A Novel *Plasmodium falciparum* Sporozoite and Liver Stage Antigen (SALSA) Defines Major B, T Helper, and CTL Epitopes[1]

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In the search for subunit vaccines that are able to induce the type of sterile, protective immunity achieved by irradiated sporozoites, there is increasing evidence that defense mechanisms directed at the intrahepatic stage and Ags expressed at this stage are critical. We have initiated a systematic search for such molecules and report here the identification and partial characterization of a novel *Plasmodium falciparum* gene encoding a 70-kDa protein, expressed in both sporozoite and liver stages (SALSA), with a vaccine potential that stems from its antigenic features. Antigenicity and immunogenicity studies were conducted in individuals exposed to malaria, in immunized mice, and in chimpanzees, using a recombinant protein and two synthetic peptides. Results show that the SALSA nonrepetitive sequence defines 1) major B cell epitopes, as shown by a high prevalence of Abs to each peptide in three African areas differing in their level of endemicity; 2) Th epitopes, as demonstrated by lymphoproliferation and IFN-γ secretion in cells from the individuals from one of the low transmission areas, as well as helper effect upon Ab secretion in mice; and 3) epitopes for cytolytic lymphocytes, demonstrated in immunized and sporozoite-challenged chimpanzees, and associated with MHC class I leukocyte Ags. The latter are of particular importance, because this is the only part of the malaria life cycle in which the parasite is located in a cell expressing class I Ags and because $CD8^+$ lymphocytes were found to be responsible for protection in experimental models. *The Journal of Immunology,* 1996, 156: 2874–2884.

Research to develop a malaria pre-erythrocytic stage vaccine stems from a very unusual and therefore striking phenomenon in the field of immunity to parasites, namely that the immunity resulting from the injection of irradiated sporozoites is a total, sterilizing resistance, allowing the vaccinee to fully resist challenge by large numbers of virulent parasites. Initially, because this state was induced by injection of sporozoites, research focused primarily on sporozoite surface molecules, and in particular on the major circumsporozoite (CS)[3] surface molecule. However, CS immunization in many formulations has always been disappointing in comparison with that obtained with attenuated sporozoites (1).

There is consequently a recognized need to investigate the potential of other pre-erythrocytic stage Ags. Growing evidence indicates that those Ags expressed during liver stage development are critical. A requirement for postsporozoite developmental Ags was indicated when only those sporozoites injected i.v. that retained the capability to transform into intrahepatic trophozoites were protective (2) and could remain in the liver for prolonged periods of time (3, 4). Parallel in vivo/in vitro experiments established a clear-cut relationship between protection and the ability of sporozoites to invade the hepatocytes (5). The destruction of these live liver trophozoites by primaquine treatment reversed protection (4).

Liver stages appear to be targets for more varied and more efficient defense mechanisms than sporozoites alone. Views have moved from a focus on Ab-dependent mechanisms directed against free sporozoites, prevalent 10 to 15 yr ago, to a focus on incorporating (based on rodent experiments) a wide range of effectors such as ADCC-like inhibition; a large number of leukocyte-derived mediators, of which the cytokines IFN-γ, IL-1, and IL-6 are the most efficient, as well as free oxygen radicals and nitric oxide intermediates; and direct cytolysis by lymphocytes of the infected hepatocytes (6). In human malaria, far less is established regarding the effectors of immunity induced by irradiated sporozoites. It is known that Abs and IFN-γ have a substantial, although subtotal, effect against *Plasmodium falciparum* (7, 8). Although direct evidence for CTL activity against infected hepatocytes is still lacking, indirect indications were obtained from epidemiologic studies (9, 10).

Several years ago, as evidence for the critical role of intrahepatocytic parasite development emerged, we initiated a detailed study of the antigenic content of *P. falciparum* pre-erythrocytic stages. We confirmed the existence of several non-CS sporozoite surface molecules (11) and developed a systematic strategy to identify, characterize, and screen liver stage-expressed molecules (12).

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[3] Abbreviations used in this paper: CS, circumsporozoite protein; LS, liver stages; LSA-1, liver stage antigen-1; SALSA, sporozoite and liver stage antigen; SI, stimulation index; IFA, immunofluorescence assay; STARP, sporozoite threonine- and asparagine-rich protein; RT, reverse transcriptase; aa, amino acid; MPES, malarial pre-erythrocytic stages.

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Table I. *Choice of discriminating sera*[a]

Sera	IFA-Sporozoite	ELISA-CS
1	<100	0.7
2	400	1.8
3	6,400	11.8
4	6,400	1.1
5	1,600	5.8
6	800	5.6
7	12,800	0.6
8	6,400	8.6
9	<100	1.9
10	25,600	0.9

[a] Results of detection of Abs directed to whole sporozoite surface molecules (IFA reciprocal titers) and to circumsporozoite Ag repeats (ELISA ratios to values from healthy controls + 3 SD). The three discriminating sera that were used to select non-cs (i.e., SALSA) Ag are underlined.

LSA-1 was the first to be reported (13); more recently we have described the sporozoite threonine- and asparagine-rich protein (STARP).

We report here the identification of a novel sporozoite and liver stage antigen (SALSA) and describe antigenicity studies in humans and immunogenicity studies in animals that demonstrate that SALSA-derived epitopes, which are not defined by a repetitive structure, have valuable immunologic properties that may contribute to an effective malarial vaccine.

Materials and Methods

Antibodies

Discriminating sera. Sera were selected as follows. Among subjects living in malaria endemic areas, we had previously observed a frequent discrepancy between the results from sporozoite surface labeling and recognition of the CS repeats (11), suggesting the prevalence of high titers of Abs to non-CS surface molecules. In a series of 150 individuals, we thus selected sera with high Ab titer to sporozoite surface components in a "wet" sporozoite immunofluorescence assay (IFA) (14), yet having low or no detectable Ab to CS, as measured by ELISA (Table I) and by Western blots (not shown). The three discriminating sera that were used to select non-CS, i.e., the SALSA Ag, are those underlined in Table I.

Two discriminating sera for identifying new liver stage Ags were selected in a similar manner. We chose individual endemic sera that had high Ab titers by IFA to *P. falciparum* liver stages and yet were very low or negative when tested by ELISA on B cell epitopes containing peptides from the LSA-1 Ag (13, 15).

Sera directed to heterologous malaria species. To assess the species specificity of the new molecule, we employed four sera from *Plasmodium vivax* infections, two from *Plasmodium ovale*, two from *Plasmodium malariae*, two from *Plasmodium cynomolgii* infections in man (16), one from *Plasmodium berghei*, and two from *Plasmodium yoelii* in mice. These sera were chosen because they contained significant amounts of Abs when assayed by IFA on Ags (sporozoites and blood stages) from the homologous species. Finally, we used three mAbs identified as specific for three non-CS sporozoite surface Ags of *P. yoelii* (17).

Tranfusion malaria sera: to assess the stage specificity of SALSA, we also used five sera from French patients who acquired *P. falciparum* blood stage infection by an accidentally contaminated blood transfusion (18). These sera were collected after treatment and had IFA Ab titers to *P. falciparum* asexual blood stages ranging from 1/600 to 1/16200, whereas they were negative when tested on *P. falciparum* sporozoites and liver stages (as described below (*Ab assays*)).

Affinity-purified antibodies. Monospecific polyclonal Abs were affinity purified onto the recombinant proteins by serial absorption of Abs from eight hyperimmune human sera that had been depleted of Abs reactive with β-galactosidase. The recombinant proteins, adsorbed on isopropylthiogalactoside-impregnated nitrocellulose filters (BA 85, Schleicher & Schuell, Dassel, Germany), were incubated serially with each of the hyperimmune sera and washed extensively. Abs were eluted at pH 2.5 in glycine buffer, neutralized, and concentrated using a Minicon apparatus (Amicon Corp., Beverley, MA).

Anti-peptide Abs were prepared by affinity purification from human sera on ELISA plates coated with each of the two synthetic peptides derived from the SALSA Ag according to the technique of Brahimi et al. (19). In this case, single sera chosen on the basis of their ELISA titer to the corresponding peptides were used.

DNA techniques

Screening of a genomic DNA library. Three of the discriminating sera shown in Table I, and two others that were positive on LS and negative on LSA-1, were employed to screen a subset of 120 *P. falciparum* genomic DNA clones. These were formerly identified as encoding mainly for pre-erythrocytic stage Ags (13); they were spotted onto nitrocellulose discs impregnated with isopropylthiogalactoside, as described above, and reacted with Abs. Following washings, the reaction was revealed with anti-human IgG peroxidase-labeled second Abs (Biosys, Compiegne, France; used at 1/500 dilution).

To determine the relationship between SALSA-DG671 and a series of other pre-erythrocytic stages recombinant clones, the ^{32}P-labeled DG671 DNA insert was hybridized in 6× SSC buffer at 65°C overnight with DNAs (transferred to a Hybond N membrane, Amersham, Buckinghamshire, U.K.) from the above subset of 120 clones encoding stage-specific pre-erythrocytic Ags (13, 12); the membranes were then autoradiographed.

Sequence analysis. λgt11+DG671 DNA was prepared from a liquid phage lysate and digested by *Eco*RI, and the gel-purified *Eco*RI insert was subcloned into pUC18. The clone pUC18+DG671 obtained was sequenced from both stands using the dideoxynucleotide chain termination method. The sequence data were obtained from *P. falciparum* clone T9/96.

PCR studies. The DNA from 23 *P. falciparum* lines and isolates (NF54, Palo Alto cultured strains, 5 isolates from Thailand, and 16 from the village of Dielmo, Senegal, West Africa) were prepared by phenol-chloroform extraction (20) and amplified by PCR using the oligonuleotides indicated in Figure 1. The PCR was performed for 40 cycles with the following sequence: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and thereafter 75°C for 5 min, using Taq polymerase (Cetus Corp., Emoryville, CA) in a Hybaid thermal reactor. The amplified products were electrophoresed in 4% Nusieve-agarose-genetic technology grade gel and examined under UV light. These were transferred to nitrocellulose and probed with ^{32}P-labeled DG671.

