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Specific T-Cell Recognition of the Merozoite Proteins Rhoptry-Associated Protein 1 and Erythrocyte-Binding Antigen 1 of *Plasmodium falciparum*

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The merozoite proteins merozoite surface protein 1 (MSP-1) and rhoptry-associated protein 1 (RAP-1) and synthetic peptides containing sequences of MSP-1, RAP-1, and erythrocyte-binding antigen 1, induced in vitro proliferative responses of lymphocytes collected from Ghanaian blood donors living in an area with a high rate of transmission of malaria. Lymphocytes from a large proportion of the Ghanaian blood donors proliferated in response to the RAP-1 peptide, unlike those of Danish control blood donors, indicating that this sequence contains a malaria-specific T-cell epitope broadly recognized by individuals living in an area with a high transmission rate of malaria. Most of the donor plasma samples tested contained immunoglobulin G (IgG) and IgM antibodies recognizing the merozoite proteins, while only a minority showed high IgG reactivity to the synthetic peptides.

Several merozoite proteins located on the surface (10, 29) and in the rhoptries (2, 18, 21) appear to play an important role in efficient merozoite invasion of erythrocytes. Furthermore, secretion of the merozoite protein erythrocyte-binding antigen 1 (EBA-1) into the medium seems to be important in facilitating the merozoite invasion (3). Because of their role in parasite multiplication in blood, merozoite proteins are malaria vaccine candidates. It has been reported that some merozoite proteins are able to induce significant protection of monkeys against malaria caused by *Plasmodium falciparum* (4, 6, 7, 20, 25). The identification of T-cell epitopes in these antigens is important in designing vaccine constructs that induce a long-lasting protective immune response against malaria by using merozoite proteins as vaccine constituents.

Some merozoite antigens are shed as exoantigens during schizont rupture and merozoite reinvasion (10, 11a, 17). These circulating antigens may induce harmful cellular immune responses; down-regulation of immune responsiveness to some of these exoantigens may be an important aspect in immunity against the clinical symptoms of malaria (23). If different T-cell epitopes could be identified, the roles of harmful and protective epitopes might be identified in future immunoepidemiological studies.

In this study, we show that the merozoite proteins rhoptry-associated protein 1 (RAP-1) and merozoite surface protein 1 (MSP-1) and synthetic peptides containing sequences of RAP-1, EBA-1, and MSP-1 are able to induce an in vitro lymphocyte response, as measured by a proliferation assay. These proteins are also recognized by immunoglobulin G (IgG) and IgM antibodies from the donors tested.

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MATERIALS AND METHODS

Study areas and donors. (i) Ghana. This study was performed in the coastal region of Ghana approximately 80 km west of Accra in an area with high seasonal transmission rates of malaria. Blood samples were obtained from 14 adults (18 to 49 years old) and 7 children (5 to 11 years old) from Gomoa Onyadze village after informed consent was obtained.

(ii) Denmark. Blood samples from eight healthy adults never exposed to malaria (control blood donors) were obtained.

Blood sampling and PBMC preparation. Peripheral blood samples were collected by venipuncture and kept in heparinized vacutainers (Becton Dickinson Ltd., Rutherford, N.J.). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Lymphoprep (Nyegaard, Oslo, Norway), washed three times in RPMI 1640 supplemented with 58.4 μg of L-glutamine per ml, 20 IU of penicillin per ml, and 20 μg of streptomycin per ml (all from GIBCO Ltd., Paisley, United Kingdom) plus 10% fetal calf serum (FCS). PBMC were frozen by controlled gradient freezing to −196°C in RPMI 1640 supplemented with 10% dimethyl sulfoxide and 20% FCS. On the day of use, PBMC were quickly thawed at 37°C and washed three times in the medium used for the assays. Cell viability after thawing was >90%, as judged by trypan blue exclusion. Plasma samples obtained following density centrifugation were stored frozen at −20°C.

Malaria antigens and synthetic peptides. Soluble *Plasmodium* antigens (SPag) of isolate G2/Gambia were isolated from in vitro cultures by affinity chromatography essentially as described elsewhere (12). Recombinant RAP-1 (rRAP-1) was produced by subcloning a fragment of the RAP-1 gene (21) encoding amino acids 23 to 294 into a pDS expression vector (30) containing six C-terminal histidine residues to aid

purification. Native MSP-1 was obtained from the K1 isolate, as described previously (8).

