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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

We have employed a 26-amino-acid synthetic peptide based on *Plasmodium falciparum* liver stage antigen-3 to evaluate improvements in immunogenicity mediated by the inclusion of a simple lipid-conjugated amino acid during peptide synthesis. Comparative immunization by the peptide in Freund's adjuvant or by the lipopeptide in saline shows that the addition of a palmitoyl chain can dramatically increase T helper (Th) cell responses in a wide range of major histocompatibility complex (MHC) class II haplotypes, to the extent that responses were induced in mice otherwise unable to respond to the non-modified peptide injected with Freund's adjuvant, and that the increased immunogenicity of the lipopeptide led to high and longer lasting antibody production (studied up to 8 months). B and T cell responses induced by the lipopeptide were reactive with native parasite protein epitopes, and a lipopeptide longer than ten amino acids was endogenously processed to associate with MHC class I and elicit cytotoxic T lymphocyte (CTL) responses. Finally, the lipopeptide was safe and highly immunogenic in chimpanzees, whose immune system is very similar to that of humans. Our results suggest that relatively large synthetic peptides, carefully chosen from pertinent areas of proteins and incorporating a simple palmitoyl-lysine, can induce not only CTL, but also strong Th and antibody responses in genetically diverse populations. Lipopeptides engineered in this way are simple to produce and purify under GMP conditions, they are well tolerated by apes, and with the enhanced immunogenicity without the need for adjuvant that we report here, they offer a quick and relatively low-cost route to provide material for human malaria vaccination trials.

1 Introduction

Due to its relative simplicity, synthetic peptide technology has been widely used to produce epitope antigens. This technology has proven extremely useful in the detailed analysis of immune responses induced by other means, and has further shown that synthetic peptides can be of value for immunization purposes [1]. However, their use for vaccine development has been hampered in practical terms by problems such as weak immunogenicity (coupled with a paucity of sufficiently potent adjuvants that can be tolerated by humans), the correct folding of complex conformational epitopes [2], and perhaps most critically, the problem of MHC restriction [3–5]. Some of these limitations have been, in part, circumvented by the use of "Freund's-like preparations" [6, 7], chemical conjugation to viral [8, 9] or bacterial [10, 11] carrier proteins, incorporation into liposomes [12], proteosomes [13], or immunostimulating complexes [14], or by synthesis of larger constructs such as multiple antigenic peptides [15] or of hybrid multiple epitope [16]. These different presentations often increased the immunogenicity of the Ag, at least with respect to antibody production, which was frequently the main parameter used to assess immunogenicity.

A major breakthrough occurred in the field of peptide-based vaccines when Rammensee and coworkers [17, 18] reported that a synthetic peptide that had been identified as a target for CTL could induce CTL when the peptide was covalently coupled with lipids. Although an influenza-specific peptide conjugated to a relatively complex lipopeptide tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P_3CSS), induced CTL activity in mice to a level similar to that obtained using live vectors, the construct was mitogenic and somewhat toxic [19, 20]. However, the recent demonstration that the simple substitution of an amino acid by a lipid-conjugated amino acid during a standard peptide synthesis could achieve the same goal [21, 22] and be relevant to viral peptides in their native state [23] has enormously increased the potential for the development of effective synthetic peptide vaccines. The applicability of such vaccines may also be enhanced by the possibilities for selected artificial association of the most relevant epitopes as a result of recent mapping studies for B, T, and CTL epi-

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Abbreviations: **LP:** Lipopeptide **NLP:** Nonlipidic peptide **LS:** Liver stage **SI:** Stimulation index **CS:** Circumsporozoite **IFAT:** Immunofluorescent antibody test **LSA:** Liver stage antigen

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topes within the major vaccine candidate molecules for critical pathogens such as hepatitis B virus (HBV), HIV, influenza virus, and malaria. These studies also indicated that several of these epitopes may often be clustered [24, 25], an observation which, in combination with the advances in presentation described above, has restimulated interest in the potential of synthetic peptide vaccines. In the context of vaccination strategies, these approaches have unique advantages in terms of cost, purity, and simplicity of construction.

