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T-Cell Immunity to Peptide Epitopes of Liver-Stage Antigen 1 in an Area of Papua New Guinea in Which Malaria Is Holoendemic

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Liver-stage antigen 1 (LSA1) is one of several pre-erythrocytic antigens considered for inclusion in a multiantigen, multistage subunit vaccine against falciparum malaria. We examined T-cell proliferation and cytokine responses to peptides corresponding to amino acids 84 to 107, 1813 to 1835, and 1888 to 1909 of LSA1 in asymptomatic adults living in an area of Papua New Guinea where malaria is holoendemic. Whereas T cells from North Americans never exposed to malaria did not respond to any of the peptides, those from 52 of 55 adults from the area where malaria is endemic had vigorous proliferation responses to one or more of the LSA1 peptides (mean stimulation indices of 6.8 to 7.2). Gamma interferon (IFN-γ) production driven by LSA1 peptides ranged from 34 to more than 3,500 pg/2 × 10^6 cells, was derived primarily from $CD8^+$ cells, and was dissociated from T-cell proliferation. The frequencies of IFN-γ response to the amino acid 1819 to 1835 and 1888 to 1909 peptides were significantly greater than that to the amino acid 84 to 107 peptide (87 and 88% versus 33% of subjects; $P < 0.0001$). In contrast to proliferation and IFN-γ, interleukin 4 (IL-4) and/or IL-5 responses to LSA1 peptides were detected in only 18% of the subjects. These data show that T-cell immunity to epitopes in the N- and C-terminal regions of LSA1 are common in persons living in this area of Papua New Guinea where malaria is endemic. The dominance of type 1 CD8 cell IFN-γ responses is consistent with a role for this T-cell population in immunity to liver-stage *Plasmodium falciparum* in humans.

Falciparum malaria continues to be major cause of morbidity and mortality in tropical areas of Africa, Asia, South America, and the South Pacific. The emergence of *Plasmodium falciparum* resistant to multiple drugs and spread of the anopheline vectors of malaria underscore the need for a vaccine against this infectious disease (2).

Residents of areas where malaria is holoendemic are repeatedly inoculated with mosquito-borne infectious sporozoites throughout life, yet immunity and partial resistance to pre-erythrocytic and erythrocytic stages of *P. falciparum* develop slowly with age and are most evident in adults, who suffer fewer episodes of malaria-related illnesses than do children. A multiantigen, multistage malaria vaccine for use in infants living where malaria is holoendemic will ideally enhance or accelerate development of appropriate immune responses to *P. falciparum* to at least the level acquired by adulthood (20, 30).

Studies of immunologically naïve volunteers afforded transient sterile immunity against challenge infection by prior injection of irradiated sporozoites (5, 32) have provided insight into the immunologic correlates of resistance against pre-erythrocytic *P. falciparum* in humans. Such persons develop T-cell proliferation responses to several pre-erythrocytic antigens (Ag), including circumsporozoite protein (CSP), sporozoite surface protein 2 (SSP2; also called thrombospondin-related anonymous protein), liver-stage antigen 1 (LSA1), and Ag expressed by blood-stage parasites (e.g., merozoite surface proteins 1 and 2) (15, 23, 26, 29, 33, 34). Malaria Ag-specific cytotoxic T lymphocytes (CTL) are also engendered by immunization with irradiated sporozoites, and CTL produced in response to peptide epitopes of vaccine candidate molecules such as SSP2 correlate with resistance to challenge infection (41, 42). Although it is not possible to evaluate directly the mechanisms of resistance to pre-erythrocytic *P. falciparum* in humans, studies of rodent malaria models demonstrate that liver-stage parasites are the target of protective immunity induced by irradiated sporozoites and that gamma interferon (IFN-γ) is involved in their elimination (7, 36). This cytokine also enhances killing of malaria-infected hepatocytes in vitro (8, 25, 28).

Several studies have examined T- and B-cell responses to pre-erythrocytic *P. falciparum* Ag in naturally infected humans. T-cell proliferation stimulated by a 19-mer peptide in the non-repeat region of CSP correlated with resistance to reinfection in three adult residents of western Kenya previously cured of malaria with chemotherapy (17). Lymphocyte proliferation responses to polymorphic regions of CSP have been noted among adults living in other areas where malaria is endemic (13, 44). CTL produced in response to peptide epitopes of SSP2, CSP, and several other pre-erythrocytic Ag were detectable in children and adults in The Gambia and Tanzania (1, 12, 18, 24, 37).

