HUMAN IMMUNE RESPONSES TO THE HIGHLY REPETITIVE PLASMODIUM FALCIPARUM ANTIGEN Pf332

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Abstract. The B and T cell responses to EB200, a repetitive part of the Plasmodium falciparum antigen Pf332, were examined in malaria-exposed Senegalese adults. Most donors had high levels of antibodies to recombinant EB200 and 17 overlapping peptides spanning EB200. Taking proliferation and/or cytokine (interferon-γ and interleukin-4) production as a measure of T cell activation, eight of the EB200-derived peptides induced responses in >40% of the donors tested. There was no general association between the different types of T cell responses measured, emphasizing the importance of including multiple parameters when analyzing T cell responses and suggesting that EB200 induces functionally distinct T cell responses. The most efficient peptide for induction of proliferative responses was one previously shown to induce T cell responses in five different H-2 congenic mouse strains primed with EB200, suggesting that this is a universal T cell epitope. The presence of multiple B and T cell epitopes in EB200, widely recognized by humans, is important since EB200 has been shown to elicit protective antibody responses in monkeys and may be considered for inclusion in malaria subunit vaccines.

The Plasmodium falciparum antigen Pf332 is of potential interest for inclusion in a subunit vaccine against the malaria parasite.1 Pf332 is a 750-kD protein expressed by late stages of intra-erythrocytic asexual parasites.2,3 Its amino acid sequence is only partially deduced (1,400 residues) and comprises undecamer repeat sequences with regularly spaced pairs of glutamic acid.2,4,5 Although these repeats share a common feature, they are highly degenerated and most motifs occur only once in the sequence of Pf332 so far deduced.2 Structurally related repeats are found in a number of P. falciparum proteins including Pf155/ring-infected erythrocyte surface antigen (RESA), Pf11.1, and glutamate-rich protein.6,8

Adult humans living in malaria-endemic regions of Africa displayed high seroprevalence and high levels of antibodies reactive with a peptide corresponding to a representative Pf332 repeat.9 Moreover, analysis of the epitope specificity of human Pf332-reactive antibodies suggested that a multitude of Pf332 repeats constitute B cell epitopes.10 The importance of Pf332-reactive antibodies for protective immunity against P. falciparum asexual blood stages is indicated by several findings. Antibodies to Pf332 repeats, raised in animals or affinity purified from human serum, inhibit in vitro parasite growth.10,11 Additionally, antibodies reactive with the Pf332-derived recombinant fragment EB200 mediate cellular elimination of parasitized red blood cells by acting as opsonins,12 and immunization of Saimiri monkeys with recombinant EB200 induced opsonizing antibodies.13

An essential feature of a malaria vaccine candidate antigen is the capacity to elicit relevant T cell responses, a prerequisite for efficient B cell responses. The T cell responses induced by EB200 have previously been examined in mice and have identified multiple T helper cell epitopes within the 134 amino acids of EB200.14 In H-2 congenic mouse strains, recognition of most of these epitopes was restricted to mice of certain H-2 haplotypes although one epitope was universally recognized. Similar H-2 restrictions have been observed when analyzing immune responses to structurally related repeats from Pf155/RESA and Pf11.1.15,16 The repeats of Pf155/RESA17 and Pf11.118 have also been shown to constitute T cell epitopes recognized by humans, whereas no previous studies have addressed human T cell responses to Pf332.

In the present study, a recombinant EB200 protein and a panel of overlapping peptides together spanning EB200 were used to analyze human immune responses elicited in malaria-primed individuals living in a holoendemic area (Dielmo, Senegal) in west Africa. The aim of the study was to define human immune responses to this part of the Pf332 antigen with regard to T and B cell specificities and to functionally characterize the T cell responses. For this purpose, peripheral blood mononuclear cells (PBMC) were examined in malaria-exposed Senegalese adults. Most donors had high levels of antibodies to recombinant EB200 and EB200-derived peptides.18 To investigate the relationship between B and T cell responses, serum was analyzed for antibody reactivity with recombinant EB200 and EB200-derived peptides.