In the field of malaria pre-erythrocytic stages, vaccine development has been strongly influenced by the observation that the injection of whole, radiation-attenuated sporozoites can induce strong protection against challenge, including *Plasmodium falciparum* in humans [26]. The extended immunization schedule and the complexity of delivery for sporozoite vaccines make such an approach unfeasible for human use. Thus, efforts are being made to determine the critical components required for pre-erythrocytic vaccines. Although studies initially emphasized the role of the circumsporozoite (CS) protein, recent results suggest that antigens expressed early in liver stage (LS) development are also likely to be critical targets for protective effector mechanisms [27–30]. Several new molecules expressed during LS development have recently been characterized, and are currently being evaluated for vaccine development [31–35].

Recently, a new pre-erythrocytic vaccine candidate, liver stage antigen-3 (LSA3), was identified ([32] and Daubersies et al., in preparation). The vaccine potential of this novel molecule was indicated by results obtained from humans that had been exposed to parasite infection, and in mice (Daubersies et al., in preparation). In the present study, we relied on previous observations [33, 36] which designated those regions located in the vicinity of repeated sequences as prone to be preferentially processed and to associate with MHC molecules. We compared the immunogenicity of a 26-mer region of LSA3 in mice and chimpanzees with that of a simple lipid-tailed modification of this peptide. Our data confirm that the lipopeptide without adjuvant can induce T helper cells, B cells, as well as CTL.

2 Materials and methods

2.1 Peptide and lipopeptide synthesis

All peptides were synthesized by the solid-phase method [37] in an automated peptide synthesizer (Applied Biosystem, model 430A, Foster City, CA) using a standard t-butyloxycarbonyl (Boc)-benzyl strategy. A Boc-L-Lys (Fmoc) was introduced in the C-terminal end of the peptide and was coupled with a palmitic acid as described [21, 22, 38]. The crude peptides were purified to >90% by reversed-phase (RP) chromatography on a 8.2 × 250 mm column (Polymer Laboratories 300Å, 20 μm, PLRP-S) eluted with an acetonitrile/25 mM triethylamine phosphate buffer (pH 9) gradient solvent system. After purification, the peptide was desalted on an 8.2 × 100 mm (Vydac 300Å, 7 μm, C4) column. 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). The lipopeptide was soluble in water at neutral pH (>2 mg/ml). The NRII peptide (LEESQVNDDIFNSLVKSVQQEQQHNV) used in this study was selected from the LSA3 *P. falciparum* T 9.96 isolate sequence (Daubersies et al., in preparation). Three peptides corresponding to known T or B cell epitopes were used as controls: the LSA-1 peptide ERRAKEKLQEQQRDLEQRKDTKK of T 9.96 strain of *P. falciparum* [34] designated LSA-J, and its lipopeptide analog (lipopeptide LSA-J), the Th2R peptide from the CS protein PSDKHIEQYLKKIKNSIS of the 7G8 strain of *P. falciparum* [5], and the ring infected erythrocyte surface antigen (RESA, Pf155) peptide (EENVEHDA)2(EENV)2 of *P. falciparum* [39].

2.2 DNA preparation, PCR amplification, and sequencing

To analyze the potential sequence polymorphism of LSA3-NRII, parasite DNA was extracted from a series of malaria-infected human blood specimens as described [40] and resuspended in 50 μl water. The LSA3-NRII peptide-containing region was amplified from 5 μl DNA sample according to the manufacturers recommendations (USB-Pharmacia, Cleveland, OH; 2 mM $MgCl_2$, 40 pmole each primer). Sequence of the primers will be reported elsewhere (Daubersies et al., in preparation). Amplification cycles were 94 °C for 3 min, 55 °C for 2 min, 72 °C for 2 min: 2 cycles; 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min: 35 cycles, followed by 72 °C for 10 min. The size of the amplification product (300 bp) was assessed by loading 10 μl of the PCR reaction on a 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, ME) containing ethidium bromide. The remaining PCR products were ethanol-precipitated, pelleted, gel-purified, and sequenced according to the manufacturer's recommendations (T7 Polymerase Sequencing kit, Amersham, Indianapolis, IN).