For RT-PCR, NF54 sporozoite RNA was prepared as described (21) and enriched using a Micro-FastTrack mRNA isolation kit according to the manufacturer's instructions (Invitrogen, San Diego). RT-PCR was performed from 1.5 μg of mRNA using the Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) and Taq polymerase (Amersham) with the same oligonucleotides and the same amplification program as described above.

Study areas and subjects

The three areas of field study have been previously described in detail (15). The individuals studied ranged in age from 1 to 75 yr. The village of Podor is located in the Northern part of Senegal, an almost desert-like part of the Sahel. The transmission of malaria by mosquitoes is seasonal, as in the other two areas, and there was estimated to be an average one infective bite per person per year (22), with relatively large yearly variations (one to five) depending on the amount of rainfall. Donse is in the savannah part of Burkina Faso, 50 km north of Ouagadougou. Malaria transmission reaches 100 infective bites/individual/year (14), which is high, although average by African standards. Ankazobe, where the population is mainly of Asian origin, is one of the rare villages in the highlands of Madagascar where transmission (10 infective bites/individual/year on average) has remained uninterrupted over the past 20 yr. There are large variations from one household to another, due to greater differences in the building materials employed as compared with the very homogeneous habitat of the other two villages, and depending also on the distance from the main breeding site, which is the rice fields. For T cell studies, after informed consent was obtained from the donors or their parents, blood was collected on Liquemine (Roche, Basel, Switzerland), a preservative-free heparin, and PBMC were processed in our laboratory in Antananarivo, Madagascar within 4 to 6 h following sampling.

Ab assays

Immunofluorescence and immunoelectron microscopy of sporozoites and liver stages. IFA on whole sporozoite surface Ags were performed using wet sporozoites, NF54 strain, and sporozoites obtained after feeding *Anopheles dirus* on gametocytes from four Thai patients (as described in Ref. 14) and expressed as the reciprocal of the last positive serum dilution. *P. vivax, P. yoelii*, and *P. berghei* sporozoites were produced in our laboratory by feeding *Anopheles stephensi* mosquitoes on patient gametocytes

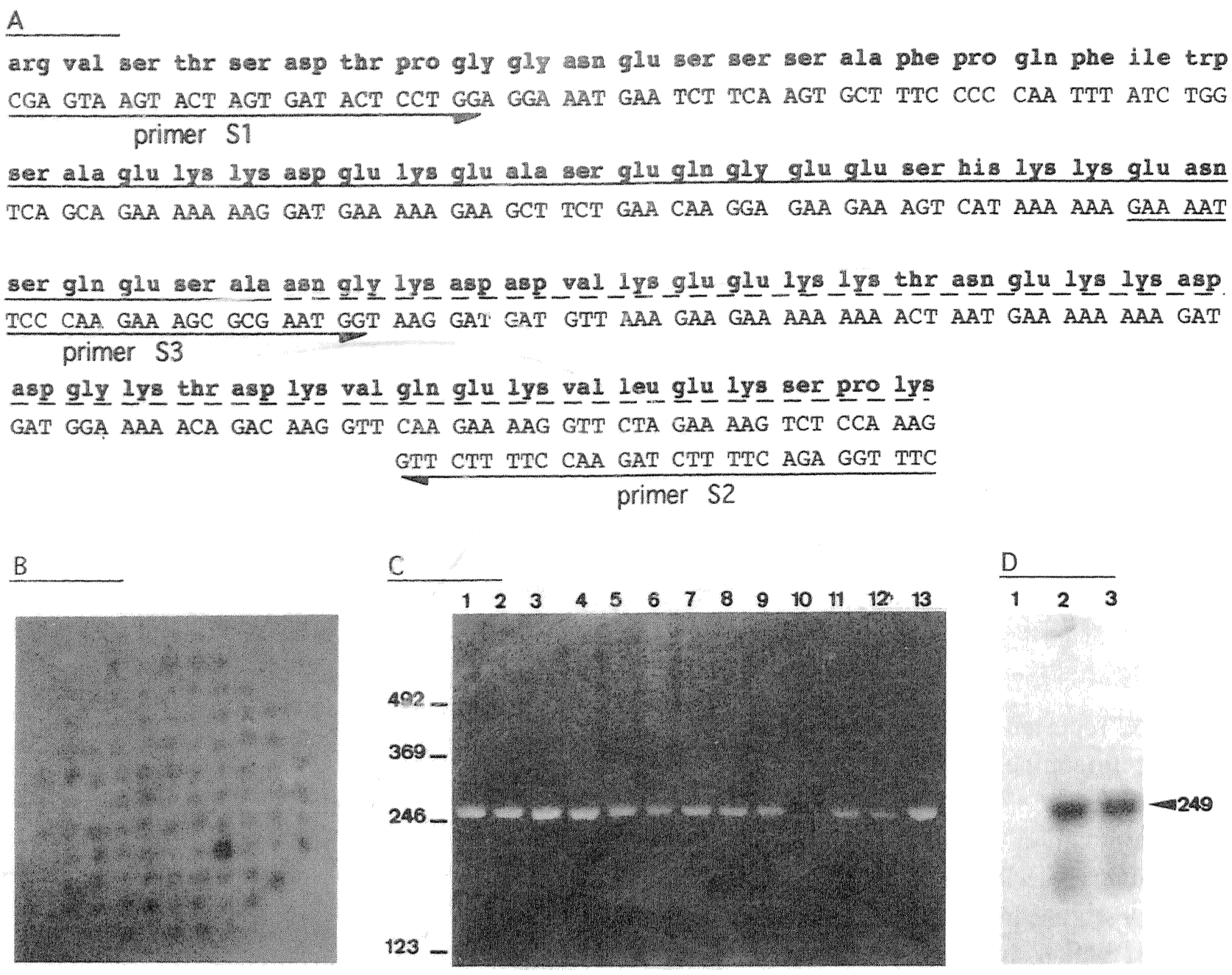

FIGURE 1. *A*, DNA and amino acid sequence from DG671 deduced from the single open reading frame. The underlined nucleotides correspond to the primers S1 and S2 used for DNA amplification by PCR, S3 being used as a probe. The underlined amino acid sequences correspond to the two synthetic peptides, SALSA-1 (solid line) and SALSA-2 (dotted line). *B*, Hybridization of the ^{32}P-labeled DG671, with the 120 clones corresponding to pre-erythrocytic-stage Ags. *C*, Results obtained by PCR with 11 (*lanes 1–9, 11–12*) of the 23 *P. falciparum* lines and isolates studied, and controls (T−, 10; T+, 13), are shown. Probing with ^{32}P-labeled primer located internally in SALSA sequence (primer S3) revealed only one band (not shown). *D*, Results obtained by RT amplification of sporozoite RNA. *Lane 1*, Negative control; PCR performed using the primers S1 and S2 with sporozoite mRNA (without RT) showing the absence of contaminating DNA. *Lane 2*, RT-PCR peformed on NF54 sporozoite mRNA using the primers S1 and S2. *Lane 3*, Control PCR with genomic NF54 DNA. After transfer to nylon, the blot was probed with primer S3.

in artificial feeders or infected mice, respectively. IFA assays with LS from the human species *P. falciparum*, *P. vivax*, and the rodent species *P. yoelii* and *P. berghei*, were performed using the materials and the techniques described in Reference 18. In addition, for *P. falciparum* LS we used schizonts derived from three distinct isolates: in the *Cebus apella* monkey, using sporozoites derived from an African isolate containing gametocytes (18); in *Saimiri sciureus*, from another wild African isolate (P. Druilhe et al., unpublished material); and in a chimpanzee infected with NF54 strain sporozoites (23). IFA on asexual blood stages was performed using acetone-fixed thin smears of either *P. falciparum* (NF54, Palo Alto)-, *P. vivax* (patient isolate)-, *P. yoelii* (17XNL strain)-, or *P. berghei* (ANKA strain)-infected RBCs. In other assays, *P. falciparum*-infected RBC were fixed with either HCl (0.1) or methanol. For all IFAs, the second FITC-labeled Ab was either goat anti-human Ig (Pasteur Institute production), or rabbit anti-mouse Ig (Biosys), diluted 1/200 in PBS supplemented with 1/10,000 Evans blue counterstain.

Ultrastructural studies were performed with *P. falciparum* sporozoites and LS material. Affinity-purified Abs as described above under *Antibodies* were allowed to react with: 1) liver sections containing 6-day-old NF54 *P. falciparum* liver forms obtained from chimpanzees (23), fixed in glutaraldehyde-paraformaldehyde, and embedded in LR white; Abs were revealed using protein A-gold particles, 10 nm in diameter (24); or 2) sections of NF54 sporozoites fixed in 0.1% glutaraldelyde/1% paraformaldehyde, embedded in Epon, and revealed with protein A-gold particles, 30 nm in diameter (25).

ELISA assays. ELISAs were performed by coating microtiter plates with either 1) a 10-μg/ml solution in PBS of the recombinant R32tet32 ($NANP_{30}$-$NVDP_2$) (a gift from Mitchell Gross, Smith Kline Beecham), or 2) a 10-μg/ml solution in Tris-buffered saline of peptides SALSA-1 (SA EKKDEKEASEQGEESHKKENSQESA), or 3) SALSA-2 (NGKDDVKE EKKTNEKKDDGKTDKVQEKVLEKSPKEF) (see also the localization of sequences in Fig. 1), and then studying the sera at a 1/100 dilution (described in Ref. 26). Results are expressed as the ratio of the mean ODs from test sera to the mean OD + 3 SD from 10 healthy individuals studied in parallel in the same plates; results are taken as positive for ratios >1.