MATERIALS AND METHODS

Study subjects and study area. Samples of venous blood were obtained after informed consent from 22 adults (mean age = 36.1 years, range = 16–87 years) living in the village of Dielmo in Senegal. Malaria transmission in this region is perennial with moderate seasonal variations.19 Samples from adult Swedish donors not exposed to P. falciparum served as controls. This study was approved by the Ministry of Public Health in Senegal and the National Ethic Committee in Sweden.

Antigens. A panel of 17 overlapping peptides (P1-P17) spanning the sequence of the Pf332-derived fragment EB20020 was synthesized (Table 1). The peptides were 16 amino acids long with 8 amino acid overlaps and were obtained from NeoSystem (Strasbourg, France), except for peptides P1, P13 and P14, which were synthesized using Fmoc chemistry as previously described.36 All peptides were C-terminally amidated with free amino termini and displayed single major peaks in reverse-phase high-performance liquid chromatography (purity = 60–90%). The peptides were de-
salted prior to use in T cell assays and were not toxic as assessed on phytohemagglutinin (PHA)-activated T cell blasts. Crude *P. falciparum* antigen extract was prepared from cultures of asexual blood-stage parasites. GST-EB200, a recombinant EB200 linked to glutathione-S-transferase (GST), and GST alone were produced as described. Preparation of PBMC and plasma collection. Venous blood (30–40 ml) was collected in heparinized vacutainer tubes. Plasma was prepared by centrifugation, aliquoted, and stored frozen. The PBMC were isolated by Ficoll Hypaque (Pharmacia Upjohn, Stockholm, Sweden) gradient centrifugation as earlier described by Perlmann and others. Enzyme-linked immunosorbent assay. Serum samples were analyzed for antibody reactivity with various antigen in an ELISA essentially as previously described. Briefly, ELISA plates (Costar, Cambridge, MA) were adsorbed overnight at 4°C with 2 µg/ml of recombinant GST-EB200 or GST, 10 µg/ml of synthetic peptides coupled to bovine serum albumin (BSA), or with 10 µg/ml of crude *P. falciparum* extract. After blocking with 0.5% BSA for 3 hr at 37°C, the wells were washed and serum diluted 1:1,000 was added and incubated for 1 hr at 37°C. The plates were washed and bound IgG was detected with alkaline phosphatase–conjugated goat antibodies to human IgG (γ-chains) (Mabtech AB, Stockholm, Sweden) using *p*-nitrophenyl phosphate as substrate. The IgG reactivity is displayed as units where 1 unit equals 1 µg/ml of human IgG determined from curves obtained with serial dilutions of human IgG in wells coated with antibody (Jackson Immunoresearch Laboratories, Westgrove, PA) to human IgG γ chain. Reactivity with EB200 was determined by subtracting the reactivity with GST from the reactivity with GST-EB200. The background reactivity with GST was low in the Senegalese sera, comparable with that of the Swedish control sera that displayed negligible reactivity with GST as well as with GST-EB200.

**Table 1**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
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<tr>
<td>P1</td>
<td>ILVEGVSTEEVGVGEK</td>
</tr>
<tr>
<td>P2</td>
<td>EVVGEKLYSEPSEIYT</td>
</tr>
<tr>
<td>P3</td>
<td>LVEEIVTEEGSGVAGEE</td>
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<tr>
<td>P4</td>
<td>EGVIAEVVEEDAFA</td>
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<tr>
<td>P5</td>
<td>TVEDAPATREEDIEE</td>
</tr>
<tr>
<td>P6</td>
<td>TEEIDIEVESVTEVEVE</td>
</tr>
<tr>
<td>P7</td>
<td>SVTVEEEVEEGPVDEEE</td>
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<tr>
<td>P8</td>
<td>EEPVDEEEVOEGTVE</td>
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<tr>
<td>P9</td>
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<tr>
<td>P10</td>
<td>TEEIIQGSEQEVVEVE</td>
</tr>
<tr>
<td>P11</td>
<td>SKVEEVEEGENSEE</td>
</tr>
<tr>
<td>P12</td>
<td>EOSNEEIEFVEEVE</td>
</tr>
<tr>
<td>P13</td>
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<td>P15</td>
<td>SGTEIELEKVASQGIE</td>
</tr>
<tr>
<td>P16</td>
<td>KVASQIEIQVDGSVTE</td>
</tr>
<tr>
<td>P17</td>
<td>VCDGSVTEQIEELF</td>
</tr>
</tbody>
</table>