2.3 Immunization of mice and collection of sera

Female mice 6 weeks old of five different inbred strains, C57BL/6 (H-2^b), SJL/J (H-2^s), BALB/c (H-2^d), DBA/1 (H-2^q) and C3H/HeJ (H-2^k), were obtained from Institut Pasteur (Paris, France). They were immunized by subcutaneous injection at the base of the tail with 50 μg of either LSA3-NRII or control LSA-J synthetic peptides or the corresponding lipopeptides, either in PBS or emulsified in Freund's incomplete adjuvant (FIA, Sigma, St. Louis, MO). Mice received one boost 3 weeks later with 25 μg of the same peptide or lipopeptide in PBS or in FIA. The mice were bled before each injection and 10 days after.

2.4 Immunization of chimpanzees and collection of sera

Four randomly selected healthy young adult chimpanzees (*Pan troglodytes*) in the colony of the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands were used. Chimpanzee Gerda was injected at days 0, 21, 93, and 217 with 100 μg of the LSA3-NRII lipopeptide in saline. Chimpanzee Dirk had been immunized three times

with 50 μg of the β-galactosidase recombinant LSA3-DG-729 protein, which encompass the LSA3-NRII sequence peptide (Daubersies et al., in preparation), prior to receiving two injections of 100 μg LSA3-NRII peptide in non-lipidic form. Dirk was immunized 28 months later twice with a 20-day interval with 100 μg LSA3-NRII lipopeptide in saline. Peptides, lipopeptides, and recombinant proteins were injected by the subcutaneous route. Control chimpanzees Bram and Fuad were selected for mismatched MHC class I, based on sequence analysis of their leukocyte antigens. Sera and PBMC were collected before and after each injection.

2.5 Antibody assays

2.5.1 ELISA

ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 0.1 ml peptide solution at 3 μg/ml in PBS pH 7.4 containing 3 % BSA. The RESA and LSA-J peptides were used as controls. The plates were washed twice in PBS with 0.01 % Tween 20 (PBS-T), blocked for 1 h in PBS-T supplemented with 1 % BSA prior to the addition of 0.1 ml serial twofold dilutions of mouse sera or chimpanzee sera at 1/100 dilution. The plates were then incubated at 37 °C for 1 h. After washing, the bound IgG were detected using peroxidase-conjugated goat anti-mouse or anti-human IgG (Biosys, Compiègne, France) added at a 1/2000 dilution (mouse) or 1/4000 dilution (human) in PBS-T. Following incubation at 37 °C for 1 h and a final wash, 50 μl 0.30 % H_2O_2 containing orthophenylenediamine dihydrochloride (Sigma), dissolved in 0.1 M citrate buffer (pH 5.0) were added to each well at room temperature. The absorbance at 492 nm was measured using a multichannel spectrophotometer (Titertek Multiskan MCC. 340). Individual mouse sera from the five groups were diluted 1/100–1/50 000 and analyzed separately. The results are expressed as the reciprocal of the serum dilution giving a signal twofold that recorded with the preimmune serum at a dilution of 1/100 (mean titers + SD). Preimmune sera from immunized chimpanzees Gerda and Dirk and from nonimmunized chimpanzees Bram and Fuad were used as negative controls, and the results are expressed as the ELISA ratio calculated as $A_{492\,nm}$ immune/$A_{492\,nm}$ pre-immune.

Isotype analysis of mouse responses was carried out using class-specific goat horseradish peroxidase conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 Ab (Southern Biotechnology Associates, Birmingham, AL) added at a 1/2000 dilution in PBS-T.

2.5.2 Immunofluorescent Ab assay (IFAT)

The reactivity of the sera against native parasite proteins from various stages of the parasite were analyzed by IFAT using either *P. falciparum* NF54 strain sporozoites (a gift of W. Eling) as described [41], sections from liver biopsies containing day 5 *P. falciparum* liver schizonts [42], or day 6.5 *P. falciparum* liver schizonts obtained from a chimpanzee [43]. IFAT-labeled anti-human IgG, IgA, or IgM (Diagnostic Pasteur, *France*), or anti-mouse (Cappel, Wester Chester, PA) diluted 1/200 were employed as second Ab.

