Induction of T Helper Type 1 and 2 Responses to 19-Kilodalton Merozoite Surface Protein 1 in Vaccinated Healthy Volunteers and Adults Naturally Exposed to Malaria

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Plasmodium falciparum malaria is a major cause of death in the tropics. The 19-kDa subunit of *P. falciparum* merozoite surface protein 1 (MSP-1₁₉), a major blood stage vaccine candidate, is the target of cellular and humoral immune responses in animals and humans. In this phase I trial of MSP-1₁₉, immunization of nonexposed human volunteers with either of the two allelic forms of recombinant MSP-1₁₉ induced high levels of antigen-specific Th1 (gamma interferon) and Th2 (interleukin 4 [IL-4] and IL-10) type lymphokines. The adjustment of the antigen dose and number of immunizations regulated the level of specificity of immune responses and Th1/Th2 bias of responses induced by vaccination. Novel conserved and allelic T-cell epitopes which induced cross-strain immune responses were identified. Importantly, responses to many of these novel epitopes were also present in adults exposed to malaria, both in east (Kenya) and west Africa (The Gambia). These data suggest that epitope-specific naturally acquired MSP-1₁₉ immune responses in endemic populations can be boosted by vaccination.

The 19-kDa subunit of *Plasmodium falciparum* merozoite surface protein 1 (MSP-1₁₉) is a major blood stage vaccine candidate (15). Proliferative and cytokine T-cell responses to recombinant MSP-1₁₉ antigen correlate with protection from subsequent episodes of clinical malaria, suggesting a critical role for T cells in protective immunity (21, 22). T cells may confer protection against blood-stage malaria through helping in the production of antibodies or by the secretion of effector lymphokines, such as gamma interferon (IFN- γ). The T helper 1 (Th1) subset secrete IFN- γ and promote cellular responses, while the T helper 2 (Th2) subset produce interleukin 4 (IL-4) and IL-10, which are important in promoting humoral immunity. IFN- γ in the presence of monocytes can inhibit malaria parasite growth in vitro (3, 4). Elevated serum IFN- γ levels are associated with protection in *Aotus* monkeys immunized with recombinant *P. falciparum* MSP-1 protein (12). In human volunteers immunized with attenuated sporozoites, IL-4 production in response to parasitized *P. falciparum* erythrocytes correlates with protection (2). In this phase I study of vaccinated healthy nonexposed volunteers, IFN- γ , IL-4, and IL-10 T-cell responses to recombinant MSP-1₁₉ antigen and peptides were

demonstrated. Novel conserved and cross-reactive allelic T-cell epitopes capable of stimulating rapid cytokine secretion were identified. Both 3D7 and FVO strains of the recombinant MSP-1₁₉ antigen could induce cross-reactive T-cell responses. Immunity induced in naive volunteers may not be relevant to populations in areas where malaria is endemic (14). IFN- γ responses to merozoite antigens are associated with resistance to malaria reinfection (18); therefore these were also evaluated in African donors in The Gambia in west Africa and Kenya in east Africa. Importantly, several T-cell epitopes identified in naive vaccinees overlapped those induced by malaria exposure in African adults. These studies suggest that naturally acquired immunity in endemic populations could be boosted by vaccination.

MATERIALS AND METHODS

Volunteers. The clinical protocol is described briefly in Results and is described in detail elsewhere (16). Informed consent was obtained from all patients, and human experimentation guidelines of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, were followed. The two malaria vaccines tested were the most divergent allelic forms of MSP-1₁₉, represented by the FVO and 3D7 strains of *P. falciparum*. Control vaccines were licensed hepatitis B virus (HBV) vaccine (Recombivax; 20 μ g/0.5-ml dose; Merck & Co.) and adult tetanus-diphtheria (Td) booster vaccine (Lederle). Vaccines were adsorbed to alum. All vaccines were administered to the deltoid muscle. The low dose was 20 μ g, while the high dose was 42 mg. There were 28 days between each immunization. Peripheral blood mononuclear cells (PBMC) were collected prior to and 14 days after the last immunization and cryopreserved

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prior to analysis. Operators were blind to the immunization regimen, with each assay containing a random selection of donors.

Adults exposed to malaria 18 to 60 years old were recruited from the villages of Brefet, The Gambia, west Africa, and villages around Kilifi, Kenya, east Africa. Studies were approved by the Ethical Committee of The Gambia Medical Research Council Joint Ethics Committee and the Gambian government and the Kenya Medical Research Institute. Gambian and Kenyan samples were collected after the wet seasons of July to October in The Gambia and July to August in Kenya. All African donors were parasite negative by microscopy of Giemsa thick and thin blood films and nonsymptomatic for malaria and other illnesses.