Immunoblotting of native and recombinant proteins. SDS-PAGE was run in a 7.5% acrylamide gel. *P. falciparum* sporozoites (NF54 strain and 3D7 clone) were obtained by dissecting salivary glands from *A. stephensi* mosquitoes infected 16 days previously with cultured gametocytes and then boiled in SDS-2-ME buffer. The reaction was revealed by 1) anti-SALSA-DG671 affinity-purified human Abs prepared as above (see *Antibodies*); 2) a serum diluted 1/100 from the chimpanzee Bart immunized by the DG671 recombinant protein, sampled before exposure to sporozoites (see below); 3) two African human sera, titrating 1/1600 and 1/6400, respectively, by IFA assay on sporozoite surface Ags, used at 1/100 dilution; 4) Abs to DG307, a control clone of the LSA-1 Ag (13) not expressed at sporozoite stage, affinity-purified from the same sera as DG671; or 5) a mAb directed to the CS protein repeats (clone NFS2) (27) diluted 1/50,000. After washings, the Western blots were incubated with ^{125}I-labeled goat anti-human IgG, IgA, or IgM (Biosys), or anti-mouse IgG (Biosys), followed by autoradiography at −70°C.

The affinity-purified human Abs to DG671 were also tested for reactivity with the set of recombinant proteins from the same 120 clones as described above (see *DNA techniques*) and then adsorbed onto nitrocellulose filter. They were revealed with anti-human IgG, IgA, and IgM Abs labeled with peroxidase and diaminobenzidine substrate.

To study the prevalence of Ab responses to SALSA, LSA, and CS recombinant molecules in 30 individuals from each of the three villages, Western blots of SALSA (clone DG671), LSA1 (clone DG307) and CS (clone DG705) recombinant proteins were performed as described above, reacted with 1/100 dilutions of each of the 90 sera, and revealed by peroxidase-labeled second Abs.

Lymphocyte assays

Proliferation assays. PBMCs from 111 individuals living in the village of Ankazobe and from 22 control, non-malaria-exposed donors from Antananarivo, were collected in preservative-free heparin (Liquemine), separated on Ficoll-Hypaque gradient, and distributed at a rate of 2.5×10^5 cells per

well in round-bottom 96-well microtiter culture dishes (Nunc, Roskilde, Denmark) filled with 200 μl of RPMI medium supplemented with 10% human AB serum, 3.7 g of sodium bicarbonate, 2 mM glutamine, 1% sodium pyruvate, and 10 mM HEPES per liter. The peptides were added at 10-μg/ml concentrations in quadruplicate wells. Control wells included no Ag, the lectin leukoagglutinin, and purified protein derivatives, both at 10 μg/ml. All plates were incubated for 6 days at 37°C in 5% CO_2 air mixture; on day 6, 100 μl of cell-free medium was removed for IFN-γ assay and replaced with 100 μl of fresh medium containig 1 μCi of [^{3}H]TdR. Incorporation was counted in a liquid scintillation spectrophotometer from cells collected on day 7. Stimulation indices (SI) were calculated as the ratio of the geometric mean cpm in quadruplicate test wells to the geometric mean of quadruplicate control wells (i.e., without Ag). A proliferation was considered positive when the SI was >2, with a Δ cpm (difference between signal and background values) >1000 cpm.

IFN-γ assay. The IFN-γ concentration in pooled supernatants from the quadruplicate wells was assessed by a two-site capture ELISA performed in duplicate using two anti-human IFN-γ (mAbs were kindly given by Mrs. Cousin, Roussel Uclaf, Paris). Wells were coated for 48 h at 4°C with 50 μl of the anti-IFN-γ mAb RU 40.2 at 5 μg/ml in PBS and further incubated with BSA (3%) in PBS as blocking buffer. Undiluted supernatant from Ag-stimulated T cells (50 μl/well) was added for 16 h at room temperature, and the wells were then washed three times with PBS-Tween, 0.1%. A second anti-IFN-γ mAb, RU 308.7, coupled to peroxidase was added in the wells at a 0.8 μg/ml concentration and left for 24 h at 4°C. The wells were washed three times and the activity was revealed after a 15-min incubation at room temperature with 100 μl of reaction substrate (OPD 1 mg/ml, H_2O_2 30%:1 μl in citrate buffer, pH 5.0), and the absorbance was read at 450 nm on a Dynatech reader. The IFN-γ content of supernatants was calculated from standard curves performed with the same culture medium, containing known amounts of IFN-γ (1000–0.1 IU/ml), included in each plate.

Cytotoxic T lymphocyte assays. CTL assays were performed using PBLs from the SALSA-immunized chimpanzees, Bart and Socrates, and four control chimpanzees (Dirk, Ruud, Cor, and Peer). PBLs were separated on Ficoll-Hypaque, resuspended in RPMI 1640 supplemented with 10% human AB serum, and restimulated by incubation of cells with either SALSA-1 or SALSA-2 peptides (10 μg/ml of each peptide) in 25-cm^2 Falcon flasks. IL-2 (rIL-2, Genzyme, Cambridge, MA) was added (20 IU/ml) at 72 h, and assays were performed after 7 days of culture at 37°C in 7.5% CO_2/air.

The ^{51}Cr release assays were conducted using peptide-pulsed cells, either autologous EBV-transformed B (EBV-B) lymphocytes or PHA blasts, as target cells. The PHA blasts were generated by stimulating 10^6 autologous PBMC with 0.5 μg/ml of L-PHA for 3 days. The target cells were incubated overnight at 37°C in the presence or absence of 20 μg/ml of individual peptide (SALSA-1, SALSA-2, or a control peptide, MSP-3b from a blood stage Ag (28)) and 3 μg/ml of human β_2-microglobulin (Sigma Chemical Co., St. Louis, MO) in RPMI medium (Life Technologies) supplemented by 20% human AB serum. After labeling with 150 μCi of $Na_2{}^{51}CrO_4$ (ICN Biomedical Inc., Irvine, CA) for 60 min at 37°C, the cells were washed three times, and 5 to 10 × 10^3 target cells were added to duplicate wells in 96-well round-bottom microtiter plates (Corning Glass, Corning, NY). Effector cells were added at various E:T cell ratios, and the plates were incubated for 5 h at 37°C in 7.5% CO_2/air. Supernatant (100 μl) was removed from each well and quantitated for ^{51}Cr using a gamma counter. The MSP3-b peptide, which was used as control, corresponds to the sequence AKEASSYDYILGWEFGGGVPEHKKEEN.

To assess class I or class II presentation of Ag, mAbs, either anti-HLA-DR (5 μg/ml of Mab L-243) (29), or anti HLA-A-B-C (1/1000 dilution of ascite supernatant of Mab W6/32, a dilution found to inhibit class I-restricted cytotoxic T cell responses) (30), or an irrelevant IgG2a Ab used as control, were added to 10^6 target cells for 1 h at 4°C and remained present throughout the 5-h CTL assay.

The percentage of specific lysis was determined as follows: % lysis = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Immunization of animals

Mice. To investigate Th activity, two groups of five outbred Swiss albino mice (Charles River, St. Aubin les Elbeuf, France) were immunized on day 0 by s.c. injection of either 50 μg of SALSA-1 peptide emulsified in CFA, or for controls, saline in CFA. They were boosted 40 days later by s.c. injection of 50 μg of the recombinant β-galactosidase-SALSA protein (clone DG671), containing both SALSA-1 and -2 peptide sequences), in IFA. Sera taken on days 0, 40, and 60 were studied in ELISA assays toward peptides SALSA-1 and -2, as described above, to investigate the occurrence of a secondary Ab response to peptide SALSA-2, thus indicating help provided by SALSA-1 priming. In further experiments, we also investigated the immunogenicity of either of the two peptides (two injections of 50 μg at 15-day intervals in CFA/IFA), or the recombinant DG671 alone (three injections of 50 μg at 15-day intervals using CFA/IFA, Titermax, or saponin as adjuvants).

Chimpanzees. Socially housed, captive bred, male chimpanzees (*Pan troglodytes*), 10 to 15 yr old, were used under veterinary supervision. As part of a series of experiments, chimpanzees Bart and Socrates received three s.c. injections of 50 μg of purified β-galactosidase-SALSA recombinant protein represented in clone DG671 adsorbed on alum and were challenged by i.v. inoculation of 28 million sporozoites from the NF54 strain. Boosting was performed 18 mo later by s.c. injection of GST-671 recombinant protein emulsified in Montanide ISA 51 as adjuvant. The cells studied in lymphoproliferative and CTL assays were taken either before, or 1, 3, 8, 9, 12, 18, and 20 mo after challenge and 30 and 65 days after the boost. Cells from chimpanzees Cor and Peer, who received three injections of control Ag consisting of the carrier molecule β-galactosidase, and from Dirk and Ruud, who were immunized by other LS molecules (all received the same 28 million sporozoite challenge at the same time), were used as controls. Preliminary data from challenge of the SALSA-immunized chimpanzees and from inhibition of sporozoite invasion assays performed with sera from immunized chimpanzees already suggest that SALSA may be involved in protective responses. The results of this and other challenges will be reported separately (A. Thomas et al., manuscript in preparation).

Results

Identification of a novel sporozoite surface molecule

To select non-CS sporozoite surface Ags, we used an approach similar in principle to that formerly used to detect clones expressing a LSA (13, 12), i.e., the screening of a *P. falciparum* expression library with polyclonal human Abs of restricted specificity. Three sera that had low or undectable levels of anti-CS protein, and yet were strongly positive in a "wet" sporozoite IFA (Table I), and two sera that were negative for LSA-1 reactivity, and yet were strongly reactive with liver stage by IFA, were used in this selective screen. From a group of 120 *P. falciparum* genomic DNA clones encoding predominantly pre-erythrocytic Ags, these sera facilitated the selection of 12 candidate clones. Stage specificity was assessed by sporozoite, liver stage, and blood stage IFA with human Abs that had been affinity purified on the clones. Due to the reported genetic restriction of CS epitopes for Th cells and the consequent low prevalence of Abs to CS among subjects from highly endemic areas (11), we wished to select only those clones expressing epitopes that were more consistently recognized by malaria-exposed individuals. A complementary set of 10 sera from both low and high endemicity areas was therefore used to study the prevalence of specific Ab response in Western blots against recombinant products. From this screening, one consistently well recognized clone, DG671, was selected and submitted to further studies.