2.6 T helper cell proliferation assays

2.6.1 Lymph node assays in mice

The draining inguinal LN of mice (two or three per group) were removed aseptically 10 days after the second antigen injection. The LN cells were washed twice with RPMI 1640 (Gibco, Courbevoie, France) and adjusted to a concentration of 3×10^6 cells/ml in RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 1 % minimum nonessential amino acids (Gibco), 5×10^{-5} M 2-mercaptoethanol (Gibco), 1.5 % heat-inactivated FCS, and 0.5 % normal mouse serum. Aliquots of 0.1 ml of the cell suspension were deposited onto flat-bottom 96-well plates (Nunclon, Roskilde, Denmark) to which 0.1 ml of a range of dilutions of either synthetic peptides or lipopeptides were added. Each test was performed in triplicate. The cell suspensions were incubated for 72 h at 37 °C in 7.5 % CO_2 in air. [^{3}H]dThd (1 μCi; Amersham, Les Ulis, France) was added to each well for 16 h before the cultures were harvested (Skatron, Lierbyen, Norway) and the incorporated radioactivity determined by liquid scintillation (LKB-Wallac, Turku, Finland). Results are expressed as the mean cpm of cell-associated [^{3}H]dThd recovered from wells containing Ag, substracted by the mean cpm of cell-associated [^{3}H]dThd recovered from wells without Ag (Δ cpm; average of triplicates). Each assay has been repeated at least twice.

2.6.2 Chimpanzee PBMC proliferation assays

Fresh PBMC from chimpanzees collected at various pre- and postinjection time points and isolated by density gradient centrifugation (Organon Teknika, Durham, NC) were cultured essentially as described above except that the medium did not contained β-mercaptoethanol and was supplemented with 10 % heat-inactivated human AB serum (Institut Jacques Boy, Reims, France), and the culture period was 6 days. Control PBMC from chimpanzees Bram and Fuad were included in each set of experiments. To assess MHC class I or class II presentation, the following mAb were used: either the anti-DR L-243 mAb (5 μg/ml) [44], or anti-HLA-A-B-C W6/32 mAb (1/1000 dilution of ascites fluid; a gift of Dr. Choppin) [45], or anti-CD4 (OKT4a), or anti-CD8 (OKT8) (Orthodiagnostic Systems, Raritan, NJ) antibodies were added at the initiation and remained present throughout the 6 days of culture. All Ab were mouse IgG2a isotype, and an irrelevant IgG2a was used as control.

2.6.3 IFN-γ assay

The IFN-γ concentration in pooled supernatants from the triplicate wells (collected on day 5) was assesed by a two-site capture ELISA performed in duplicate as described [33, 35], using the anti-IFN-γ mAb RU 40.2 for capture and the mAb RU 308.7, coupled to peroxidase, as the second mAb. The IFN-γ content of supernatants was calculated from standard curves performed on culture medium containing known amounts of IFN-γ, included in each plate.

2.7 Cytotoxic T lymphocyte assays

2.7.1 Standard CTL assay

CTL assays were performed using PBMC from immunized chimpanzees Dirk and Gerda and two control chimpanzees Bram and Fuad. PBMC resuspended in RPMI 1640 supplemented with 10% human AB serum were restimulated by incubation with either LSA3-NRII or control peptides (10 μg/ml of each peptide) in 25-cm^2 Falcon flasks. Recombinant (r)IL-2 (20 IU/ml; Genzyme, Cambridge, MA) was added at 72 h and assays performed after 7 days of culture at 37 °C in 7.5% CO_2/air. The ^{51}Cr-release assays were carried out using peptide-pulsed PHA blasts (generated with 0.5 μg/ml of L-PHA for 3 days). These target cells were incubated overnight at 37 °C in the presence or absence of 20 μg/ml test peptide LSA3-NRII or LSA-J control peptide, and 3 μg/ml human β_2-microglobulin (Sigma), in RPMI 1640 (Gibco) supplemented with 20% human AB serum. After labeling with 150 μCi Na_2[^{51}Cr]O_4 (ICN Biomedical Inc., Irvine, CA) for 60 min at 37 °C, the cells were washed three times, and 5×10^3–1×10^4 target cells were added to triplicate wells in 96-well round-bottom microtiter plates (Corning, NY). To reduce the nonspecific cytolytic activity of NK cells during the ^{51}Cr-release assay, the human erythroleukemia NK-sensitive cell line K562 was added in 20-fold excess of labeled targets. Effector cells were added at effector:target (E:T) cell ratios of 40:1. The plates were incubated for 5 h at 37 °C in 7.5% CO_2 in air and 100 μl supernatant was quantitated for released ^{51}Cr using a gamma counter. The percent specific lysis was determined as: percent specific lysis = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. For CTL assays in C3H/HeJ mice, the cell line RDM4 (H-2^k, CBA) was used as target cells.