Antigens. Both 3D7 and FVO allotypes of the MSP-1₁₉ protein were expressed as recombinant fusion proteins in *Saccharomyces cerevisiae* with two T helper epitopes from the tetanus toxoid protein (P30P2) expressed amino terminal to MSP-1₁₉ (16). Recombinant MSP-1₁₉ proteins did not induce IFN- γ , IL-4, or IL-10 ELISPOT responses in 20 malaria-naïve donors (not shown).

A computer program based on eluted pool sequence data of HLA class II alleles DR1, DR2, DR3, DR4, DR5, DR6, and DR7 was used to scan the MSP-1₁₉ antigen for candidate T-cell epitopes that fitted the respective HLA class II peptide motifs (described in reference 17). Four pairs of allelic 3D7/FVO variant peptides (M1901/M1902, 1633 to 1652; M1903/M1904, 1642 to 1657; M1905/M1906, 1681 to 1696; M1907/M1908, 1689 to 1704) and three conserved C-terminal peptides (M1909, 1675 to 1690; M1910, 1714 to 1728; M1911, 1722 to 1744) were synthesized (Sigma-Genosys, Cambridge, United Kingdom) and reconstituted in sterile phosphate-buffered saline (PBS). The peptides did not induce IFN- γ production in PBMC from 20 malaria-naïve donors (not shown). Peptides were used at a predetermined supraoptimal concentration of 25 μ g/ml.

Ex vivo ELISPOT assays. PBMC were separated from venous blood by density gradient centrifugation on Ficoll (Pharmacia, Oslo, Norway) at 800 $\times$ g for 20 min. They were washed three times in RPMI 1640 medium and resuspended at 4 $\times$ 10⁶ per ml in RPMI 1640 supplemented with 5% heat-inactivated human AB serum (John Radcliffe Hospital Blood Bank, Oxford, United Kingdom), 2 mM glutamine, 100 μ g of streptomycin/ml, and 100 U of penicillin/ml (all from Gibco Paisley, United Kingdom). ELISPOT assays were performed on these PBMC as described previously (10). Briefly, 0.4 million PBMC/well were cultured at 37°C in a humid CO₂ incubator with antigens for 16 h in 96-well flat-bottom nitrocellulose plates (MAIP 45S; Millipore, Molsheim, France) coated with anti-IFN- γ , anti-IL-4, or anti-IL-10 monoclonal antibodies (Mabtech, Nadra, Sweden). Plates were washed six times with phosphate-buffered saline with 0.05% Tween 20 (Sigma, Irvine, Calif.) and incubated at room temperature for 2 h with the corresponding biotinylated second antibodies, followed by a washing as described above and a 2-h incubation with a streptavidin-alkaline phosphatase conjugate. After the washing, a precipitable alkaline phosphatase substrate (Bio-Rad, Hercules, Calif.) was added. Individual lymphokine-producing cells were enumerated by a computerized system and expressed as spot-forming units (SFU) per million PBMC. An ELISPOT response was positive if the chi-squared comparison of the numbers of lymphokine-positive cells in the test well and the control well based on a Poisson distribution at 4 $\times$ 10⁵ cells per well yielded a *P* value <0.05 (24). Probability plots based on a normal approximation to the Poisson distribution were very close to a straight line for either a whole-protein response (*n* = 22 replicates for TT) or a single CD8⁺ peptide response (*n* = 22 replicates for the immunodominant FluA2 epitope from the influenza virus nucleoprotein) (not shown). This indicated that the standard deviation was not significantly different from the square root of the mean, and the theoretical Poisson distribution predicted the observed error of the ELISPOT, suggesting that assay performance errors were much smaller than the predicted sampling errors and may be ignored. Most responses also yielded a *P* value <0.001, and these are indicated below. Responders to a peptide pool (pool 1: M1901, -03, -05, -07, and -10; pool 2: M1902, -04, and -06; pool 3: M1908, -09, -10, and -11) were retested to identify the stimulatory peptides in the pool.

Statistical methods. The numbers of positive responders for the different vaccination regimens were compared using chi-squared tests adjusted for small sample sizes where appropriate. Comparisons of mean SFU between vaccine groups (responders only) were performed using the Student *t* and Mann-Whitney tests. Due to the original vaccine trial design (16), tests were not independent. In particular, the effects of vaccine dosage and number of doses could not be addressed separately since volunteers receiving low-dose vaccination were more likely to receive three doses. Due to the small numbers of volunteers, no corrections for multiple comparisons were made.