Microculture plates (Limbro, Flow Laboratories, New Haven, CT) 1 × 10^5 cells in 100 µl were added together with 100 µl of synthetic peptides (final concentration = 2 µg/ml) or medium alone (negative control). As positive control, PHA was used at a final concentration of 10 µg/ml. All cultures were set up in triplicate, incubated for 5 days at 37°C in an atmosphere of 5% CO₂ and pulsed with 1 µCi of *H*-thymidine (specific activity = 2 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) for 18 hr. Cellular incorporation of *H*-thymidine was measured by liquid scintillation counting. Mean ± SEM incorporation, in the absence of antigen, in T cells from 20 donors was 1.279 ± 177 cpm (95% confidence limits; range = 907–1611). Samples having a stimulation index (SI) ≥ 2.5 were considered positive as described earlier by Kabilan and others. The SI was calculated by dividing the cpm for test samples with the cpm for medium control samples.

**ELISPOT assay.** The ELISPOT assay was performed as described previously. Briefly, 100 µl of monoclonal antibody (MAb) to human IFN-γ (1-D1K) or IL-4 (82-4) (Mabtech AB) were adsorbed to nitrocellulose plates (Millipore, Millipore Corp., Bedford, MA) at a concentration of 15 µg/ml in sterile phosphate-buffered saline (PBS). The plates were incubated overnight at 4°C and washed with PBS. The PBMC were diluted in medium as above to a concentration of 2 × 10^6 cells/ml and incubated with or without peptides for 4 hr in 5% CO₂ at 37°C. Resuspended cells (2 × 10^5 cells in 100 µl) were added to ELISPOT wells in duplicate. After incubation for 36 hr at 37°C, the cells were washed away with PBS and 100 µl/well (0.1 µg) of biotinylated MAb to IFN-γ (7-B6–1) or IL-4 (12–1) (Mabtech AB) were added and incubated for 1 hr at room temperature. Subsequently, the plates were washed with PBS, incubated for 1 hr with streptavidin–alkaline phosphatase (Mabtech AB), and washed again with PBS. Finally, 100 µl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution (Bio-Rad, Hercules, CA) were added and incubated at room temperature until dark spots emerged (1–2 hr). Development was stopped by washing with water. The plates were dried and spots were counted using a dissection microscope (40×). Cytokine responses were considered positive when the number of spots, each indicating one cytokine-producing cell, was greater than the mean ± 2 SEM of nonstimulated cells from the same individual. With the exception of one donor in each assay, the mean medium control backgrounds were 4.5 spots/2 × 10^4 cells (range = 0–17 spots) for IFN-γ and 0.6 spots/2 × 10^4 cells (range = 0–3 spots) for IL-4. The donors with abnormally high background levels were donor 3 (IFN-γ = 54 spots/2 × 10^6 cells) and donor 28 (IL-4 = 22.5 spots/2 × 10^6 cells).

**Statistical analysis.** Statistical evaluation was performed using linear regression analysis.

**RESULTS**

None of the 22 Senegalese donors had symptoms of acute malaria at the time samples were taken, and none were parasitemic as assessed by blood smears. However, all individuals had antibodies to *P. falciparum* when tested for antibody reactivity with a crude *P. falciparum* extract in the ELISA (mean = 71.8 units, range = 18.3–205 units), indi-
cating exposure to *P. falciparum* parasites (Figure 1a). In 21 Swedish individuals never exposed to malaria parasites, the background level was 9 units (range = 6.3–11.1 units).

**Antibody reactivity with EB200 and EB200-derived peptides.** The antibody reactivity was analyzed in plasma samples from the same donors as analyzed for cellular responses. Recombinant EB200 protein and 17 overlapping peptides corresponding to the sequence of EB200 were used to determine the presence of specific IgG. All but one (donor 41) of the Senegalese sera reacted significantly with recombinant EB200, although the levels of reactivity varied (Figure 1b). A significant correlation was seen between antibody levels to crude *P. falciparum* extract and antibody levels to EB200 (r = 0.697, P < 0.0003).