2.7.2 Blocking by antibodies of cytolytic activity

To assess class I or class II presentation of antigen, either anti-DR (5 μg/ml mAb L-243), or anti-HLA-A-B-C (1/1000 dilution of ascites fluid containing mAb W6/32, a dilution found to inhibit class I-restricted CTL responses) [45], or an irrelevant IgG2a antibody used as control, were added to 10^6 target cells for 1 h at 4 °C and remained present throughout the 5 h of the CTL assay.

2.7.3 Depletion of CD4$^+$ and CD8$^+$ T cells *in vitro*

A fraction from the peptide-stimulated effector cells were depleted by using anti-CD8 or anti-CD4 mAb coated on Dynabeads according to manufacturer's specifications (Dynal, Great Neck, NY). More than 95% of the cell populations were effectively depleted as evaluated by IFA and flow cytometry. These were used as effectors in the CTL assay as described above.

3 Results

3.1 Peptide selection

Within the LSA3 sequence, the repeat region shows a very strong propensity to helical organization using the Garnier-Robson method [46]. This region indeed contains an extraordinary, regular spacing of valine or isoleucine, alternating with acidic or proline residues. The disposition of the hydrophobic clusters on the surface of this putative helix provides a hydrophobic seam that gradually shifts from one face of the helix to the other, probably related to coiled-coil packing, although there are skips in its organization around glycine and proline residues (Fig. 1).

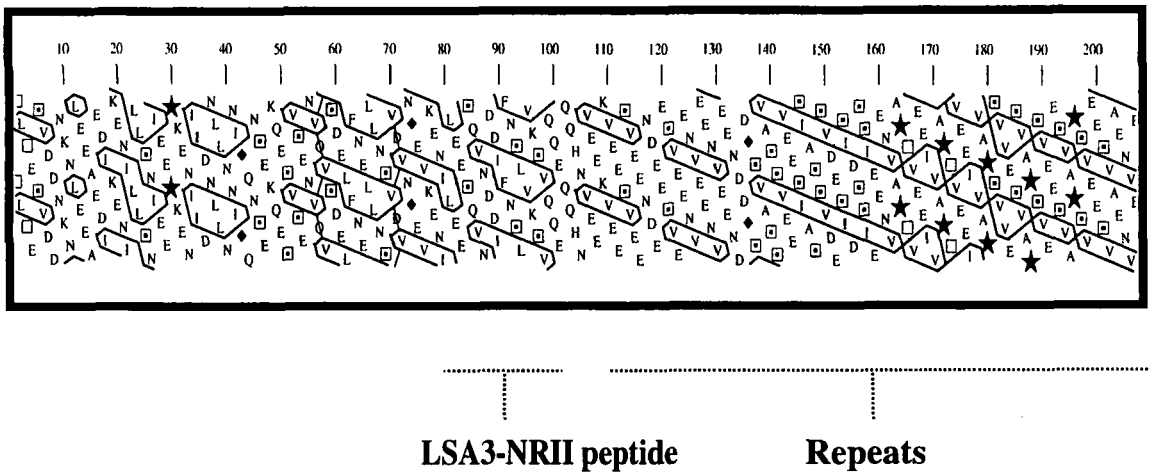

Figure 1. Hydrophobic cluster analysis plot of the N-terminal region of the LSA3 protein: hydrophobic residues are contoured. Some residues are represented by symbols: P (closed star) – a known helix-breaker, G (closed diamond) T (open rectangle) and S (open rectangle with closed rectangle inside). The amino acid sequence is written diagonally from left to right such that there are seven residues per line and the last three or four residues are alternately also displayed on the following line. Since a classical α helix contains 3.6 residues per turn and is typified by the regular spacing of hydrophobic residues, this sequence representation enables efficient visualization of an unfolded α helical sequence [65]. The LSA3-NRII peptide (underlined) is located near the long highly amphipathic segment.