Synthetic peptides containing putative T-cell epitopes on malaria antigens were constructed. The MSP-1 peptide sequence, YKLNFYFDLLRAKL, corresponds to the sequence of a peptide previously reported to stimulate lymphocytes (5). The RAP-1 peptide (SPSSTKSSSPSSTKSSS) includes two serine-rich repeats of a region previously reported to be potentially antigenic (21). EBA-1 peptide A (LKSHMNRESDDGELYDENS) (26) and EBA-1 peptide B (TLTKEYEDIVLKSHMNRESDD) were selected to include amphipatic regions of a sequence of EBA-1 reported to bind to erythrocytes (26).

Peptides were synthesized manually using the fluorenylmethoxy-carbonyl (Fmoc)-polyamide strategy with preactivated pentafluorophenyl-esters and couplings in dimethylformamide (1). Deprotection was performed in 20% piperidine in dimethylformamide. Acylation was performed until no free amino groups on the resin remained, as judged by the disappearance of yellow color caused by dihydroxybenzotriazine. After synthesis, solid-phase-bound peptides were cleaved by a combination of trifluoroacetic acid, water, and scavengers, precipitated by diethyl ether, redissolved in aqueous acetic acid, filtered, freeze-dried, analyzed, and purified by reverse-phase high-pressure liquid chromatography.

Lymphoproliferation assay. Each well of 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) received 10^5 PBMC in 150 μl of RPMI 1640 supplemented with 15% heat-inactivated pooled normal human serum, 58.4 μg of L-glutamine per ml, 20 IU of penicillin per ml, and 20 μg of streptomycin per ml. Samples (20 μl each) of antigen (SPag at final dilutions of 1:75 and 1:150; RAP-1 and MSP-1 at concentrations of 2.5 and 1 μg/ml, respectively; peptides at final concentrations of 20 and 5 μg/ml) were added to test cultures, whereas control cultures received 20 μl of medium.

The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 7 days and pulsed with [^{3}H]thymidine (20 μl per well) (New England Nuclear, Boston, Mass.) (1.85 mBq/ml) for the last 24 h of incubation. Cultures were harvested onto glass-fiber filters, and the incorporation of [^{3}H]thymidine into DNA was determined by liquid scintillation spectrometry. All tests were done in triplicate. For each set of triplicate samples, the mean incorporation (kilocounts per minute [kcpm]) was recorded. Unstimulated cultures were always included as controls. The results were expressed as Δkcpm ($kcpm_{stimulated} - kcpm_{unstimulated}$).

Antibody reactivities with merozoite proteins and synthetic peptides. Samples (0.1 μg per well) of rRAP-1, native MSP-1, or multimeric forms of the synthetic peptides were used to coat Maxisorp plates (Nunc). The multimeric peptides were prepared using immunostimulating complex (ISCOM) technology (15, 32). Plasma samples were diluted 1:100, incubated for 1 h, washed, and incubated with peroxidase-conjugated rabbit anti-human IgG or IgM for 1 h before addition of the substrate, 1,2-phenylenediamine (DAKO, Copenhagen, Denmark) and reading of the plates at 492 nm. On each enzyme-linked immunosorbent assay (ELISA) microtiter plate, one positive-control serum sample and one negative-control serum sample were assayed as well as control wells without serum (background level). All tests were done in duplicate.

To account for day-to-day variation, results were calculated as ELISA units (EU): $[(OD_{sample} - OD_{background})/(OD_{positive\ control} - OD_{background})] \times 100$, where OD is optical density (492 nm). Reactivity was considered to be positive when EU was > EU_{mean} + 2 standard deviations, where EU_{mean} was the mean value from 34 Danish controls.

