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High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new *Plasmodium falciparum* pre-erythrocytic molecules

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Abstract

We have investigated the immunogenicity in chimpanzees of twelve synthetic peptides derived from four new *Plasmodium falciparum* molecules expressed at pre-erythrocytic stages of the human malaria parasite. These parasite molecules were initially selected through their ability to be recognized by stage restricted human antibodies. Twelve 20- to 41-mer peptides representing potential human B- or T-cell epitopes were selected from these proteins, and synthesized. Six of these were modified by a C-terminal lipidic chain in order to re-inforce their immunogenicity. Strong B- and T-helper cell responses were induced in chimpanzees by lipopeptides injected without adjuvant and by peptides in Montanide. All twelve peptides induced $CD4^+$ T-cell proliferative responses, as well as the secretion of IFN-γ (some of them at very high levels) and eleven peptides induced antibody responses. The immune responses elicited in this way were reactive with native parasite proteins, as shown by recall studies with sporozoite stage proteins, and proved to be long-lasting (up to 10 months after immunization). Our results support the strategy employed to select these four new malarial antigens and the corresponding peptides, and suggest that the immunizing formulations are both efficient and clinically acceptable.

Keywords: Immunogenicity; Lipopeptides; *Plasmodium falciparum*

1. Introduction

Effective vaccination is the most cost-effective solution to the enormous mortality caused by malaria (currently 1.5–2 million deaths per year). The urgent need for a vaccine is emphasized by the rapidly deteriorating global picture for malaria. The spread of drug-resistant parasites could soon lead to an 8-fold increase in the mortality rate [1,2].

Vaccines aimed at preventing the pre-erythrocytic development of the human malaria parasite *Plasmodium falciparum* are thought to be attainable because immunization with radiation-attenuated sporozoites (IRRD-SPZ) induces protective immune response in humans against infective sporozoites (reviewed in [3]). The protection induced seems to require incomplete intrahepatocytic development of liver forms resulting from the injection of IRRD-SPZ [4]. Thus, molecule(s) expressed during liver stage parasite development may bear epitopes which are crucial in the induction of protection [4–6]. This hypothesis was recently experimentally supported by the successful induction of a

Abbreviations: Tp cell responses, T-cell proliferative responses; PEPS, peptides; LPEPS, lipopeptides; LS, liver stage; SI, stimulation index; CS, circumsporozoite protein; IFAT, Immunofluorescent antibody test; IRRD-SPZ, radiation-attenuated sporozoites.

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protective immunity by immunization with a *P. berghei* liver stage extract, as well as by abrogation of the *P. berghei* IRRD-SPZ induced immunity when the liver forms were eliminated by primaquine [7,8].

We have therefore focused on the isolation and characterization of parasite Ags present during liver stage development of *P. falciparum*. To this end, the screening of a genomic expression library with stage-restricted human antibodies was initiated [9,10]. To date four new Ags have been identified: Liver Stage Ag-1 (LSA1) [9,11]; Sporozoite Threonine and Asparagine-Rich Protein (STARP) [12,13]; Sporozoite And Liver Stage Ag (SALSA) [14] and Liver Stage Ag-3 (LSA3) ([10,15]; Daubersies et al., submitted). Using predictions of T cell epitopes in LSA1, SALSA, STARP and LSA3 [11,12,14–16], twelve regions were selected from these four molecules. The corresponding synthetic peptides proved to be highly antigenic, since they were found to be the target of both humoral and cellular immune responses in individuals from high and low endemicity malaria areas ([11,14,15]; Daubersies et al., submitted; Brasseur et al., in preparation). A critical question for vaccine development was whether they were also immunogenic. Our choice of approach in addressing this question was strongly influenced by two factors, namely (i) that lipopeptides derived from the LSA3 molecule had proven highly immunogenic [15,16] and (ii) that chimpanzees had proven to be highly reproducible models for pre-erythrocytic malaria vaccine research. Indeed, results obtained using LSA3 have recently shown that protection can be induced in the chimpanzee using simple and very well tolerated formulations (Daubersies et al., submitted). This encouraged us to extend our studies of immunogenicity to a wider range of peptides derived from the four new Ags.

The chimpanzee, *Pan troglodytes* provides an important model for pre-erythrocytic vaccine development [17]. It has the immune system closest to humans [18,19] and apart from humans, is uniquely fully receptive to the pre-erythrocytic stages of *P. falciparum* [17,20]. Preliminary studies using individual peptides derived from LSA1 ([11]; L. BenMohamed, 1987 unpublished data), from LSA3 ([15]; Daubersies et al., submitted), from SALSA ([14]; L. BenMohamed 1987 unpublished data), and from STARP [12,13] had shown immunogenicity to varying degrees in mice. Their vaccine potential was investigated by analyzing immunogenicity in chimpanzees. The maximal information was obtained from the fewest number of experimental animals by use of combinations of peptides derived from either SALSA, STARP, or LSA1 with those derived from the LSA3 molecule, since the latter appears to be one with greatest potential ([10,15,16]; Daubersies et al., submitted).

On the basis of previous findings that covalent modification of peptides by a simple fatty acid enhances dramatically their immunogenicity in the absence of adjuvant [15,21,22], the strategy of introducing a C-terminal palmitoylysylamide residue (K(Pam)-NH2) [23] was extended to six of the selected peptides. Thus these six lipopeptides (LPEPS) were injected without adjuvant, i.e. in saline. With the perspective of employing the chimpanzees as a pre-clinical screen, the remaining six non-lipid tailed peptides (PEPS) were also included and adjuvated by Montanide ISA 51, a new oil in water adjuvant, because it has already been used in humans [24]. Data obtained in five outbred chimpanzees confirm the value of the approach since very satisfactory results in terms of antibody production, T cell proliferative responses (Tp cell responses), and IFN-γ production were obtained.

2. Materials and methods

2.1. Antigens

2.1.1. Synthetic peptides and lipopeptides

The sequence localization and the numbers of amino acids of the six peptides and the six lipopeptides derived from the four *P. falciparum* pre-erythrocytic Ags (T. 9. 96 strain): LSA1 [10,11], LSA3 ([10,15,16]; Daubersies et al., submitted), SALSA [14] and STARP [12,13] are shown in Table 1. All peptides were synthesized by the solid-phase method on a benzhydrylamine resin (Applied Biosystem, Foster City, USA) using a standard *t*-butyloxycarbonyl (Boc)-benzyl strategy, and systematically acetylated at the end of the synthesis [15]. A Boc-L-Lys (Fmoc) was introduced in the C-terminal end of the peptide and was coupled to a palmitic acid as previously described [25]. The crude peptides and lipopeptides were purified by reversed-phase chromatography. Peptides and lipopeptides were checked for homogeneity by analytical RP-HPLC and for identity by amino acid-analysis and molecular mass determination on a Bio Ion 20 plasma desorption mass spectrometer (Bio Ion AB, Uppsala, Sweden). Purity of the peptides and lipopeptides was around 95 and 90% respectively. An exception was the STARP lipopeptide, which was found to be totally insoluble in water medium. To prevent aggregation, the crude STARP lipopeptide (100 mg) was solubilized with TFA in the presence of SDS (600 mg), precipitated with ether, and then solubilized with water. Excess SDS was eliminated by extensive dialysis. The synthesis of the STARP-derived mixotope was performed as described [13,26]: accurately weighted equimolecular amounts of appropriate protected amino acids were used in coupling reactions when degenerated sites were required. The first coupling was performed with 0.5 mmol total amount of the introduced

BOC amino acids. A second coupling, using 2 mmol total amount, was then systematically performed.

As controls, four peptides corresponding to T-and B-cell epitopes from irrelevant malaria proteins were included: The Th2R, PSDKHIEQYLKKIKNSISTE peptide from the N-terminal non-repetitive region of the circumsporozoite Ag (CS) of *P. falciparum* (7G8 strain), [27] and the MSP3-C peptide, AKEASSY-DYILGWEFGGGVPEHKKEEN of *P. falciparum* (T 9/96 clone), [28] were used as control T cell epitopes. The Ring Infected Erythrocyte Surface Ag (Pf155/RESA) peptide (EENVEHDA)2(EENV)2 of *P. falciparum* [29] and the ((NANP)4(NVDP)2)2 from the repetitive region of *P. falciparum* CS (7G8 strain), were used as control B-cell epitopes.

