Differential Antibody Responses to *Plasmodium falciparum* Invasion Ligand Proteins in Individuals Living in Malaria-Endemic Areas in Brazil and Cameroon


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**Abstract.** Antibody responses to malaria invasion ligands and proteins on the merozoite surface have been shown to interfere with red cell invasion and correlate with immunity to malaria. The current study is the first to characterize the antibody responses to EBA-140 and EBA-181, *Plasmodium falciparum* invasion ligands implicated in the alternative pathways of invasion, in age-matched populations of individuals living in endemic areas in both Brazil and Cameroon. Antibody responses to the proteins screened were different between populations. The African individuals reacted strongly with most fragments of these two EBAs, while the majority of the individuals from Mato Grosso, Brazil, reacted weakly and those from the Amazon had elevated responses to these EBA proteins. When compared with the responses against MSP-1α and EBA-175, it appeared that the Brazilian population has a variable ability to recognize *P. falciparum* invasion ligand proteins and that these responses are distinct from the African population.

**(INTRODUCTION)**

Falciparum malaria is one of the most significant causes of morbidity and mortality in the developing world, with up to 500 million new cases annually. With the increase in antimalarial drug resistance, there is a growing requirement for an effective malaria vaccine. Epidemiologic surveys performed in areas of high malaria transmission have shown that persons who are continuously exposed to repeated malaria infection gradually develop clinical immunity. Experiments with antibodies purified from the sera of African adults who were clinically immune to malaria and given by passive transfer to susceptible children have established that immunoglobulin G (IgG) is at least a main component of defense against the asexual blood stage of *Plasmodium falciparum*. Thus, antibodies that target the asexual blood-stage parasites seem to be of central importance, and several blood-stage antigens have been implicated as targets for protection. Vaccine development will be facilitated by studying the naturally acquired immune responses that mediate protection.

Antibodies may act in different ways, by preventing merozoite invasion of red blood cells (RBCs), by attacking infected RBCs and facilitating phagocytosis, or by preventing cytoadhesion of infected RBCs. Molecules important to all 3 of these processes have been identified as candidate vaccines, but those involved in merozoite invasion have been the most studied. Repeated cycles of merozoite invasion of RBCs quickly amplify the blood-stage parasitemia, and this contributes to symptomatic disease. Successful invasion requires successful receptor–ligand interactions on the RBC surface. These invasion ligands can be present on the merozoite surface (MSP family) or be harbored in apical organelles like the micronemes (EBL family) and the rhoptries (RH family).

*P. falciparum* uses a wide variety of RBC receptors for invasion. Erythrocyte-binding antigen 175 (EBA-175) was the first member of the erythrocyte-binding ligand (EBL) family characterized and shown to bind to the major glycoprotein found on human erythrocytes, glycophorin A (GPA), during invasion. Recombinant fragments of EBA-175 are recognized by human sera from malaria-endemic areas. Antibodies raised in rabbits against EBA-peptide 4 blocked binding of native EBA-175 to human erythrocytes and inhibited merozoite invasion in *vitro*. Additionally, IgG1 antibodies to EBA-175 peptide 4 are associated with protection against clinical malaria. We and others have shown that the majority of *P. falciparum* parasites in the endemic areas of India and Brazil are not dependent on neuraminidase-sensitive invasion pathways, of which the interaction of EBA-175 with GPA is the major pathway. Importantly, the targeted disruption of the *eba-175* gene was not lethal but instead caused switching to a GPA-independent alternative invasion pathway. The parasite ligands that mediate the alternative invasion pathways are postulated to belong to the EBL and RH families of invasion ligands. Although most of the immunogenicity studies have focused on EBA-175, little is known regarding the acquisition of natural antibodies to the other members of the EBL family. The rationale behind this study was to evaluate for the first time the role of EBA-140 and EBA-181, parasite ligands used in the alternative invasion pathways as targets of naturally acquired immune responses. Such data will be relevant to the design of vaccines aimed to inhibit erythrocyte invasion by merozoites. Additionally, study of the naturally acquired antibodies present in endemic populations may reflect the choice of invasion ligands that locally circulating parasites use, which in turn may be dictated by the repertoire of host RBC receptors available in that specific study population.