The prevalence rate of IgG specific for the EB200-derived peptides (P1-P17) varied between 80% and 100% except for peptides P11 and P15, which were recognized by only half of the donors, respectively (Figure 2). Although the prevalence of seropositivity was generally high, the levels varied. The highest geometric mean antibody level was seen with P6 (92.8 units, range = 12.3–291.8 units). The peptides P2, P3, P9, and P12 also gave high geometric means ranging from 37.1 to 43.1 units.

**Lymphoproliferative responses.** In vitro proliferative responses to the EB200-derived peptides were analyzed using PBMC obtained from 18 Senegalese subjects. The frequency of responders was generally low (Table 2), as was the level of the responses (Figure 3). The highest overall prevalence induced by peptides P3 and P8 were 44% and 28% of the donors, respectively, with a mean ± SEM SI of 2.7 ± 0.5 (range = 0.4–7.2) and 2.0 ± 0.5 (range = 0.5–11.3), respectively (Table 2 and Figure 3). Peptides P4 and P9 elicited a positive response in 22% of the donors. Peptides P2 and P7 induced proliferative responses in 17%, whereas the rest of the peptides tested induced lower or no responses at all in the donors tested. The PBMC from Swedish individuals never exposed to malaria were also tested and did not respond to any of the peptides.

**Number of IFN-γ- and IL-4-producing cells.** When the number of cells producing IFN-γ in response to the EB200-derived peptides was estimated in 21 of the Senegalese individuals, the most frequent responses (28%) were induced by P1, P13, and P17 (Table 2), with a mean ± SEM number of spots of 4.4 ± 1.4, 2.6 ± 0.8, and 2.5 ± 4.3/2 × 10³ cells, respectively (Figure 3). Peptides P3, P7, P8, and P16 induced secretion of IFN-γ in 24% of the individuals tested, while the other 10 peptides induced a response in less than 20% of the individuals.

The frequency of IL-4 responders was estimated in 15 individuals. The most frequent responses were induced by peptide P17, which induced a positive response in 67% of the donors (Table 2), although the mean ± SEM number of IL-4-producing cells was rather low (3.2 ± 1.1/2 × 10³ cells) (Figure 3). The overall prevalence of positive responses was 27–40% for the peptides P2, P6-P9, and P14-P16 and lower for the rest of the panel (Table 2).

**Association between proliferative, IFN-γ, and IL-4 responses.** Taking proliferation and/or cytokine secretion as a measure of T cell activation, all peptides elicited a positive response in 27–67% of the donors. The most frequent responses were triggered by peptides P17, P3, and P8, which induced responses in 67%, 54%, and 54% of the individuals, respectively (Table 2). More than 40% of the individuals tested responded to the peptides: P9 (50%); P4 and P7 (45%); P1 and P6 (41%), whereas the remaining peptides induced responses in less than one-third of the donors. Among those who did respond, 13–67% did so by production of IL-4 (mean = 26%, range = 13–67%) while 19% (range = 5–28%) and 11% (range = 0–40%) responded by production of IFN-γ or proliferation, respectively (Table 2).
FIGURE 2. Individual B and T cell responses against EB200-derived peptides. Thresholds for proliferative responses (stimulation index
[SI], interferon-γ [IFN-γ], or interleukin-4 [IL-4] release) as well as peptide-specific antibodies were calculated as described in the Material
and Methods. Cellular responses (SI, IFN-γ, and IL-4): light bars = negative response; gray bars = positive response; — = not tested. Humoral
responses (Ab): light bars = ≤ background; gray bars = > background (up to 30 units/ml); black bars = > 30 units/ml.

The association between proliferative, IFN-γ, and IL-4 re-
sponses was assessed in 10 individuals from whom enough
cells were recovered to test for all parameters and who re-
sponded positively to PHA in all three assays. There was no
correlation between proliferation and cytokine production,

TABLE 2
Frequency of T cell responses to peptides from the EB200 fragment
of Pf332*

<table>
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<tr>
<th>Peptide</th>
<th>Proliferation (n = 18)</th>
<th>IFN-γ (n = 21)</th>
<th>IL-4 (n = 15)</th>
<th>Total (n = 22)</th>
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<tr>
<td>P17</td>
<td>6</td>
<td>18</td>
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* IFN-γ = interferon-γ, IL-4 = interleukin-4; n = number of donors tested. Values are
the % of positive responders. Only donors that responded positively to phytohemagglutinin are included.