2.1.2. Sporozoite preparations

P. falciparum (NF54 strain), *P. yoelii* (17XNL strain) and *P. berghei* (ANKA strain) sporozoites were aseptically prepared, as previously described [15,30], resuspended in RPMI medium and stored at minus 70°C until use.

2.2. Animals and immunization protocol

A total of nine healthy adult chimpanzees (*Pan troglodytes*), were randomly selected from the colony of Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands (Table 2). They were used under standard conditions meeting institutional guidelines for the human care and use of laboratory animals after independent approval by an animal care and use committee. The chimpanzees had distinct MHC background which were determined by sequencing of class I and class II locus but cannot be classified according to the human nomenclature since it is one of the rare genes which is fully different from the human genes. Therefore the class I and class II types are not shown (R. Bontrop et al., 1994 unpublished data). Prior to immunization, chimpanzees did not have pre-existing B- and T cell responses to the *P. falciparum* peptides under investigation.

Chimpanzees were immunized s.c. three times at 4-week intervals, in one site (over the shoulder blade) with a mixture of peptides (100 μg per injection of each peptide) emulsified in Montanide ISA-51 adjuvant (SEPPIC, Quai d'Orsay, France) and in a second site (over the other shoulder blade) with a mixture of lipopeptides in PBS (100 μg per injection of each lipopeptide). Three chimpanzees received LSA3 in association with either LSA1 (Demi), SALSA (Karlien) or STARP (Iris) PEPS and LPEPS, as shown in Table 2. In addition, we also studied two additional chimpanzees (Dirk and Bart)

Table 1
Peptides derived from four pre-erythrocytic *P. falciparum* molecules (LSA1, LSA3, SALSA and STARP), selected for synthesis[a]

Antigen	Peptide Name	Residues	Sequence	N[ber]of A.A.
LSA1	**LSA1-REP**	**187-227**	Ac-LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ	41
	LSA1-J	**1613-1636**	Ac-ERRAKEKLQEQQSDLEQRKADTKK ***K(Pam)-NH2***	24
	LSA1-NR	**1633-1659**	Ac-DTKKNLERKKEHGDILAEDLYGRLEIP	27
	LSA1-TER	**1686-1719**	Ac-NSRDSKEISIIEKTNRESITTNVEGRRDIHKGHKGHL	37
LSA3	**LSA3-CT1**	**24-43**	Ac- LLSNIEEPKENIIDNLLNNI ***K(Pam)-NH2***	20
	LSA3-NRI	**60-85**	Ac- DELFNELLNSVDVNGEVKENILEESQ	26
	LSA3-NRII	**81-106**	Ac- LEESQVNDDIFNSLVKSVQQEQQHNV ***K(Pam)-NH2***	26
	LSA3-RE	**183-210**	Ac-VESVAPSVEESVAPSVEESVAENVEESV	28
SALSA	**SALSA-1**	**23-49**	Ac- SAEKKDEKEASEQGEESHKKENSQESA ***K(Pam)-NH2***	27
	SALSA-2	**50-83**	Ac- NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPK	34
STARP	**STARP-R**	**359-378**	Ac-STDNNNTKTISTDNNNTKTI ***K(Pam)-NH2***	20
	STARP-M	**512-531**	Ac-STDNNNTNTI STDN NNTNTI ***K(Pam)-NH2*** (L T NTIKA S IT N; D D NL D T; K K K K K)	20

[a] Animals were injected subcutaneously, in one site, with a mixture of lipopeptides in saline, and in another site, with a mixture of peptides emulsified in Montanide ISA-51 adjuvant. Chimpanzees Demi, Karlien and Iris were immunized with LSA3 peptides and lipopeptides in combination with, respectively, either LSA1, or SALSA or STARP peptides and lipopeptides (showed as Shaded areas in Figs.1, 2 and 3). Chimpanzees Cor, Peer, Bram and Fuad served as controls.

immunized either with LSA3 in association with LSA1 (Dirk) or with SALSA (Bart). Dirk had previously received the β-galrecombinant proteins DG-729 S from LSA3 (which encompass the LSA3-NRI, LSA3-NRII, LSA3-RE sequences) and DG-536 from LSA1 (which encompass the LSA1-REP, LSA1-J, LSA1-NR and LSA1-TER sequences) both adsorbed on alum. Bart had previously received the β-Gal DG-671 (which encompass the SALSA-1 and SALSA-2 sequences) and SALSA-1 and SALSA-2 on alum. One year later the immune responses had significantly decreased, and 4 years later, responses were no longer detectable in either chimpanzee. At this time Dirk was re-immunized with LSA1 and LSA3 PEPS and LPEPS as described above for Demi, and Bart was re-immunized with SALSA derived PEP and LPEP. For Dirk and Bart the immune responses were compared with Day 0 of peptide immunization (Table 2).

Four chimpanzees (Cor, Peer, Bram and Fuad) which served as non-immunized controls were injected in the same conditions as Demi, Karlien, Iris, Dirk and Bart with 50 μg of control Ag consisting of the β-Gal carrier molecules, with Montanide adjuvant alone or with PBS (Table 2).

2.3. Assessment of T lymphocyte responses

2.3.1. Proliferation assays

Tp cell responses were performed as previously described [15]. Briefly, venous blood was collected from immunized or control chimpanzees and PBMC were isolated by centrifugation on Ficoll–Hypaque density gradient (Pharmacia LKB, Uppsala, Sweden). PBMC (2×10^5 cells/well) were cultured in flat-bottomed 96-well plates (Costar, Cambridge, MA) alone or with either individual peptide (at 1, 3, 10, 30, 90 μg/ml) or frozen-thawed sporozoites (at 10, 100, 1000 or 5000 SPZ/well) for 5 days in RPMI-1640 medium supplemented with 10% human AB^+ serum. One μCi of [^{3}H]thymidine (Amersham, Les Ulis, France) was added to each well, for the final 16 h of culture. Cells were harvested (Skatron, Lierbyen, Norway) and the incorporated radioactivity determined by liquid scintillation (LKB-Wallac, Turku, Finland). Results are expressed as Δ cpm (Δ cpm = cpm in the presence of antigen-cpm without antigen) or as stimulation indices (SI = cpm in the presence of antigen/cpm without antigen). *Mycobacterium tuberculosis* PPD (10 μg/ml); PHA (5 μg/ml) and Leukoagglutinin A (5 μg/ml) were used as positive controls.

Table 2
Scheme of immunization of chimpanzees[a]

Chimpanzees	Antigens	Lipopeptides +	Peptides	Recombinant proteins
Immunized animal				
Demi	LSA3 and LSA1	LSA3-CT1-*K(Pam)-NH2* LSA3-NR11-*K(Pam)NH2* LSA1-J-*K(Pam)-NH2*	LSA3-NR1 LSA3-RE LSA1-REP LSA1-NR LSA1-TER	–
Iris	LSA3 and STARP	LSA3-CT1-*K(Pam)-NH2* LSA3-NR11-*K(Pam)-NH2* STARP-R-*K(Pam)-NH2* STARP-M-*K(Pam)-NH2*	LSA3-NR1 LSA3-RE	–
Karlien	LSA and SALSA	LSA3-CT1-*K(Pam)-NH2* LSA-3-NR11-*K(Pam)NH2* SALSA-1-*K(Pam)-NH2*	LSA3-NR1 LSA3-RE SALSA-2	–
Dirk	LSA3 and LSA1	LSA3-CT1-*K(Pam)-NH2* LSA3-NR11-*K(Pam)NH2* LSA1-J-*K(Pam)-NH2*	LSA3-NR1 LSA3-RE LSA1-REP LSA1-NR LSA1-TER	β-GAL-536[b] β-GAL-729 S
Bart	SALSA	SALSA-1-*K(Pam)-NH2*	SALSA-2	β-GAL-671
Control animals				
Cor	None	–	–	β-GAL
Peer	None	–	–	β-GAL
Bram	None	–	–	GST
Fuad	None	–	–	PBS

[a] The amino acids sequence of each peptide, as single letter code, the corresponding position and the number of amino acid are shown. Lipopeptides are elongated with an additional lysine residue linked to a palmitic acid molecule (*K(Pam)-NH2*).
[b] Parasite protein fused to β-galactosidase (β-GAL).