The focus of the current study was on LSA1, an ~200-kDa pre-erythrocytic *P. falciparum* Ag expressed during hepatic schizogony (14, 45). LSA1 is detectable in the parasitophorous vacuole of chimpanzee liver sections and, unlike several other pre-erythrocytic Ag (e.g., CSP and SSP2), is expressed only by liver-stage *P. falciparum* (14). The potential importance of LSA1 as a vaccine candidate was suggested by the observation that a peptide corresponding to amino acids (aa) 1786 to 1794

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of the molecule bound to HLA-B53, which in Gambians is associated with resistance to severe malaria morbidity (16). Epitopes of this molecule were demonstrated to be immunogenic by Krzych et al. (23), who reported that peripheral blood mononuclear cells (PBMC) from three North Americans vaccinated with irradiated *P. falciparum* sporozoites proliferated when stimulated with one N-terminal and two C-terminal peptides corresponding to the nonrepeat regions of LSA1. With respect to responses by naturally infected individuals, Fidock et al. (9) found that 22 to 48% of residents of an area of Madagascar where malaria is endemic had PBMC which proliferated and/or made IFN-γ when stimulated with a series of LSA1 peptides corresponding to aa 1613 to 1719. The current study was undertaken to determine more completely the nature of T-cell and B-cell immunity to LSA1 in persons naturally exposed to malaria. We examined responses by asymptomatic adults living in the Wosera area of Papua New Guinea, where falciparum malaria is holoendemic and associated with decreased morbidity and duration of patent infection in adults compared with children.

MATERIALS AND METHODS

Human subjects. Volunteers were recruited from two villages (Miko 1 and Miko 2) in the Wosera area of East Sepik Province, Papua New Guinea. Study subjects were selected by using the following critiera: (i) lack of a history of fever or other symptoms attributable to malaria within the previous 2 months; (ii) membership in different nuclear families, (iii) life-long residency in the Wosera area, and (iv) lack of a history of ingestion of antimalaria chemotherapy within the previous 2 months.

Malariametric indices, morbidity, and mortality in the Wosera area have been described previously in detail (10, 11). *P. falciparum*, *P. vivax*, and *P. malariae* infections are endemic, the former being predominant (55% of all malaria infections). The highest prevalence of *P. falciparum* asexual parasitemia occurs in 5- to 9-year-old children, and the intensity of parasitemia decreases with age (from a mean of >1,000 asexual forms/μl of blood in 1- to 4-year olds to <700 in adults older than 20 years). The duration of an episode of patent *P. falciparum* infection in adults is 53 to 57 days, compared with 146 days in 1- to 4-year-old children.

Fifty-five subjects (25 women and 30 men) ranging in age from 17 to 88 years (median, 25 years) participated. All volunteers were asymptomatic at the time blood was drawn and had an axillary temperature of <37.6°C, and <10% had a spleen palpable by physical examination. Malaria infection status of the study subjects was established by thick and thin smears of finger prick blood samples, which were read by trained microscopists employed by the Papua New Guinea Institute of Medical Research. Smears were repeated at a 6-month interval (May and November 1995) for 38 subjects who agreed to donate blood for diagnosis of malaria infection on two occasions. To determine whether LSA1 peptides stimulated proliferation and cytokine production by lymphocytes from persons not sensitized to malaria Ag, blood was obtained from six healthy North American adults >25 years of age who had never been exposed to malaria.

Informed consent was obtained from all subjects prior to venipuncture. Ethical approval for this study was granted by the appropriate medical authority of Papua New Guinea and the Institutional Review Board for Human Studies at University Hospitals of Cleveland, Case Western Reserve University.

PBMC preparation, proliferation, and cytokine assays. Blood was anticoagulated in heparin and transported from the field to laboratories in Maprik within 2 h of venipuncture. PBMC were separated from whole blood by Hypaque-Ficoll density gradient centrifugation. Proliferation assays were performed in quadruplicate at 2×10^5 cells/200 μl of culture medium (RPMI 1640 containing 10% fetal bovine serum, 100 U of penicillin, and 1 μg of gentamicin/ml). [^{3}H]thymidine incorporation was measured by addition of the radiolabel (1 μCi) for the final 18 h of a 96-h period of incubation at 37°C in 5% CO_2 in air (6). Results are expressed as a stimulation index (SI), which is the mean counts per minute incorporated by PBMC stimulated by peptide or Ag divided by an aliquot of cells in culture medium alone.