In this paper, the naturally acquired antibody responses to different regions of these two relatively unstudied invasion ligands, EBA-140 and EBA-181, were analyzed in populations of individuals living in endemic areas of Brazil and Cameroon, known to be of differing endemicity.

**(MATERIALS AND METHODS)**

**Study areas, subjects, and blood sample collection.** We analyzed groups of subjects who had been exposed to malaria transmission in endemic areas in both Brazil and Cameroon.
Two study groups from hypoendemic regions for malaria in Brazil were studied: The serum samples of the first group were collected in 1995 from 17 adult male individuals (median age = 25 years) living in Peixoto de Azevedo, a municipality of Mato Grosso state, located in the southern part of the Amazon region in Brazil. The individuals were migrant gold mining workers without documented ethnicity who had come to the local health service center, run by the Ministry of Health, and were found to be *P. falciparum* positive and *P. vivax* negative. These individuals resided in the endemic region for only a few years and had no more than 1–3 malaria episodes. The serum samples of the second group were collected in 2004 and originated from individuals who have lived in the Amazon region (Macapá, Belém, Porto Velho, and Rio Branco) in Brazil for most of their life and have reported multiple episodes (range 1 to >4 episodes) of *P. falciparum* malaria infection and were *P. vivax*-negative at the time of blood collection. This group consisted of 37 individuals (4 females and 33 males; median age = 25 years, range 18–76 years).

The serum samples from West Africa were collected in 1996 and 1997 and originated from individuals who live in villages in the Kumba region, an area of hyperendemic malaria in the southwest province of Cameroon. The individuals who participated in the study were born or had resided for >10 years in villages: Marumba I, Marumba II, Boa Bakundu, Bombanda, and Bombele. The group consisted of individuals who were both *P. falciparum* blood smear positive and negative at the time of bleeding. The Cameroon study group comprised 180 individuals (118 males and 62 females; median age = 17 years; range, 3–75 years). An age-matched subset of these individuals was selected (*N* = 28; median age = 32 years; range 8–38 years) to compare their responses with those from the individuals from Brazil.

All blood samples were obtained from volunteers with informed consent using protocols approved by both local and NYBC IRB committees.

**Recombinant malaria protein expression and purification.** Recombinant malaria proteins were expressed in *Escherichia coli* BL21 (DE3) (Sigma, St. Louis, MO) as fusion proteins with glutathione *S*-transferase (GST) using the pGEX vector (Amersham Biotech, Piscataway, NJ).

The 3D7 strain of *P. falciparum* was used as template to derive the various fragments that were used for cloning and expression. Fragments of the erythrocyte-binding proteins EBA-140 and EBA-181 were expressed as soluble GST-fusion proteins and corresponded to fragments within the following regions: portion (aa 350–440) of the Duffy-binding-like domain (DBL) also known as region II, portions of region VI (aa 799–963) and the C-terminal region (aa 967–1140) of EBA-140, and portions of the N-terminal (aa 33–171) and the C-terminal (aa 1235–1345) of EBA-181. These regions do not exhibit sequence polymorphism among the various *P. falciparum* strains. Recombinant proteins were purified by affinity chromatography on glutathione agarose (Sigma) as previously described. Control GST was purified from *E. coli* BL21 transformed with the pGEX vector alone. Fusion proteins were assessed by Coomassie blue staining of SDS-PAGE gels, and protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

Full length EBA-140 region II (aa 141–756; the 3D7 allele, INKK) and EBA-175 region II (aa 145–760; 3D7 allele) were expressed in yeast and were a kind gift from D. Narum and L. Miller (LMV, NIH). The 19-kDa processed fragment of the merozoite surface protein 1, MSP-1_19, was obtained as a recombinant yeast protein from the MR4 (ATCC) and also corresponds to the 3D7 allele. These 3 recombinant proteins were properly folded.