In order to assess if Tp cell responses were MHC class II dependent, we used either the mAb L-243, a mouse IgG2a specific for the non-polymorphic determinants of human MHC class II molecule (HLA-DR) or the mAb W6/32 a mouse IgG2a, specific for the monomorphic determinants on human MHC class I molecule (HLA-A, B, and C) or a control IgG2a mAb (M-18) [15]. The mAbs were a gift from Dr. Choppin, (IGMC, Paris, France). They were added at the initiation of the in vitro PBMC culture at 1:200 and remained present throughout the 6 days culture period.

2.3.2. IFN-γ induction

PBMC were incubated in 24-well plates (Costar, Cambridge, MA) at 3×10^6 cells/well in a total volume of 1 ml of the medium described above. Cell cultures were stimulated with each individual peptide (10 μg/ml), with PPD (10 μg/ml) or with PHA (5 μg/ml). The IFN-γ concentration in supernatants (SNs) of triplicate wells was assessed separately by a two-site capture ELISA performed in duplicate by using anti-IFN-γ mAb RU 40.2 for capture and the mAb RU 308.7, coupled to peroxidase, as the second mAb [11,14,15]. Anti-IFN-γ mAb were kindly supplied by Mrs. Cousin, Roussel Uclaf, France. The IFN-γ content of SNs was calculated from standard curves performed on culture medium containing known amount of IFN-γ. Readings were compared with the international human NIH IFN-γ standard Gg23-901-530. Samples which IFN-γ concentration was greater than 2 IU/ml were considered positive [11,14,15].

2.4. Antibody assays

2.4.1. ELISA

Peptide-specific antibodies were determined using ELISA assay as described previously [15]. Optimal conditions for Ab detection were determined for each peptide, by using a panel of ten malaria endemic areas sera and ten control sera, three coating buffers (Tris, pH 7.4; Carbonate, pH 9.6 and PBS, pH 7.4); three coating temperatures (4°, 25° and 37°) and three peptide concentrations (1, 3 and 10 μg/ml). ELISA plates (Nunc-Immuno Plate II, Nunc) were coated overnight with individual tested or control peptides. The plates were washed twice, blocked for 1 h in PBS supplemented with 1% BSA prior to addition of 0.1 ml of 1/100 dilution of a chimpanzee serum. The bound IgG were detected using peroxidase-conjugated goat anti-human IgG (Biosys, Compiègne, France). Pre-immune sera from immunized chimpanzees as well as sera from non-immunized chimpanzees were used as negative controls. Results are expressed as ELISA-RATIO calculated as followed: OD_{492nm} post-immune sera/OD_{492nm} pre-immune sera. The mean ELISA-RATIO of duplicates + SD are shown. ELISA-RATIO > 1 are considered as indicative of a specific IgG Ab response [11,14,15].

2.4.2. Immunofluorescent Ab assay

The reactivity of the sera against native parasite proteins from various stages of the parasite was analyzed by IFA as previously described [15]; using either (i) Glutaraldehyd-fixed *P. falciparum* sporozoites (a gift of Dr. W. Eling) [30], or (ii) Carnoy-fixed sections from liver biopsies containing day 5 *P. falciparum* liver schizonts obtained from a Cebidae monkey (*Cebus apella*) [31]. FITC-conjugated goat anti-human IgG, -A, -M (Diagnostic Pasteur France) diluted 1/200 was employed as second antibody.

3. Results

3.1. Peptide selection

Putative T-cell epitopes able to bind MHC class II molecules were selected on the argument that T-cell and B-cell epitopes have frequently been observed to cluster within a limited region of Ags [32–36]. The four Ags of interest were therefore screened (i) for B-cell epitopes on the basis of conventional predictive conformational or surface accessibility criteria, focusing on putative α-helical regions, because this type of organization is accessible to an acceptable mimicry by synthetic peptides, and (ii) for putative hinge regions, downstream these α-helices, because such regions are physicochemically more accessible to proteolysis than constrained, organized structures (e.g. α-helices) [37]. Twelve sequences were selected, and the corresponding peptides were synthesized (Table 1). These peptides were deliberately of medium size (20–41 aa), because T-cell determinants corresponding to separate MHC-restriction elements have been found to frequently overlap [32,35,36]. The actual occurrence of B- and T-cell recognition motifs was then evaluated by testing their reactivity with antisera and PBMC from infected individuals in several African settings [11,12,14,15]; (Daubersies et al., submitted; Brasseur et al., in preparation). No significant Tp cell responses and no IFN-γ responses to any peptide or lipopeptide was observed in pre-immune lymphocyte cultures from chimpanzees, nor in cultures from twenty malaria-naive individuals indicating a lack of mitogenic effect (data not shown).

3.2. T cell responses from immunized chimpanzees

3.2.1. PEPS and LPEPS induce Tp cell responses in chimpanzees

The Tp cell responses to recall peptides following the third injection of the mixture of synthetic PEPS

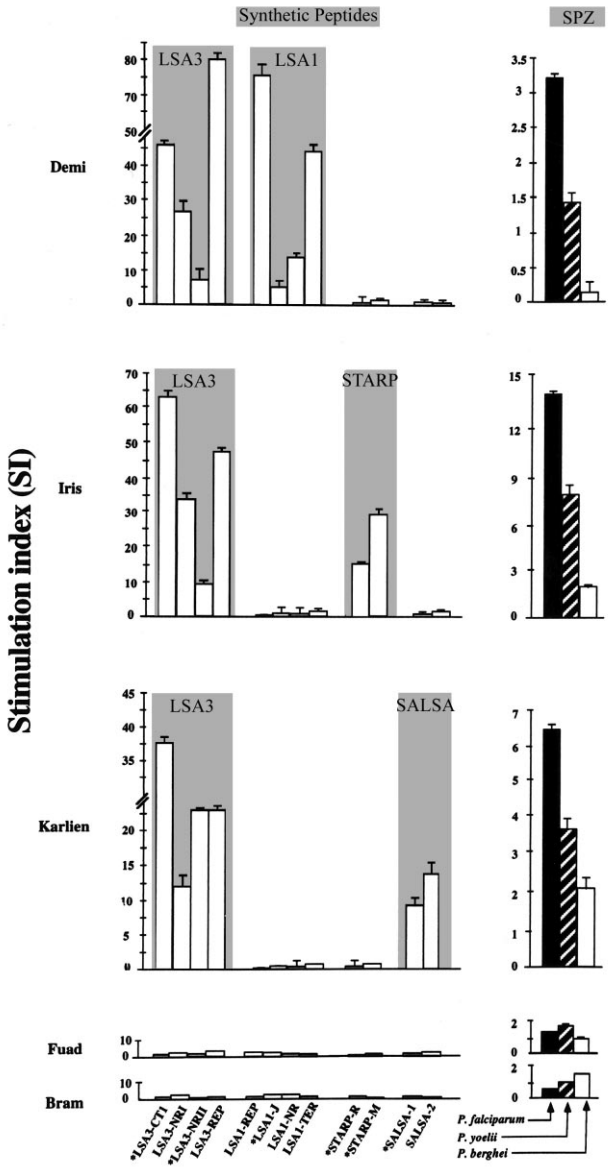

Fig. 1. Anti-peptide (left) and anti-SPZ (right) Tp cell responses of immunized chimpanzees recorded 4 weeks after the third immunizing doses. PBMC were prepared from each animal and incubated either with the peptides from immunizing molecules (Shaded areas) or with peptides which were not used for the immunization (left panels) or with *P. falciparum, P. yoelii* or *P. berghei* SPZ Ag 100 SPZ per 2.10^5 cells (unshaded in right panels). Experimental and control wells were runned in triplicate. These data are representatives as they were reproduced in other experiments obtained from two additional blood samples. The results are presented as stimulation indices (SI). The mean background cpm of responses in control post-immune-cultures without Ag, were 2250 cpm for Demi, 2876 cpm for Iris, 3260 cpm for Karlien, 4215 cpm for Fuad and 2018 cpm for Bram. Asterisks (*) in front of peptides in the *x*-axis indicates that a lipopeptide form was used for in vivo immunization.

and LPEPS were assessed. As shown in Fig. 1 (left panels), specific and strong Tp cell responses were induced by all the peptides. Each of the immunized animals showed a significant T cell reactivity to more than one peptide, indicating that each of these peptides bears at least one T cell epitope recognized by the chimpanzee immune system.

