Plasmodium falciparum Liver Stage Antigen-1 Is Well Conserved and Contains Potent B and T Cell Determinants[1]

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We have previously identified a *Plasmodium falciparum* liver stage-specific Ag (LSA-1) found to encode tandem 17 amino acid repeats harboring B cell determinants. Here we extend this study in terms of sequence analysis, protein localization, and immunologic properties. Analysis of the N- and C-terminal regions of LSA-1 from the T9/96 clone reveals high sequence conservation with LSA-1 from NF54. This 200-kDa protein is detected throughout liver schizogony and accumulates in the parasitophorous vacuole space. In our investigation of T and B cell responses to LSA-1, we have focused on both the area of the C-terminal, nonrepetitive "hinge" region and the conserved repetitive region and derived synthetic peptides. These were found to contain major B and T cell determinants. High prevalences and elevated Ab levels to LSA-1, directed primarily, although not exclusively, to the repetitive region, were detected in sera of individuals from one moderately high and two low transmission malaria-endemic areas (prevalences of 97%, 75, and 77%, respectively). In one of these low transmission areas, secretion of the cytokine IFN-γ, known to inhibit malaria liver stages, and T cell proliferation were detected in PBMC of 22 to 48% and 6 to 20%, respectively, of individuals in response to separate LSA-1 peptides. These results complement the recent finding of conserved CTL epitopes in LSA-1 and support the assertion that immune responses to LS Ag are involved in protection against malaria pre-erythrocytic stages. *The Journal of Immunology,* 1994, 153: 190.

Investigation of the pre-erythrocytic stages of the human malaria parasite *Plasmodium falciparum* has long been guided by the key studies demonstrating that full protection against these stages could be induced in humans by immunization with radiation-attenuated sporozoites (reviewed in Ref. 1). Results from these various studies in humans and animal models led to the hypothesis that this protection was dependent on the ability of sporozoites to invade host hepatocytes and develop into young LS[6] forms (reviewed in Ref. 2), as was recently experimentally supported (3). Thus, a number of years ago we set out to explore the antigenic composition of *P. falciparum* at the LS. This led to the identification of the LS Ag (LSA-1) (4), which to date is the only Ag described that is expressed solely at this stage. The importance of this Ag was recently reinforced by the work of Hill et al. (5), who demonstrated the presence, in LSA-1, of CTL epitopes potentially involved in protection against disease.

In this study, we extend our characterization of LSA-1 in terms of sequence analysis, protein structure and intrahepatic localization as well as its immunologic properties. Initially, this Ag had been found to contain a region of

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[6] Abbreviations used in this paper: LS, liver stage; LSA, liver stage-specific Ag; LSA-1, liver stage specific Ag-1; SI, stimulation index; LeuA, leukoagglutinin; PPD, purified protein derivative; CS, circumsporozoite; IFAT, immunofluorescent Ab test; aa, amino acid; CD, circular dichroism; AU, arbitrary units; PV, parasitophorous vacuole; PVM, PV membrane; R, arginine; S, serine.

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tandem 17 aa repeats that encoded strong B cell epitopes. Because immunogenic determinants have been frequently detected in nonrepetitive regions of *Plasmodium* Ags, in addition to those contained in the repeat sequences (6–9), and in view of the wide range of T and B cell-mediated effector mechanisms found in experimental models to operate upon *Plasmodium*-infected hepatocytes (reviewed in Refs. 10 and 11), we decided to focus our attention on identifying and immunologically analyzing nonrepetitive and conserved repetitive regions of this molecule. We present data showing that sequence of this Ag is highly conserved between the Thai strain T9/96 and the African strain NF54 (12) and demonstrate the presence, in both the repetitive and nonrepetitive regions, of major T cell and B cell epitopes recognized by individuals from diverse endemic regions.

Materials and Methods

Sequence analysis of rLSA-1 clones

DNA was prepared from lysates of λgt11 rLSA-1 clones (DG307, DG29f, and DG536) isolated from a *P. falciparum* (clone T9/96) genomic expression library (4). *Eco*RI-liberated inserts were then gel purified, subcloned into the vector M13 mp18 and sequenced. A total of 310 bp of additional 3′ nonrepetitive sequence extending from the known 3′ sequence in DG536 was then generated in the following way: 2×10^7 phage from the T9/96 library were subjected to standard PCR, using (at 1 μM final concentrations) LSA-1-specific primers (3′1 and, subsequently, 3′2) and a λgt11 primer flanking the *Eco*RI cloning site. High m.w. fragments were gel purified and subcloned into a pUC or pCR (Invitrogen, San Diego, CA) vector. LSA-1-positive clones were identified by hybridization with an internal oligomer and sequenced. Newly available sequence from the NF54 strain (12) was then used to complete the 3′ end of the gene (amplified using oligomers 3′3 and 3′4) and to obtain the sequence of the 5′ nonrepetitive region (using primers 5′1 and 5′2). The PCR primers used are as follows (+ and − designates coding and noncoding strand, respectively; nucleotide positions are as indicated in Fig. 1A): 5′1 (+, 47–70); 5′2 (−, 505–528); 3′1 (+, 5243–5266); 3′2 (+, 5410–5434); 3′3 (+, 5553–5575); and 3′4 (−, 5799–5822). By DNA hybridization, two LSA-1 clones (DG750 and DG443) were subsequently found to contain the majority of the 5′ nonrepetitive region as well as a long stretch of repeat units and were sequenced. The final sequence was determined from both strands using the dideoxynucleotide chain-termination method and in the case of cloned PCR fragments was always determined from multiple clones.

Hybridization analysis

Genomic *P. falciparum* DNA (5 μg) was digested with Mung bean nuclease, using the conditions outlined in (13) and size fractionated on a 0.8% agarose gel. Southern hybridization was performed using standard techniques (14).

Synthetic peptides

LSA-1 peptides were synthesized by the stepwise solid phase technique (15) in an automated peptide synthesizer (Applied Biosystems, Foster City, CA, model 430A) using a standard butyloxycarbonyl (BOC)-trifluoroacetic acid method on a benzhydrylamine resin (Applied Biosystems). After synthesis, the N-terminal butyloxycarbonyl-protecting group was removed and the peptide acetylated using acetic anhydride. Final deprotection and cleavage of the peptidyl resins was performed by the "high" hydrogen fluoride procedure, for 1 h at 4°C. The cleaved deprotected peptides were precipitated with cold diethylether and then dissolved in 5% acetic acid and lyophilized. Crude peptides were purified by reverse phase HPLC on a 5-μ, 300 Å Nucleosil C18 (Macherey Nagel, Rochester, N.Y.) column (1 cm × 0.5 cm), using an acetonitrile/water/0.5% trifluoroacetic acid solvent system. Peptides were checked for homogeneity by analytic reverse phase-HPLC and for identity by aa analysis, and their molecular mass was verified on a Bio Ion 20 plasma desorption mass spectrometer (Bio Ion AB, Uppsala, Sweden). Circular dichroism (CD) measurements were used to assess the α-helicity of peptides dissolved in 0.1 M Nacl or 90% trifluoroethanol, as described (16). CD results were reported in terms of mean residue ellipticity, expressed in degree decimol^{-1} cm^2. Peptide sequences are shown in Figure 1A for the peptides LSA-J, LSA-NR, and LSA-Ter and in Table I for the peptide LSA-Rep (previously referred to as LSA41) (16). Note that the LSA-Rep sequence is present three times in the NF54 strain and at least once in the T9/96 strain. The *P. falciparum* CS peptide Th2R (PSDKHIEQYLKKIKNSIS; aa 326–343 of the 7G8 strain) was a kind gift of Mitch Gross, (SmithKline Beecham, Philadelphia, PA). The LSA-1 repeat peptide LSA-Rep was FITC-labeled using FITC (1 mg/mg of peptide), in a water/pyridine (200 μl/400-μl) solvent mixture for 24 h. After dilution with water, excess reagent was removed by extraction with ethyl acetate. The aqueous layer, containing the labeled peptide, was then lyophilized.

Table I. *Antigenicity of LSA-1 repeats depends on α-helicity*

LSA-1 Repeat Peptide Sequences	CD[a]	Ag[b]
LAKEKLQGQQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ[c]	α	+
GQQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ	α	+
EQQSDLEQERLAKEKLQ	α	+
GQQSDLEQERLAKEKLQ	α	+
LEQERLAKEKLQ	rc	−
RLAKEKLQ	rc	−
EQQSDLEQERL	rc	−
EQQSDLEQ	rc	−
KEKLQEQQSDLEQERLA[d]	rc	−

[a] CD measurements of peptide conformation adopted in aqueous solution. α, alpha helix. rc, random coil (absence of helical organization).
[b] Antigenicity of peptides in ELISA assays.
[c] LSA-Rep peptide sequences.
[d] LSA 13–29 peptide sequences.

Abs

Six-week-old female BALB/c mice were immunized s.c. with 15 μg of purified rβ-gal+DG307 emulsified in CFA, and boosted at 4 and 6 wk with the same material in IFA. Rabbits and chimpanzees were similarly immunized with rβ-gal+DG307 (50 μg/dose) or rβ-gal+DG536 (200 μg/dose), respectively. In a similar manner, BALB/c mice were also immunized with the synthetic peptides LSA-Rep or LSA-J (50 μg/dose). Human Abs from pooled human immune sera were affinity purified from the synthetic peptide LSA-Rep, as described by Brahimi et al. (17) or from recombinant or wild-type λgt11 clones, according to Reference 18.