**Measurement of antigen-specific antibody responses.** Human serum samples were analyzed for IgG1 and IgG3 reactivity to the recombinant malaria proteins by a defined enzyme-linked immunosorbent assay (ELISA), as described previously. The subclass of immunoglobulin determines antibody function (e.g., complement fixation or the activation of phagocytes), and in humans, immunoglobulin G1 (IgG1) and IgG3 are important mediators of malaria parasite clearance. To determine which *E. coli*-expressed EBA protein fragments were antigenic, we performed an initial ELISA screen using the various recombinant EBA fragments with a small group of sera from Cameroon. Based on their antigenicity, for all further experiments we focused on the antibody responses generated to 3 EBA-140 protein fragments: portion of region II, region VI and the C-terminal region, and to the C-terminal region fragment of EBA-181.

Microtiter ELISA plates (Costar, Corning Life Sciences, Corning, NY) were coated with 1 μg/mL of recombinant protein diluted in 0.05 M carbonate buffer, pH 9.6. After incubation overnight at 4°C, the plates were washed 5 times with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST) and blocked with blocking buffer (3% BSA in PBST) for 1.5 hr at 37°C. Serum samples were pre-incubated with *E. coli* extract prior to dilution and incubation with the bound antigen to remove antibodies to potential *E. coli* contaminants in the recombinant protein preparations. Serum samples, diluted 1:500 in blocking buffer, were reacted with the bound antigens by incubating for 2 hr at 37°C, in duplicate wells. The bound IgG1 and IgG3 antibodies were detected after incubation for 1 hr at 37°C with mouse monoclonal antibodies against different human IgG subclasses (Hybrdoma Reagent Laboratory, Baltimore, MD) diluted 1:1000 in blocking buffer, followed by incubation for 1 hr at 37°C with horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:1250 in blocking buffer. Tetramethylbenzidine (Sigma, St Louis, MO) was used as the substrate for all ELISAs, and the optical density (OD) was read at 450 nm on a SpectraMax 190 ELISA Reader (Molecular Devices, Sunnyvale, CA).

Each serum sample was tested against GST as a negative control for the GST-fusion proteins. Positive responders were defined as those that gave an OD value greater than the cut-off OD, which was defined as the mean plus 3 standard deviations of OD values from control sera. If the cut-off was <0.1, positive responders were defined as those with an OD value > 0.1. Control sera used were collected from 5 healthy adult volunteers living in New York who had never been exposed to malaria. Samples from the different study sites were run in parallel to ensure reliable comparison of ODs.

**Statistical analysis.** The χ² test was used to compare proportions of antibody responders in different groups, whereas the differences in the immunoglobulin levels between groups were compared using the two-tailed nonparametric Mann-
Whitney U test. Spearman’s rank correlation test was used to test the significance of the correlation between the age of the individuals and their antibody responses, with the correlation coefficient being expressed as r. A P value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad PRISM software (GraphPad Software Inc., San Diego, CA).

