IgG3 anti-SE47 responders, whereas 6 of 9 children with fever were high IgG1 anti-SE50A responders. Although the sample sizes do not allow statistical comparisons, it is remarkable that low IgG3 anti-SE47 responses are associated with fever and that IgG1 anti-SE50A responses in general are not correlated with fever.

Relationship between antibodies against SE47 and SE50A and parasite density. It was also of interest to determine in children aged $< 15$ years if there was any relationship between levels of IgG3 anti-SE47 and IgG1 anti-SE50A on the one hand, and parasite density on the day of the study on the other (Figure 3). Twenty-one percent (7 of 34) of the IgG3 anti-SE47 high responders (OD $> 0.50$) had parasite densities $> 1,000$ parasites/μL. By contrast, 42% (22 of 53) of IgG3 anti-SE47 low responders (OD $< 0.50$) had parasite densities above 1,000 parasites/μL. This difference in the frequency of high parasite densities between IgG3 anti-SE47 high and low responders was significant ($P = 0.043$). Furthermore, the geometric mean parasite density was lower among the IgG3 anti-SE47 high responders (389; 95% CI, 203–746) compared with the low responders (848; 95% CI, 524–1,374) although the difference was not statistically significant ($P = 0.067$). By contrast, the proportions with parasite densities $> 1,000$ parasites/μL were comparable among high and low IgG1 anti-SE50A responders (31.3%; 15 of 48) versus 36.8% (14 of 38; $P = 0.451$); the geometric mean parasite density was also comparable between high and low IgG1 anti-SE50A responders (650; 95% CI, 355–1,191; versus 654; 95% CI, 379–1,129; $P = 0.98$). Thus, there was an association between high IgG3 anti-SE47 responses and lower prevalence and levels of par-

### Table 1

<table>
<thead>
<tr>
<th>Subjects</th>
<th>IgG subclass antibody response</th>
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<tbody>
<tr>
<td></td>
<td>IgG3 anti-SE47</td>
</tr>
<tr>
<td>Children</td>
<td>44/160 (27.5%)†</td>
</tr>
<tr>
<td>Adults</td>
<td>61/125 (48.8%)†</td>
</tr>
<tr>
<td>All subjects</td>
<td>105/291 (36.1%)</td>
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</tbody>
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* IgG = immunoglobulin.
† The difference between the prevalence of IgG3 anti-SE47 high responders in children ( $< 15$ years old) and adults ( $\geq 15$ years old) was statistically significant ($P < 0.0001$).
‡ The difference between the prevalence of IgG1 anti-SE50A high responders in children and adults was not statistically significant ($P > 0.05$).
Figure 2. Scatter plot of individual optical density (OD) values for immunoglobulin (Ig) G3 anti-SE47/H11032 and IgG1 anti-SE50A antibodies in nonfebrile and febrile Atopi Parish children aged < 15 years. The children were grouped into fever (n = 9) and nonfever (n = 31) groups, depending on whether the body temperature on the day of the cross-sectional study was above 37.5°C. The dashed line indicates the cutoff OD value for delineating high and low IgG1 and IgG3 responders. The bars indicate the arithmetic mean OD values of the fever and nonfever groups.

Discussion

There is a growing body of evidence from immunization experiments in nonhuman animal model systems that SERA4 might be a useful malaria asexual blood stage vaccine antigen.11–15 A recent report confirmed that SE47/H11032 is a viable component of a DNA-based human malaria vaccine.21 However, there is virtually no detailed information available on human immune responses to SERA4 by residents of malaria-endemic regions. This gap urgently needs to be filled in the current efforts to develop asexual blood stage vaccines against P. falciparum. In order to investigate human humoral immune responses to SERA4 in a malaria-endemic region, recombinant constructs corresponding to the 47- and 50-kDa fragments of SERA4 (SE47/H11032 and SE50A) were used as antigens in IgG subclass-specific ELISAs with serum samples from Atopi Parish in Apac District, northern Uganda. Atopi Parish is a region of intense seasonal and perennial malaria transmission.19