The PBMC from chimpanzee Demi, immunized with the LSA1 PEPS and LPEPS, together with LSA3, responded to all four LSA1 peptides, the highest stimulation indexes being seen with LSA1-REP and LSA1-TER peptides. The specificity of the responses was demonstrated by the lack of responses to LSA1 peptides in the chimpanzees Iris and Karlien, which were not immunized with LSA1 and in the controls Fuad and Bram. This strong immunogenicity is further supported by the results obtained in chimpanzee Dirk, which had previously shown a weak Tp cell response following a first immunization with the corresponding recombinant protein (unpublished data). Dirk was re-immunized with the same mixture of LSA1 PEPS and LPEPS as Demi, and responded more strongly than earlier. Dirk had stimulation indexes of 2.5, 22.4, 45.0 and 46.2 to LSA1-REP, LSA1-J, LSA1-TER and LSA1-NR, respectively. Both STARP lipopeptides proved to be effective immunogens since they induced specific and significant Tp cell responses in chimpanzee Iris. The SALSA immunized animal (Karlien) showed a Tp cell response to both SALSA-1 and SALSA-2 peptides. This was confirmed by results obtained in a second animal (Bart) which showed a strong response following re-injection with the same SALSA peptides (stimulation indexes of 14.3 and 40.5 to SALSA1 and SALSA2, respectively). Finally Tp cell responses were elicited to all four LSA3 peptides and were consistently induced in each of the three immunized chimpanzees, Demi, Iris and Karlien. The magnitude of Tp responses against LSA3 peptides was among the highest, in agreement with the results obtained in Dirk following immunization with these PEPS/LPEPS and with the results recorded previously in an animal injected with the LSA3-NRII lipopeptide alone (Gerda) [15]. The T cell responses induced in this way proved to be long-lived since they remained detectable in samples obtained 8 months after immunization and tested in the same manner (i.e. in Dirk SI decreasing from 13.9 to 10.1 for LSA3-NRII, from 30.1 to 28.5 for LSA3-RE).

No significative response was detected when the PBMC from the immunized animals were incubated with an heterologous pre-erythrocytic peptide (Fig. 1) or with other control peptides (Th2R and MSP-3C) (data not shown), showing the specificity of Tp cell responses and the lack of cross-reactive T cell epitopes between these Ags. The intensity of the Tp cell response against PPD, PHA as well as Leukoagglutinin A was similar for the control and immunized animals (data not shown). Since some PEPS and LPEPS induced Tp cell responses of the same magnitude as the Tp cell responses to PPD (e.g. LSA3-CT1, LSA3-

RE and LSA1-REP) this suggests that they are strongly immunogenic. As expected, these Tp cell responses were MHC class II dependent as shown by the blocking effect of an anti-HLA-DR mAb though not of a mAb against HLA-A, B, and C. (e.g. Tp response against SALSA-1 in chimpanzee Bart; SI of 14.3 without mAb yielding SI of 1.4 and 11.5 using an anti HLA-DR and anti HLA-A, B, and C respectively).

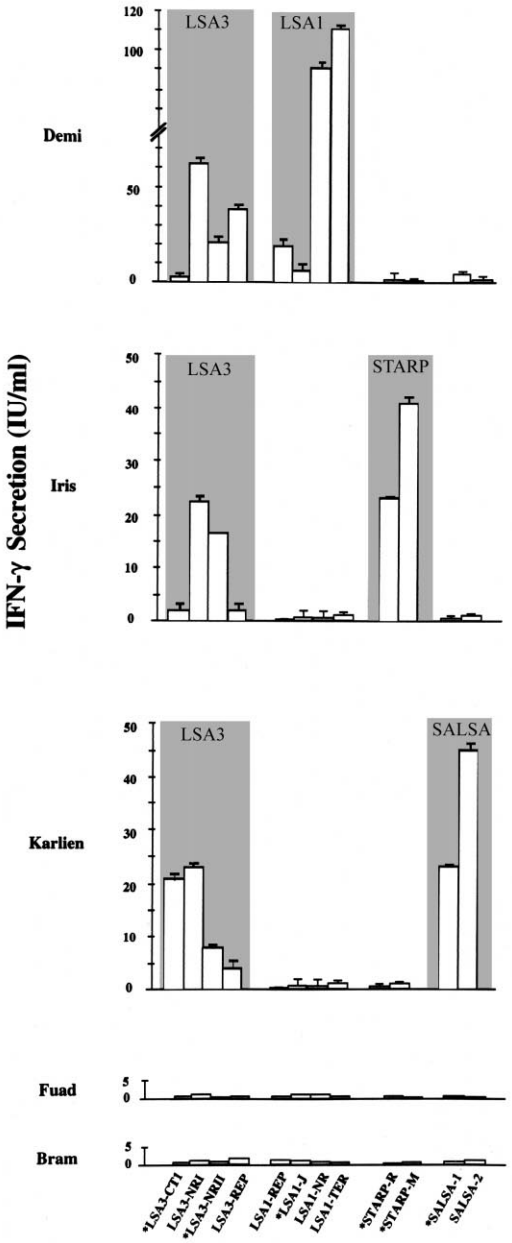

Fig. 2. Secretions of IFN-γ by PBMC from immunized and control chimpanzees in response to various peptides. IFN-γ responses of PBMC from animals were assessed on two separate occasions, before immunization and following PEPS and LPEPS immunization. Cell cultures were stimulated with PPD (10 μg/ml) or with individual peptides (30 μg/ml). The IFN-γ concentrations were assessed in three days SNs as described in Section 2, results are expressed as IU + SD. Asterisks (*) in front of peptides in the *x*-axis indicates that a lipopeptide form was used for in vivo immunization.

3.2.2. IFN-γ production following synthetic PEPS and LPEPS immunization

Because IFN-γ is a most potent lymphokine inhibiting the intrahepatic development of the parasite, we also investigated whether these liver stage Ags would be able to stimulate T-cells to IFN-γ production. As shown in Fig. 2, specific secretion of IFN-γ frequently at high amounts, was found upon in vitro re-stimulation with all peptides, though not in all animals, in contrast to Tp cell responses.

The PBMC recovered from the chimpanzee Demi secreted substantial amounts of IFN-γ upon in vitro re-stimulation with both LSA1-NR and LSA1-TER peptide whereas lower but significant amounts were induced by LSA1-J and by LSA1-REP. The PBMC recovered from the LSA1 re-immunized chimpanzee (Dirk) also showed IFN-γ responses against the various LSA1 peptides (IFN-γ: 29.7, 31.1, 80.4, 95.8 IU/ml in response to LSA1-J, LSA1-REP, LSA1-NR and LSA1-TER, respectively). The animal injected with STARP (Iris) showed specific and significant amount of IFN-γ upon in vitro challenge of PBMC with both STARP peptides. Both SALSA-1 and SALSA-2 peptides proved also to be effective at inducing an IFN-γ responses in chimpanzee Karlien; this was confirmed in the re-immunized chimpanzee Bart (IFN-γ 9.7 and 38.6 IU/ml in responses to SALSA-1 and SALSA-2, respectively).