Affinity-purified human Abs on DG671 were found to be strongly reactive by IFA with the surface of sporozoites from NF54 strain (see below) showing an evenly distributed labeling over the entire surface, which was also detectable in four additional sporozoite isolates from Thailand (not shown).

Sequence analysis of the DG671 clone showed that the 249-base pair fragment contains only one open reading frame, encoding an 83-aa nonrepeat stretch (Fig. 1*A*). PCR amplification of DNA from this region in 5 Asian and 2 African culture-adapted strains, and in 16 isolates from Senegal, showed no size polymorphism (e.g., Fig. 1*C*). The SALSA polypeptide is rich in glutamic acid (17%), lysine (19%), and serine (13%), but contains no methionine or cysteine and tyrosine. Although, in contrast with other *Plasmodium* proteins, SALSA does not contain repeated sequences, a typical pattern consisting of two acidic residues (Asp or Glu) clustured between two lysine residues is found five times (KKDEK, KD DVK, KEEKK, KDDGK, KVLEK). No homology with known *P.*

falciparum Ags at >30% level was found when NBRF (release 30) and Swissprot (release 23) data banks were screened. A 62% homology at the nucleotide sequence level was found with the human cytoplasmic β-actin pseudogene, although no structural homology with this or other proteins was found at the protein level.

Specificity of the synthetic peptides derived from SALSA

The structure of the 83-aa polypeptide was analyzed according to the method described by Chou and Fassman (31), which revealed that two areas had a high tendency to adopt an α-helical conformation. Therefore two nonoverlapping peptides of 27 (SALSA-1) and 34 (SALSA-2) amino acids, covering these two areas of high amphipathicity, were synthetized. Human Abs, affinity purified on the recombinant 83-aa SALSA protein, reacted strongly in ELISA assays with both peptides. Anti-peptide Abs, prepared by affinity purification from human sera on ELISA plates coated with the synthetic peptides, reacted by IFA with native Ag on sporozoites and liver stages. Anti-SALSA-1 Abs prepared in this way did not react with SALSA-2 in Elisa, and vice versa (not shown). Each peptide therefore represented at least one native, non-repeat B cell epitope, in contrast with many other malarial epitopes with a repetitive nature that has been postulated to represent one means of immunologic evasion (32).

Evidence for expression at sporozoite and liver stages

In Western blots from NF54 sporozoite extracts, human Ab affinity purified on DG671, and Abs raised in the chimpanzee, labeled a sporozoite protein of M_r = 70 kDa (Fig. 2, *lanes D*, *D'*, and *F*); control Abs purified in the same manner on the LSA-1 recombinant protein DG307, and on DG705, a clone containing many CS tetrapeptide repeats, did not. Abs to the 70-kDa protein represented a frequent and quantitatively important Ab specificity in many sera (see e.g., Fig. 2, *lanes A* and *B*). RT-PCR experiments with sporozoite RNAs confirmed the pattern of stage-specific expression indicated by Ab assays (Fig. 1*D*)

The surface location of the DG671 Ag, reproducibly indicated by IFA (Fig. 3*A*), was also shown at the ultrastructural level using immunogold labeling (Fig. 3, *C* and *D*). By this means, the Ag appeared regularly distributed over the parasite surface and was not detected in the cytoplasm. In the same assay, the location and number of gold particles were similar when using one of our selective African sera (diluted 1/100), which had a high IFA titer to sporozoite surface Ags (1/6 400) although no detectable Abs to CS repeats by ELISA and Western blots. Compared with the pictures obtained when using an anti-CS mAb, the results suggest that both Ags have the same location, although the DG671 Ag is possibly less abundant than the CS.

This Ag could also be detected in *P. falciparum* liver stages (Fig. 3*B*), and for this reason was named sporozoite and liver stage antigen (or SALSA). It gave the peripheral type of labeling formerly described (18) in 5-day-old liver forms, which correspond to the content of the parasitophorous vacuole. In fully mature 6.5- and 7-day-old forms, it was further distributed between the pseudo-cytomeres, similar to LSA1 (15), and between, or possibly on, the liver stage merozoites. This differs clearly from CS, which is located only on the outer membrane of the parasitophorous vacuole in decreasing amounts as the parasite matures. At the ultrastructural level (Fig. 3*E*), the SALSA appeared to be confined to the granular, or fluffy, material present in the parasitophorous vacuole, which in fully mature forms gets in between the pseudocytomeres and finally around the merozoites. In mouse malaria, this material is found to be preferentially taken up by macrophages surrounding liver forms (33). The amounts of Ab-labeled material increased markedly during liver schizogony, suggesting that SALSA synthesis starts at the sporozoite stage and augments throughout liver schizogony.

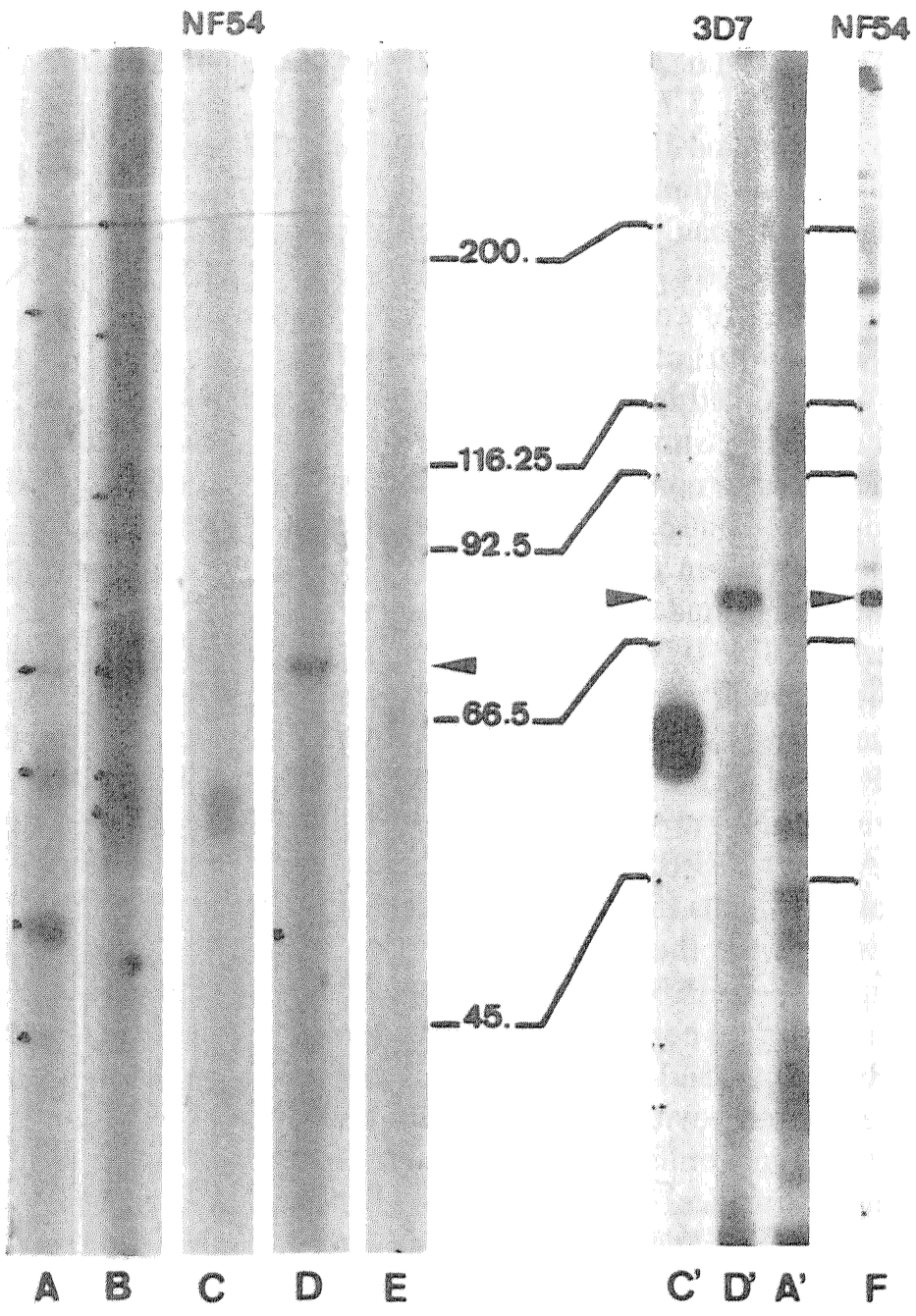

FIGURE 2. Identification of a 70-Kda SALSA protein in sporozoites. Western blot of NF54 (*lanes A–E*) and 3D7 (*C'–A'*) sporozoites. *Lanes A*, *A'*, and *B* were reacted with two African human sera (1/100), *C* and *C'* with anti CS-repeat Mab NFS2, *D* and *D'* with affinity-purified human Abs to SALSA DG671, *E* with control affinity-purified human Abs to LSA-1 clone DG 307, and *F* with the serum of chimpanzee Bart immunized with DG671 (1/100). Arrows indicate the 70-kDa SALSA protein.

We screened liver stages in *C. apella, S. sciureus*, and *P. troglodytes*, obtained, respectively, from two different African patient isolates, and the NF 54 culture strain. In each case, all the parasites observed (at least 30 schizonts/strain) were equally well labeled by the monospecific anti-SALSA Abs. This indicates that polymorphism of the Ag is restricted for the major B cell epitopes in the parasite clones constituting these isolates, since it contrasts, for example, with the variable expression of another as yet uncharacterized Ag, DG775, used as control, which was detectable among only 8% of the same liver stage parasites (P. Druilhe and B. Galey, unpublished observations).