For cytokine assays, 2×10^6 PBMC with or without peptide, Ag, or mitogen (see below) were incubated for 48 h in culture medium, and supernatants were stored in liquid nitrogen before transport to Cleveland. IFN-γ, interleukin 4 (IL-4), and IL-5 were measured by two-site enzyme-linked immunosorbent assay (ELISA) as previously described (21).

In experiments to determine the cellular source of IFN-γ, CD4 and CD8 cells were purified from PBMC by positive selection using immunomagnetic beads as previously described (22). Positively selected cells were suspended in culture medium, and 10^4 autologous PBMC depleted of CD4 cells were added as antigen-presenting cells. The LSA1 aa 1813 to 1835 or 84 to 107 peptide was added at 10 μg/ml (see below), and cytokine production was measured. Positive selection resulted in >98% purity of CD4 and CD8 cells by immunofluorescence.

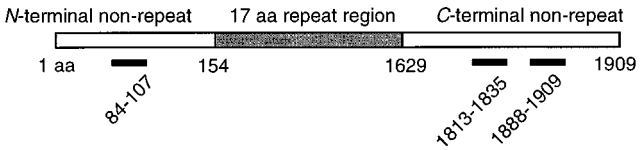

FIG. 1. Peptide epitopes of LSA1. The numbers under the bar diagram indicate the amino acid sequences of the peptides used in this study. The sequences are based on *P. falciparum* NF54.

LSA1 peptides, antigens, and mitogens. Three peptides in the N- and C-terminal nonrepeat regions of LSA1 shown to stimulate proliferation of PBMC from North Americans inoculated with irradiated *P. falciparum* sporozoites (23) were studied. These peptides also have been predicted to contain T-cell epitopes based on their amphipathicity (27). Amino acid sequences were deduced from the nucleotide sequence of *P. falciparum* NF54 (45).

Peptides were produced by solid-phase synthesis, purified by reverse-phase chromatography, and suspended in sterile phosphate-buffered saline, pH 7.0 (37). The N-terminal peptide (aa 84 to 107) included LTMSNVKNVQTNFK SLLRNLGVS, the first C-terminal peptide (aa 1813 to 35) included NENLD DLDEGIEKSSEELSEEKI, and the second C-terminal peptide (aa 1888 to 1909) included DNEILQIVKELSEKITKYFMKL (Fig. 1). All were used at a concentration of 10 μg/ml, since dose-response experiments established that this was optimal for stimulation of PBMC from adults in the Wosera area (no responses were observed when the concentration was <0.1 μg/ml). Streptolysin O (SLO) (10 μg/ml) and phorbol myristate acetate (50 ng/ml) plus ionomycin (1 μg/ml) were included as Ag and mitogen controls in the cytokine studies. Phytohemagglutinin at a concentration of 1 μg/ml was used as a positive control for the proliferation studies. A 17-mer peptide corresponding to the EQ repeat in the central region of LSA1 (Fig. 1) was also used for Ab determinations.

Ab determinations. Levels of antibodies (Ab) to the N- and C-terminal peptides and the EQ repeat in the central region of LSA1 (EQQSDLEQER LAKEKLQ) (45) were measured by ELISA. The first three peptides were dissolved in 0.05 M sodium bicarbonate, pH 9.6, to a concentration of 10 μg/ml, and 50 μl was added to Immunolon 4 flat-bottom plates (Dynatech, Chantilly, Va.). Following incubation overnight, washing, and blocking in 5% (wt/vol) powdered milk and 1% gelatin in water, duplicate 50-μl serum samples diluted 1:4 in blocking solution were added to wells and incubated for 1 h at room temperature. After extensive washing, 50 μl of alkaline phosphatase-conjugated anti-human immunoglobulin G (Jackson ImmunoResearch, West Grove, Pa.) diluted 1:10,000 was added and removed after 1 h, and then the substrate *para*-nitrophenyl phosphate was added in accordance with the manufacturer's (Sigma Chemical Co., St. Louis, Mo.) instructions. Optical density at 405 nm was measured. To measure Ab to the EQ repeat, peptide was bound to Immunolon 1 plates pretreated with poly-L-lysine. The cutoff value for a positive response was defined as serum which produced an optical density greater than or equal to the mean plus 3 standard deviations of sera from nine North Americans who had never been exposed to malaria.