Although there were differences between the LSA3 immunized animals in the amounts of IFN-γ produced, substantial amounts were induced by all four LSA3 peptides. The LSA3-NRI and LSA3-NRII peptides corresponding to well-conserved non-repeated sequences were found to be the most consistent stimulators of IFN-γ secretion with reproducibly high levels induced by LSA3-NRI peptide, in three animals (Fig. 2) as well as after the re-immunization of Dirk (IFN-γ: 134.0, 25.3, 8.8, 4.7 IU/ml in response to LSA3-NRI, LSA3-NRII, LSA3-RE and LSA3-CT1, respectively). As for the Tp cell response, the IFN-γ production was abrogated by anti-class II, but not by anti-class I mAbs (i.e. in chimp Demi IFN-γ response against LSA-3-NRII peptide; IU of 22.6 without mAb reduced to IU of 3.8 and 18.5 when anti-HLA-DR and anti-HLA-A, B, and C were added, respectively).

IFN-γ secretion and Tp cell responses were found to be associated, although this was not universal. Indeed, PBMC from chimpanzee Demi showed moderate proliferative response but yielded high amounts of IFN-γ, upon stimulation with LSA1-NR peptide. Conversely, LSA3-RE peptide failed to stimulate IFN-γ production in both chimpanzees Iris and Karlien, whereas high levels of proliferation were seen in these animals.

On an individual basis, the production of IFN-γ in response to PPD, PHA and Leukoagglutinin (Leu A) was higher than that induced by peptides in each animal. The IFN-γ response was also peptide-specific since no IFN-γ production was observed in the presence of heterologous peptides. As previously reported in humans living in several malaria endemic areas [11,12,14,15], these results confirm the presence, within these Ags, of epitopes for IFN-γ secreting T cells.

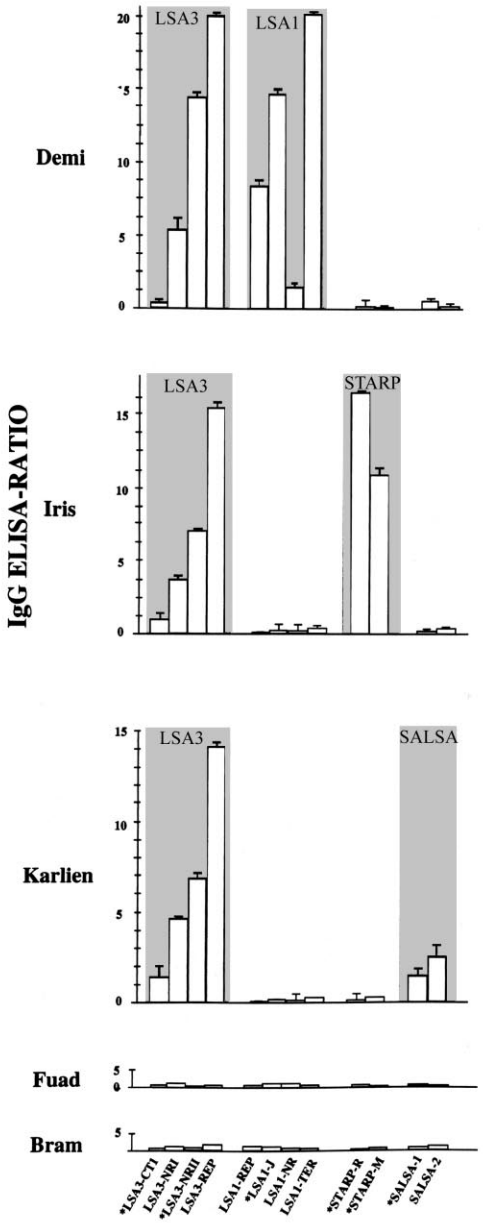

Fig. 3. Antibody responses of chimpanzees against various pre-erythrocytic stage peptides in sera collected 4 weeks after the third PEPS and LPEPS immunization. Results are expressed as ELISA-RATIO (see Section 2). The means of triplicate wells from two separate ELISA assays are presented. Asterisks (*) in front of peptides in the *x*-axis indicates that a lipopeptide form was used for in vivo immunization.

Specific CD8+ cytotoxic T cell responses (CTL) were also generated by these peptides and lipopeptides in chimpanzees and will be reported elsewhere.

3.3. Humoral immune responses of chimpanzees following injection with PEPS and LPEPS mixture

3.3.1. Antibody responses to recall Ags

As shown in Fig. 3, 4 weeks after the third injection, PEPS and LPEPS immunized animals displayed significant Ab responses. Antibodies were detected against all peptides tested except LSA3-CT1, confirming in the chimpanzee that all peptides except one define at least one B-cell epitope. As was the case for the Tp cell responses, the intensity of the IgG Ab responses varied depending on the peptide. Interestingly, the pattern of response was similar in the three animals immunized with LSA3 in contrast to what was observed with Tp cell responses. Although repetitive peptides were generally targeted by strong IgG Ab responses (i.e. LSA3-RE, LSA1-J and STARP-R), some of the non-repeti-

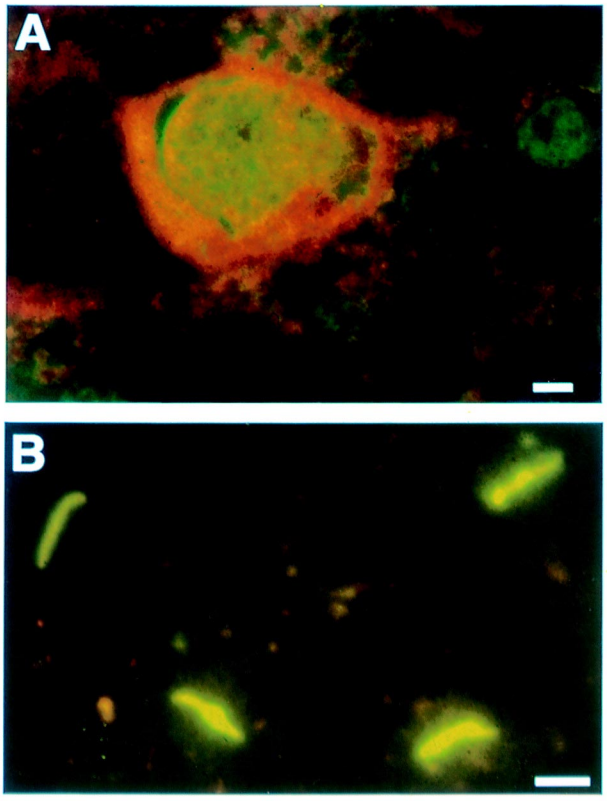

Fig. 4. Reactivity with the native parasite protein of antibodies induced by the mixture of liver stage peptides and lipopeptides in chimpanzees: Iris (lower picture) and Demi (lower picture). Sera were tested by IFAT on *P. falciparum* sporozoites isolated from salivary glands of infected *A. stephensi* mosquitoes (lower picture) or on sections of 5 day-old *P. falciparum* liver stages obtained in *Cebus apella* (upper picture). The chimpanzees sera were taken 1 month after the third injection.

tive peptides were also targeted by high IgG responses (i.e. LSA3-NRII and LSA1-TER). As expected, the level of response increased with the number of immunizations (data not shown).

Chimpanzee Demi showed the strongest Ab responses to the repeat peptides, but high responses were seen against two of the non-repeat peptides. This is in agreement with the results obtained in the re-immunized chimpanzee Dirk which showed a similar pattern of response (ELISA-Ratio = 3.1 and 17.0 to peptide LSA3-NRII and LSA3-RE, respectively). STARP-immunized chimpanzee Iris responded with a higher level to the consensus sequence (STARP-R) than to the mixed-epitope sequence (STARP-M). Sera from both SALSA immunized animals (Karlien and Bart) contained low but significant amounts of Abs to SALSA-2 peptide. Bart showed an additional response to SALSA-1 peptide (data not shown) which was absent from Karlien (Fig. 3).

The antibodies detected are specific since no response was measurable either to the heterologous peptides (e.g. the 4 LSA1 peptides in Iris and Karlien) (Fig. 3), or to the control peptides (RESA and (NANP)4(NVDP)2)2) (data not shown), and no response was detected in control animals receiving adjuvant only.