IFA on *P. falciparum* erythrocytic stages with DG671 Abs that were strongly reactive against sporozoites was consistently negative with six culture-adapted strains and six patient isolates, whether acetone, methanol, or hydrochloric acid fixed. In Western blots, a weakly labeled 41-kDa polypeptide was detected. This probably results from cross-reactivity with a blood stage epitope exposed under denaturating conditions, because five sera from patients who acquired *P. falciparum* malaria through accidental blood transfusion were nonreactive to SALSA despite high titers of Abs to blood stages proteins.

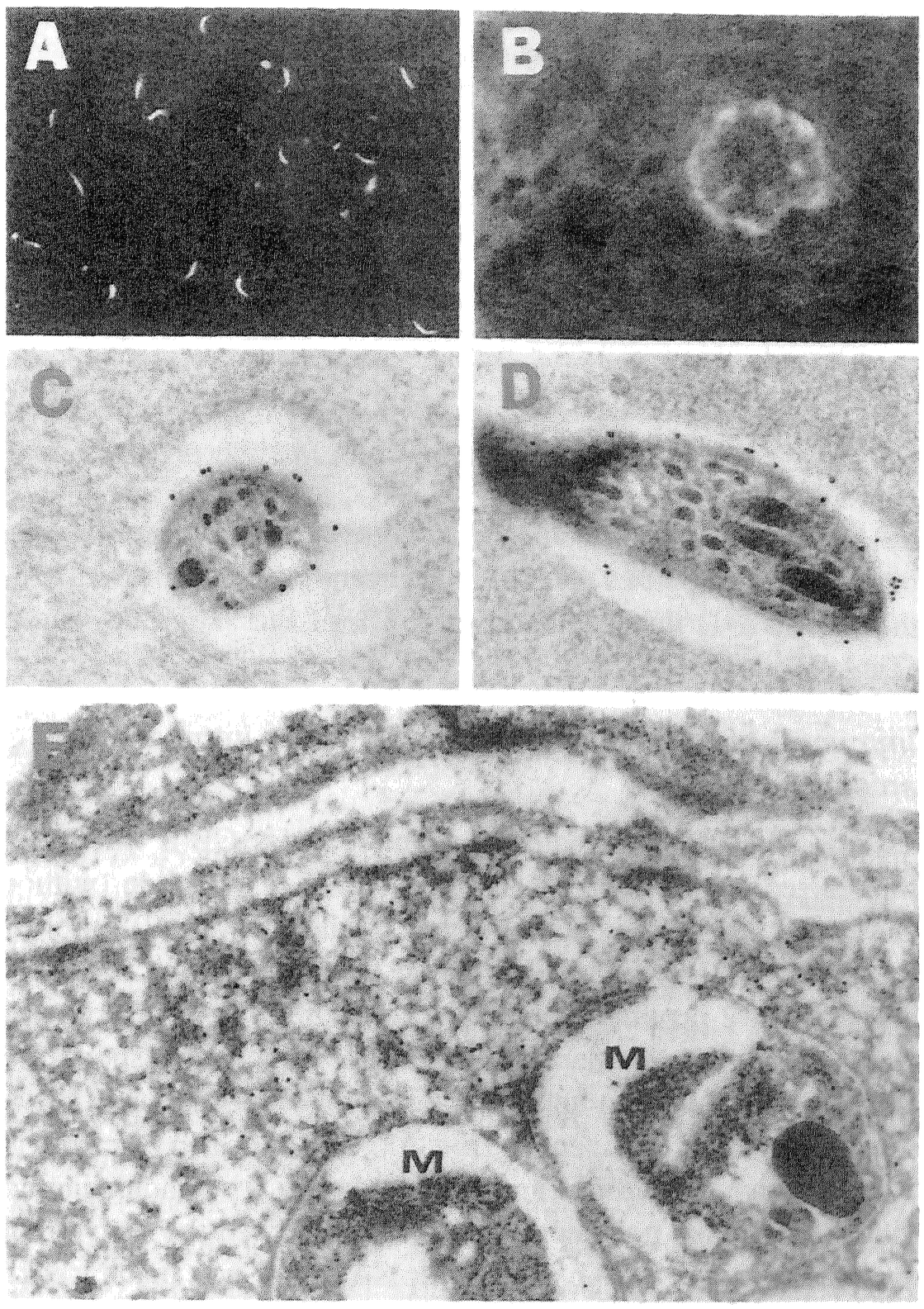

FIGURE 3. Localization of SALSA in *P. falciparum* sporozoites (*A*, *C*, and *D*) and liver stages (*B* and *E*). Affinity-purified Abs to SALSA were allowed to react: *A*, with the surface of *P. falciparum* sporozoites (NF54 strain) in a wet IFA assay; *B*, with liver forms from Carnoy's-fixed biopsies obtained in *Cebus* monkeys, revealed by FITC-labeled Abs; *C* and *D*, with NF54 sporozoite sections revealed with protein A-gold 30-nm particles; *E*, liver forms of *P. falciparum* (NF54) revealed using protein A-gold particles of 10-nm size; the gold particles are distributed in the granular material present between the parasitophorous vacuole membrane and the two liver stage merozoites (M), which can be seen in the figure, in fully mature liver forms obtained in the chimpanzee.

Species specificity

IFA was negative on sporozoites of *P. vivax* and on sporozoites and liver stages of the rodent species *P. yoelii* and *P. berghei*. This was confirmed by analysis of reactivity of the DG671 Ag with sera from heterologous malarial infections. In Western blots, the β-galactosidase-DG671 recombinant protein was not recognized by human sera after *P. vivax* (four sera), *P. ovale* (two sera), *P. malariae* (two sera), and *P. cynomolgii* (two sera) infection, or by mouse sera after *P. berghei* and *P. yoelii* infections (three sera containing significant amounts of Abs to Ags of the homologous species). In addition, three mAbs identified as specific for three non-CS sporozoite surface Ags of *P. yoelii* (17) showed no cross-reactivity with DG671.

The relationship between SALSA and other Ags of the pre-erythrocytic phase was investigated. Abs to SALSA did not react with CS construct DG705 (a clone containing a long stretch of NANP repeats) or with DG176 (a clone encoding part of CS region I). These Abs also failed to react and SALSA DNA failed to hybridize with the other 119 pre-erythrocytic-stage recombinant clones that were screened (Fig. 1*B*).

Antigenicity: B and T cell responses in exposed individuals

The high prevalence of Abs in man was one of the original criteria of selection for SALSA. This was further evaluated in 269 individuals, ages 1 to 75 yr, originating from three areas of Africa that have distinct levels of transmission, and was compared with responses to CS repeats, LSA-1, and blood stages Ags. Results are detailed in Figure 4. The proportion of individuals having detectable Abs specific for either one or the other SALSA peptide was found to be high as compared with the other Ags studied, particularly in the area of Podor (Senegal), which is an area of very low endemicity by African standards (it was estimated that mosquitoes inject virulent sporozoites from 1 to 5 times/year/individual) (22). This suggests that low amounts of the native SALSA protein are capable of inducing an immune response in humans. The overall prevalence of responses in all age groups varied from one area to the other, from 64 to 90% for SALSA-1 peptide, from 82 to 88% for SALSA-2, and from 53 to 65% for the recombinant R32tet32 from the CS protein (Fig. 4, *1A*, *2A*, and *3A*), whereas prevalences ranged from 65 to 80% for anti-Resa and from 76 to 91% for whole anti-blood stage Ags measured by IFA (not shown). Similarly, the overall mean titers of Abs directed to the above three molecules were 2.63 to 3.84, 4.45 to 5.70, and 1.89 to 2.58, respectively (for details per age group, see Fig. 4, *1B*, *2B*, and *3B*). In each area there was, as is the case of responses to many malarial Ags, an age-dependent increase of both prevalence and titers of Abs, particularly among the younger subjects. From one area to the other, there was a correlation between either prevalence or mean titer, and the mean number of sporozoite inoculations in the area: prevalence and titers are lower in Podor (Senegal), where individuals receive an average of 1 to 5 infective bites, than in Ankazobe (Madagascar), where transmission is only slightly higher (10 infective bites/individual/year on average), while in Donse (Burkina Faso), where transmission averages 100 infective bites/individual/year, a larger proportion of children harbor SALSA-specific Abs. These data confirm that SALSA-1 and SALSA-2 aa sequences define one or several B cell determinants. Those defined by SALSA-2 appear to be more immunogenic when presented by the parasite, since there are higher prevalences and higher Ab titers against this peptide in the two areas of lower endemicity and in each age group. Ab prevalences were also assessed by Western blot against DG671 recombinant protein, using a subgroup of 30 individuals spanning all age groups, from each of the above three areas. Ab prevalences measured in this way were 87, 90, and 95%, respectively, of the individuals studied in Podor, Ankazobe, and Donse (not shown).