Statistics. Differences in the frequencies of proliferation and cytokine responses to peptides were compared by using the χ^2 test. Quantitative differences in the level of IFN-γ production were evaluated by the Student's *t* test using log-transformed data.

RESULTS

Proliferation of PBMC stimulated with N- and C-terminal LSA1 peptides. PBMC from six healthy North American adults never exposed to malaria did not proliferate when incubated with 1, 5, 10, or 15 μg of any of the three peptides per ml (SI of 1.0 versus 3.1 to 11.1 for aliquots of cells stimulated with phorbol myristate acetate plus ionomycin). In contrast, PBMC from 52 of 55 Wosera area residents proliferated (SI, ≥3.0) in response to one or more of the peptides (Table 1). The mean SIs for PBMC stimulated with aa 84 to 107, 1813 to 1835, and 1888 to 1909 peptides were 6.8, 6.5, and 7.2, respectively. Phytohemagglutinin-stimulated PBMC from the Wosera area subjects had SIs of 3.6 to 202.8.

Cytokine responses to LSA-1 peptides. PBMC from the six North American controls did not make IL-4, IL-5, or IFN-γ in response to any of the LSA1 peptides. There were enough cells from 38 of the Wosera area subjects to study IL-4 and IL-5 responses to all three peptides and the SLO and mitogen

TABLE 1. Proliferation of PBMC stimulated with LSA1 peptide epitopes

LSA1 epitope (aa)[a]	No. of positive responses/total	Mean SI ± SE (range)
84–107	45/55[b]	6.8 ± 0.7 (0.8–25.1)
1813–1835	45/55	6.5 ± 0.6 (0.6–21.6)
1888–1909	41/54	7.2 ± 0.7 (0.8–24.2)

[a] Peptides were used at a concentration of 10 μg/ml, and PBMC were maintained in culture for 96 h. [^{3}H]thymidine was included in the culture for the final 18 h before harvesting. PBMC incubated in medium alone incorporated <1,000 cpm.
[b] A positive response represents an SI of >3.0.

controls. Cells from seven (18%) of these individuals made IL-4 in response to one or more LSA1 peptides (range of positive responses, 20 to 320 pg/2 × 10^6 cells), and only one donor had PBMC which made IL-5 (204 pg/2 × 10^6 cells). Control cultures showed that 14 and 16 of 38 subjects, respectively, had PBMC that made IL-4 and IL-5 in response to SLO. PBMC from 37 of 38 individuals made IL-4 (>400 pg/2 × 10^6 PBMC) and IL-5 (>100 pg/2 × 10^6 PBMC) when stimulated with phorbol myristate acetate plus ionomycin.

PBMC from these 38 subjects and an additional 15 persons were examined for IFN-γ production in response to one or more of the LSA1 peptides (there were not enough cells from two donors to examine responses to all three peptides). Sixteen (33%) of 48 persons had PBMC which produced IFN-γ in response to the N-terminal aa 84 to 107 peptide, and the frequencies of response to the aa 1813 to 1835 and 1888 to 1909 peptides were 87 and 88%, respectively (46 of 53 and 38 of 43 subjects, respectively; $P < 0.0001$ compared with the aa 84 to 107 peptide) (Fig. 2). The range of IFN-γ production among positive responders was similar for the three peptides (34 to >3,800 pg/2 × 10^6 cells). Stimulation with SLO or phorbol myristate acetate plus ionomycin resulted in IFN-γ production of 487 to >3,800 (upper limit of the assay) and >3,800 pg/2 × 10^6 cells, respectively.

There was no correlation at the individual level between the level of IFN-γ production and lymphocyte proliferation induced by a given LSA1 peptide. Moreover, because positive proliferation responses (SI of ≥3.0) were observed for all but 1 of the 53 subject whose PBMC were evaluated for IFN-γ production, there was no significant correlation between positive IFN-γ and proliferation responses.