or between the two cytokine responses measured (Figure 2).
Only in three of the donors could proliferation and cy-
tokine production be induced simultaneously by the same
peptide (Figure 2). In general, there was no association be-
tween total T cell responses (proliferation, IFN-γ, and IL-4
production) and antibody responses to the same or adjacent
peptides. Thus, both IL-4- and IFN-γ-producing cells, al-
though frequently negatively associated in individual donors,
could be found in donors having anti-peptide serum anti-
bodies (Figure 2).

DISCUSSION

The purpose of the present study was to investigate cel-

ular and humoral immune responses to peptides correspond-
ing to EB200 in west African adults naturally primed against
P. falciparum. EB200 is derived from the highly repetitive
malaria antigen Pf332, a subunit vaccine candidate, and is
composed of 13 repeat entities made up of pairs of glutamic
acid regularly interspersed by mainly hydrophobic amino ac-
cids. Many of the degenerate repeats in Pf332 are likely to
constitute B cell epitopes. However, no information is
available on the existence of T cell epitopes within Pf332
recognized by individuals naturally primed by P. falciparum.
In the present study, peptides corresponding to EB200
were used to investigate proliferative T cell responses, and
the ELISpot was used to determine the number of IFN-γ-
and IL-4-producing cells in peripheral blood of P. falcipa-
rum-primed donors from one village with perennial malaria transmission in Senegal. We chose to measure the production of IFN-γ and IL-4 since these cytokines are generally believed to be a reflection of Th1 and Th2 type responses, respectively.26,27 In addition, the ELISPOT assay makes it possible to analyze the responding T cells in terms of both numbers and functional heterogeneity.

All Senegalese individuals tested exhibited seroreactivity with crude malaria antigen, indicating exposure to *Plasmodium falciparum.*28 Most donors also displayed reactivity with recombinant EB200, and there was a strong correlation between reactivity with crude malaria antigen and recombinant EB200. However, the individual level of antibodies varied considerably; whether this is a reflection of the time passed since last exposure,13 interethnic variation,29 or random stochastic variation is not known.30

The individual T cell responses to the various peptides varied markedly. Cells from most donors could be stimulated above background by more than one peptide, but none of the peptides elicited a positive response in all donors. Of the peptides tested, P3 and P8 induced significant proliferative responses in 44% and 28% of the donors, respectively. The adjacent peptides P2 and P4 as well as P7 and P9 induced proliferative responses in 17–22% of the donors, indicating the presence of separate as well as overlapping T cell epitopes.

When we determined whether the peptides could elicit production of IFN-γ or IL-4, the most frequent IFN-γ responses were obtained with peptides P1, P13 and P17, while the most frequent IL-4 responses were seen with the peptides P9 and P17. Although prominent T cell responses were encountered in some donors, the average responses were low, as has been seen when assessing cytokine responses to antigens from other pathogens.31 This may be explained by a low frequency of antigen-specific T cells in the peripheral blood,32 which in turn may relate to the fact that antigen-reactive T cells home to other organs.33 In individual donors, no correlation was seen between proliferation and cytokine production. There was also no correlation between the number of IFN-γ- and IL-4-producing cells. This confirms previous findings that proliferating, IFN-γ- and IL-4-producing cells frequently belong to functionally different subsets and emphasizes the importance of including measurements of several different T cell activities when screening donors for responsiveness to a given epitope.34–40 Since the studies described here were performed with unseparated T cell preparations (i.e., mixed CD4+ and CD8+ cells), we cannot exclude that functionally distinct CD8+ cells were also stimulated.41 These qualities will be further investigated by comparing T cell activation with phenotype characterization of the responding cells. Nonetheless, the combined use of proliferation, IFN-γ, and IL-4 assays can increase the sensitivity of detection of T cell activation. The overall frequency of responders increased to between 54% and 67% when a measure of T cell activation was a positive response in either proliferation, IFN-γ, and/or IL-4 for peptides P17, P3, and P8, respectively. Similar frequencies were obtained when analyzing peptides corresponding to the structurally related repeats of Pf155/RESA;42 whereas reported T cell reactivities with Pf11.1 repeats were lower.16