Lymphocyte studies in 111 individuals from the low transmission area of Ankazobe in Madagascar revealed the presence of T cell-stimulatory epitopes within both SALSA-1 and -2 peptides. The prevalence of T cell responders to SALSA-1 or SALSA-2 was of the same order of magnitude as to the CS Th-2r epitope (Table II). Proliferative responses to the two SALSA peptides were in several instances dissociated, probably due to individual MHC restriction, so that in total 22.5% of the cohort investigated showed a significant lymphocyte proliferation to either one or both of the peptides. Results were negative in 22 nonexposed controls who were studied in parallel, indicating that the peptides had no mitogen or superantigen activity (not shown). IFN-γ secretion was measured to provide an additional marker of T cell Ag stimulation, and also because it is, to date, the most potent cytokine against liver stage development. The proportion of individuals responding

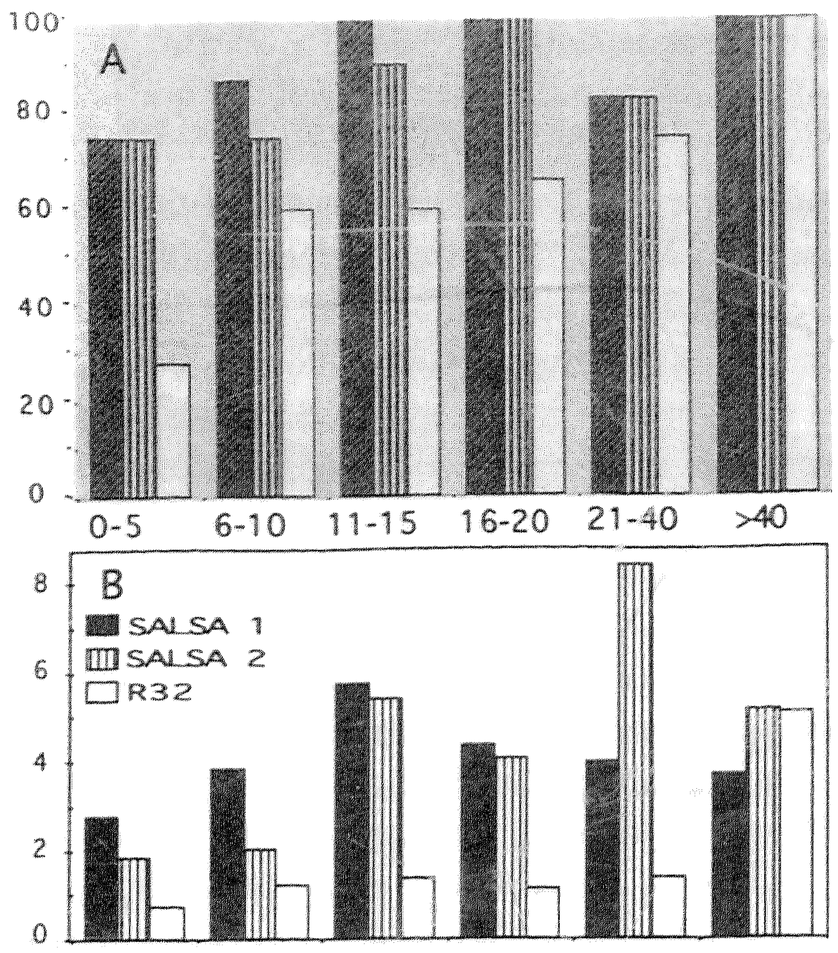

Fig 4-1 : DONSE

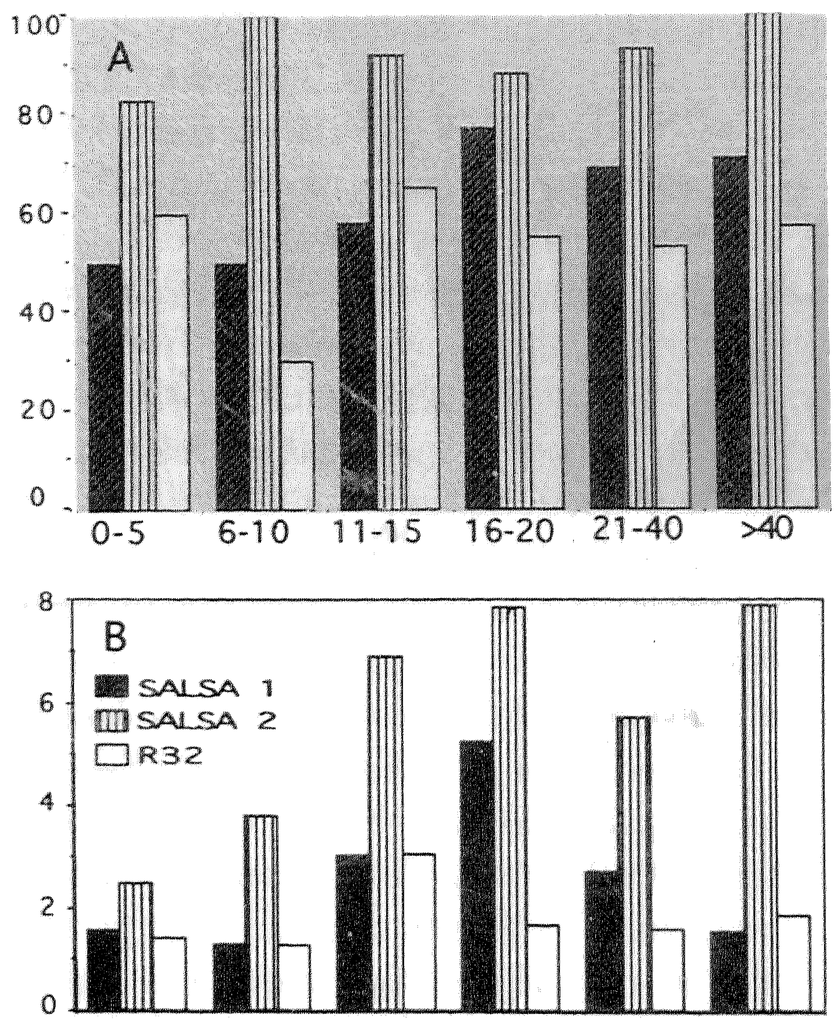

Fig 4-2 : ANKAZOBE

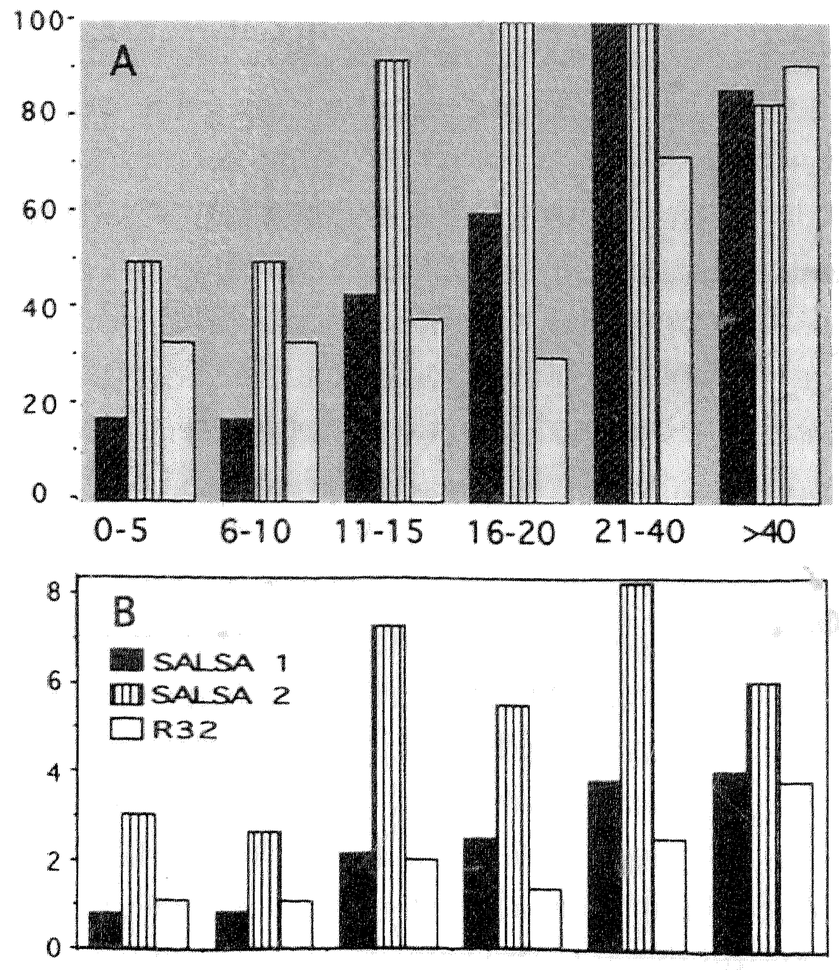

Fig 4-3 : PODOR

Table II. *Prevalence of lymphocyte responses to SALSA and Th2r peptides among exposed individuals from Ankazobe, Madagascar*

	Proliferation (SI > 2)	IFN-γ Secretion (>2 IU/ml)
Th2r	12/85 (14.1%)	9/26 (34.1%)
SALSA-1	17/111 (15.3%)	12/50 (24%)
SALSA-2	14/111 (12.6%)	22/50 (44%)
SALSA-1 and /or SALSA-2	25/111 (22.5%)	26/50 (52%)

by specific secretion of IFN-γ was greater than those showing proliferative responses and was significantly higher for SALSA-2 than SALSA-1, the total reaching 52% of the subgroup studied (Table II).

Immunogenicity: B, Th, and CTL activity in immunized animals

To investigate Th cell activity, outbred Swiss CB 1 mice were immunized by injection of 50 μg of SALSA-1 peptide (without carrier) on day 0. They were boosted on day 40 with 50 μg of the purified DG671 recombinant protein (containing both SALSA-1 and SALSA-2 peptide sequences). The high IgG Ab response towards SALSA-2 peptide indicated that a secondary response occurred, presumably via help mediated by cells primed with the aa sequence of SALSA-1 peptide (Fig. 5). Mice sera also reacted with *P. falciparum* sporozoites and liver stages by IFA, indicating additionally that the polypeptide properly mimicked the native molecule. In contrast, the Ab response to SALSA-1 was much weaker (Fig. 5*A*). The relative dominance of SALSA-2 was underlined when, after two injections in outbred mice of 50 μg of each peptide, SALSA-2, but not SALSA-1, induced Ab strongly reactive with the original peptide and with sporozoite and liver stages (mean ELISA ratio, 9.5; IFA, 1/400). Three immunizations with the β-galactosidase-fused DG671 protein induced Abs reacting with both peptides.