Culturing and immunoblotting of liver stage parasites

The culture of *P. falciparum* LS was performed as previously described (19). Briefly, human hepatocytes were isolated from biopsies by collagenase perfusion and seeded in monoculture at 5×10^5 hepatocytes/35-mm petri dish. From 24 to 48 h later, 5 to 10 aseptically dissected pairs of *P. falciparum* (NF54) sporozoite-infected salivary glands were introduced into half of the cultures, the other half serving as a noninfected control. Cultures were maintained 6 days with daily medium replacement before harvesting cells. To perform a single immunoblotting experiment, cells had to be pooled from over 40 dishes prepared in five separate experiments in which late stage LS forms were successfully obtained. A number of other culture experiments had to be aborted because of lack of parasite maturation. Standard immunoblotting was performed and the bound IgG detected using ^{125}I-labeled goat anti-human IgG (Biosys, Compiegne, France).

Immunofluorescent and immuno-electron microscopy of in vivo derived LS material

After i.v. sporozoite inoculation, liver biopsies containing young, immature, or fully mature *P. falciparum* liver schizonts were obtained, respectively, from a squirrel monkey (*Saimiri sciureus*) at day 3, a Cebidae

monkey (*Cebus apella*) at day 5 (20), and a chimpanzee (*Pan troglodytes*) at day 6 (21). Immunofluorescent Ab tests (IFAT) were performed on Carnoy-fixed material (20). Positive IFAT reactions on hepatic schizonts were verified by phase-contrast microscopy and subsequent Giemsa staining of the schizonts. For immuno-electron microscopy, day 6 chimpanzee liver biopsies were immersion-fixed with 0.1% glutaraldehyde/2% paraformaldehyde, dehydrated in ethanol, and embedded in LR White resin (Polysciences, Cambridge, UK). Ultrathin sections were blocked with 1% BSA and 1% cold fish gelatin in 0.1 M phosphate buffer (pH 7.4) for 30 min. Grids were incubated overnight at 4°C with anti-LSA-1 Abs and, after thorough washings, incubated with protein A gold (10 nm) for 1 h. After final washing they were dried, followed by contrasting with 2% uranyl acetate.

Study areas and donors

Three areas of contrasting epidemiologic status were investigated. 1) Donsé, Burkina Faso (West Africa): this rural village of 500 inhabitants is situated in a savannah area of Upper Volta, 50-km north of the capital. Malaria is seasonal with high parasite transmission rates (22). 2) Podor, northern Senegal: sera were collected from two neighboring rural villages (whose population totaled 3000) in the vicinity of Podor, 10-km south of the Senegal river. This is a very dry Sahel area with a brief transmission season characterized by relatively low transmission rates (by African standards) (23). 3) Ankazobé, Madagascar: this small town of 5000 inhabitants is situated on the outlying regions of the Highlands plateau. Malaria has always persisted at low levels in this town, in contrast to other settings in the Central Highlands plateau (for example Manarintsoa) where malaria had been eradicated for many years. In the agricultural areas of Donsé and Podor, living conditions are essentially homogeneous with all houses consisting of mud walls, open windows, and thatched roofs. In contrast, in Ankazobé the greater socio-economic disparity is reflected in the variety of housing construction, ranging from mudwall houses similar to those found in Donsé and Podor to houses with brick and plaster walls, glass windows and tiled roofs. The respective malaria transmission rates are approximately 100 infectious bites/individual/year for Donsé and 1 to 5 for Podor and Ankazobé. Individual donors ranged in age from 1 to 75 and were chosen so as to have an even distribution over the different age groups, except in the case of Ankazobé where a moderately greater proportion of adults were chosen for T cell studies. After informed consent, venous blood was collected into heparinized vacutainers. Plasma was harvested after centrifugation, aliquoted, and stored at −80°C until use. For the Ankazobé population sample, no donors were adjudged to be suffering clinical malaria at the time of sampling. Samples were collected over the period of February to October 1991. PBMC were separated on Ficoll gradients within 4 to 6 h of sampling.

Lymphocyte assays

These were performed essentially as previously described (24), using round bottom, 96-well plates in which PBMC were seeded at 2×10^5 cells (in 200 μl)/well. Preliminary experiments had determined that 10 μg/ml was the most appropriate concentration for all the test peptides concerned (LSA-Rep, LSA-J, LSA-NR, LSA-Ter, and Th2R). Leukoagglutinin (LeuA) and *Mycobacterium tuberculosis* purified protein derivative (PPD) were used as positive controls. Sixteen hours before harvesting cells onto glass fiber filters, 100 μl of cellfree supernatant were removed from each well, saved for estimation of IFN-γ concentration, and replaced with 100 μl of complete culture medium containing 1 μCi of [^{3}H]TdR. DNA incorporation was determined by liquid scintillation spectrometry. The stimulation index (SI) was calculated as the mean cpm of triplicate test cultures/mean cpm of triplicate control (unstimulated) cultures. A positive lymphoproliferative response was assigned to samples for which the SI was >2 and the difference in cpm between test and control samples was >1000 (24). Control PBMC from individuals with no history of exposure to malaria were included in each set of experiments. Identical plates from each individual were also run in which human rIL-2 (Genzyme, Cambridge, MA) was added at a concentration of 25 U/ml at day 4 of culture. The IFN-γ concentration was measured in pooled supernatants from wells in which rIL-2 had not been added and was assessed by a two-site capture ELISA test previously described (24). Briefly, IFN-γ content was calculated from standard curves obtained using known amounts of IFN-γ diluted in culture medium on the same plate. Readings were compared with the international human National Institutes of Health IFN-γ standard Gg23-901-530. The detection limit of this test was 0.25 IU/ml. Peptide-stimulated supernatants containing >2 IU/ml of IFN-γ were considered positive. In the rare cases in which IFN-γ production was detected in control, unstimulated PBMC, samples were excluded from analysis.

ELISA assays

ELISA plates (Nunc-Immuno Plate II, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of peptide solution: LSA-Rep, LSA-Ter, or R32tet32 at 10 μg/ml in PBS (pH 8.0); LSA-J at 10 μg/ml in Tris-buffered saline (pH 7.4); or LSA-NR at 1 μg/ml in Tris-buffered saline. The purified recombinant protein R32tet32 (25), containing the CS repeats NANP and NVDP, was used as a comparative control. Human sera were tested in duplicate at a dilution of 1/100 and bound IgG was detected using peroxidase-conjugated goat anti-human IgG (Biosys) as described in Reference 17. Initially, each peptide was tested separately on a control group of 40 serum samples from healthy French blood donors with no history of exposure to malaria. Six serum samples reproducing the mean and SD of the control group were selected and tested on each plate. IgG Ab levels were defined in arbitrary units (AU), where the number of AU was calculated by dividing the mean OD_{492} value of the test serum by the mean +3 SD of the six control serum samples from the same plate. AU values ≥1 were considered as indicative of a specific IgG Ab response. Considering the large number of individuals tested, this manner of estimating Ab levels was considered preferable to titrating every sera on the various peptides. Sera tested from Ankazobé were obtained the day of PBMC preparation. For competitive inhibition assays, hyperimmune sera, diluted 1/100, were incubated overnight at 4°C with test inhibitory peptides at concentrations ranging from 10^{-1} to 10^5 ng/ml. These samples were then processed as standard ELISA assays, using LSA-Rep- or LSA-J-coated plates. Results for each peptide tested at different concentrations were expressed as percentage of inhibition, calculated as follows: % inhibition = (OD_{492} (immune serum incubated with test peptide at test concentration) − OD_{492} (background, serum dilution buffer))/(OD_{492} (immune serum incubated in the absence of peptide) − OD_{492} (background, serum dilution buffer)) × 100.

Statistical methods

All statistical tests were performed on $\log_e$-transformed data, according to accepted convention (26). The Pearson test was used to examine the correlation between 1) Ab or lymphoproliferative responses and age and 2) quantitative responses to pairs of peptides. The analysis of variance (ANOVA) F-test was used to compare mean Ab responses between age groups. The McNemar X^2 test of symmetry was used to examine the

FIGURE 1. *A*, partial nucleotide and deduced amino acid sequence of the LSA-1 gene, clone T9/96. Nucleotide sequence data reported in this paper have been submitted to the EMBL data base with the accession numbers 230319 and 230320 (5′ and 3′ extremities, respectively). Nucleotide positions whose sequence varies between T9/96 and NF54 are underlined and NF54 aa variants are indicated above the T9/96 aa sequence. The numbering used conforms with that used for the NF54 strain (12). The sequence of the three peptides LSA-J (aa 1613–1636), LSA-NR (aa 1633–1659), and LSA-Ter (aa 1686–1719) is underlined. LSA-Rep, derived from the DG307 clone, maps to the inner segment of the repeat region (between aa 319 and 1417). Asterisks delineate the central repetitive region (aa 154–1629). Clones DG750, DG443, and DG536 span nucleotides 159–993, 159–1034, and 4327–5289, respectively. *B*, abridged aa structure showing the relative placement of the above mentioned peptide sequences.