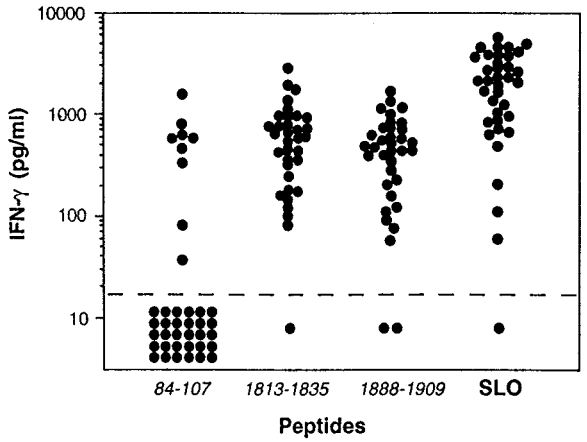

FIG. 2. LSA-1 peptide-driven IFN-γ production by PBMC from Wosera area subjects. Duplicate preparations of PBMC were incubated with 10 μg of peptide per ml, and IFN-γ in supernatants after culture for 48 h was determined by ELISA.

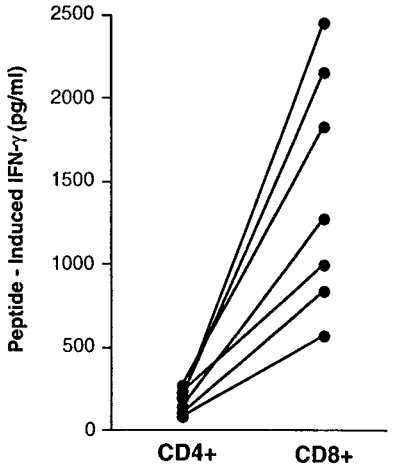

FIG. 3. IFN-γ production by $CD4^+$ and $CD8^+$ cells in response to an LSA1 peptide corresponding to aa 1813 to 1835. Lines connecting two points represent values for cells from one person. Lymphocytes were purified by positive selection using immunomagnetic beads, and 10^6 CD4 or CD8 cells were coincubated with 10^5 antigen-presenting cells (PBMC depleted of both CD4 and CD8 cells) plus the aa 1813 to 1835 peptide at 10 μg/ml for 48 h.

T-cell subsets producing IFN-γ. Blood was obtained from seven additional subjects, and CD4 and CD8 subsets were separated by positive selection prior to stimulation with the aa 1813 to 1835 peptide. As shown in Fig. 3, CD8 cells produced 4.3- to 12.8-fold more IFN-γ than did CD4 cells. Comparable results were obtained when cells from one of the subjects were stimulated with the aa 84 to 107 peptide (CD4 and CD8 subsets made 132 and 2,744 pg of IFN-γ, respectively).

Comparison of T-cell responses by persons with thick blood smears negative or positive for *P. falciparum*. Thirty-eight subjects were examined twice at a 6-month interval for blood-stage infection. Eleven persons had negative thick blood smears for asexual-stage *P. falciparum* on both occasions, 5 had a positive blood smear at one time point, and 22 had positive blood smears at both time points (80 to 1,250 asexual parasites/μl of blood). With respect to T-cell proliferation, there was no difference between the frequencies of response to the N- or C-terminal peptides in the group of 11 individuals with negative smears and the 27 subjects who had positive thick blood smears on one or two occasions. In contrast, there was a higher frequency of IFN-γ responses to the aa 84 to 107 peptide by PBMC from persons with negative smears than by PBMC from those having positive smears (Table 2; 10 of 11 versus 9 of 27, $P < 0.001$). This difference was specific for the aa 84 to 107 peptide, since the proportions of individuals whose PBMC made IFN-γ following stimulation with the aa 1819 to 1835 and 1888 to 1909 peptides, SLO (Table 2), and phorbol myristate acetate plus ionomycin (data not shown) were similar for the two groups. There was no correlation between the presence or absence of asexual parasitemia by thick blood smear and IFN-γ production driven by any of the LSA-1 peptides when persons were categorized according to whether or not they had a *P. vivax* or *P. malariae* blood-stage infection.

Levels of Ab to LSA1 peptides in serum. Immunoglobulin G Ab to the peptides corresponding to the N- and C-terminal peptides and the 17-aa central repeat region were detected in sera from 40 to 50% of the subjects, but there was no correlation between this and *P. falciparum* asexual parasitemia (Table 2).