The liver stages are immunologically unique in the life cycle of *Plasmodium* sporozoites, because they develop within a host cell that expresses MHC class I molecules. The evidence for $CD8^+$-mediated cytotoxicity in rodents suggests that target Ag(s) can be adequately transported and suitably expressed on the host cell membrane. Cytolytic activity was therefore sought in lymphocytes from two chimpanzees, Bart and Socrates, who received three injections of the purified SALSA recombinant protein adsorbed on alum and were subsequently exposed to live sporozoites.

Specific Abs and strong Th cell responses were recorded in samples taken before and 1, 3, 8, and 12 mo after exposure to live sporozoites. Abs towards SALSA-1 and SALSA-2 were detected after immunization (e.g., ELISA ratios of 9.1 and 24, respectively, in Bart and labeling of the 70-kDa protein in Western analyses; Fig. 2, *lane F*), decreased in the following months and were boosted by the challenge. Stronger T cell responses towards SALSA-2 than SALSA-1 were recorded at various time points

FIGURE 4. Mean prevalence (*A*) (gray background) and titers (*B*) (clear background) against SALSA-1 (black columns) and SALSA-2 (vertical stripes) peptides, and recombinant R32tet32 (clear columns) in: Donse (Burkina Faso; 4-*1*), Ankazobe (Madagascar; *4-2*) and Podor (Senegal; *4-3*) populations. Prevalence (*A*) is expressed as the percentage of positive individuals in each age group. Titer (*B*) is expressed in arbitrary units representing the ratio of experimental OD value to healthy controls + 3 SD. Age groups ranges are shown on the abscissa.

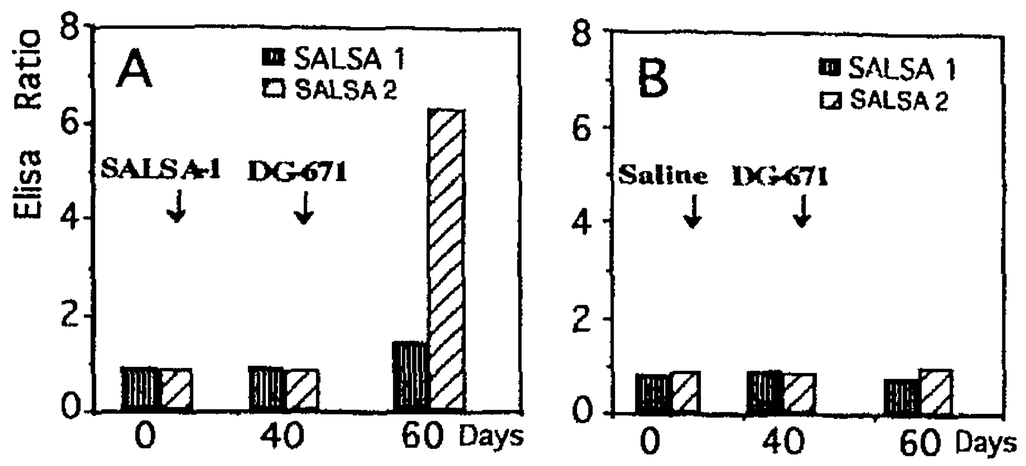

FIGURE 5. Th activity of SALSA-1 peptide. Outbred Swiss mice primed with peptide SALSA-1 (*A*) or saline (*B*) were boosted with the recombinant SALSA protein DG671 (containing both SALSA-1 and -2 peptide sequences). Ab responses were measured by ELISA against SALSA-1 (vertical stripes) and SALSA-2 (hatched columns) peptides. The high response to SALSA-2 at day 60 is suggestive of a secondary type of response, indicating Th activity provided by SALSA-1.

(e.g., SI of 45 and 15, respectively, in the Bart sample taken 8 mo after challenge were representative of the values seen throughout). In contrast, both assays were negative in two control animals immunized with the β-galactosidase carrier molecule alone and in two chimpanzees immunized with other LSAs before and after the challenge.

For CTL assays, PBLs from Bart were taken 9 mo after challenge, and cell lines were established by in vitro stimulation with SALSA-1 and 2 peptides for 7 days before performing the assay. A potent, peptide-specific cytolytic effect was detected for SALSA-2- and, to a lesser extent, for SALSA-1-stimulated cells against autologous PHA blasts (Fig. 6, *A* and *B*). The lysis was peptide specific, as negative results were obtained with control peptide-pulsed target cells (Fig. 6, *C* and *D*), and with SALSA-2-specific effector cells towards SALSA-1-pulsed targets (not shown in the figure). In the cells from the other immunized chimpanzee, Socrates, a similar cytolytic activity towards SALSA-1-pulsed cells was observed (Fig. 6*F*), whereas no CTL activity to SALSA peptides was detected in material taken at the same time points from the four control animals, despite the fact that they had received a similar sporozoite challenge.

In samples taken from Bart 18 and 20 mo after challenge, CTL activity was still detectable (specific lysis 60–30% in the two succesive assays). The animal was then immunized again with SALSA material, and in samples taken 30 days after this boost the CTL activity was restored to the high initial levels (Fig. 6*E*). This result was confirmed in cells from a second sample drawn at 65 days (not shown). In both cases, responses were clearly shown to be class I-restricted, as in the presence of Mab W6/32 the CTL activity specific of each peptide was totally abolished (Fig. 6*E*, black columns), whereas this was not the case when using anti-HLA-DR Abs (not shown).

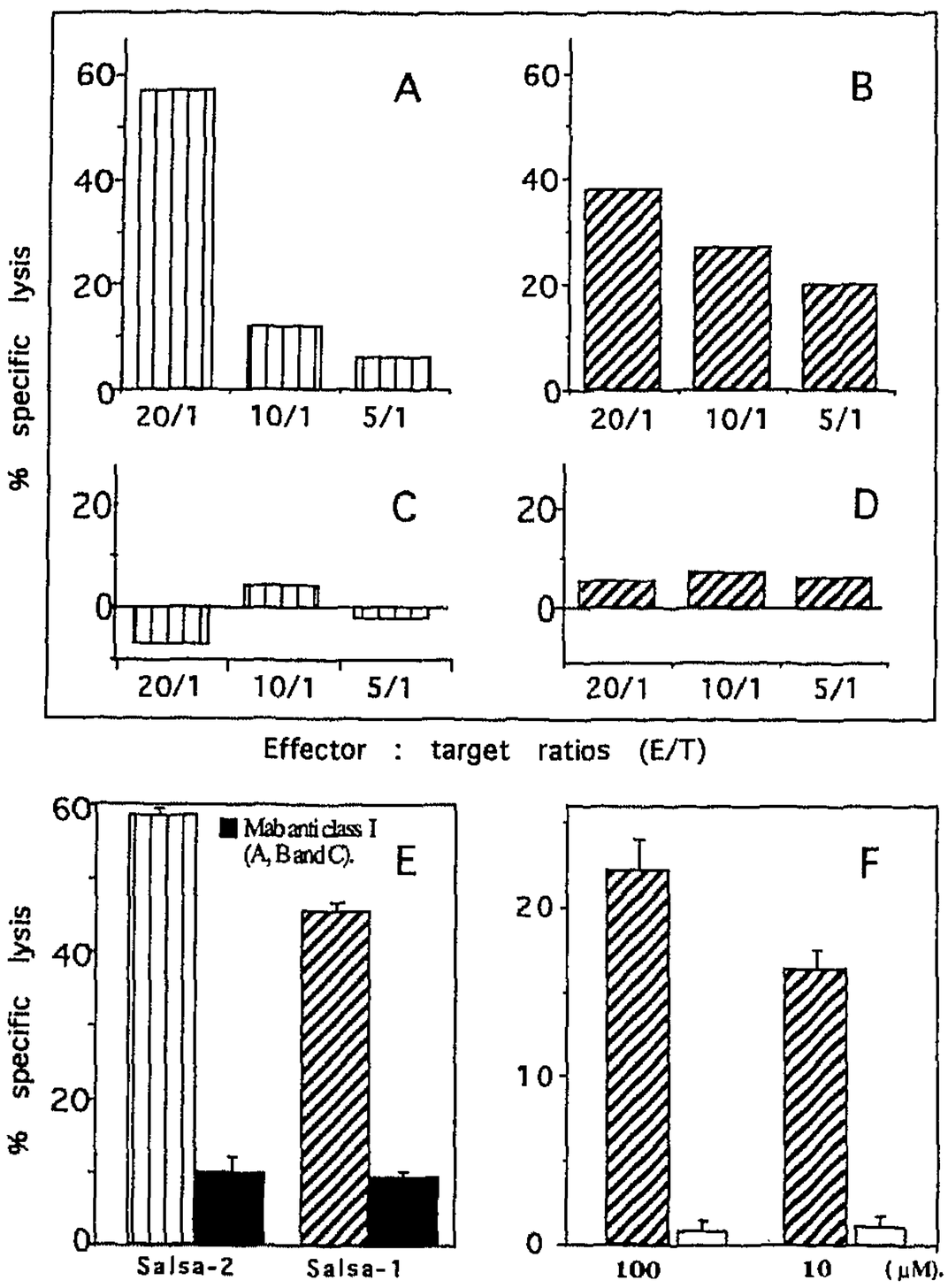

FIGURE 6. Identification of CTL epitopes in SALSA-1 and -2 peptides. *A* through *D*, Specific release at various E:T ratios with Bart cells stimulated by SALSA-2 peptide, directed toward SALSA-2-pulsed autologous PHA blasts (*A*) compared with (*C*) SALSA-2 effector cells with autologous cells pulsed with an irrelevant 27-aa peptide derived from the *P. falciparum* blood stage Ag MSP3. In a similar fashion, specific CTL response in the chimpanzee Bart was also evident against SALSA-1 (*B*) in comparison with the control peptide (*D*). Both SALSA-1 and -2 peptide responses are MHC class I restricted (*E*). CTL responses to SALSA-1 peptide obtained in cells from the chimpanzee Socrates (*F*) (hatched columns indicate SALSA-1-pulsed targets; clear columns indicate control, LSA-1-pulsed targets) (shown is a single E:T of 20:1 and two peptide concentrations for pulsing target cells, 100 and 10 μM). All results are expressed in percentage of specific release.