FIG. 1. Response of vaccinated donors to recombinant MSP-1₁₉ antigen. Shown are comparisons of results for the 3D7 strain (shaded bars) versus the FVO strain (open bars) (A), two (shaded bars) versus three (open bars) immunizations (B), and low-dose (shaded bars) versus high-dose (open bars) vaccinations (C). Values are average numbers of antigen-induced lymphokine-secreting cells (SFU) per million PBMC less the background SFU per million PBMC $\pm$ SE. Only donors with responses significantly different from background (*P* < 0.05) were included. The average backgrounds $\pm$ SE for these donors were 83 $\pm$ 23, 60 $\pm$ 14, and 10 $\pm$ 5 SFU/10⁶ PBMC for IL-10, IFN- γ , IL-4, respectively. Numbers above bars, numbers of antigen-positive vaccinated donors/total numbers of donors tested. All donors were assessed 14 days after the final immunization. *, significant (*P* < 0.05) difference in the number of responders between immunization groups.

RESULTS

Clinical protocol. Forty healthy adults not exposed to malaria were divided into four study groups (*n* = 32) and one control group (*n* = 8). The control group received HBV vaccine (*n* = 4) or Td booster (*n* = 4). The first two study group (*n* = 16) received the low dose of either of the two malaria vaccines (*n* = 8 for 3D7 and FVO), while the remaining two study groups (*n* = 16) received the high dose of either type (*n* = 8 for 3D7 and FVO). Of the 16 donors receiving the low dose, two had two immunizations of the malaria vaccine while 14 had three immunizations. Of the 16 donors receiving the high dose, 10 had two immunizations while 6 had three immunizations. All donors were assessed 14 days after the last immunization for IFN- γ , IL-4, and IL-10 production in response to recombinant MSP-1₁₉ antigens and peptides. The comparison immunization regimens were 3D7 versus FVO, two doses versus three doses, and low dose versus high dose.

Responses of vaccinated volunteers to recombinant MSP-1₁₉ antigens. Immunization with MSP-1₁₉ induced IFN- γ , IL-4, and IL-10 T-cell responses to recombinant antigens in 9 of 32

FIG. 2. Response of vaccinated donors to MSP-1₁₉ peptides. Shown are comparisons of results for the 3D7 strain (shaded bars) versus the FVO strain (open bars) (A), two (shaded bars) versus three (open bars) immunizations (B), and low-dose (shaded bars) versus high-dose (open bars) vaccinations (C). Results are presented as in Fig. 1. The average backgrounds for these donors $\pm$ SE were 39 ± 9 , 24 ± 6 , 3 ± 0.5 SFU/10⁶ PBMC for IL-10, IFN- γ , and IL-4, respectively. Donors were initially tested with nonoverlapping MSP-1₁₉ peptide pools (not shown), and responders were retested with individual MSP-1₁₉ peptides.

(28%), 4 of 32 (13%), and 6 of 22 (27%) of the volunteers tested, respectively (Fig. 1). One of the eight control immunized donors reacted to the recombinant antigen (not shown). Prior to immunization, 1 of 24 (4%), 1 of 24 (4%), and 1 of 11 (9%) of the study group donors responded by IFN- γ , IL-4, and IL-10 production, respectively (not shown). Different T-cell lymphokine secretion patterns were observed in response to recombinant MSP-1₁₉ protein after the various immunization protocols (Fig. 1). More volunteers responded by IFN- γ production to recombinant antigen after three than after two immunizations ($P < 0.04$) (Fig. 1B). Low-dose immunization induced more volunteers to produce IL-10 in response to recombinant antigen than high-dose immunization ($P < 0.05$) (Fig. 1C). IL-10-secreting T cells were most frequently produced in response to recombinant antigen (experimental average $\pm$ standard error [SE]: 219 ± 52 SFU/10⁶ PBMC), followed by IFN- γ (180 ± 31 SFU/10⁶ PBMC), and IL-4-secreting T cells (28 ± 4 SFU/10⁶ PBMC). No statistical difference in the number of lymphokine-producing cells among the different immunization regimens was found.