A

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   1                                                                M  K  H  I  L  Y  I  S  F  Y  F  I  L  V  N  L  L  I  F  H  I  N
  47  aaagtatacatcttcctttctttacttcttaaaATGAAACATATTTTGTACATATCATTTTACTTTATCCTTGTTAATTTATTGATATTTCATATAAAT
                                                                                                                                K
  23  G  K  I  I  K  N  S  E  K  D  E  I  I  K  S  N  L  R  S  G  S  S  N  S  R  N  R  I  N  E  E  N  H  E
 145  GGAAAGATAATAAAGAATTCTGAAAAAGATGAAATCATAAAATCTAACTTGAGAAGTGGTTCTTCAAATTCTAGGAATCGAATAAATGAGGAAAATCACGAG

  57  K  K  H  V  L  S  H  N  S  Y  E  K  T  K  N  N  E  N  N  K  F  F  D  K  D  K  E  L  T  M  S  N  V  K
 247  AAGAAACACGTTTTATCTCATAATTCATATGAGAAAACTAAAAATAATGAAAATAATAAATTTTTCGATAAGGATAAAGAGTTAACGATGTCTAATGTAAAA

  91  N  V  S  Q  T  N  F  K  S  L  L  R  N  L  G  V  S  E  N  I  F  L  K  E  N  K  L  N  K  E  G  K  L  I
 349  AATGTGTCACAAACAAATTTCAAAAGTCTTTTAAGAAATCTTGGTGTTTCAGAGAATATATTCCTTAAAGAAAATAAATTAAATAAGGAAGGGAAATTAATT
                                                                                                  *           T
 125  E  H  I  I  N  D  D  D  D  K  K  K  Y  I  K  G  Q  D  E  N  R  Q  E  D  L  E  E  K  A  A  K  E  K  L
 451  GAACACATAATAAATGATGATGACGATAAAAAAAAATATATTAAAGGGCAAGACGAAAACAGACAAGAAGATCTTGAAGAAAAAGCAGCTAAAGAAAGTTA
                 L           L                                                       S
 159  Q  G  Q  Q  S  D  S  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  L  A  K  E  K  L
 553  CAGGGGCAACAAAGCGATTCAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACTTGCTAAAGAAAAGTTG
                          L
 193  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  L  A  K  E  K  L
 655  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACTTGCTAAAGAAAAGTTG

 227  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L
 757  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTG
                          R
 261  Q  E  Q  Q  S  D  L  E  Q  E  R  L  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  D  R  L  A  K  E  K  L
 859  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACTTGCTAAAGAAAAGTTACAAGAGCAGCAAAGCGATTTAGAACAAGATAGACTTGCTAAAGAAAAGTTG
                                   K
 295  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  R  L  Q  E  Q  Q  S  D  L.....
 961  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAGGTTGCAAGAACAACAAAGCGATTTAGA.....

                                                                                                  E     R
1417  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  D  R  L  A  K  E  K  L
4327  CAAGAACAACAAAGCGATCTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGATAGACTTGCTAAAGAAAAGTTA
                          R                                                                       L
1451  Q  E  Q  Q  S  D  L  E  Q  E  R  L  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L
4429  CAAGAGCAGCAAAGCGATTTAGAACAAGAGAGACTTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATCTAGAACAAGAGAGACGTGCTAAAGAAAAGTTG
                    R                                                                             E     R
1485  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  D  R  L  A  K  E  K  L
4531  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGATAGACTTGCTAAAGAAAAGTTA
                                   L     N                          R
1519  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L
4633  CAAGAGCAGCAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTG
                          R
1553  Q  E  Q  Q  S  D  L  E  Q  E  R  L  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L
4735  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACTTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTG
                          L                          R                          L
1587  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L  Q  E  Q  Q  S  D  L  E  Q  E  R  R  A  K  E  K  L
4837  CAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTGCAAGAACAACAAAGCGATTTAGAACAAGAGAGACGTGCTAAAGAAAAGTTG
                    *                                                                             V
1621  Q  E  Q  Q  R  D  L  E  Q  R  K  A  D  T  K  K  N  L  E  R  K  K  E  H  G  D  I  L  A  E  D  L  Y  G
4939  CAAGAGCAGCAAAGAGATTTAGAACAAAGGAAGGCTGATACGAAAAAAAATTTAGAAAGAAAAAAGGAACATGGAGATATATTAGCAGAGGATTTATATGGT

1655  R  L  E  I  P  A  I  E  L  P  S  E  N  E  R  G  Y  Y  I  P  H  Q  S  S  L  P  Q  D  N  R  G  N  S  R
5041  CGTTTAGAAATACCAGCTATAGAACTTCCATCAGAAAATGAACGTGGATATTATATACCACATCAATCTTCTTTACCTCAGGACAACAGAGGGAATAGTAGA

1689  D  S  K  E  I  S  I  I  E  K  T  N  R  E  S  I  T  T  N  V  E  G  R  R  D  I  H  K  G  H  L  E  E  K
5143  GATTCCAAGGAAATATCTATAATAGAAAAAACAAATAGAGAATCTATTACAACAAATGTTGAAGGACGAAGGGATATACATAAAGGACATCTTGAAGAAAAG

1723  K  D  G  S  I  K  P  E  Q  K  E  D  K  S  A  D  I  Q  N  H  T  L  E  T  V  N  I  S  D  V  N  D  F  Q
5245  AAAGATGGTTCAATAAAACCAGAACAAAAAGAAGATAAATCTGCTGACATACAAAATCATACATTAGAGACAGTAAATATTTCTGATGTTAATGATTTCAA

1757  I  S  K  Y  E  D  E  I  S  A  E  Y  D  D  S  L  I  D  E  E  E  D  D  E  D  L  D  E  F  K  P  I  V  Q
5347  ATAAGTAAGTATGAGGATGAAATAAGTGCTGAATATGACGATTCATTAATAGATGAAGAAGAAGATGATGAAGACTTAGACGAATTTAAGCCTATTGTGCAA

1791  Y  D  N  F  Q  D  E  E  N  I  G  I  Y  K  E  L  E  D  L  I  E  K  N  E  N  L  D  D  L  D  E  G  I  E
5449  TATGACAATTTCCAAGATGAAGAAAACATAGGAATTTATAAAGAACTAGAAGATTTGATAGAGAAAAATGAAAATTTAGATGATTTAGATGAAGGAATAGAA

1825  K  S  S  E  E  L  S  E  E  K  I  K  K  G  K  K  Y  E  K  T  K  D  N  N  F  K  P  N  D  K  S  L  Y  D
5551  AAATCATCAGAAGAATTATCTGAAGAAAAAATAAAAAAAGGAAAGAAATATGAAAAAACAAAGGATAATAATTTTAAACCAAATGATAAAAGTTTGTATGAT

1859  E  H  I  K  K  Y  K  N  D  K  Q  V  N  K  E  K  E  K  F  I  K  S  L  F  H  I  F  D  G  D  N  E  I  L
5653  GAGCATATTAAAAAATATAAAAATGATAAGCAGGTTAATAAGGAAAAGGAAAAATTCATAAAATCATTGTTTCATATATTTGACGGAGACAATGAAATTTTA

1893  Q  I  V  D  E  L  S  E  D  I  T  K  Y  F  M  K  L  -
5755  CAGATCGTGGATGAGTTATCTGAAGATATAACTAAATATTTTATGAAACTATAAaaggttatatattt
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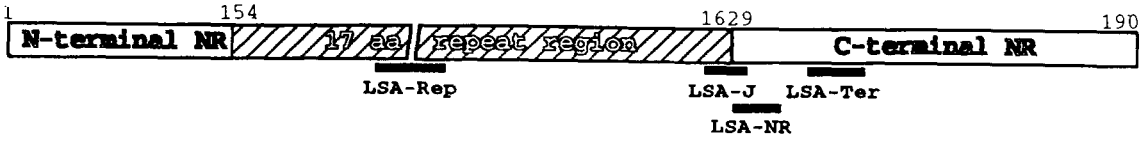
B



relationship between presence or absence of positive response to pairs of peptides.

Results

Isolation and sequence characteristics of the 3′ and 5′ regions of the LSA-1 gene (strain T9/96)

Screening of a genomic *P. falciparum* expression library with stage-restricted human sera previously led us to identify 120 cloned fragments, whose corresponding Ags would seem to be expressed in pre-erythrocytic stages (27). DNA hybridization and serologic analysis were used to classify these clones into families, of which the largest, comprising 26 clones, corresponds to cloned regions of the LSA-1 gene. All 26 clones were found to contain repetitive sequence encoding tandem highly conserved 17-aa repeats, a major B cell determinant well recognized by malaria-endemic populations (4, 27). We subsequently focused our attention on identifying the sequence of the nonrepetitive regions. Starting from the hypothesis that these regions may also contain minor B cell epitopes which could serve as a means of selection, we screened large numbers of African sera collected in high-transmission areas. This led to the identification of seven sera nonreactive with LSA-1 repeat peptides though having high IFAT titers to LS. These sera recognized 12 of the 26 LSA-1 clones, notably DG536. All 12 clones were found to contain sequence mapping to within the first 330 bp of the 3′ nonrepetitive region in addition to a variable number of repeats. The remaining sequence of the 3′ and 5′ nonrepetitive regions was subsequently determined using PCR-based approaches.

Figure 1*A* shows our 5′ and 3′ sequence data for the LSA-1 gene in the T9/96 clone and comparison with the NF54 sequence (12). The nonrepetitive regions are remarkably well conserved between the two strains, with only one nucleotide substitution in each of the 5′ and 3′ nonrepetitive regions (which in total consist of 1.30 kb). Both substitutions are nonsynonymous. The repetitive region shows greater sequence divergence, with 40 nucleotide substitutions (out of 1.14 kb), of which 21 are nonsynonymous. These substitutions are, however, not randomly distributed along the repeat unit. Interestingly, this nonrandom distribution was virtually identical in both T9/96 and NF54. At the aa level, no variants were found in either strain for over half of the residues forming this unit (E*QQSDLEQERRAKEKLQ*; the invariant residues are italicized). Variant residues almost always involved the same aa substitution (position 1*: E → G; 4*: S → R; 6: L → S; 9*: E → D; 11*: R → L; 13: K → N; 15: K → T or K → R; in this case the most frequently variant residues are indicated with an asterisk). Notably, for codons 4, 9, and 11, over 80% of the nucleotide substitutions were nonsynonymous. These LSA-1 repeats contain a highly conserved motif, KEK. This motif, also present in a number of blood-stage proteins, has been implicated in merozoite binding to erythrocytes before invasion (28). However, after incubation of noninfected erythrocytes with an FITC-labeled LSA-1 repeat peptide, LSA-Rep, we could not detect any specific fluorescence associated with the erythrocyte surface.