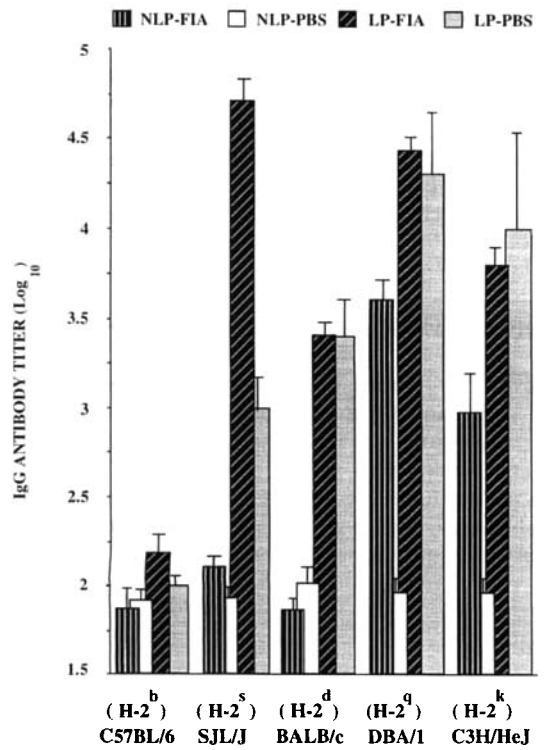

Figure 2. IgG antibody titers induced by LSA3-NRII LP in C57BL/6, SJL/J, BALB/c, DBA/1, and C3H/HeJ mice. Groups of five mice were immunized (50 μg on day 0, and a boost of 25 μg on day 20) with either the LSA3-NRII peptide in saline (open bars) or in FIA (vertical stripes), or with the LSA3-NRII lipopeptide in saline (grey) or in FIA (hatched). Antibody titers in the sera collected 10 days after the second injection were determined by ELISA. Values are the reciprocal of the dilution of serum giving a signal at least twice that recorded with the pre-immune serum at a dilution of 1/100. Each individual mouse serum was analyzed separately. Results from a group of five mice are expressed as the mean titer ± SD.

Based on the interest in potential T cell epitopes in pre-erythrocytic stage molecules, we focused our attention on possible hinge regions between the repetitive and nonrepetitive areas of the molecule. These regions are probably less constrained than other parts of the molecule, and therefore more readily accessible to proteolysis, an event that precedes T cell epitope presentation in association with MHC molecules [33, 36]. B and T cell epitopes have frequently been observed to cluster within a limited region in antigens from a wide range of organisms [24, 47]. Multiple epitopes corresponding to different HLA class I restrictions were also observed in a relatively small region of HIV [24, 25, 48]. For these reasons, the localization of B epitopes may serve as a guide to regions in which clusters of T helper or CTL epitopes can be found.

Since repetitive regions of malarial antigens frequently have proven to contain B cell epitopes [49], and such is also the case for LSA3 (Daubersies et al., in preparation), the epitope cluster analysis led us to search for T epitopes in the immediate vicinity of the repeat region, and from the hydrophobic cluster analysis plot four peptides were selected. The prediction that one Th cell epitope would include the sequence QQEQQ in position 100–104 was confirmed by showing that the peptide LSA3-NRII, which includes this sequence, defined a strong Th epitope: in 67 individuals from the holoendemic areas of Dielmo, Senegal [50], 59.6% showed specific lymphoproliferative and 48.3% IFN-γ secretion when challenged *in vitro* with this peptide (Daubersies et al., in preparation). In contrast to the repeat region, this peptide defined only a minor B cell epitope.

3.2 The addition of palmitic acid has a profound effect upon the Ab responses induced by the peptide in mice

The LSA3-NRII lipopeptide (LP) as well as the corresponding non-lipidic peptide (NLP) were tested for Ab induction after two subcutaneous injections with or without FIA, in five different strains of mice (Fig. 2).