Although the immune responses decreased 8 months after immunization, these responses were still detectable at significant levels (i.e. in chimpanzee Bart ELISA-RATIO decreasing from 5.5 to 3.1 for SALSA-1 and from 11.3 to 9.7 for SALSA-2 over eight months after immunization).

3.4. Relevance of peptide-induced-immune-responses to the native parasite proteins

3.4.1. T cells responses to sporozoite antigens

The ability of an immunogen to prime the immune system in such a way that a secondary response will be induced when the pathogen is introduced is of great importance for vaccine purposes. Since LSA3, SALSA and STARP Ags are expressed on the sporozoite surface, we assessed whether immune T cells induced by these Ags would be boosted upon in vitro challenge with the whole parasite. The PBMC obtained 2, 4 and 12 weeks after the final immunization of animals with LSA3, SALSA and STARP responded vigorously to *P. falciparum*-SPZ (NF54 strain), i.e. significant Tp cell responses (Fig. 1, right panels), and high concentrations of IFN-γ (Fig. 2, right panels), were obtained. These parasite-induced cell responses were class-II, but not class-I, dependent (data not shown). This suggests that recognition of Ags normally associated with the SPZ occur in lymphocytes from immunized chimpanzees. However, since the chimpanzees were immunized with a mixture of Ags these results do not allow determination, except for Demi, of the Ag (s), or the epitope (s) responsible for the proliferative response observed in this assay. It should be noted that a small amount of SPZ Ag (as low as 100 SPZ per 2.10^5 PBMC) proved sufficient to induce such high responses, suggesting that the immunogen formulations used are efficient in presenting properly conformed SPZ T cell epitope (s). No significant Tp cell responses against SPZ were observed in pre-immune lymphocyte cultures from all animals, indicating a lack of intrinsic mitogenic effect of the sporozoites (data not shown). The response is specific since it was not obtained when PBMC from the immunized animals were incubated (i) with *P. berghei* sporozoites (Figs. 1 and 2), which do not have obvious homologues of the molecules employed in this study (Daubersies et al., submitted) (ii) nor with salivary gland extracts from non-infected mosquitoes (not shown). The Tp cell responses against *P. falciparum* sporozoites were MHC class II dependent as shown by the blocking effect of an anti-HLA-DR mAb though not of a mAb against HLA-A, B, and C. (e.g. in chimpanzee Bart; SI of 13.3 without mAb reduced to SI of 3.1 and 12.9 when anti HLA-DR and anti HLA-A, B, and C were added, respectively).

3.4.2. Antibodies induced by immunization bind to sporozoites and liver stage forms

Similarly the potential recognition by antibodies of native structures on parasite was investigated with sera from immunized chimpanzees by immunofluorescence assay (IFAT) upon sporozoite and liver forms. Sera from all immunized chimpanzees, but not from controls, strongly reacted with both pre-erythrocytic stages (Fig. 4), but were non-reactive with *P. berghei* sporozoites (data not shown). This suggests that the synthetic peptides used for immunization share properly conformed antigenic determinants with the natural proteins expressed in sporozoite and liver stages.

3.5. P. falciparum LSA3 molecule induce B- and T-cell responses which cross-react with P. yoelii pre-erythrocytic stages

In addition, in vitro stimulation of PBMC with *P. yoelii* SPZ (17 XNL strain), but not with *P. berghei* SPZ (ANKA strain) nor with salivary gland extracts from non-infected mosquitoes, induced a significant Tp cell response in two out of three animals (Fig. 1) and substantial secretion of IFN-γ in all three animals (Fig. 2). Similarly, sera from all LSA3 immunized animals were positive by IFAT on both sporozoite and liver stage of *P. yoelii* (Not shown, pattern similar to that shown in Fig. 4). This result confirms our previous observation that the LSA3-NRII peptide shows

cross-reactive B- and T-cell epitopes with the *P. yoelii* homologue [15]. The responses detected are very likely due only to LSA3 since, in the same conditions, no response was obtained with cells and sera from chimpanzees immunized only with SALSA (this study and [14], with LSA1 alone [11] or with STARP alone (P. Millet and L. BenMohamed, 1996 unpublished data).

4. Discussion

We have studied in the chimpanzee, a non-human primate whose immune system is very close to that of man, the immunogenicity of twelve synthetic peptides derived from four molecules expressed at pre-erythrocytic stages of the human malaria parasite *P. falciparum*. Using as immunogens combinations of lipopeptides in saline and peptides in Montanide, specific Ab and cellular responses to these four new molecules were elicited which proved relevant to the native epitopes, and long-lasting.

Among numerous problems in the development of a peptide-based malarial vaccine, it has long been considered that a main limitation is insufficient immunogenicity, itself being generally related to parasite molecular polymorphism, and to the genetic restriction of immune responses observed in outbred populations. The problem of variable and usually low immunogenicity of the vaccine candidates currently developed has been stressed in many reports [27,38–41]. Examples are provided by research on the development of a subunit synthetic or recombinant vaccine using the circumsporozoite protein (CS) of *P. falciparum* [27,42–46]. In order to reach consistent responses and to attempt to achieve sustained and long lasting immune responses, CS-derived peptides were coupled with large recombinant "carrier" molecules as source of T-cell epitope(s) (e.g. Tetanus toxoid, PPD or HBsAg) [44,45,47], or required either complex presentation (proteosomes or liposomes) [44,46] or toxic adjuvants (e.g. Freund) [47,48]. Two human vaccine trials using NANP-repeats of the *P. falciparum* CS covalently fused to a sequence encoded by a tetracycline resistance gene that was read out in frame as "carrier" epitope (referred to as $R32tet_{32}$) [42,43] or to tetanus toxoid using alum as adjuvant generated low and variable titers of antibodies [45], even though both preparations had proven very immunogenic in mice [27,38]. Similarly the synthetic hybrid peptides described by Patarroyo, representing epitopes from sporozoite and erythrocyte stages of malaria, conjugated and adsorbed to Alum, induced detectable immune responses in only about half of vaccinated humans [49,50].

In sharp contrast a consistent and frequently very high immunogenicity has been obtained with all twelve peptides selected for this study. This has been confirmed in other chimpanzees immunized in another Primate Center (Luty et al., submitted), as well as in Aotus monkeys [16], and is in agreement with preliminary data obtained in mice [15]. The selection employed to identify both the four molecules characterized and the specific peptides, may have particularly favored the selection of immunogenic molecules: (i) based on epitope conservation in twelve sporozoite isolates and on the consistency and longevity of natural human immune responses [15]. (ii) Based on the ease of epitope expression in prokaryotic systems as judged during initial screening by antibody of a bacteriophage expression library implying that pre-erythrocytic peptides did not need the full protein environment to define a B-cell epitope [9,10]. (iii) Based on proximity to predicted hinge regions, a process which although to some extent empirical, has proven in practice to be as successful as other predictors such as mathematical algorithms [11,14,15]. (iv) Based on the synthesis of medium size peptides, rather than epitope-sized peptides, as in many instances, it has been shown that T-cell epitopes overlap and segregate within a relatively small area of a given molecule [35,36]. (v) Finally, in the particular case of STARP which, in contrast to the remaining Ags, is known to present polymorphism in its repeat sequence, we have relied on the previously described "mixotope" strategy to enhance the immunogenicity. This consists in producing a convergent combinatorial library of peptides, or mixotope, obtained in a single synthesis by introducing sequence degenerations, representing, in the initially described model, the variability of an hypervariable antigenic viral determinant [26]. The situation for STARP is different from that of the above viral Ags, since STARP contains a repetitive domain, corresponding to the repetition of a series of degenerated ten amino acids units, which was used as a basis for the design of a mixotope containing all the described, and the potentially possible as yet undescribed, degeneracies provided they permit to keep the same secondary structure [13].

The results obtained in outbred apes, as well as the high B- and T-cell antigenicity observed in the field, lend support to the above described empirical approach as it is unlikely that out of twelve random peptides, 12 out of 12 and 11 out of 12, can define universal T- and B-cell epitopes, respectively.