To estimate the size of the LSA-1 gene in the *P. falciparum* T9/96, a Southern blot was made of T9/96 genomic DNA digested by Mung bean nuclease which, under appropriate formamide concentrations, liberates gene-sized fragments (13). Hybridization with a LSA-1 probe detected a large fragment of approximately 5.3 and 5.2 kb under conditions of digestion in 40% and 45% formamide, respectively (Fig. 2*A*). As this is smaller than the known NF54 gene size (5.7 kb), this would suggest that the two strains differ in the total number of repeats, as has been reported for many other repetitive *P. falciparum* Ags (29).

Molecular features of the LSA-1 gene product

In general, access to the LS is extremely limited, particularly so for *P. falciparum* in which major logistical problems are compounded by the very low output of in vitro and in vivo models. However, by pooling *P. falciparum* sporozoite-infected hepatocyte cultures from five separate experiments in which late stage liver schizonts were successfully obtained in vitro, enough material was obtained for three wells on a SDS-polyacrylamide gel. Immunoblotting, using Abs directed to LSA-1 repeats, identified the molecular mass of LSA-1 as being 200 kDa (Fig. 2*B*).

IFAT using a series of anti-LSA-1 Abs on LS forms of different maturity reveal that this protein can be detected even from the early stages of liver schizogony, which for *P. falciparum* lasts 6 to 7 days. Initially, in day 3 (i.e., four- to eight-nuclei stage) and small day 5 forms (Fig. 3, *a* and *b*, respectively), the LSA-1 protein is located in the parasitophorous vacuole (PV) space, which is delineated by the inner plasmalemma and the outer PV membrane (PVM) and which forms a distinct ring separating the parasite cytoplasm from the host hepatocyte. At a later stage, the Ag appears also to infiltrate the spaces between the pseudocytomeres of the developing schizonts, as the plasmalemma forms deep invaginations into the parasite cytoplasm (Fig. 3*c*), and is subsequently seen localized around the cytomeres, just before the individualization of merozoites (Fig. 3*d*). The ultrastructural localization of LSA-1 was revealed by immunogold labeling of fully mature forms, which showed this protein to be associated with the fluffy or flocculent material present in the PV that surrounds the emerging exo-erythrocytic merozoites (Fig. 3*e*). Notably, labeling of LSA-1 was always seen within the boundaries of the PVM and never in the hepatocyte cytoplasm or on the hepatocyte surface. However, the anti-LSA-1 repeat Abs used were dependent on the repeats adopting their natural α-helical structure (see below) and thus we cannot rule out the possibility that some LSA-1 material ends up as linearized, thus undetectable, peptide

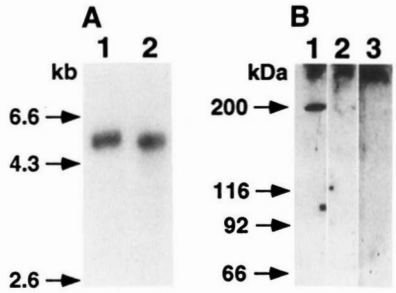


FIGURE 2. *A*, estimation of the LSA-1 gene size. *P. falciparum* (clone T9/96) genomic DNA was Mung bean digested under conditions of 40% (*lane 1*) and 45% formamide (*lane 2*) and the resulting blot hybridized with a radiolabeled DG307 probe. *B*, Immunoblot detection of the LSA-1 polypeptide in infected hepatocytes. Protein extracts from primary cultures of *P. falciparum* (strain NF54)-infected human hepatocytes were run on a 7.5% SDS-polyacrylamide gel under reducing conditions and labeled with human antisera affinity-purified from the λgt11 rLSA-1 clone DG307 (*lane 1*) or wild-type λgt11 (*lane 2*). Human anti-DG307 antisera were also tested on a protein extract of control noninfected human hepatocytes (*lane 3*). The DG307 insert encodes 4 tandem repeats of 17 aa derived from the LSA-1 repetitive region (4).

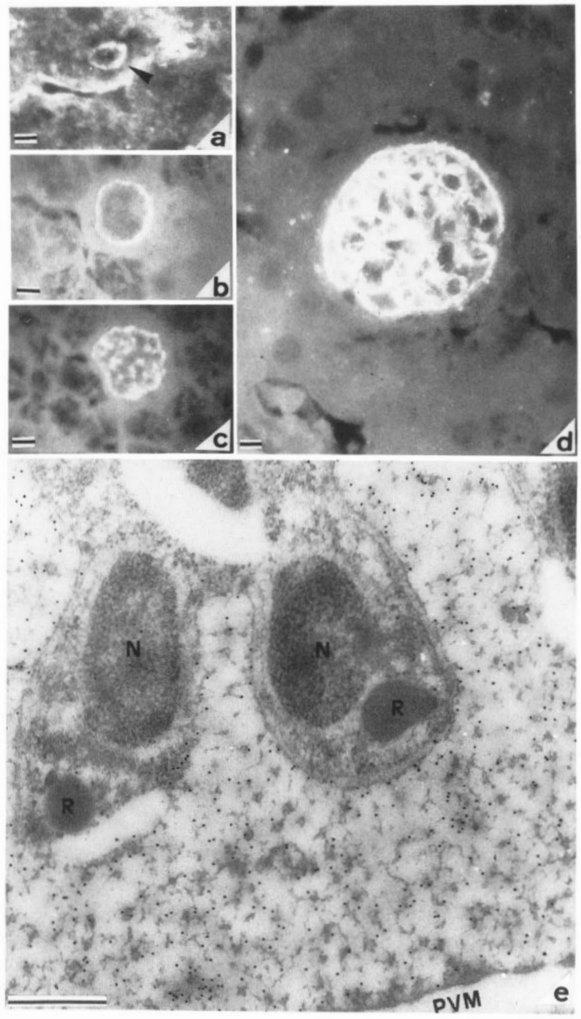


FIGURE 3. Localization of LSA-1 during liver schizogony. Anti-LSA-1 antisera were tested by IFAT on day 3 (*a*), day 5 (*b*, *c*), and day 6 (*d*) *P. falciparum* LS forms. For *a* the serum used was monospecific human anti-LSA-Rep antisera and for *b* to *d* mouse anti-β-gal+DG307 antisera. Identical labeling results were obtained with anti-β-gal+DG307 or anti-β-gal+DG536 sera raised in rabbits and chimpanzees, respectively, as well as mouse anti-LSA-J antisera (data not shown). These Abs were repeatedly negative, by IFAT and Western analysis, on both asexual blood stages and sporozoites. *E*, immunoelectron micrograph of a mature (day 6) *P. falciparum* liver schizont labeled with human polyclonal antisera affinity-purified from the λgt11 clone DG29f (a repeat-encoding member of the LSA-1 clone family). LSA-1 is seen in the floccular material, surrounding young emerging exoerythrocytic merozoites with their clearly visible rhoptries (R) and nucleus (N). Also visible is the PVM separating the LS forms from the host hepatocyte. Control serum purified from the nonrecombinant λgt11 clone was negative. *a* to *d*, bar represents 10 μm. *e*, bar represents 0.5 μm.

fragments transported outside the PV, where association with MHC class I molecules could occur.

Overall, the intensity of labeling seen with anti-LSA-1 Abs appears to increase progressively throughout liver schizogony, which suggests that LSA-1 is being actively synthesized during this stage. This contrasts with the CS protein which, although remaining present around the parasite surface, become less and less detectable in later stages (our unpublished observations) (30).

With the aim of defining potential epitope-bearing regions, the protein structure was analyzed using the hydrophobic cluster analysis plot described by Gaboriaud et al. (31) as well as helical propensity and hydrophobic moment modelization (32, 33). These predict that the long repetitive region, comprising nearly 1500 residues in NF54 (aa 154–1629), is organized as an uninterrupted stretch of α-helices. Directly following this, a hinge region can be predicted from Gly 1645 to Gly 1685. This is immediately followed by a putative α-amphipathic helix (aa 1686–1709) upstream to a second major bend of the molecule (aa 1710–1729). Both distal parts of the molecule contain several putative α-amphipathic helices, alternating with bend or β-sheet segments. Figure 4 shows the hydrophobic cluster analysis plot of part of the N-terminal and C-terminal regions of LSA-1. The essentially regular spacing, every seven residues, of hydrophobic amino acids in the LSA-1 repeats, which is constant throughout the entire length of the repeat region, is strongly evocative of an α-helical structure. As a comparison, Figure 4*C* shows similar longitudinal distribution of hydrophobic residues in a region of the M5 protein of *Streptococcus pyogenes* with a known α-helical coiled-coil structure (reviewed in Ref. 34).