TABLE 2. T-cell IFN-γ and Ab responses among subjects with thick blood smears negative or positive for asexual-stage *P. falciparum*[a]

Response	No. of negative smears/total	No. of positive smears/total
IFN-γ response to:		
aa 84–107 peptide	9/27 (35–1,860)[c]	1/11[b] (496)
aa 1813–1835 peptide	27/27 (81–2,200)	10/11 (105–3,250)
aa 1888–1909 peptide	27/27 (150–1,950)	11/11 60–2,000
SLO	17/19 (150–3,500)	11/11 (70/3,500)
Ab response to:		
17-aa repeat	12/27[d]	7/11
aa 84–107 peptide	7/27	5/11
aa 1813–1835 peptide	14/27	8/11
aa 1888–1909 peptide	7/27	6/11

[a] Blood smears were prepared twice over a 6-month interval.
[b] $P < 0.0001$ versus the group with negative blood smears.
[c] Values in parentheses are ranges of IFN-γ production in picograms per 2×10^6 PBMC.
[d] A positive Ab response represents an optical density greater than or equal to the mean ± 3 standard deviations for nine North American control subjects.

DISCUSSION

Along with several asexual blood-stage Ag and CSP, immunogenic molecules expressed during the liver stage of *P. falciparum* infection are potential components of a multistage human malaria vaccine (20, 30, 39). Dissection of the precise mechanisms of resistance and immune responses to liver-stage *P. falciparum* in humans is a formidable problem, since there is no direct means of evaluating parasite elimination in the liver and the number of well-characterized liver-stage Ag is relatively small compared with that of the blood-stage Ag. The problem is compounded in studies of residents of areas where malaria is endemic, since these individuals have developed partial resistance to asexual blood-stage parasites. Studies of various rodent malaria models indicate that CD8 cells and CTL directed at liver-stage Ag are involved in elimination of hepatic schizonts (38) and that, in vaccination experiments, CD4 cells are required (40). IFN-γ is a key cytokine in vaccine-induced elimination of liver-stage parasites in rodent malaria (8, 28). Although it is not known with certainty whether analogous responses are important in human falciparum malaria, immunologically naïve persons immunized with irradiated *P. falciparum* sporozoites and residents of areas where malaria is endemic have T cells which proliferate and produce IFN-γ in response to liver-stage Ag and CSP and CTL directed against liver-stage Ag and CSP (1, 12, 13, 17, 18, 24, 26, 44).

The current study examined the antigenicity of LSA1, a vaccine candidate molecule expressed exclusively during hepatic schizogony. The data demonstrate that T cells from more than 90% of adults living in an area of Papua New Guinea where malaria is holoendemic proliferate in response to one or more peptide epitopes in the N- and C-terminal nonrepeat regions of LSA1. These proliferation responses were remarkable in that the mean SIs exceeded 6.0. Previous reports of proliferation responses to LSA1 in populations living where malaria is endemic have been limited to Africa. Fidock et al. (9) observed positive responses to a series of LSA1 peptides in 6 to 20% of subjects from Madagascar (the cutoff value for a positive response was 2.0, and many of the positive responses were detected only when IL-2 was added to the cell cultures). There may be several reasons why we observed higher frequencies and levels of proliferation in response to LSA1 in the Wosera area. First, the peptides used in the Madagascar and Papua New Guinea studies correspond to different and nonoverlapping regions of the molecule (the former were peptides corresponding to aa 1553 to 1719, whereas we used those corresponding to aa 84 to 107, 1813 to 1835, and 1888 to 1909). Second, residents of the Wosera area experience a high degree of boosting by repeated exposure to sporozoites and liver-stage *P. falciparum*. Transmission of malaria in this area of Papua New Guinea is intense and occurs year round, with minor seasonal variability (10, 11), whereas there is marked seasonal fluctuation in Madagascar, especially in highland areas (35). Third, our observations were limited to adults, while persons examined in Madagascar were between the ages of 1 and 75 years. Ongoing studies in the Wosera area indicate that the frequency of T-cell responses to LSA1 is substantially lower in 1- to 4-year-old children (<10%) than in adults (4a). Levels of T-cell proliferation in response to aa 84 to 107, 1819 to 1835, and 1888 to 1909 peptides observed in Papua New Guinea were also equivalent to and, in most cases, greater than that reported for three of four North Americans immunized with irradiated sporozoites (SI range, ~2.0 to 5.5) (23). The relatively vigorous response by naturally infected Papua New Guineans is consistent with repeated boosting with liver-stage Ag, whereas recipients of irradiated sporozoites experience a single, abortive liver-stage infection.