The modified peptide synthesis leading to the production of lipid-tailed peptides has been a strategy first defined in order to induce the production of CTL by synthetic compounds without the need to rely on live vectors [51]. The capacity of exogenous proteins or peptides associated with lipidic compounds to prime CTL has been well documented, although the precise mechanism is not fully understood. It is usually hypothesized that the lipidic component leads to

increased transmembrane passage of the protein or the peptide and thus to higher cytosolic concentrations [15,21,22] and this has received recently experimental support (BenMohamed et al., submitted). It has recently been recognized that the same process can also increase the immunogenicity in terms of class II dependent responses [52]. Our initial results obtained with the LSA3-NRII lipopeptide [15], are supported by the present results with five additional lipopeptides. They indicate that this strategy can provide immunogenic molecules that do not have a requirement for adjuvant. Thus, major hurdles in synthetic peptide vaccine development, i.e. insufficient immunogenicity and the requirement for powerful adjuvants [48], may be overcome by the use of lipid-tailed peptides. Taken together with technical progress in the production of long peptides (i.e. 100–120 aa) [53], this increases the likelihood that peptide based immunization can provide alternatives to more complex and fastidious strategies based on cloning, expression and purification of recombinant proteins [54,55].

In the case of LSA-3 peptides, which was used in four animals the immune responses obtained were found to be relatively homogeneous differing only in the intensity of the response in each animal, this despite the fact that the animals had proven distinct MHC background (R. Bontrop et al., 1994 unpublished data). Three animals responded to all four LSA3 peptides in a similar fashion. Another chimpanzee, Nuria, immunized in a separate study (Luty et al., submitted) also responded to all four LSA3 peptides. An additional indication came from the chimpanzee Dirk who had earlier received recombinant proteins and was re-immunized with the peptides at a time when immune responses were no longer detectable. In the case of peptide LSA3-NRII the same was also found in chimpanzee Gerda [15]. Thus, in total six animals (for the LSA3-NRII peptide), and five animals (for the remaining three LSA3 peptides), showed strong T cell responses to components of LSA3. These results obtained in outbred apes suggest in addition that each peptide defines at least one multi-restricted epitope or a series of adjacent or overlapping T-cell epitopes restricted by distinct class II Ags. Although the design of the study does not allow us to distinguish between these three hypotheses, these results underline the clinical potential of this set of peptides and of the vaccine formulation employed.

For some of the peptides, the intensity of Tp cell responses did not directly correlate with IFN-γ secretion levels. This result is in-keeping with previous studies [11,14], and has been attributed to genetic restriction in Ag recognition and mainly to stimulation of different T lymphocyte subpopulations which may differ by IFN-γ secretion[56].

When injected in combination with other peptides (chimpanzees Dirk, Demi, Karlien and Iris) the LSA3-NRII peptide induced increased Ab and cellular responses when compared to those recorded previously in an animal injected with the LSA3-NRII peptide alone (i.e. more than 3-fold higher for proliferative T-cell responses) [15]. This may correspond to individual variations in the ability to mount a strong immune response, or to the fact that the Ags were combined in the immunizing protocol we employed [57]. It is possible that the cytokine profile induced by one of the peptides can modulate the immunogenicity of co-injected peptides, as reported previously [58]. However, there are strong indications that the high immunogenicity obtained is a feature of each individual peptide because single peptide immunizations which were performed in monkeys and in mice also induced high immune responses [11,12,14–16]; Luty et al., submitted).

The immunogen formulations derived from four new malarial molecules display several valuable features: (i) the long persistence of B- and T-cell responses suggests that major T-cell epitopes in these Ags are capable of eliciting good memory responses; (ii) the production of IFN-γ is of interest since it is known to efficiently stimulate the elimination of *P. falciparum* liver forms even at low concentrations [59]; (iii) the induction of responses specific to native determinants is also of importance, and suggests that the epitopes defined by the peptides are similar to those generated by the in vivo processing of the whole parasite. In contrast to responses to many malarial proteins we show here that high levels of antibodies not only to the repetitive sequences but also to several non-repetitive epitopes could be elicited by experimental immunization.

In conclusion, we show here that a strategically selected mixture of synthetic peptides constitutes a valuable alternative to the classical recombinant protein strategy [54,55] or to very long synthetic polypeptides [53] since, despite their relatively small size, they generated B-and T-cell responses to all component peptides. These results, obtained in non-human primates phylogenetically close to man, help to further characterize the most promising molecules, and the most immunogenic epitopes within them, and have important implications for the design of an optimized *P. falciparum* pre-erythrocytic subunit vaccine. Experimental results obtained with peptide based vaccines derived from viral molecules already indicate that protection can be achieved against viruses [60] and recent results obtained with LSA3 derived PEPS and LPEPS induced consistent protection in chimpanzee (Daubersies et al., submitted). Although it is not yet clear which of the numerous defense mechanisms ([6,61] and references therein) will prove effective against a *P. falciparum* sporozoites challenge, it is encouraging to ob-

serve, as described here, that these molecules can induce a wide range of immune responses.