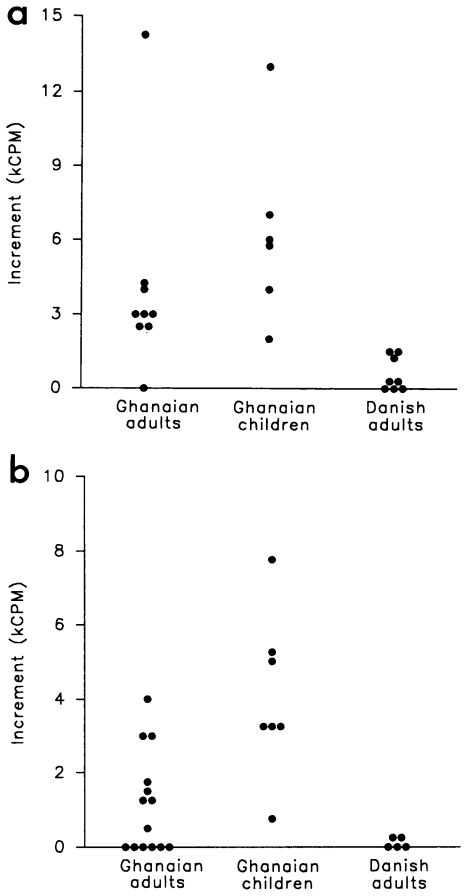

FIG. 1. Lymphocyte proliferative responses to RAP-1. Proliferative responses of PBMC to rRAP-1 (a) and the RAP-1 peptide (b) after 7 days in culture (Δkcpm). Each point represents the mean response of triplicate tests from one individual.

Statistical analysis. The Kruskal-Wallis one-way analysis of variance by ranks was used in comparisons of lymphoproliferative responses.

RESULTS

Immune reactivity to RAP-1. Lymphocytes from all the Ghanaian children and from most of the Ghanaian adults proliferated in response to RAP-1 (Fig. 1a). Lymphocytes from a substantial fraction of the Ghanaian donors also proliferated in response to the RAP-1 peptide (Fig. 1b). The responses to rRAP-1 and to the RAP-1 peptide were significantly higher in the children than in the adults ($P < 0.05$). The responses of the Danish donors to rRAP-1 and to the RAP-1 peptide were significantly lower than those of the Ghanaian children and adults ($P < 0.05$). None of the control blood donors showed proliferation in response to the RAP-1 peptide.

All Ghanaian plasma samples showed IgG reactivity and many showed IgM reactivity to rRAP-1, while none of the Danish donors showed IgG or IgM reactivity (Fig. 2a and b). In contrast, of all the donors tested, only 5 Ghanaian adults showed IgG reactivity to the RAP-1 peptide (Fig. 2c), and 2

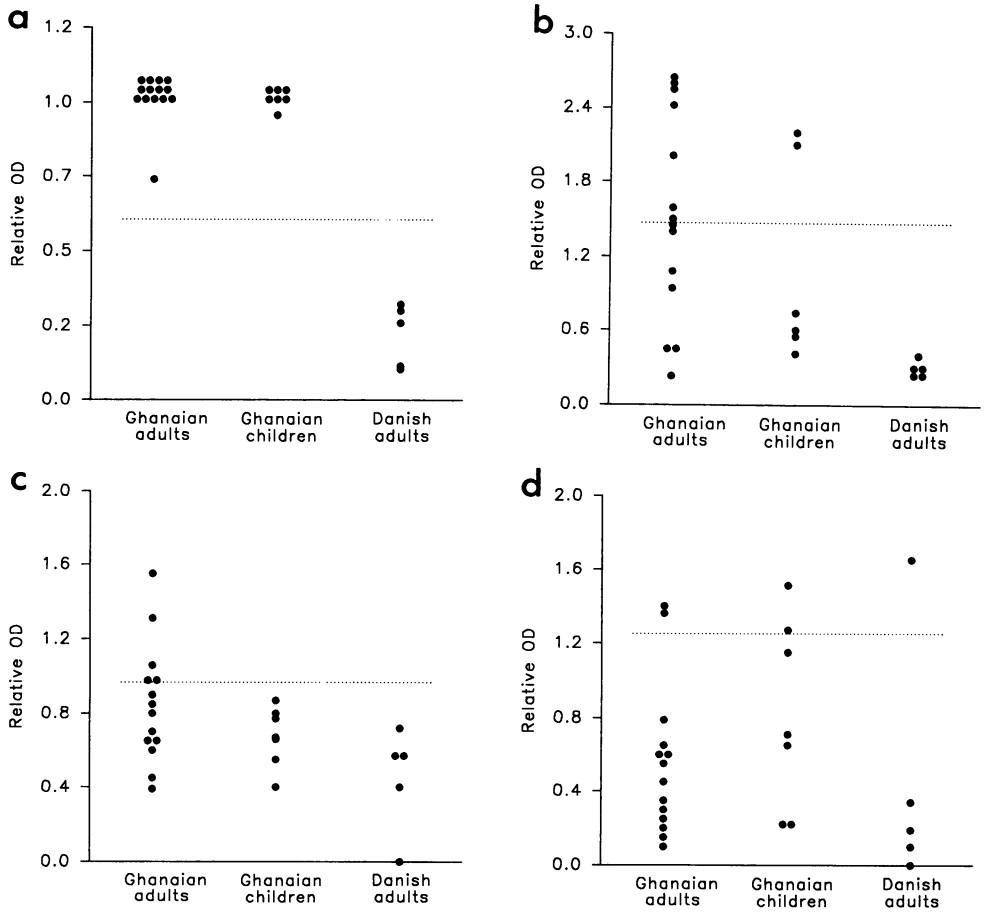

FIG. 2. Antibody reactivities to RAP-1. Plasma IgG (a and c) and IgM (b and d) reactivities to rRAP-1 (a and b) and the RAP-1 peptide (c and d). Cutoff levels are indicated by the horizontal lines.