RESULTS

Differential antibody responses to EBA-140 and EBA-181 fragment proteins in individuals living in Brazil and in Cameroon. Analysis of the IgG1 and IgG3 responses to different fragments of EBA-140 and EBA-181 in individuals from Mato Grosso and the Amazon region, Brazil, and Cameroon revealed a dramatic difference in both the prevalence and magnitude of responses between the populations (Figure 1; Table 1). Whereas the Cameroon individuals mounted an efficient antibody response to most of the recombinant EBA-140 and EBA-181 proteins (percent positive responders: IgG1, 38.1–96.4%; IgG3, 65–100%), the antibody response to the same panel of proteins in residents of Mato Grosso was much lower (percent positive responders: IgG1, 5.9–29.4%; IgG3, 5.9–29.4%) (Figure 1A:B; Table 1). Only 1 serum sample from Mato Grosso in each assay showed a strong response (OD > 0.3) to the EBAs. All of the IgG1 and IgG3 responses in Mato Grosso, with the exception of the IgG1 response mounted to a fragment within the EBA-140 region II, were significantly lower than the responses in Cameroon (EBA-140 proteins—full-length region II P < 0.001 for both, region II fragment P < 0.001 IgG3, region VI fragment P = 0.9716 IgG1 and P = 0.0034 IgG3, C-terminal fragment P = 0.0087 IgG1 and P < 0.001 IgG3; EBA-181 C-terminal fragment P < 0.001 for both). The EBA-140 proteins corresponding to full-length region II, fragment of region VI, and fragment of the C-terminal were found to be the most antigenic ligands for the Mato Grosso population (11.8–29.4% positive responders), although the reactivity was much lower than found in the African population (80–100% positive responders). In the African population, the full-length region II fragment, the RBC-binding domain of EBA-140, is the most immunoreactive in all individuals, regardless of age.

Interestingly, when we analyzed the profile of antibodies present in other populations of the Brazilian Amazon, residing in Macapá, Belém, Porto Velho, and Rio Branco, the antigen-specific antibody responses differed between the 2 endemic regions within Brazil (Figure 1B:C). The antibody responses in these Amazonian individuals, to the same panel of EBA proteins, were elevated (percent positive responders: IgG1, 7.7–30.8%; IgG3, 28.5–69.2%) when compared with those from Mato Grosso, although lower in magnitude than the responses in the Cameroonian sera (Table 1). Amazonian individuals had significantly higher antibody responses than individuals from Mato Grosso to EBA-181 C-terminal fragment (P < 0.001 for both IgG1 and IgG3), EBA-140 region II fragment (P < 0.001 IgG1 and P = 0.0027 IgG3), EBA-140 region VI fragment (P = 0.0524 IgG1), and EBA-140 C-terminal fragment (P = 0.0051 IgG3). Regardless, the antibody responses in the Amazon individuals was significantly lower than the responses in the Cameroonian individuals to EBA-140 full-length region II (P < 0.001 for both IgG1 and IgG3), EBA-140 region II fragment (P = 0.0162 IgG1 and P = 0.0003 IgG3), EBA-140 region VI fragment (P = 0.0088 IgG3), and EBA-140 C-terminal fragment (P = 0.0006 IgG1 and P < 0.001 IgG3). Antibody responses to the EBA-181 C-terminal fragment in the Amazonian individuals were also lower than in the Cameroonian individuals; however, these differences were not statistically significant (P = 0.145 IgG1 and P = 0.176 IgG3).

Antibody responses to MSP-119 and EBA-175 region II. To determine if the low humoral response seen in the Mato Grosso population against the EBA proteins was antigen-specific, we examined the antibodies present in these endemic populations to 2 other known highly antigenic malaria proteins: the GPA-binding domain of EBA-175, EBA-175 region II, another member of the EBL family and the 19-kDa processed fragment of the merozoite surface protein 1 (MSP-119). These results are presented in Table 1 and Figure 2. In both the Mato Grosso inhabitants and the individuals from Cameroon, the IgG3 responses to MSP-119 were prevalent (76.5% IgG3-positive responders in Mato Grosso versus 95% IgG3-positive responders in Cameroon; P = 0.08 using χ² analysis). The prevalence of the IgG1 responses against MSP-119 was, however, significantly lower in Mato Grosso inhabitants than in the individuals from Cameroon (70.6% IgG1-positive responders in Mato Grosso versus 100% IgG1-positive responders in Cameroon, P = 0.007 χ² test) (Table 1). Both the IgG1 and IgG3 antibody responses to MSP-119 by individuals