Consistent with the present study, the existence of functionally different T cell responses in human malaria has been reported previously.39,43 The reasons why some individuals mount a Th1-type of immune response, others a Th2 response, and still others a Th0 response to defined malarial antigens are not clear. Differences such as parasite polymorphism, host genetic factors, stage of immunity, antigen dose, or other unidentified factors may explain those differential responses.43 The biologic relevance of these findings is not clear. Earlier data with peptides from Pf155/RESA indicated a role of peptide induced IL-4 production in the regulation of certain anti-Pf155/RESA peptide-specific antibody responses.39 The role of IL-4 in the regulation of certain anti-malarial-specific antibodies is also indicated by the recently described association between an increased ratio of IL-4/IFN-γ-producing cells and the presence of elevated anti-malarial IgE levels.44 In the present study, donors having anti-peptide serum antibodies could be induced to produce IFN-γ and IL-4. However, IFN-γ and IL-4 have been shown to regulate isotype switching to different subclasses differently.45 Thus, further analysis between different cytokine profiles and antibody isotypes should include studies of different immunoglobulin subclasses. The importance of differ-
different subclasses in immune protection to malaria has been reported.46

All donors tested for proliferation as well as for secretion of IFN-γ and IL-4 showed T cell responses to one or several peptides. However, some of the donors that were not tested in all three assays due to a shortage of biologic material did not respond to any peptide. This is unlikely to be due to lack of previous exposure since all but one of the donors had measurable levels of antibodies reactive with EB200. If one considers the large size and repetitive nature of Pf332, it is more likely that donors who did not respond at the T cell level failed to do so because they recognize T cell epitopes within Pf332 that are located outside the EB200 region or because they were not tested in all three assays. The absence of a response may also be due to a too low proportion of specific cells as indicated by the fact that also responding donors displayed low responses in general.

Previous immunization of H-2-congenic mouse strains with recombinant EB200 demonstrated that T cell responses to several EB200-derived epitopes were genetically restricted by certain H-2 class II haplotypes.14 Human leukocyte antigen (HLA) class II typing was performed on all donors used in this study. However, there was no evidence for an association between human major histocompatibility complex (MHC) class II haplotypes and responsiveness to certain epitopes, which is consistent with what has been observed with other asexual blood-stage antigens of the P. falciparum malaria parasite.47–50 This is not surprising in view of the fact that humans are highly polymorphic at the MHC class II loci whereas inbred mice express a very limited set of the H-2 class II molecules. Still, some resemblance between the analyses of T cell responses to EB200 in mice and humans was seen. Peptide P3 was universally recognized as a T cell epitope by five different H-2 congenic mouse strains primed with recombinant EB200,14 and in the present study, it was the most well-recognized peptide in the proliferation assay.

Data from recent studies in twins suggests that genetic factors other than HLA class II molecules are also of importance in the regulation of both T cell and humoral responses against defined P. falciparum antigens.51,52 However, the nature and relative role of these non-MHC-encoded genes in the regulation of antigen specific T and B cell responses remains to be elucidated.

In summary, our data show that the EB200 fragment of Pf332 contains several T and B cell epitopes recognized by human populations. The data also indicate that measurement of cytokine production, in addition to proliferation, provides complementary information regarding T cell responses, as well as increases the sensitivity for the detection. Thus, as pointed out previously,39 including assays for functional T cell responses is essential when assessing cellular immune functions, thereby resulting in a more appropriate selection of epitopes to be included in subunit vaccines, as well as in large-scale epidemiologic studies of malaria immunity.

Acknowledgments: We thank Dr. Denise Mattei for providing the plasmid encoding GST-EB200, Dr. Klavs Berzins for helpful discussions, Dr. Eleanor M. Riley for critical reading of the manuscript, and El Hadji Malik Fall and Ababacar Diouf for excellent technical assistance.

Financial support: This work was supported by grants from the UNDP/World Bank/WHO Special Program for Research in Training in Tropical Diseases, the Swedish Agency for Research Cooperation with Developing Countries (SAREC), the Swedish Medical Research Council (MFR), and the Swedish National Board for Laboratory Animals.

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REFERENCES