of 14 adults, 2 of 7 children, and 1 of 5 Danish donors showed IgM reactivity (Fig. 2d). The levels of IgG in plasma and the lymphocyte responses to the peptide were not significantly associated (data not shown).

Immune reactivities to EBA-1 synthetic peptides. Lymphocytes from Ghanaian adults and children showed statistically significant higher responses to EBA-1 peptide A than lymphocytes from the Danish controls ($P < 0.05$) (Fig. 3a). There was no difference in the proliferative responses to EBA-1 peptide B from Ghanaian and Danish blood donors (Fig. 3b). Of all the blood donors tested, only one Ghanaian child showed IgG reactivity to EBA-1 peptide B and none showed IgG reactivity to EBA-1 peptide A (data not shown). In contrast, 9 of 14 adults, 4 of 7 children, and 1 of 5 Danish blood donors showed IgM reactivity to peptide B (Fig. 3d), and 5 of 14 adults, 6 of 7 children, and 1 of 5 Danish blood donors showed IgM reactivity to peptide A (Fig. 3c).

Immune reactivities to MSP-1 and SPag. Lymphocytes from Ghanaian adults and children showed statistically significant higher responses to native MSP-1 than lymphocytes from the Danish controls ($P < 0.05$) (data not shown). Lymphocytes from some of the Danish control blood donors also proliferated in response to the MSP-1 peptide (Fig. 4a). There was no significant difference between Ghanaian and Danish blood donors in reactivity to the MSP-1 peptide.

Four of 14 Ghanaian adults and 2 of 7 Ghanaian children showed IgG reactivity and 2 adults and 3 children showed IgM reactivity to native MSP-1, while only one Ghanaian adult showed IgG reactivity and one Ghanaian child showed IgM reactivity to the MSP-1 peptide (data not shown).

Lymphocytes from Ghanaian blood donors showed a statistically significant higher response to SPag than lymphocytes from the Danish blood donors ($P < 0.05$) (Fig. 4b).

No association between IgG and IgM reactivity and proliferation to any of the peptides was detected.

DISCUSSION

Merozoite proteins are exposed to the immune system both as surface proteins on merozoites and as exoantigens circulating in the blood. The merozoite proteins may stimulate T lymphocytes to liberate gamma interferon, which may enhance the antiparasite activity of macrophages (16). Furthermore, activated T lymphocytes may induce a secondary IgG response to the merozoites, blocking their invasion of erythrocytes. However, T lymphocytes may also be harmful by producing gamma interferon, which augments the tumor necrosis factor production associated with pathology (23). This study reports T-lymphocyte-stimulating activities of three merozoite proteins which have been implicated as malaria vaccine candidates.