The pattern of cytokine production following stimulation with LSA1 peptides indicated a dominant type 1 response. IL-4 or IL-5 production induced by any of the three LSA1 peptides was observed in only 18% of subjects, whereas 88% had PBMC which produced IFN-γ. IFN-γ responses to various LSA1 peptides encoded by aa 1553 to 1719 have also been observed in 6 to 20% of malaria-exposed individuals from Madagascar (IL-4 and IL-5 production was not described) (9), and to our knowledge, production of this or other cytokines in response to these or other LSA1 peptides has not been reported for humans immunized with irradiated sporozoites. The current study demonstrates that the predominant source of IFN-γ produced by PBMC driven with LSA1 is $CD8^+$ cells. This finding is consistent with the notion that malaria-specific CD8 cells recognize peptide epitopes of liver-stage Ag that are processed and presented by HLA class I-bearing hepatocytes. Since elimination of *P. falciparum* in hepatocytes may be mediated or augmented by T cells restricted by HLA class I (12, 18, 29, 41, 42), it will be important in future studies to ascertain whether the observed CD8 cell IFN-γ, as well as CTL, responses to these peptides differ according to HLA types common in the Wosera area. These experiments are more properly done with 9-mer peptides than the 20- to 30-mer peptides used here, since multiple major histocompatibility complex class I-restricted responses may be elicited by the latter constructs. In any case, measurement of peptide-driven IFN-γ production by CD8 cells may provide a convenient and biologically relevant correlate of immunity to various pre-erythrocytic Ag for field studies of human malaria given the fact that the CTL precursor frequency for liver-stage Ag is low (31) and performance of CTL assays is technically laborious and requires HLA class I-matched targets. In this context, Bottius et al. (3) recently examined IFN-γ production by PBMC stimulated with the pre-erythrocytic Ag SALSA-1 and -2. Secretion exceeding 2 IU (~20 pg in our assay) was noted in 26 of 50 subjects from an area of Madagascar where malaria is endemic.

We do not know why IFN-γ responses to the aa 84 to 107 peptide are less frequent than those to the two C-terminal peptides. It is possible that there are nonsynonymous mutations in this region of LSA1, thereby accounting for lack of sensitization to a peptide synthesized on the basis of the NF54 sequence. Polymorphisms in the N terminus and other regions

of LSA1 have been described in *P. falciparum* isolates from various areas where malaria is endemic, including Papua New Guinea (43). We have recently identified two nonsynonymous mutations at position 2 of the N-terminal peptide (aa 85) in *P. falciparum* isolates from the Wosera area (4a). Alternatively, it is possible that CD8 cell IFN-γ responses to 9-mer epitopes contained within the aa 84 to 107 peptide are restricted by HLA class I alleles that are less common in the Wosera area. HLA-restricted T-cell immunity has been documented for peptide epitopes of other pre-erythrocytic Ag, such as SSP2 (24, 41, 42).

Finally, Ab levels and T-cell proliferation and cytokine responses to the various LSA1 peptides were compared for groups of adults who had thick blood smears negative for *P. falciparum* infection on two occasions over a 6-month interval with those who had positive smears at one or both time points. There was a significantly greater frequency of IFN-γ responses to the aa 84 to 107 peptide in the group with negative smears compared to those with positive smears ($P < 0.0001$). The correlation was specific for IFN-γ production stimulated by the aa 84 to 107 peptide, since blood smear status was not significantly associated with T-cell proliferation, IFN-γ production in response to the aa 1813 to 1835 or 1888 to 1909 peptide, levels of Ab to the flanking or central repeat regions of LSA1, or cytokine and proliferation responses to SLO. The significance of this correlation in terms of resistance to pre-erythrocytic infection cannot be established on the basis of data currently available to us. Ascertainment of blood-stage infection by thick blood smear alone is insensitive relative to PCR-based methods of diagnosis (4), and in residents of areas where malaria is endemic, a relatively high level of acquired resistance to blood-stage infection cannot be distinguished from resistance to pre-erythrocytic parasites. Future efforts will thus be directed at measuring peptide-driven IFN-γ responses in persons cured of blood- and liver-stage *P. falciparum* infections with chemotherapy and evaluating the relationship between the time to reinfection and the level of T-cell immunity to LSA1 and other pre-erythrocytic Ag. These studies will ideally be done with foreknowledge of the subjects' HLA types and employ highly sensitive PCR-based methods to detect asexual blood-stage infection.

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