Circular dichroism (CD) studies were then conducted to test the predicted α-helical nature of the LSA-1 repeats,

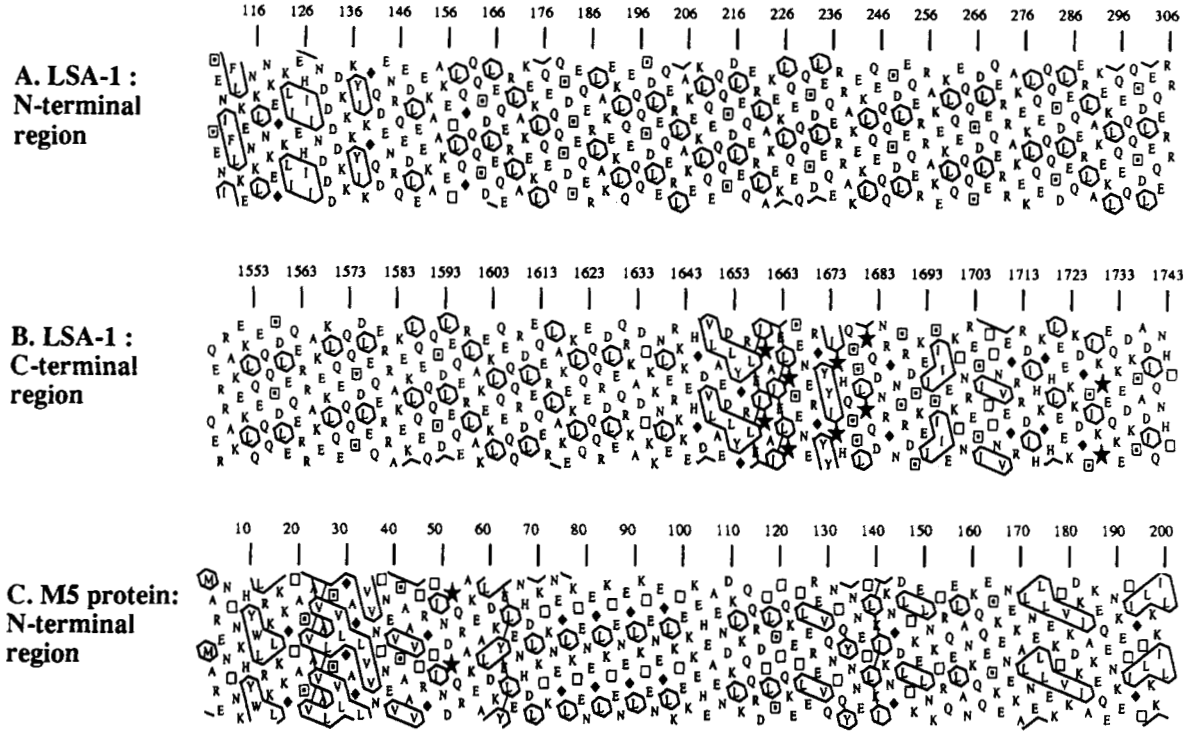


FIGURE 4. Hydrophobic cluster analysis plot of N-terminal (*A*) and C-terminal (*B*) segments of the *P. falciparum* LSA-1 protein (strain NF54) and of the N-terminal region (*C*) of the *Streptococcus pyogenes* M5 protein (Manfredo strain) (58). Hydrophobic residues are contoured. Some residues are represented by symbols: P (★)—a known helix-breaker, G (◆), T (□), and S (▭). The amino acid sequence is written diagonally from left to right such that there are seven residues per line and the last three or four residues are alternatively displayed on the following line. Because a classical α-helix contains 3.6 residues/turn and is typified by the regular spacing of hydrophobic residues, this sequence representation enables efficient visualization of an unfolded α-helical sequence (31). The 17 amino acid repeat units of LSA-1 commence at residue 154 of the N-terminal segment (AKETL. . ., in *A*) and continue through until residue 1629 of the C-terminal segment (. . . DLEQ, in *B*). *C*, this region of the M5 protein includes the signal peptide (residues 1–42) and part of the extracellular domain (residues 43–200). The α-helical region begins with the heptapeptide repeats spanning residues 66 and 100.

using a series of synthetic peptides from the repeat region. Figure 5 shows the CD spectra for the repeat peptide LSA-Rep, dissolved in either aqueous or trifluoroethanol medium. Both spectra were characteristic of a significant helical organization, with minima at 206 to 208 and 222 to 223 nm and a positive value at 198 to 200 nm. This peptide showed a helical content of 25% and 74% in aqueous or trifluoroethanol medium, respectively. CD results for the set of tested LSA-1 repeat peptides are summarized in Table I. This shows that significant α-helical organization could be adopted in aqueous solution by as little as a single repeat unit, however, this was lost when the single unit (in the frame EQQSDLEQERLAKEKLQ) was displaced. Furthermore, antigenicity studies revealed that only those peptides capable of assuming an α-helical organization were recognized by human hyperimmune antisera (Table I). Additional evidence in support of the conformational dependence of LSA-1 repeat B cell epitopes has been recently obtained by studies in which the addition of the helix-promoting residue α-aminoisobutyric acid to the extremities of a nonorganized displaced repeat peptide (known as LSA13-29; see Table I) restored both α-helical conformation and antigenicity (H. Gras, L. BenMohamed, M. Bossus, A. Londono, B. Barbier, P. Druilhe, and A. Tartar, manuscript in preparation).

In our search for T cell epitopes, we focused our attention on the hinge region of LSA-1, located just downstream of the repetitive region, for the following reasons: 1) such regions are physicochemically more accessible than constrained, organized structures (e.g., α-helices) are to proteolysis, an event that precedes T cell epitope presentation in association with MHC molecules (reviewed in Ref. 35). This increased accessibility has been shown, for example, in classical experiments demonstrating enzymic digestion of the hinge regions of myosin (36) and Ig (37); 2) B and T cell epitopes have frequently been observed to cluster within a limited region in Ags from a wide range of organisms (38–41). According to our hypothesis, strong T cell epitopes were likely to be found in the immediate vicinities of this hinge, neighboring the

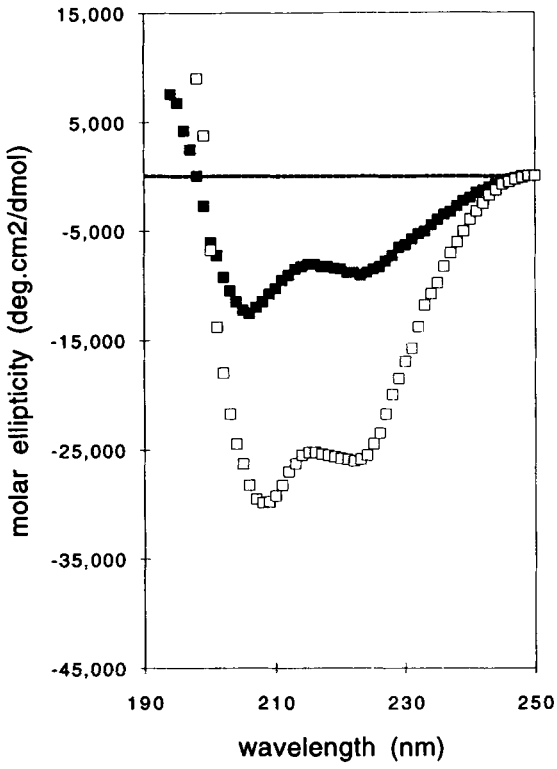


FIGURE 5. CD spectra of the LSA-1 peptide LSA-Rep. This shows the molar ellipticity of this peptide dissolved in aqueous (■) or trifluoroethanol (□) solution. A molar ellipticity of -35.7 degree decimol^{-1} cm^2 at 222 nm was taken as representing 100% helicity (16).

repeat fragment that is known to contain strong B cell epitopes. Three peptides were thus chosen from this area (LSA-J, LSA-NR, and LSA-Ter; see Fig. 1, *A* and *B*). LSA-J represents the junction of the last repeat unit and the next seven residues in the C-terminal nonrepetitive region. Notably, this last repeat unit contains the previously mentioned aa variant at position 4, S → R. LSA-NR represents part of the hinge region and LSA-Ter is from the putative α-amphipathic region downstream. These three peptides were deliberately long (24–34 aa) because T cell determinants corresponding to separate MHC-restriction elements have been frequently found to overlap (11, 42). In parallel, we studied the repeat peptide LSA-Rep (Table I), chosen as a representative of the repeat region.

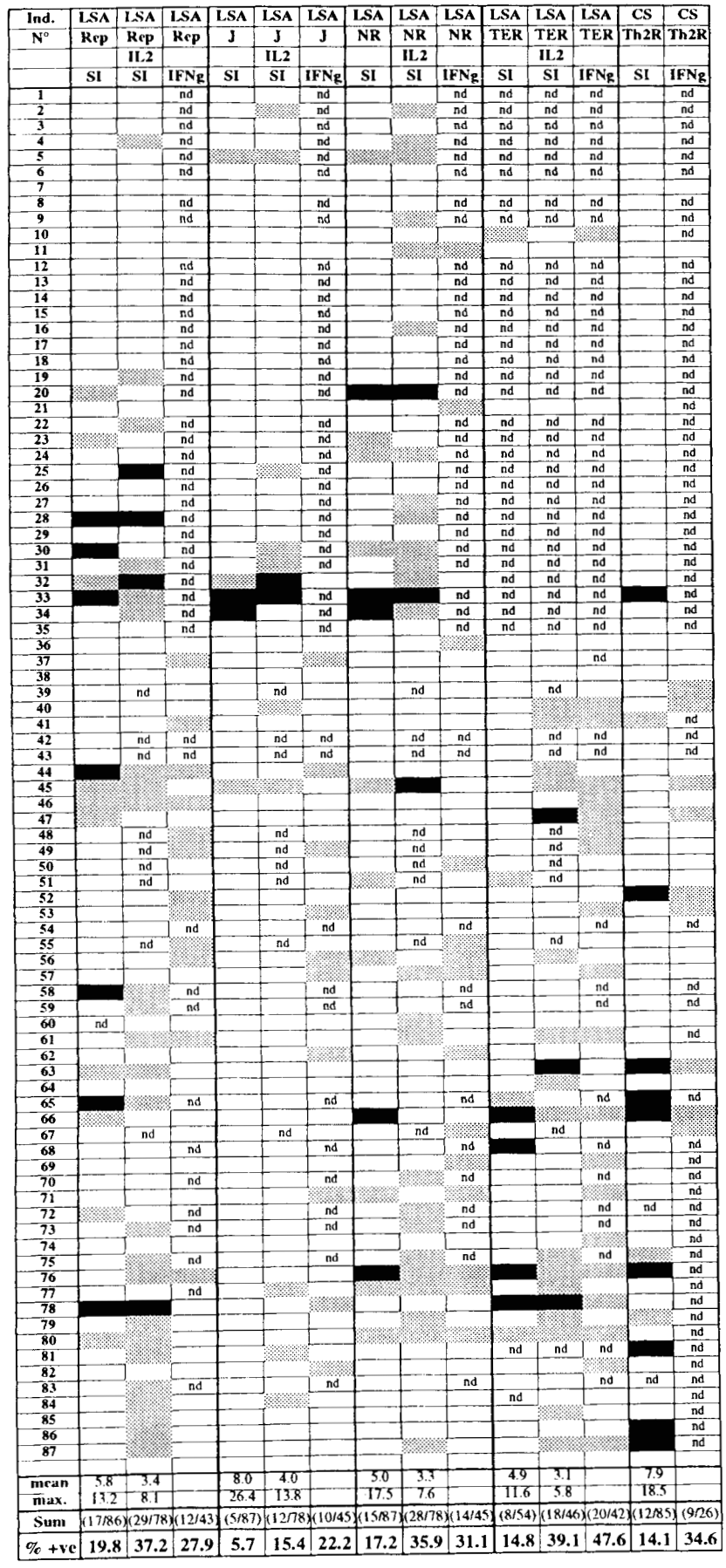
T and B cell responses to LSA-1 in naturally-exposed individuals