Figure 1. Comparison of isotype-specific antibody responses to EBA-140 and EBA-181 fragment proteins in age-matched individuals living in (A) Kumba, Cameroon (N = 28), (B) Mato Grosso, Brazil (N = 17), and (C) the Amazon, Brazil (N = 39). Median values are indicated by horizontal bars.
Prevalence and magnitude of antibody responses to EBA fragment proteins and MSP-1_19 from age-matched individuals living in Kumba, Cameroon,* Mato Grosso, Brazil,† and the Amazon, Brazil‡ (measured by ELISA)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Kumba, Cameroon</th>
<th>Mato Grosso, Brazil</th>
<th>Amazon, Brazil</th>
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<tbody>
<tr>
<td></td>
<td>IgG1 (%)</td>
<td>IgG3 (%)</td>
<td>IgG1 (%)</td>
</tr>
<tr>
<td>EBA-181</td>
<td>38.1 (0.02-0.51)</td>
<td>65.0 (0.29-0.93)</td>
<td>5.9 (0.001)</td>
</tr>
<tr>
<td>aa1233-1345</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBA-140</td>
<td>96.4 (1.23)</td>
<td>100 (1.77)</td>
<td>11.8 (0.014)</td>
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<tr>
<td>EBA-140</td>
<td>20.0 (0.14)</td>
<td>70.8 (0.14)</td>
<td>5.9 (0.001)</td>
</tr>
<tr>
<td>aa335-440</td>
<td>(0.05)</td>
<td>(0.13-0.71)</td>
<td></td>
</tr>
<tr>
<td>EBA-140</td>
<td>80.0 (0.14)</td>
<td>80.0 (1.33)</td>
<td>11.8 (0.001)</td>
</tr>
<tr>
<td>aa799-965</td>
<td>(0.81)</td>
<td>(0.2-0.50)</td>
<td></td>
</tr>
<tr>
<td>EBA-140</td>
<td>80.0 (0.27)</td>
<td>81.8 (0.79)</td>
<td>29.4 (0.050)</td>
</tr>
<tr>
<td>aa967-1140</td>
<td>(0.11-0.42)</td>
<td>(0.63-1.16)</td>
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</tr>
<tr>
<td>EBA-175</td>
<td>92.9 (1.19)</td>
<td>100 (1.34)</td>
<td>11.8 (0.001)</td>
</tr>
<tr>
<td>aa145-760</td>
<td>(0.90-1.46)</td>
<td>(1.07-1.72)</td>
<td></td>
</tr>
<tr>
<td>MSP-1_19</td>
<td>100 (1.42)</td>
<td>95.2 (1.59)</td>
<td>70.6 (0.84)</td>
</tr>
<tr>
<td></td>
<td>(1.03-1.54)</td>
<td>(1.19-1.89)</td>
<td>(0.45-1.17)</td>
</tr>
</tbody>
</table>

* 28 serum samples collected from Kumba, Cameroon.
† 17 serum samples collected from Mato Grosso, Brazil.
‡ 39 serum samples collected from Amazon, Brazil.
§ Percentage of positive responders (% positive sera) are defined as those with an OD value greater than the cut-off OD which was defined as the mean ± 3 standard deviations for the ODs of control sera or OD values > 0.1.
¶ Median ODs are shown with 95% confidence interval (CI).

Notably, we found a significantly lower prevalence of both IgG1 and IgG3 responses to EBA-175 region II (11.8% IgG1-positive responders and 5.9% IgG3-positive responders) in Mato Grosso, which was in sharp contrast to the 93% IgG1-positive responders and 100% IgG3-positive response in Cameroon (P < 0.0001 for both) (Table 1). Both the IgG1 and IgG3 antibody responses to EBA-175 region II by individuals in Mato Grosso were significantly lower than the responses mounted by Cameroon individuals (P < 0.0001 for both IgG1 and IgG3) (Figure 2).

Although the responses to the EBA proteins in the Amazon individuals were elevated compared with those from Mato Grosso (Figures 1 and 2), both IgG1 and IgG3 responses to MSP-1_19 in individuals from the Amazon region were significantly lower than those in Mato Grosso (P = 0.0438 IgG1 and P = 0.0006 IgG3) (Figure 2).