RAP-1 is a rhoptry protein made of 82- and 65-kDa

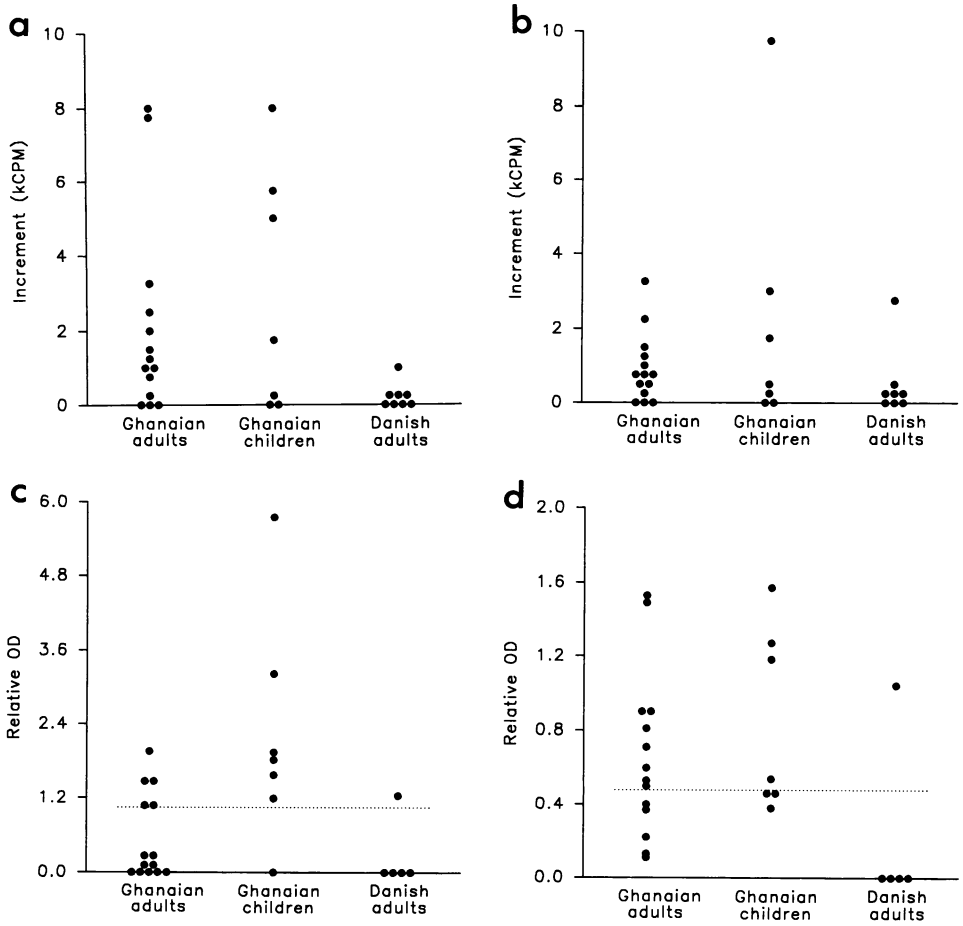

FIG. 3. Immune responses to the EBA-1 peptides. Proliferative responses of PBMC to EBA-1 peptide A (a) and EBA-1 peptide B (b) after 7 days in culture (Δkcpm). Each point represents the mean response of triplicate tests from one individual. Plasma IgM reactivities to EBA-1 peptide A (c) and EBA-1 peptide B (d). Cutoff levels are indicated by the horizontal lines.

components involved in the invasion process (21). RAP-1 forms a complex with another antigen, RAP-2, and affinity-purified protein preparations containing RAP-1 and RAP-2 protected monkeys from malaria caused by *P. falciparum* (19, 20). Purified RAP-1 and a synthetic peptide containing a serine-rich sequence found in the 82-kDa component of RAP-1 but not in the processed 65-kDa component of RAP-1 (21) induced a proliferative response in most of the Ghanaian blood donors tested. Lymphocytes from several Ghanaian blood donors proliferated in response to rRAP-1 without showing any response to the peptide, indicating that this peptide contains only one of several T-cell epitopes on the protein. Interestingly, lymphocytes from none of the Danish blood donors proliferated in response to the peptide. It is possible that the peptide sequence may be unique to the malaria parasite or that the Ghanaian blood donors have major histocompatibility complex proteins that are more efficient in binding the peptide than those in the Danish donors. The sequence of the peptide is conserved between two *P. falciparum* isolates (11, 18a). Ghanaian children showed the strongest proliferative responses to the recombinant protein and the peptide, indicating that T-cell recognition of the rhoptry protein occurs early in life. In contrast, only Ghanaian adults showed IgG reactivity to the RAP-1 peptide, indicating that the lymphocyte proliferative response and the IgG response are regulated differently. None of the Danish blood donors showed IgG reactivity with either rRAP-1 or the RAP-1 peptide. All Ghanaian blood donors showed IgG reactivity, and most of them showed IgM reactivity with rRAP-1. rRAP-1 may thus be useful in evaluating specific antibody responses to the malaria parasite.

EBA-1 is a merozoite protein located in the micronemes (27). Peptide A containing an EBA-1 sequence induced specific proliferative responses in some of the Ghanaian donors tested. The peptide sequence is from a region which binds to the erythrocyte surface. Antibodies against this region inhibit merozoite invasion of erythrocytes (26). This region is conserved between different parasite isolates (26). The presence of a T-cell epitope in this region was predicted by an amphiphilic plot of the sequence, which makes this antigenic region very attractive for inclusion into a malaria vaccine. None of the Ghanaian or Danish blood donors showed IgG reactivity to peptide A, but about 52% of the tested Ghanaian blood donors showed an IgM response, indicating that this sequence may also be a target for a T-cell-independent antibody response. Both EBA-1 peptides were recognized by IgM antibodies more often than the RAP-1 and MSP-1 peptides were.