Preliminary studies on the four LSA-1 peptides showed that they did not induce any positive lymphoproliferative response (when cells were incubated either in the presence or absence of rIL-2) or IFN-γ secretion when incubated with PBMC from 16 French donors never exposed to malaria (data not shown). Subsequently, these peptides were tested in lymphoproliferation assays of PBMC from inhabitants of the low transmission region of Ankazobé. As a control, PBMC were simultaneously tested for their lymphoproliferative response to the mitogen LeuA and the Ag PPD. Positive responses were assessed by comparison with control wells without Ag. Results were retained for analysis only for those 87 individuals whose PBMC produced a positive proliferative response to LeuA and/or PPD.

Specific lymphoproliferative responses were observed for every LSA-1 peptide, indicating that each of the four chosen sequences contained T cell determinants (Fig. 6). For each of the peptides LSA-Rep, LSA-NR and LSA-Ter, positive lymphoproliferative responses were detected in 15–20% of individuals. LSA-J-specific proliferative responses were detected in 6% of individuals. In comparison, 14% of individuals showed positive lymphoproliferative responses to a Th2R peptide (strain 7G8) representing an immunodominant T cell antigenic region of the CS protein (43). The addition of rIL-2 to Ag-stimulated PBMC cultures, which has been previously shown to partially reverse T cell proliferative unresponsiveness to malaria Ags in semi-immune individuals (44), did indeed reveal that PBMC from a markedly higher proportion of the population could respond at significant levels to the LSA-1 peptides. Under these conditions, the prevalence of responders was in the order of 36 to 39% for each of LSA-Rep, LSA-NR, or LSA-Ter. This, in spite of the sizable increase in background cpm, which in some cases may have masked a positive proliferative response (such cases were nonetheless retained for analysis). Note that the majority of the responders in the standard proliferation assay were also positive in the rIL-2 assay.

Because IFN-γ secretion is a marker of T cell activation frequently dissociated from proliferative response (45) and in view of the known susceptibility of *P. falciparum* LS to IFN-γ (46), the ability of T cells to secrete this cytokine in response to stimulation with LSA-1 peptides was tested, using a capture ELISA. Responses were considered positive when >2 IU/ml of IFN-γ were secreted into Ag-stimulated PBMC supernatants from standard lymphoproliferation assays. Overall, positive responses were detected in a large proportion of individuals, ranging from 22% for LSA-J to 48% for LSA-Ter.

In all, 32% of the population sample from this low transmission region showed a positive proliferative response to at least one of the LSA-1 peptides in the standard assay (no rIL-2). This proportion increased to 73% when proliferative responses in the presence or absence of rIL-2 were considered. A total of 73% of the population sample was similarly found able to secrete IFN-γ in response to one or more LSA-1 peptides. Statistically, no significant associations were observed for any of the test peptides between either 1) IFN-γ secretion and T cell proliferation; 2) age and either of these two modes of T cell response; or 3) presence or absence of detectable *P. falciparum* blood stage parasites at the time of sampling and



Ind.	LSA	LSA	LSA	LSA	LSA	LSA	LSA	LSA	LSA	LSA	LSA	LSA	CS	CS
N°	Rep	Rep	Rep	J	J	J	NR	NR	NR	TER	TER	TER	Th2R	Th2R
		IL2			IL2			IL2			IL2			
	SI	SI	IFNg	SI	SI	IFNg	SI	SI	IFNg	SI	SI	IFNg	SI	IFNg
mean	5.8	3.4		8.0	4.0		5.0	3.3		4.9	3.1		7.9	
max.	13.2	8.1		26.4	13.8		17.5	7.6		11.6	5.8		18.5	
Sum	(17/86)	(29/78)	(12/43)	(5/87)	(12/78)	(10/45)	(15/87)	(28/78)	(14/45)	(8/54)	(18/46)	(20/42)	(12/85)	(9/26)
% +ve	19.8	37.2	27.9	5.7	15.4	22.2	17.2	35.9	31.1	14.8	39.1	47.6	14.1	34.6

FIGURE 6. Individual responses to LSA-1 and CS peptides in Ankazobé, Madagascar. IL-2: SI calculated for sample wells to which exogenous rIL-2 was added. Proliferative responses were considered positive if the SI was ≥2 and the δcpm exceeded 1000. □: negative response; ▨: positive response with $2 \leq SI < 5$; ■: positive response with SI ≥5. For IFN-γ, □: negative response; ▨: positive response (supernatant quantity ≥2 IU/ml). PBMC from native Malagesh individuals with no known history of malaria and with no detectable Abs to malaria Ags were tested in parallel, with and without rIL-2, and were consistently negative in terms of either T cell proliferation or IFN-γ secretion in response to the LSA-1 peptides. Background cpm were: 1) in standard assays without rIL-2: geometric mean = 877; range = 157–4,147; 2) in rIL-2 assays: geometric mean = 2,236; range = 238 to 12,740).

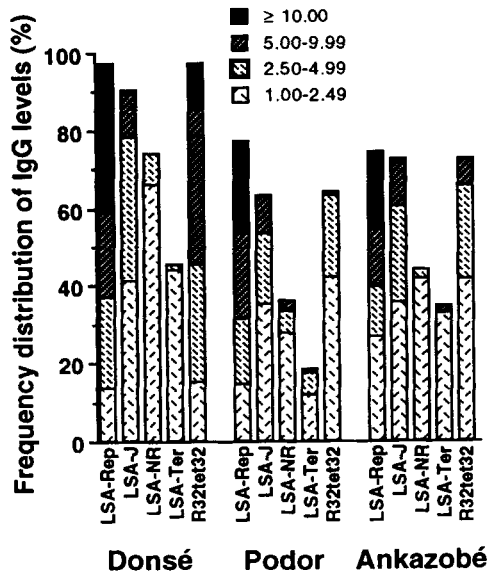


FIGURE 7. IgG Ab prevalence data for LSA-1 and CS Ags. Sample sizes were 73 from Donsé, 109 from Podor, and 98 from Ankazobé. Individual IgG levels were calculated in AU (as defined in *Materials and Methods*), where AU values ≥1 were considered as indicative of a specific Ab response. The overall frequency of Ab carriers equals the sum of the frequency of Ab carriers whose IgG levels fell into one of the four strata indicated.

T cell proliferative response profiles (i.e., background counts, responses to peptides, or control stimuli).

Sera from a total of 280 individuals living in the high transmission region of Donsé (Burkina Faso) or the low transmission regions of Podor (Senegal) or Ankazobé (Madagascar) were studied by ELISA. Ab responses were detected for all four LSA-1 peptides, indicating that they all contained B cell epitopes. As shown in Figure 7, the Ab prevalence was consistently the highest for the 2.5-repeat peptide LSA-Rep, reaching 97% in Donsé (the two nonresponders both being young children), 77% in Podor, and 75% in Ankazobé. Elevated prevalence rates were also observed with R32tet32, containing the CS repeats and with LSA-J, containing the C-terminal variant repeat. Individual Ab levels were classified into four strata (≥1, ≥2.5, ≥5, or ≥10, where ≥1 signifies a positive response; corresponding to low, medium, high, or very high Ab levels) as a means to compare relative amounts of Ab to each peptide. In all three population samples, markedly higher levels of Abs were found against LSA-Rep than against all other LSA-1 peptides or against R32tet32. Considering the low transmission rates prevailing in Podor and Ankazobé, it was notable that very high Ab levels were found against LSA-Rep. The nonrepeat peptides, LSA-NR, and to a lesser extent, LSA-Ter, contained B cell epitopes that are relatively minor as compared with those present in the repeat regions. LSA-NR nevertheless was recognized by 74% of individuals from Donsé, although only a few individuals had medium to high Ab levels.

Comparison of mean IgG Ab levels to the separate peptides in the three population samples showed that these were consistently higher in the high transmission area of Donsé (Table II). Furthermore, for each of the three endemic areas, the highest Ab levels were recorded for LSA-Rep. Statistical comparison of the mean Ab levels to each peptide in the separate age groups (divided into: ≤ 5, 6 to 10, 11 to 20, 21 to 40, and > 40 years of age) was then performed, using the analysis of variance (ANOVA) test. This revealed that Ab levels to each peptide increased significantly with age in both the Donsé and Podor population samples, although no such significant age-related increase was recorded in the Ankazobé sample (Table II). This is illustrated for the repeat-based Ags LSA-Rep and R32tet32 in Figure 8. In Donsé, the mean Ab level to LSA-Rep was already high in children under the age of five and climbed rapidly later in life, with no signs of reaching a plateau. In Podor, Ab levels to LSA-Rep and R32tet32 were generally low in children, rising at a significantly faster rate for LSA-Rep than for R323tet32 in later years. The lack of a significant increase in Ab levels to LSA-Rep or R32tet32 was clearly evident in Ankazobé. This is presumably related to the large socio-economic heterogeneity and diversity in the housing construction found in Ankazobé (as opposed to the essentially homogeneous situation in Donsé and Podor), leading to some Ankazobé individuals being only relatively rarely exposed to *Anopheles* bites, as evidenced by the high proportion of Ab nonresponders in adults (≥21 years) (for LSA-Rep: 24% nonresponders in Ankazobé vs 0% and 2% in Donsé and Podor, respectively; for R32tet32: 24% vs 0% and 14%, respectively). Indeed, in Ankazobé it would seem that individuals in some housing groups are almost never exposed to *P. falciparum* malaria (our unpublished observations). Recent evidence from a heterogeneous population in a malaria-endemic Sri Lankan area has indeed shown that individuals living in better constructed houses have a significantly lower parasite exposure rate (47).