Responses of vaccinated volunteers to MSP-1₁₉ peptides. After immunization with MSP-1₁₉, cytokine-secreting T cells in response to MSP-1₁₉ peptides were found in 19 of 32 (59%) donors for IFN- γ , 13 of 32 (41%) for IL-4, and 9 of 25 (36%) for IL-10 (Fig. 2). None of the eight control-immunized donors

reacted to any of the MSP-1₁₉ peptides. Before immunization, 1 of 27 (4%), 0 of 24 (0%), and 1 of 11 (9%) of the study group donors responded by IFN- γ , IL-4, or IL-10 secretion, respectively (not shown). Individual MSP-1₁₉ peptides induced at least one of the three lymphokines in 81% (26 of 32) of immunized donors. High frequencies of IL-10-secreting T cells in response to MSP-1₁₉ peptides (average $\pm$ SE: 31 ± 7 SFU/10⁶ PBMC) were observed; frequencies of IFN- γ - and IL-4-secreting T cells were lower: 26 ± 3 and 9 ± 1 SFU/10⁶ PBMC, respectively (Fig. 2). No difference in the number of lymphokine-producing cells or the number of responders among the different immunization regimens was found. Immunization with vaccine strain FVO produced significantly more specific Th2 (IL-4 and IL-10) peptide responses than immunization with 3D7 ($P < 0.03$). This result should be taken with caution as there was intralot variation of antigen in the FVO formulation of vaccine. No other differences were found by comparing Th1 and Th2 peptide responses among the different immunization regimens.

Cross-reactivity to recombinant MSP-1₁₉ antigen. MSP-1₁₉ is the most conserved region of the MSP-1 protein, with only 4 amino acid differences within its 96 residues. We investigated whether different vaccination regimens induced strain-specific or cross-reactive responses to MSP-1₁₉ antigens or peptides. The higher vaccine dose was found to induce more strain-specific Th1 antigen responses than the lower dose ($P < 0.015$). Similarly, three doses induced more strain-specific Th1 antigen responses than two doses ($P < 0.05$). A high dose was also better at inducing cross-reactive Th1 antigen responses than a low dose ($P < 0.03$). As noted in Materials and Methods, these three tests were not independent since low-dose volunteers were more likely to receive three doses. No other differences in cross-reactive Th1 responses were found by comparing vaccine strains or numbers of immunizations. There were no differences in Th2 antigen responses among the different immunization regimens.

Correlation of different MSP-1₁₉ immune responses by vaccinated volunteers. Serum antibody levels and proliferation in response to recombinant MSP-1₁₉ antigen in this trial have been previously described (16). Whether lymphokine responses to MSP-1₁₉ antigen or peptides could be correlated with previous reported antibody or proliferative responses was investigated. In vaccinated volunteers, a significant independent correlation ($P < 0.015$) between IL-4 responses to recombinant 3D7 antigen and antibody responses was found. No other correlation between different immune responses was found.

Selected MSP-1₁₉ peptides are recognized by west and east African adults. Novel T-cell epitopes identified in vaccinated American volunteers may not be relevant to populations where malaria is endemic (14). It was important to determine whether T-cell epitopes induced by the vaccine were also presented during natural malaria infection in endemic populations. We therefore tested IFN- γ T-cell responses to these peptides in adults exposed to malaria from two different areas where malaria is endemic: The Gambia, in west Africa, where malaria is seasonal with a low-to-medium transmission rate, and Kenya, in east Africa, where malaria is holoendemic with a medium-to-high transmission rate (13). Most of the peptides

FIG. 3. Response by IFN- γ production to individual MSP-1₁₉ peptides in vaccinated volunteers and in malaria-exposed donors in The Gambia, west Africa, and Kenya, east Africa. Only donors with IFN- γ responses to peptides significantly different from background ($P < 0.05$) as in Fig. 1 were scored positive. Thirty-two vaccinated volunteers and 25 Kenyan donors were tested. In The Gambia, peptides M1901 to M1908 were tested in 21 donors, while M1909 to M1911 were tested in 14 donors. The mean IFN- γ responses $\pm$ SE prior to background subtraction to peptides were 50 ± 3 , 16 ± 1 , and 15 ± 2 SFU/10⁶ PBMC in vaccinees, Gambians, and Kenyans, respectively, with backgrounds of 24 ± 6 , 3 ± 1 , and 5 ± 2 SFU/10⁶ PBMC, respectively. Values of P for all donors scored as responders for peptides were <0.05 compared to background. Further analysis of individual peptides showed that P values for 100% of responder vaccinees compared to background were <0.001 specifically for peptides M1903, M1904, and M1906 and that P values for $>50\%$ of responder vaccinees were <0.001 for peptides M1902, M1905, M1907, M1908, M1909, and M1910. In a similar analysis, P values for the response of 100% of Gambian volunteers to peptides M1902 and M1903 and of 100% of Kenyan volunteers to peptides M1907 and M1908 were both <0.001 compared to background.