Discussion

To date, attempts to develop a malaria vaccine have primarily focused on the asexual blood stage and on CS, the major sporozoite surface Ag, whereas the liver stage of parasite development has attracted relatively little interest. Through rodent malaria models, it has recently become clear that LS are susceptible to attack by a variety of immune effectors, including the direct and indirect effects of Ab and T cells. The main difficulties in exploiting this progress for the development of a pre-erythrocytic stage vaccine are twofold: 1) the malaria pre-erythrocytic stage (MPES) antigenic repertoire remains largely unknown; and 2) protective mechanisms vary widely between the rodent/*Plasmodium* combinations used. None of the combinations is considered to be more relevant to the human situation than the others (6, 34). Thus it is still not clear which molecule(s) inducing which mechanism(s) has the most promise against *P. falciparum* (or *P. vivax*) MPES in humans. The novel MPES molecule that we identify here is a sporozoite surface and liver stage Ag that is a potential target for several protective defense mechanisms, particularly in view of its ability to induce B, Th, and CTL responses that may act successively on sporozoites and LS.

We have undertaken molecular characterization sufficient to demonstrate that SALSA does not represent any of the *P. falciparum* genes reported to date. It does not contain any repeated motif. The region of the SALSA gene we have characterized was chosen, apart from its stage expression, based on Ab detection, yet was found to contain valuable T cell determinants. The prevalence of Ab in a limited selective panel of sera was a complementary initial

selection criterion, and further Ab studies confirmed that the molecule contains potent B cell epitopes. Indeed, the proportion of individuals carrying Abs directed to each peptide is relatively high in all three transmission areas studied and in each age group. The prevalence and titer of Abs were consistently higher to SALSA-2 than to SALSA-1 in the three areas, indicating that the former peptide defines a major B cell epitope.

One of the remarkable features of those SALSA B cell determinants is that, in contrast to many of the *P. falciparum* proteins characterized to date, they are not defined by repeats. Indeed, in most molecules, such as MSA1, MSA2, GLURP, etc., from blood stages, or CS, LSA-1, or STARP from pre-erythrocytic stages, the repeat region is the main target of Abs relative to nonrepeated regions (15, 35). Moreover, long series of repeats can bind more Ab molecules than single, nonrepeated epitopes. Nevertheless, our study shows that the prevalence and titer of Abs to SALSA exceeds that directed to CS repeats, a recognized dominant epitope on sporozoites, to RESA repeats, or to STARP repeats (V. Pasquetto, D. A. Fidock, E. Badell, W. Eling, H. Gras-Masse, and P. Druilhe, manuscript in preparation), are similar to the dominant repeated epitope of LSA-1 in the same study population (15) and are well above the prevalences of Abs directed to nonrepeated regions of CS (11) or LSA-1 (15).

For the analysis of B cell responses, the two areas of low malaria transmission (by African standards) of Podor and Ankazobe provide a more discriminative tool than the "standard" savanna hyperendemic area of Donse. Results for these two areas, particularly in young children, reveal the remarkable immunogenicity of SALSA, when considering that individuals receive about 1 to 10 infective bites per year and that each inoculation is made of about 10 sporozoites only, of which about one-half can be expected to transform into liver schizonts (23, 36). Nevertheless, more than one-half of the younger children in these two areas have detectable Abs to SALSA peptides. This is consistent with the identification of Th cell epitopes in the same molecule. A similarly high prevalence of Ab responders to SALSA-2, more than to SALSA-1, was also observed in other regions of Africa, reaching 85% of all age groups in the Senegalese village of Dielmo (J. L. Sarthou et al., manuscript in preparation) and above 90% in adults living in Kilifi in Kenya (J. Sherwood et al., unpublished data).

From a functional point of view, Abs directed to sporozoite surface molecules, such as CS and STARP, have been shown to reduce in a dose-dependent manner the rate of sporozoite invasion into hepatocytes (7) (V. Pasquetto, D. A. Fidock, E. Badell, W. Eling, H. Gras-Masse, and P. Druilhe, manuscript in preparation). The identification of SALSA extends the range of Ags that can be targeted by Abs at the sporozoite level. Preliminary studies in our laboratory indicate that anti-SALSA peptide Abs can significantly reduce sporozoite invasion, thus confirming the surface-accessible location of the molecule (V. Pasquetto et al., manuscript in preparation).

SALSA being expressed on sporozoites, and during liver stage development, offers possibilities for both humoral and cellular immune targeting. In view of the potential role for $CD8^+$ lytic targeting of SALSA, and the frequent requirement for T cell help in the induction of such responses, we investigated T cell proliferative responses to the SALSA peptides in humans, chimpanzees, and mice.

The induction of Th cell activity by SALSA-1 peptide was clearly indicated when boosting with the recombinant molecule induced strong anti-SALSA-2 responses. The presence of determinants for human T cells was shown by the induction of specific proliferative responses and IFN-γ secretion in individuals from the endemic area of Ankazobe, but not in control non-malaria exposed-donors. Both regions of the molecule predicted to adopt an α-helical conformation were able to stimulate T cells, as demonstrated previously for similarly conformed regions of LSA-1(15). The prevalence of responders to SALSA-1 and SALSA-2 peptides was of the same order of magnitude as that observed to the Th2-R peptide from CS in the same area, or in previous studies in other areas (37), and close to those observed for various LSA-1 peptides in the same population (15). The dissociation of responses to the peptides confirms that they are not cross-reactive and may reflect individual class II ability to associate with the T cell determinants, thus suggesting that they may act in a complementary manner in distinct individuals. These results are in general agreement with other T cell studies with SALSA peptides performed in the high transmission areas of Dielmo (J. L. Sarthou et al., manuscript in preparation) and of Djoumouna in the Congo (Ph. Brasseur et al., manuscript in preparation) where high to very high prevalences were found. Although the proportion of responders is influenced by a large number of factors and may vary greatly over time in successive studies of the same individuals (38), this suggests that the relatively low prevalence seen in Ankazobe is more likely related to the low level of transmission than to a genetic restriction of responses to the peptides. A potential polymorphism of T cell determinants in the SALSA sequence of isolates from Madagascar or a MHC class II restriction of $CD4^+$ responses from the population of Madagascar (mainly of Asian origin) are alternative hypotheses, which will need to be addressed by further studies.

Of particular importance for MPES Ags is the higher prevalence of individuals responding to both peptides by IFN-γ secretion, which is the most potent of the cytokines thus far reported to block the intrahepatic development of rodent (39–41), primate (42), and human *P. falciparum* malaria (8) and is involved in defenses induced by irradiated sporozoites (40). IFN-γ responses did not parallel proliferative responses, as has been previously found with other molecules (15, 38, 43). Even though the DG671 insert represents only a small part of the SALSA protein, it is remarkable that more than 50% of the individuals studied responded to it by either proliferation or IFN-γ production.

Our studies in immunized chimpanzees also show that SALSA can 1) elicit and 2) be a target for CTL lymphocytes. This result is particularly significant following recent data obtained in experimental malaria, as well as naturally exposed human populations, pointing to CTL activity as a most critical defense against LS. Since the initial observation that μ-suppressed mice can be effectively protected by immunization with irradiated sporozoites (44), there has been increasing evidence in favor of a major role of T cells, particularly of the $CD8^+$ subset(40, 45–48).

In individuals of diverse MHC haplotypes protected by irradiated sporozoites, it is clear that CTLs must be directed to a range of epitopes from various LS-expressed molecules. The identification of two regions of the SALSA molecule bearing epitopes targeted by CTL cells expands the range of potential epitopes for this critical defense mechanism. CTL activity was detected in a consistent manner in successive samples, and although the level of lysis was variable over time, it was found to be peptide specific, genetically restricted, and class I dependent.

In view of the complex set of immunization and challenges in the chimpanzees studied, the induction pathway for these cells is not clear. Immunization by soluble molecules is not generally thought to allow Ag presentation by the endogeneous pathway such that association with class I molecules occurs. The ability of SALSA to induce Th cells and the critical role of Th in all types of B cell- and T cell-dependent responses leads us to believe that the major $CD8^+$ CTL expansion occurred as a secondary type of response at the time of parasite challenge. The long-term persistence of circulating CTLs is in keeping with this hypothesis, given

that Th cells would be expected to play a role in the sustained production of both Ab and $CD8^+$ T cells specific for the Ag.

In conclusion, the identification of the SALSA protein confirms the existence of additional Ags on the surface of sporozoites and provides further information on the antigenic content of *P. falciparum* liver stages. Preliminary data from immunized chimpanzees point to a protective role for SALSA, suggesting that we have identified an additional vaccine candidate for pre-erythrocytic stage immunization. SALSA has attractive antigenic features, including 1) a high frequency of B and T cell responders in individuals with various genetic background and 2) the presence of epitopes for both Th and T cytotoxic cells within the limited regions studied. As well as the high prevalence of responders, the detection of gene fragments of similar size in over 20 isolates suggests that the gene is well conserved among *P. falciparum* isolates. SALSA expression at two successive stages of parasite development may allow for a wider array of immune effectors to act against a single parasite product (for example invasion-inhibition of sporozoites by Abs, opsonization by macrophages, Ab-dependent cellular cytotoxicity, lymphocyte cytotoxicity on infected liver cells, and IFN-γ-mediated blockade of LS development). The presence of epitopes that are potent stimulators of T cell activities is a critical feature in favor of this newly identified Ag.

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