After pooling the data from the 280 individuals, a weak correlation was found between Ab responses to the peptides LSA-Rep and LSA-J (r = 0.50), LSA-J and LSA-NR (r = 0.45) and LSA-NR and LSA-Ter (r = 0.62). Nevertheless, after classification of individuals into positive or negative responders, McNemar χ^2 tests showed a clear dissociation between individual responses to any two peptides. The presence of two distinct B cell epitopes in LSA-Rep and LSA-J, as suggested by the finding that 24% of the individuals having Abs to LSA-Rep were negative for LSA-J, was confirmed by peptide competition ELISAs. Preincubation of hyperimmune serum (with high Ab levels to both of these peptides) with increasing concentrations of one peptide in soluble form resulted in increasing inhibition of recognition of the same peptide (when bound to the ELISA plate), although it had no inhibitory effect on Ab recognition of the other (bound) peptide (Fig. 9). LSA-NR, included in the assay because it shares a 4-aa overlap with LSA-J, showed a marginal inhibition of LSA-J only

Table II. *Mean IgG levels to LSA-1 and CS peptides in the three regions of differing malaria transmission*

Peptides	Donsé		Podor		Ankazobé	
	Mean[a]	Prob[b]	Mean	Prob	Mean	Prob
LSA-Rep	8.88 (0.7–22.8)	<0.01	5.50 (0.3–15.9)	<0.001	5.39 (0.3–22.8)	ns[c]
LSA-J	2.80 (0.6–9.4)	<0.05	2.06 (0.2–12.2)	<0.001	2.38 (0.3–10.7)	ns
LSA-NR	1.38 (0.4–4.2)	<0.05	1.15 (0.3–8.0)	<0.001	1.09 (0.3–4.4)	ns
LSA-Ter	1.06 (0.3–3.1)	<0.05	0.79 (0.2–6.0)	<0.001	1.00 (0.3–6.4)	ns
R32tet32	5.63 (0.8–23.5)	<0.01	1.66 (0.5–5.7)	<0.001	2.13 (0.5–8.4)	ns

[a] Arithmetic mean of IgG levels in the population samples (with observed minimum and maximum levels indicated between parentheses).
[b] Probability that the sets of IgG levels recorded in younger age groups do not differ significantly from those recorded in older age groups. A $p < 0.05$ indicates that IgG levels do increase, on average, with age.
[c] Not significant.

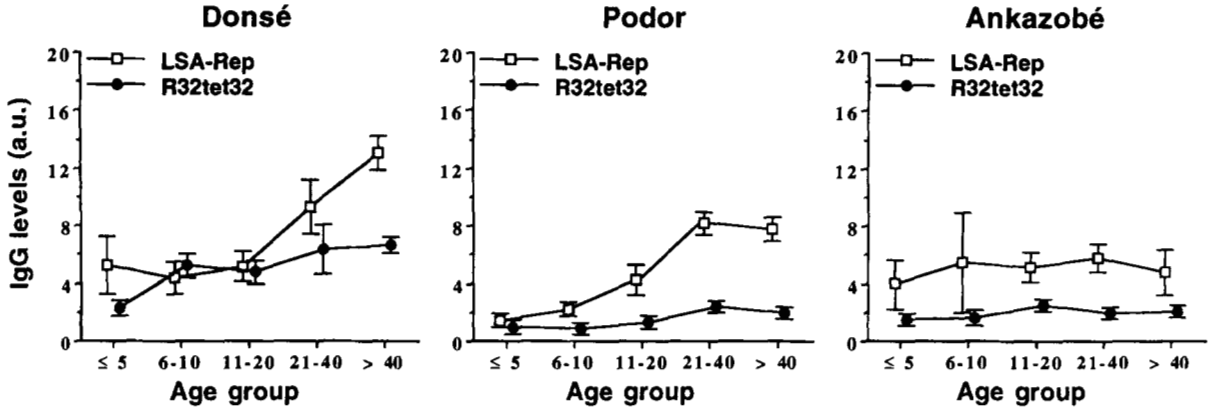


FIGURE 8. LSA-Rep and R32tet32 mean IgG Ab levels, per age group, in the population sample from (*left*) Donsé, (*middle*) Podor, and (*right*) Ankazobé. SE bars are indicated.

at high concentrations and no inhibition of LSA-Rep. It is likely that the novel B cell epitope in LSA-J is caused by the presence of the arginine (R) variant in the place of the usual serine (S) at position 4 of the repeat unit of this peptide. Conversely, competition assays run using two 17-aa repeat peptides differing only in their first residue (E → G; see Table II), revealed that this particular frequently occurring substitution did not engender a distinct B cell epitope (data not shown).

Because the Ankazobé Ab ELISA assays were performed on sera collected the day of blood sampling and PBMC preparation, we compared Ab and T cell (proliferation and IFN-γ secretion) responses. Some degree of correlation was observed between T cell proliferation to LSA-NR (in the presence of rIL-2) and Ab response to either LSA-J ($r = 0.462$, $p < 0.02$), or LSA-NR ($r = 0.417$, $p < 0.03$), although no other correlations were observed. The subset of 18 individuals for which the entire set of T cell and Ab tests had been performed was then analyzed. Of the 15 individuals showing an Ab response to our LSA-1 peptides, 13 also had a detectable T cell response to at least one of the four peptides. Conversely, of the 16 individuals having a detectable T cell response, 13 had Abs to 1 or more LSA-1 repeat peptides. None of these individuals showed a total absence of both Ab and T cell responses to this set of peptides.

To examine whether LSA-1 is the immunodominant LS Ag, we compared our Ab data for the major B cell epitope LSA-Rep with results from previous IFAT titration studies on whole *P. falciparum* LS, performed for 36 and 30 sera from Podor and Donsé, respectively (unpublished data). These IFAT studies had revealed that all sera, from both the low and high transmission regions, contained Abs to LS Ags (Podor: geometric mean of end point titers = 1/2,600; range = (1/200, 1/25,600); Donsé: geometric mean = 1,2,500; range = (1/800, 1,6,400)). For the Podor sera, 28 of the 36 LS-positive sera had Abs to LSA-Rep and there was a good overall correlation between IFAT LS

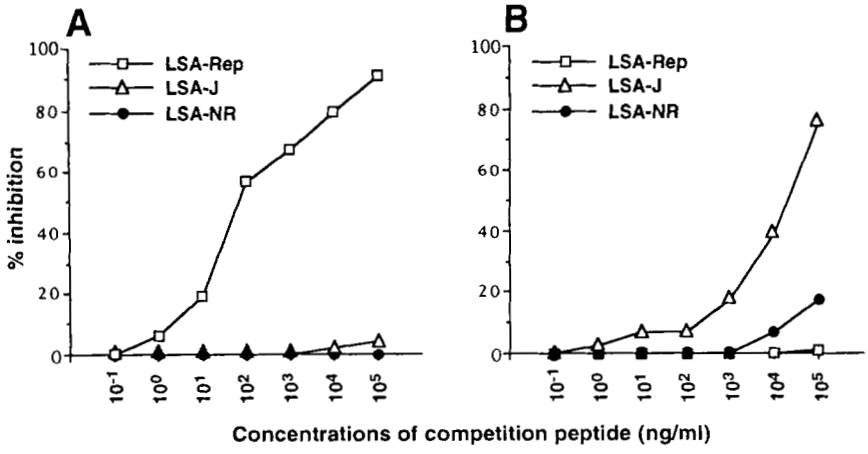


FIGURE 9. Competitive inhibition ELISAs. ELISA wells were coated with (*A*) LSA-Rep or (*B*) LSA-J and incubated with the hyperimmune serum SHI5 in the presence of different concentrations of the test inhibitory peptides LSA-Rep (□), LSA-J (△), or LSA-NR (●). Percentage of inhibition at each test concentration was calculated with respect to the OD_{492} value of the hyperimmune serum incubated in the absence of test inhibitory peptides. No inhibition was ever recorded with the test peptide LSA-Ter. Results are shown for one of three comparable experiments using different hyperimmune sera.

titers and Ab levels to this LSA-1 repeat peptide ($r = 0.64$, $p < 0.001$). In contrast, in Donsé, where natural sporozoite infections are 20 to 100 times more frequent, 28 of the 30 sera also had Abs to LSA-Rep, however, there was no correlation with IFAT LS titers ($r = 0.03$, n.s.), suggesting the co-dominance of other LS Ags.

Discussion

Until recently, the LS of the life cycle of the malaria parasite has attracted little attention. This was caused largely by the difficulty of obtaining LS parasites, particularly in the case of the human malaria pathogens, and to the earlier widely held assumption that this stage was immunologically "silent." It is now evident, however, that immunologic mechanisms can act upon this stage (10, 11). In this report we contribute to current knowledge about the human LS by showing that LSA-1, the only known *P. falciparum* Ag that is expressed solely during this stage, does induce a wide range of immune responses in a large proportion of malaria-exposed individuals. Furthermore, our data indicate that LSA-1 is actively synthesized during liver schizogony and is highly conserved, in the N-terminal and C-terminal regions, at least between the strains T9/96 and NF54.