Antibody responses to the C-terminal regions of EBA-181 and EBA-140 in Cameroonianas correlate with age. Screening a small sample of adult individuals from Cameroon (N = 28, median age = 32 years) revealed that the most antigenic fragments of the EBA proteins, in addition to EBA-140 region II, were the C-terminal regions of both EBA-181 and EBA-140.
EBA-140 (Figure 1). We extended our study to a larger population \((N = 180, \text{median age} = 17)\) to perform an age correlation study in the Cameroonian population. Both the IgG3 antibody responses to the C-terminal regions of both EBA-181 and EBA-140 were slightly increased with age \((r = 0.193, P = 0.01\) and \(r = 0.186, P = 0.012, \text{respectively})\) (data not shown). EBA-140 region II-specific IgG3 responses were highly elevated in all Cameroon individuals, at a 1:500 dilution, regardless of age (data not shown).

In addition, none of the antibody responses in Cameroon were correlated to active infection with *P. falciparum* as indicated by a positive blood smear (data not shown).

**DISCUSSION**

Malaria is a unique example of an infection where people exposed multiple times to the blood-stage parasite acquire immunity with age and/or number of episodes. This ability to combat malaria is an important adaptive trait of populations living in endemic areas. The detection of significant differences in the expression of this trait and the identification of the factors involved should improve the understanding of the host–parasite relationship and thus lead to advances in control strategies.

This is the first study to examine the humoral response to the invasion ligands EBA-140 and EBA-181 induced by natural infection. It is believed that effective immunity to *P. falciparum* blood-stage parasites involves the acquisition of inhibitory antibodies targeted to various antigens of the invasive merozoite. The obligatory interaction between invasion ligands and their cognate receptors on the RBC represents a potential target for inhibition by vaccine-induced antibodies. Some of the current most promising vaccine candidates are indeed such invasion ligands as exemplified by MSP-1, AMA-1, and EBA-175.\(^{28}\) In this study, recombinant proteins representing different fragments of *P. falciparum* EBA-140 and EBA-181 were used to analyze the profile of naturally acquired human antibodies to these proteins. The amino acid sequence homology between region II, which is the RBC-binding domain, or region VI of the 2 EBL proteins is very low, \(< 25\%\) and \(< 20\%\), respectively. The similarity between the EBA members of the EBL family is attributed to their conserved structures.\(^{29}\) Although we recognize that the tested recombinant proteins may not represent all the conformational epitopes on the native EBLs, this study serves as a springboard for a future large-scale analysis of the antibody responses in these two populations.

We found that the Mato Grosso individuals did not mount a strong humoral response against these two EBA ligands. This was in contrast to the African individuals in whom, considerable IgG1 and IgG3 antibodies were detectable to all the recombinant EBA-140 and 180 fragments tested. In addition to the full-length region II of EBA-140, a fragment of the C-terminus of both EBA-140 and EBA-181 was also the most antigenic in this Cameroonian population. However, both populations mounted equally robust responses to the various MSPs (data not shown), which have been shown to be antigenic in a variety of different endemic populations.\(^{30-33}\) When the same sera were analyzed for immunoreactivity to the GPA-binding domain of EBA-175 (EBA-175 region II), a dominant member of the EBL family, only a few Mato Grosso individuals had detectable antibody responses. Thus, the Mato Grosso population had an apparently dichotomous response to the 2 sets of ligands, the MSPs versus the EBLs.

Further analysis of a second set of Brazilian individuals living in the Amazon revealed that the individuals from Mato Grosso were not typical of the Brazilian immune response, as elevated EBA-140 and EBA-181 antibody responses were found in other regions of the Amazon, although with much lower responses than those found in the Cameroonian population. Regardless, most of the Amazonian population was still nonresponders to the EBA-175 region II protein similarly as those from Mato Grosso.