were recognized by at least one Gambian donor (Fig. 3). In The Gambia, IFN- γ responses to any of the allelic variant epitopes (i.e., M1901 to M1908) in 5 of 21 (24%) of responders were observed. There were few positive IFN- γ (3 of 30, 10%) responses to the conserved C terminus epitopes (M1909 to M1911). In Kenya 5 of 25 (20%) responded by IFN- γ production to any one of the epitopes. In contrast to results for The Gambia, there were no responses to the allelic variant epitopes (M1901 to M1906) and IFN- γ reactivity clustered to C terminus.

DISCUSSION

Immunization of naive volunteers with recombinant MSP-1₁₉ antigen induced high levels of antigen-specific Th1 (IFN- γ) and Th2 (IL-4 and IL-10) type lymphokines. Responses to recombinant MSP-1₁₉ antigen were similar to responses to MSP-1₁₉ peptides (IFN- γ $>$ IL-10 $>$ IL-4). Novel T-cell epitopes within MSP-1₁₉ capable of inducing rapid IFN- γ , IL-4 and IL-10 production in vaccinated volunteers were identified. There was minimum reactivity in preimmunized or control-immunized donors. This is in contrast to reported proliferation assays (16) and may be due to the detection of different effector T cells.

Since the dominant lymphokine induced in response to vaccination was IFN- γ , we tested for IFN- γ reactivity in response to our MSP-1₁₉ peptides in donors naturally exposed to ma-

laria. Indeed, naturally acquired reactivity was found in both The Gambia and Kenya. The number of responders exposed to malaria identified by lymphokine reactivity to these MSP-1₁₉ epitopes was similar to those from previous studies in The Gambia and Kenya measuring T-cell proliferation in response to overlapping MSP-1₁₉ peptides (15 to 30%) (8, 25). By comparison, immune responses to MSP-1 peptides other than the 19-kDa subunit of the larger MSP-1 protein could induce IFN- γ responses in >80 and 27% of donors from The Gambia and Kenya, respectively (17).

The use of alum as a vaccine adjuvant is associated with the induction of Th2 responses, and recent studies with mice show that alum induces antigen-specific Th2 responses in the absence of IL-4 (5). Indeed, we observed higher frequencies of IL-10-producing T cells than of IL-4-producing T cells in vaccinees. Hypersensitivity was noted in a proportion of vaccinees, suggesting a Th2 bias (16). The use of alum as an adjuvant may need to be balanced against such possible side effects. Another concern for recombinant protein vaccines administered in alum is intralot variability. Indeed, in the present study, this was observed for the FVO formulation (16). Vaccines based on selected peptide epitopes may help overcome such problems.

The highly conserved MSP-1₁₉ protein is hydrophobic, and it has been proposed that its C-terminal amino acid residues are glycosyl-phosphatidylinositol (GPI) anchors (11) and are unavailable for antigen processing and presentation. Conserved peptides M1910 and M1911 span the putative GPI anchor residues, and, in both vaccinated and malaria-exposed donors (of both east and west Africa), these peptides induced lymphokine responses. In fact, IFN- γ responses to MSP-1₁₉ peptides in Kenyan donors were clustered at the C terminus. Thus, putative GPI anchors within MSP-1₁₉ do not prevent their processing to elicit T-cell responses in both vaccinated donors and donors naturally exposed to malaria.

In summary, immunization of human volunteers not exposed to malaria with recombinant MSP-1₁₉ antigen induced high frequencies of Th1- and Th2-secreting cells. Novel MSP-1₁₉ T-cell epitopes capable of stimulating rapid lymphokine secretion in both vaccinated volunteers and those naturally exposed to malaria were identified. Thus, MSP-1₁₉ T-cell responses could be primed in naive unexposed donors and could be boosted in malaria-exposed donors through vaccination. The use of the highly conserved MSP-1₁₉ protein to induce blood stage protection may circumvent problems such as antigen polymorphism and altered peptide ligand antagonism (20). In this phase I vaccine study, cross-reactive lymphokine responses were induced by using specific vaccination regimens. Although further work is necessary to find the optimal safe immunogenic delivery system for human vaccination, the T-cell studies presented here suggest that MSP-1₁₉ may both induce and restimulate cross-strain specific immunity.

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