By repeatedly cultivating *P. falciparum* LS parasites in vitro, so as to obtain sufficient parasite material, we have been able to determine the molecular mass of the LSA-1 Ag as being 200 kDa. Only this single polypeptide was labeled with Abs to LSA-1 and its size agrees closely with that predicted from the gene length. This is the first report of the molecular mass of a *P. falciparum* Ag in the LS. The study of LS forms at different stages of maturity reveals that LSA-1 can be detected from the early (trophozoite) stage of intrahepatic development onward and is found in increasing quantities as the parasite matures. LSA-1 was localized, by immunoelectron microscopy, to the flocculent material present in the parasitophorous vacuole (PV) space. We previously observed that this material is initially contained in small vacuoles present in young LS forms, which subsequently fuse to the plasmalemma and open and pour their contents into the PV space (48, 49). Interestingly, this flocculent material, some of which may adhere to the merozoite, was found to form a stroma in which the hepatic merozoites are released, leading to the hypothesis that it may facilitate their passage to the sinusoidal blood stream and subsequent invasion of erythrocytes (50). It is noteworthy that our protein sequence analysis, supported by circular dichroism studies, have shown that the LSA-1 repetitive region forms an uninterrupted stretch of α-helices which, assuming a helical rise of 1.5Å per aa, is extraordinarily long (ca. 220 nm). This size is compatible only with fibrous structures. Further evidence in favor of the fibrous nature of the LSA-1 polypeptide is provided by the regular spacing of hydrophobic residues all along this stretch, strongly evocative of a relatively flexible α-helical coiled-coil stem. This organization is comparable with that of M proteins, protruding fimbriae of about 50 nm in length that are seen on the surface of Lancefield group A streptococci, and whose role, interestingly, is to protect bacteria from C-mediated phagocytosis (reviewed in Ref. 34).

Analysis of the sequence data for strains T9/96 (of Thai origin) and NF54 (of likely African origin) shows that the LSA-1 gene sequence is highly conserved, both in the repetitive and nonrepetitive regions. No small insertions, or deletions, or strain-specific V regions were observed in contrast to some polymorphic blood stage Ags (29). The nonrepetitive regions are remarkably well conserved for a *P. falciparum* Ag, with nucleotide substitutions occurring in only two positions out of the 1.3 kb, both giving rise to aa substitutions. A recent report of partial sequence of the C-terminal nonrepetitive region (residues 1660–1761, which include the LSA-Ter peptide) of the Honduras clone HB3 (51) also reveals 100% aa identity between these three separate strains. Comparing T9/96 and NF54, the

significantly higher frequency of aa variants in the repetitive region, in comparison with the nonrepetitive regions (5.6% vs 0.5%, respectively; $\chi^2 = 213, p < 0.005$) would suggest that selective forces are acting differentially upon these two regions. Interestingly, diversity in the repeat units is restricted to a limited subset of aa residues, with a high proportion of nonsynonymous substitutions, and generally involves only two possible variants at each of these positions. Because these repeats are now known to contain both B and T cell determinants, it would be revealing to know whether these variants preferentially create new T cell or B cell epitopes, as this may provide evidence as to what type of immune pressure could be operating on LSA-1 repeats.

Our search for LSA-1 T cell determinants outside the repeat region has focused on the C-terminal hinge. Analysis of T cell responses to three peptides from this region (LSA-J, LSA-NR, and LSA-Ter) and to the repeat peptide LSA-Rep, in individuals from the low transmission area of Ankazobé revealed that indeed all four chosen LSA-1 sequences do contain T cell determinants. These determinants are, thus, present in both C-terminal nonrepetitive regions and in the repeats themselves. Responses were the most elevated for the peptides LSA-Rep, LSA-NR and LSA-Ter, being detected in 15 to 20% of individuals in the standard proliferation assay and in 36 to 39% of individuals in rIL-2-supplemented assays. In all, PBMC from 73% of individuals showed a positive proliferative response to at least one of the four LSA-1 peptides. The dissociation of responses to the four LSA-1 peptides would suggest that these epitopes can act in a complementary manner in individuals of diverse MHC haplotypes. Recent studies performed in endemic regions of much higher endemicity in the Congo and Senegal showed that individuals from these countries also recognized T cell determinants in all four LSA-1 peptides (manuscript in preparation). Furthermore, the prevalence of responders varied significantly with time, although it could reach very high levels with, in particular, the peptides LSA-Rep and LSA-NR being recognized by the majority of the population samples in one study. The lower prevalence of responders in Ankazobé could be caused by the lower transmission rates or to the longer interval between blood collection and initiation of PBMC cultures.

The four peptides studied represent only a small region of the LSA-1 molecule and almost certainly represent only a subset of the total T cell determinants present in LSA-1. Nevertheless, the prevalence and magnitude of positive proliferative responses to three of the four LSA-1 peptide studies compared favorably with those observed with a Th2R peptide (strain 7G8), identified as being one of the major human T cell determinants of the CS protein (6). The prevalence of responders to this latter peptide in the low transmission area of Ankazobé is coherent with previous findings on Th2R proliferative responses from other endemic regions (reviewed in Ref. 52). In contrast to the CS Th2R region, which is known to be highly polymorphic (11, 53), present evidence suggests that the identified C-terminal LSA-1 T cell determinants are well conserved.

IFN-γ production in response to LSA-1 peptides was also studied because IFN-γ is the most potent cytokine known to act upon *P. falciparum* liver schizogony in vitro and is effective even at very low concentrations (0.1 IU/ml) (46). Moreover, IFN-γ-mediated inhibition of LS is one of the few mechanisms whose effect has been confirmed in vivo in both primate and rodent models (reviewed in Ref. 54). LSA-1 peptides were found to induce Ag-specific IFN-γ secretion in PBMC of a large proportion of individuals, ranging from 22% for LSA-J to 48% for LSA-Ter. Secretion of this cytokine in response to LSA-1 Ag stimulation was dissociated from the T cell proliferative response in the PBMC of individual donors. Such a dissociation is frequently found with *P. falciparum* Ags (55). Considering the subset of 18 individuals for which all tests were performed, it was remarkable that in this low transmission region, 17 of these individuals responded either by IFN-γ production or T cell proliferation to at least one LSA-1 peptide.

In this study we confirm and extend our preliminary observations (4) that the LSA-1 repeat units encode major B cell determinants. Indeed, even in the low transmission areas of Podor and Ankazobé the proportion of Ab carriers to LSA-Rep exceeded 75%. Our Ab data, together with the fact that repeats were contained in all of the original 26 Ab-selected LSA-1 clones, indicates that the repeat region contains the major B cell determinants in LSA-1. These are, however, not the only B cell determinants, as evidenced by the finding of a notable B cell epitope in the C-terminal peptide LSA-NR, Abs to which were found in 74% of individuals in the high transmission area of Donsé. In terms of both prevalence and mean IgG Ab levels, the response to the repeat peptide LSA-Rep in the study populations exceeded that observed with the comparative Ag R32tet32, encoding the CS repeats. The prevalence rates of Ab responders to CS repeats in our study is, nevertheless, in good agreement with those observed in high transmission and low transmission areas of Burkina Faso (56), of comparable transmission rates with Donsé and Podor, respectively.

Further evidence indicating that LSA-1 is highly immunogenic in exposed individuals comes from the finding that Abs to LSA-1 repeats were found in half of the children under 5 yrs of age in the low transmission areas of Podor and Ankazobé. Given that in these two areas the number of infectious bites/individual is in the range of one to five per year, with an estimated average of ca. 10 sporozoites entering the bloodstream (57), a proportion of which are unlikely to reach host hepatocytes, it is remarkable that the LSA-1 Ag is capable of inducing such a high degree of immunologic response. This is consistent with our finding of potent T cell epitopes in LSA-1.

Preliminary studies aimed at identifying the phenotype of the lymphocytes that proliferated in response to these LSA-1 peptides would indicate that both $CD8^+$ and $CD4^+$

lymphocytes were present in the population of proliferating cells. Typing of blastic cells using either anti-CD4/anti-CD8 or anti-CD4/anti-CD11 mAb (Coulter) on Ag-stimulated PBMC of two individuals showed that a substantial proportion of the responding T cells were of the CD8 phenotype (28 to 36% of the total proliferating lymphocytes in response to either LSA-J, LSA-NR, or LSA-Ter). Studies to address this issue in greater detail are presently ongoing.

How the LSA-1 Ag is presented to the immune system is currently unknown. This could conceivably occur either after transport of LSA-1 material outside of the distended hepatocyte, or as a consequence of host defense-driven lysis of the infected cell interrupting normal liver schizogony, or by contact with the LSA-1 containing stroma in which the thousands of mature hepatic merozoites emerge.

Recent work has demonstrated that a wide range of B and T cell-dependent effector mechanisms can operate upon infected hepatocytes. Nevertheless, the majority of these mechanisms have been described in experimental models and are known to vary depending on the host/parasite combination (reviewed in Refs. 10, 11, and 54). In the case of *P. falciparum* in humans, the relative importance of these effector mechanisms still needs to be clarified. Our present study demonstrates that the purely LS-specific Ag LSA-1 contains potent B and T cell epitopes. This complements the recent finding (5) of two MHC class-I restricted CTL epitopes near the C-terminal extremity of LSA-1 (aa 1786–1894 and aa 1850–1857, situated more than 60 aa downstream of our most N-terminal peptide LSA-Ter), which were found to be conserved in a wide range of field isolates and are equally conserved in T9/96 and NF54. Whichever mechanisms turn out to be the most critical for *P. falciparum*, it is clear that LSA-1 is capable of eliciting a wide range of immune responses, namely, Ab production, T cell proliferation, IFN-γ secretion, and CTL activity. That such a repertoire of responses are generated against this Ag in individuals living in endemic regions would suggest that they may participate in providing naturally acquired partial protection against pre-erythrocytic stages, which by optimal immunization may be enhanced to a state of full protection.

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