We put forward 2 hypotheses to explain these differences:

1. Exposure to the parasite in terms of both overall years living in endemic areas and number of malaria infections has a direct effect on antibodies to various parasite antigens. A careful look at the history of malaria in Brazil reveals many epidemics associated with migration of a nonimmune population to malaria-endemic areas.\(^{34-36}\) Malaria in Brazil, which accounts for 90\% of the cases in South America, has increased 3-fold during the past decade, and nearly 631,000 cases were reported in 2002. Of these infections, 80\% are *P. vivax* and 20\% are *P. falciparum* infections. Almost all reported cases of malaria in Brazil originate in the Amazon region, where migrant populations and poor access to diagnosis and treatment are major barriers to effective control of malaria. Migrant populations help to spread parasites throughout the endemic regions and also contribute to the spread of the disease in malaria-free areas, thus increasing the risk for malaria outbreaks. It is believed that malaria in Mato Grosso could have originated from such migrant populations and is characterized by a hypoendemic pattern of infection, due mainly to its low demographic index. Thus, in Mato Grosso, antibodies against only the immunodominant antigens like MSP-1\(_{19}\) and other MSPs were found, as exposure to *P. falciparum* in these inhabitants would not be as high or repeated as in the rest of the Amazon (relatively stable population), whose inhabitants are subjected to multiple malaria episodes in short periods of time. Malaria in the Brazilian Amazon, on the other hand, was characterized by elevated antibody responses to all parasite ligands, including both MSP-1\(_{19}\) and the EBLs, although overall magnitudes were less than what was found in Africa. This substantial difference in exposure levels to the parasite thus impacted antibody response to these 2 sets of ligands in these 2 Brazilian populations. Future studies in a larger population in the Brazilian Amazon could confirm this hypothesis.

2. The repertoire of host RBC receptors available in specific populations will, over time, select for expression of specific parasite ligands that can mediate successful RBC invasion, and thus the immune responses to such ligands may be differential, correlating to their expression. In a recent study in Tanzania,\(^{37}\) it was shown that expression levels of some of EBA and PIRH ligands correlated with the specific invasion pathway that the corresponding parasites used for invasion. The authors also hypothesized that the ligand expression patterns may represent frequency-dependent selection either by polymorphic host receptors or by immune responses. Therefore, our results, showing differences in the immunoreactivity, could be also linked to RBC heterogeneity between these 2 populations which could have dictated parasite choice of invasion pathways and thus expression of specific EBLs. We have pre-
viously characterized the invasion pathways used by field isolates from Mato Grosso and found that EBA-175-mediated invasion was not the pathway of choice for the majority of these strains. Although we do not yet know the preferred invasion pathways used by isolates from the regions of the Amazon, the low anti-EBA-175 response we see in both Brazilian populations could allude to a common low usage of the EBA-175-GPA invasion pathway. On the other hand, a study in Gambia found that invasion via GPA and EBA-175 was frequently used by field strains, and if this is reflective of malaria on the whole in Africa, then the high anti-EBA-175 reactivities that were observed in the Cameroonian population could be predicted. A study in Western Kenya found that 98.7% of samples tested had detectable total IgG against EBA-175 region II, while in 2 areas of West Africa—the Gambia and Nigeria—the proportion of individuals with antibodies against the same molecule was 60–70%. Notably, a retrospective analysis of naturally acquired antibodies to a synthetic peptide from EBA-175 peptide 4 carried out in Gabon has shown that the overall prevalence rates of antibodies to a linear epitope of EBA-175, EBA-175 peptide 4, were 85.2% and that the antibody response was associated with immunity against clinical malaria. Moreover, antibodies to EBA-175 peptide 4 were inhibitory in growth-inhibition assays in vitro. Future studies using full-length EBA-181 region II recombinant protein, that is functional in binding to RBCs, in growth-inhibition assays with serum samples containing differential patterns of anti-EBA antibodies on laboratory and field isolates from distinct geographic areas that have defined invasion pathways, in addition to determining antibody responses by ELISA, would discern the role of receptor heterogeneity on the EBA ligand-specific antibody responses.

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