There was a remarkable differential IgG subclass response to SERA4 constructs, with IgG3 antibodies predominating the response to SE47/H11032 and IgG1 antibodies predominating the response to SE50A. However, there were virtually no responses by the noncytophilic IgG2 and IgG4 subclasses. The cytophilic IgG1 and IgG3 subclasses are thought to be involved in antimalarial immunity mediated by monocytes through antibody-dependent cytotoxic inhibition.22–24 The predominance of the cytophilic IgG1 and IgG3 subclasses in human antibody responses to defined SERA4 molecules in residents of a malaria-hyperendemic region does provide some, albeit indirect, insights about the possible role of these antibodies in natural P. falciparum infections. In view of the lack of responses by the noncytophilic subclasses, our subsequent analyses focused on IgG3 anti-SE47/H11032 and IgG1 anti-SE50A antibodies.

It seems that SERA4, or at least the SERA4-derived constructs used in this study, are poorly immunogenic under natural conditions of P. falciparum infection in humans. A significantly high proportion of the adult residents of Apac were low responders to both SE47/H11032 and SE50A. The proportion of IgG3-SE47/H11032 high responders increased by almost 2-fold from 28% to 49% between childhood and adulthood. Thus, increased exposure to P. falciparum infection over many years appears to be necessary in order to have a significant proportion of IgG3-SE47/H11032 high responders in a village in an hyperendemic region. The poor immunogenicity of SERA4 have significant implications for the design and development of an asexual blood stage malaria vaccine based on this protein.

Our data suggest that IgG3 anti-SE47/H11032 probably confers
antiparasite immunity in older subjects. This assertion is supported by the finding that the prevalence of parasitemia and geometric mean parasite density was lower in IgG3 anti-SE47 high responders compared with low responders, although this difference did not attain statistical significance for geometric mean parasite density. Furthermore, high IgG3 anti-SE47 responses appeared only by 9–10 years of age, when clinical immunity and antiparasite immunity becomes apparent in this endemic region. These field observations might be explained by the laboratory observations that immunization of experimental animals with SE47 protected against a challenge infection and that anti-SE47 antibodies inhibited parasite growth in vitro by agglutination of merozoites and by complement-dependent lysis of schizonts. By contrast, immunization of rats with SE50A did not induce significant levels of parasite growth inhibitory antibodies, although SE50A was highly immunogenic. A similar observation was obtained by immunizing BALB/c mice with SE50A (Pang XL and Horii T, unpublished results). The fact that IgG1 anti-SE50A high responses appear by 1 year of age and persist into adulthood suggests that IgG1 anti-SE50A might be a marker of infection. Indeed, we observed a slightly higher proportion of IgG1 anti-SE50A high responders in children compared with adults. The high IgG1 anti-SE50A responses in very young children without clinical or antiparasite immunity is probably in response to the antigenic pressure exerted by intense sporozoite inoculations in Atopi Parish.

We are aware that the cross-sectional nature of the present study does not permit us to draw definite conclusions about the role that the IgG3 anti-SE47 and IgG1 anti-SE50A antibodies play in the host-parasite interaction. In order to resolve this important question, a longitudinal cohort study of P. falciparum parasitemia and reinfection rates in school-going children at Atopi Primary School and a hospital-based morbidity case-control study in children < 5 years old in Apac Hospital are currently under way.

One study of 35 serum samples from Gambians and Ugandans reported vigorous humoral responses against the cysteine protease-like domains of P. falciparum SERA and the serine repeat protein homologue. IgG1 was the predominant subclass in the serum samples of the African subjects. We have confirmed the predominance of the IgG1 subclass response to the cysteine protease-like domain (SE50A) and extended these observations to the predominance of IgG3 subclass response to SE47, the putatively protective amino terminal fragment, in a greater number (> 300) of Ugandan subjects.

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