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References

[1] Marsh K. Malaria disaster in Africa. Lancet 1998;352:924.
[2] Trape J-F, et al. Impact of chloroquine resistance on malaria mortality. CR Acad Sci Paris 1998;321:689–97.
[3] Clyde DF. Immunity to *falciparum* and *vivax* malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. Bull WHO 1990;68(Suppl.):9–12.
[4] Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Druilhe P. Protection against malaria induced by irradiated sporozoites. Lancet 1990;335(8691):721.
[5] Druilhe P, Marchand C. From sporozoite to liver stages: the saga of the irradiated sporozoite vaccine. In: McAdam K, editor. New strategies in parasitology. Edinburgh: Churchill Livingstone, 1989. p. 39–48.
[6] Druilhe P, Renia L, Fidock DF. Immunity to the liver stages. In: Sherman I, editor. Malaria. Washington, DC: ASM Press, 1998. p. 513–43.
[7] Rénia L, Rodrigues MM, Nussenzweig V. Intrasplenic immunization with infected hepatocytes: a mouse model for studying protective immunity against malaria pre-erythrocytic stage. Immunology 1994;82:164–8.
[8] Scheller LF, Azad AF. Maintenance of protective immunity against malaria by persistent hepatic parasites derived from irradiated sporozoites. Proc Natl Acad Sci USA 1995;92:4066–8.
[9] Guerin-Marchand C, et al. A liver stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. Nature 1987;329:164–7.
[10] Marchand C, Druilhe P. How to select *Plasmodium falciparum* pre-erythrocytic antigens in an expression library without defined probe. Bull WHO 1990;68(Suppl.):158–64.
[11] Fidock DA, et al. *Plasmodium falciparum* liver stage-specific antigen-1 is well conserved and contains potent B and T cell determinants. J Immunol 1994;153:190–204.
[12] Fidock DA, et al. Cloning and characterization of a novel *Plasmodium falciparum* sporozoite surface antigen, STARP. Molec Biochem Parasitol 1994;64:219–32.
[13] Pasquetto P, et al. *Plasmodium falciparum* sporozoite invasion is inhibited by naturally-acquired or experimentally-induced polyclonal antibodies to the STARP antigen. Eur J Immunol 1997;27:2502–13.
[14] Bottius E, et al. A Novel *Plasmodium falciparum* sporozoite and liver stage antigen (SALSA) defines major B, T helper, and CTL epitopes. J Immunol 1996;156:2874–84.
[15] Benmohamed L, et al. Lipopeptide immunization without adjuvant induces potent and long-lasting B, T helper, and cytotoxic T lymphocyte responses against a malaria liver stage antigen in mice and chimpanzees. Eur J Immunol 1997;27:1242–53.
[16] Perlaza BL, et al. Immunogenicity of four *Plasmodium falciparum* preerythrocytic antigens in *Aotus lemurinus* monkeys. Infect Immun 1998;66(7):3423–8.
[17] Thomas AW, Slierendregt B, Mons B, Druilhe P. Chimpanzees and supporting models in the study of malaria pre-erythrocytic stages. Mem Inst Oswaldo Cruz 1994;89:111–4.
[18] Haynes BF, Dowell BL, Hensley LL, Gore I, Metzgar RS. Human T cell antigen expression by primate T cells. Science 1982;215:298–300.
[19] Geluk A, et al. Evolutionary conservation of major histocompatibility complex-DR/Peptide/T cell interactions in primates. J Exp Med 1993;177:979–91.
[20] Meis JFGM, et al. *Plasmodium falciparum*: studies on mature exoerythrocytic forms in the liver of the chimpanzee, *Pan troglodytes*. Exp Parasitol 1990;70:1–11.
[21] Martinon F, et al. Immunization of mice with lipopeptides bypasses the prerequisite for adjuvant. Immune response of BALB/c mice to human immunodeficiency virus envelope glycoprotein. J Immunol 1992;149(10):3416–22.
[22] Bourgault I, et al. Simian immunodeficiency virus as a model for vaccination against HIV: induction in rhesus macaques of GAG- and NEF specific cytotoxic T lymphocytes by lipopeptides. J Immunol 1994;152:253–61.
[23] Déprez B, et al. Pimelautide or Trimexautide as built-in adjuvants associated with an HIV-1-derived peptide: synthesis and in vivo induction of antibody and virus-specific cytotoxic T-lymphocyte-mediated response. J Med Chem 1995;38:459–65.
[24] Turner JL, Trauger RJ, Daigle AE, Carlo DJ. HIV-1 immunogen induction of HIV-1-specific delayed-type hypersensitivity: results of a double-blind, adjuvant-controlled, dose-ranging trial. AIDS 1994;8:1429–35.
[25] Déprez B, Sauzet JP, Boutillon C, Martinon F, Tartar A, Sergheraert C, Guillet JG, Gomard E, Gras-Masse H, Comparative efficiency of simple lipopeptide constructs for in vivo induction of virus-specific CTL. Vaccine 1996;14,375–82.
[26] Estaquier J, et al. The Mixotope: a combinatorial peptide library as a T cell and B cell immunogen. Eur J Immunol 1994;24:2789–95.
[27] Good MF, et al. Construction of synthetic immunogen: use of new T-helper epitope on malaria circumsporozoite protein. Science 1987;235:1059–62.
[28] Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. Blood 1994;84(5):1594–602.
[29] Troye-Blomberg M, et al. T and B cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 1989;143(9):3043–408.
[30] Brahimi K, et al. Fast immunopurification of small amounts of specific antibodies on peptides bound to ELISA plates. J Immunol Methods 1993;162:69–75.
[31] Druilhe P, Miltgen F, Boulard Y, Landau I. Exo-erythrocytic schizogony of *Plasmodium falciparum* in monkey *Cebus apella*. Molec Bioch Parasit 1982;108(Suppl.):125–33.
[32] Kabilan L, et al. T-cell epitopes in Pf155/RESA, a major candidate for a *Plasmodium falciparum* malaria vaccine. Proc Natl Acad Sci USA 1988;85:5659–63.
[33] Auriault C, et al. Epitopic characterization and vaccinal potential of peptides derived from a major antigen of *Schistosoma mansoni* (Sm28 GST). Pept Res 1991;4(1):6–11.
[34] Estaquier J, et al. Determination of B-cell epitopes of nef HIV-1 protein: immunogenicity related to their structure. Molec Immunol 1992;29(11):1337–45.
[35] Higgins JA, Thorpe CJ, Hayball JD, O'Hehir RE, Lamb JR. Overlapping T-cell epitopes in the group I allergen of

Dermatophagoides species restricted by HLA-DP and HLA-DR class II molecules. J Allergy Clin Immunol 1994;93:891–9.
[36] Meister GE, Roberts CGP, Berzofsky JA, De Groot AS. Two novel T cell epitope prediction algorithms based on MHC-binding motifs; comparison of predicted and published epitopes from Mycobacterium tuberculosis and HIV protein sequences. Vaccine 1995;13:581–91.
[37] Landry SJ. Local protein instability predective of helper T-cell epitopes. Immunol Today 1997;18:527–32.
[38] Good MF, Kumar S, Miller LH. The real difficulties for malaria sporozoite vaccine development: non responsiveness and antigenic variation. Immunol Today 1988;9(11):351–5.
[39] Etlinger HM, et al. Assessment in humans of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, *Plasmodium falciparum*. J Immunol 1988;140(2):626–33.
[40] Nardin EH, Barr PJ, Heimer E Etlinger HM. Genetic restriction of the murine humoral response to a recombinant *Plasmodium vivax* circumsporozoite protein. Eur J Immunol 1988;18:1119–22.
[41] Udhayakumar V, et al. Antigenic diversity in the circumsporozoite protein of Plasmodium falciparum abrogates cytotoxic-T-cell recognition. Infect Immun 1994;62(4):1410–3.
[42] Ballou WR, Rothbad J, Wirtz RA, Gordon DM, Williams JS, Gore RF, Schneider I, Hollingdale MR, Beaudoin RL, Malloy WL, Miller LH, Hockmeyer WT. Immunogenicity of synthetic peptides from the circumsporozoite protein of *Plasmodium falciparum*. Science 1985;228:996–9.
[43] Ballou WR, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Lancet 1987;1:1277–81.
[44] Fries LF, et al. Safety, immunogenicity, and efficacy of a *Plasmodium falciparum* vaccine comprising a circumsprozoite protein repeat region peptide conjugated to *Pseudomonas aeruginosa* toxin A. Infect Immun 1992;60(5):1834–9.
[45] Herrington DA, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Nature 1987;328:257.
[46] Heppner DG, et al. Safety, immunogenicity, and efficacy of *Plasmodium falciparum* repeatless circumsporozoite protein vaccine encapsulated in liposomes. J Infect Dis 1996;174:361–6.
[47] Stoute JA, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. New England Journal of Medicine 1997;336:86–91.
[48] Gupta RK, Siber GR. Adjuvants for human vaccines-current status problems and future prospects. Vaccine 1995;13:1263–76.
[49] Amador R, et al. Safety and immunogenicity of the synthetic malaria vaccine SPf66 in a large field trial. J Infect Dis 1992;166:139–44.
[50] Noya OY, Berti de G Alarcon Noya B. A population-based clinical trial with the SPf66 synthetic *Plasmodium falciparum* malaria vaccine in Venezuela. J Infect Dis 1994;170:396.
[51] Deres K, Schild H, Karl-Heinz W, Jung G, Rammenensee H-G. In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. Nature 1989;342:561–4.
[52] Wiesmüller KH, et al. The antibody response in BALB/c mice to the *Plasmodium falciparum* circumsporozoite repetitive epitope covalently coupled to synthetic lipopeptide adjuvant. Immunology 1991;72:109.
[53] Roggero MA, et al. Synthesis and immunological characterization of 104-mer and 102-mer peptides corresponding to the N- and C-terminal regions of the *Plasmodium falciparum* CS protein. Mol Immunol 1995;32:1301–9.
[54] Sturchler D, et al. Safety, immunogenicity, and pilot efficacy of *Plasmodium falciparum* sporozoite and asexual blood-stage combination vaccine in Swiss adults. Am J Trop Med Hyg 1995;53:423–31.
[55] Etlinger HM, et al. Ability of recombinant or native proteins to protect monkeys against heterologous challenge with *Plasmodium falciparum*. Infect Immun 1991;59:3498–503.
[56] Surcel H, et al. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 1994;81:171–6.
[57] De Kroon AIPM, McConnell HM. Enhancement of peptide antigen presentation by a second peptide. Proc Natl Acad Sci 1993;90:8797–801.
[58] Redhead K, et al. Combination of DTP and *Haemophilus influenzae* type b conjugate vaccines can affect laboratory evaluation of potency and immunogenicity. Biologicals 1994;22:339–45.
[59] Mellouk S, et al. Inhibitory effect of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures. J Immunol 1987;139:4192–5.
[60] Friede M, et al. Selective induction of protection against *influenza* virus infection in mice by a lipid-peptide conjugate delivered in liposomes. Vaccine 1994;9:791–7.
[61] Hoffman SL, Franke ED, Hollingdale R, Druilhe P. Attacking the infected hepatocyte. In: Hoffman SL, editor. Malaria vaccine development. Washington: ASM publications, 1996. p. 40.