MSP-1 is a well-characterized parasite protein present in

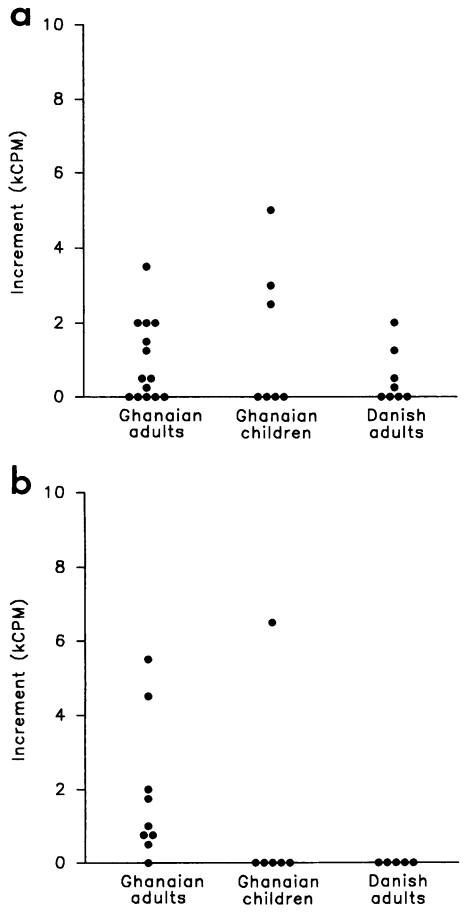

FIG. 4. Lymphocyte proliferative responses to the MSP-1 peptide and SPag. Proliferative responses of PBMC to the MSP-1 peptide (a) and SPag (b) after 7 days in culture (Δkcpm). Each point represents the mean response of triplicate tests from one individual.

the precursor form on the surface of schizonts and present after processing on the surface of merozoites in the form of several noncovalently associated fragments (14). The MSP-1 protein consists of conserved, variable, and semivariable sequences (31) and has been found to protect monkeys against malaria caused by *P. falciparum* (4, 6, 7, 9, 25). Native MSP-1 and a synthetic peptide containing a conserved sequence of MSP-1 (13) induced proliferative responses in some of the Ghanaian and Danish blood donors tested in our study. T-cell responses of exposed and nonexposed donors to MSP-1-derived sequences have been reported previously (22, 24, 28). T-cell clones reactive with the MSP-1 peptide studied here have also previously been reported. These T-cell clones responded to 11 different *P. falciparum* cultures and recognized the MSP-1 peptide in association with HLA-DQ molecules (5). As the T-cell clones were produced in nonexposed blood donors, it was not surprising that some of the Danish blood donors responded to this peptide. Proliferative responses to this peptide of nonexposed blood donors have previously been reported (33), and it has been argued that there is no major difference between exposed and nonexposed donors in reactivity to malaria antigens. We confirm the responsiveness of nonexposed blood donors to the MSP-1 peptide, but our results with RAP-1 and EBA-1 show that lymphoproliferative responses of the exposed blood donors to some malaria T-cell epitopes are indeed specific.

The lymphocyte proliferative in vitro responses to different peptides containing sequences from various merozoite proteins were not correlated, probably because of different major histocompatibility complex restriction patterns to the peptides. Antigen diversity and variation in B-cell epitopes are important restrictions in antibody responses to various fragments of proteins. The peptides tested in this study contained sequences which are conserved; antigen diversity and variation of T-cell epitopes are thus unlikely to explain the T-cell unresponsiveness of some of the tested donors. This reasoning is further substantiated by the fact that the Ghanaian children responded to the most RAP-1 and the RAP-1 peptide, although they are unlikely to have been exposed to as many parasite isolates as the Ghanaian adults. Interestingly, most of the Ghanaian children did not respond to SPag, the mixture of exoantigens, although this preparation contains both RAP-1 and MSP-1, indicating that other antigens may down-regulate the lymphocyte response to these merozoite proteins.

In conclusion, we have detected in vitro proliferative responses to specific peptide sequences from three different merozoite proteins of *P. falciparum*, all potential vaccine candidates. Some of the sequences were not recognized by Danish control blood donors. The T-cell-stimulating sequences of the merozoite proteins may induce both protective and harmful immune responses during malaria infection.

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