NLP injected with FIA induced strong Ab responses only in DBA/1 and C3H/HeJ mice, whereas no Ab responses could be induced in C57BL/6 or BALB/c mice, and only very moderate titers were observed in SJL/J. In contrast, the administration of the LP with FIA induced not only higher levels of response in the same two responding strains of mice, but most notably induced responses in the three other strains, including the two which were nonresponders to the peptide in the absence of the lipopeptide modification (*i.e.* BALB/c and C57BL/6). Levels of IgG antibodies were high in four of five strains and were low but detectable in C57BL/6. For instance, titers were 150 times higher in SJL/J mice using LP than those obtained using NLP.

It was further shown that the improvement brought by the palmitoyl-lysine component was not dependent on adjuvant; in the four highly responding strains of mice, a strong response could also be induced by immunization with LP injected with PBS (*i.e.* without FIA). In three of the four strains, the response was equivalent in intensity to the response to the same antigen in the presence of FIA. Only C57BL/6 did not respond. The improved immunogenicity of the LP was particularly clear since, in the absence of adjuvant, NLP did not induce detectable antibody response in any strain of mice. It is noteworthy that both the subcutaneous and intravenous routes of administration of LP induced responses that were comparable in all five strains of mice (data not shown).

The patterns of isotypes investigated using class-specific Ab were found to be similar for LP and NLP immunizations with or without FIA, as all IgG isotypes could be detected, IgG1 being always predominant (data not shown).

3.3 LSA3-NRII induces long-lasting Ab responses in mice

When studied 6 months and 8 months after immunization, the Ab responses were no longer detectable in those mice that had been immunized with NLP in FIA. In contrast, the Ab responses induced by LP were associated with the induction of a long-lasting memory, since these Ab could be detected in LP immunized mice for up to 8 months (longer delays were not tested) with only a minor decrease

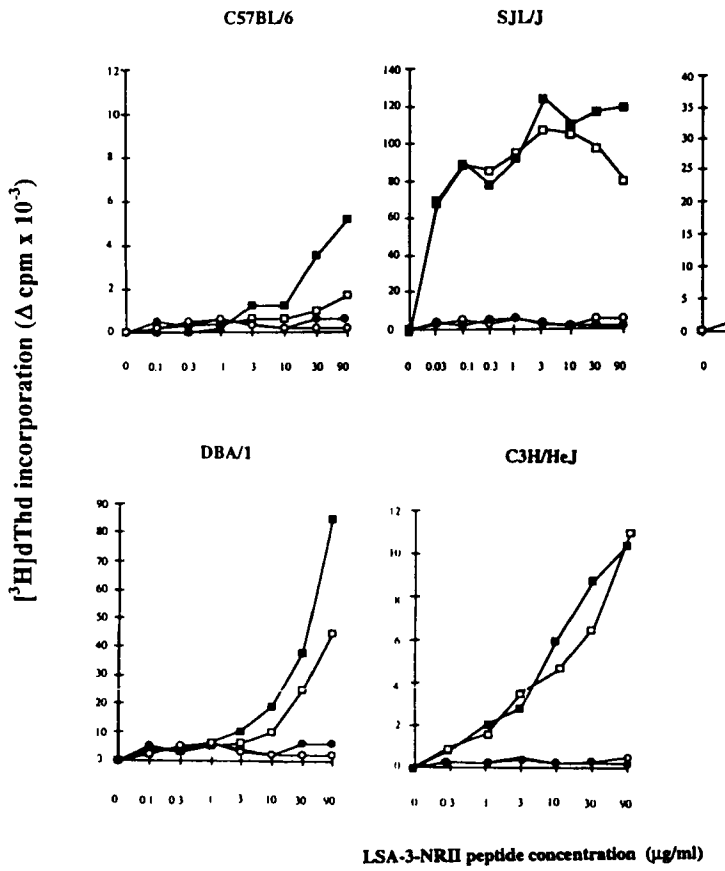

Figure 3. Proliferative T cell responses induced by LSA3-NRII LP in various strains of mice. On day 0, C57BL/6, SJL/J, BALB/c, DBA/1, and C3H/HeJ mice were injected subcutaneously with 50 μg LSA3-NRII peptide in FIA (open squares), with LSA3-NRII LP in saline (solid squares), with control peptide LSA-J in FIA (open circles), or with control LSA-J LP in saline (solid circles). The mice were boosted on day 20 with 25 μg of the same immunogen. Inguinal lymph node cells, obtained 10 days after the last injection, were stimulated *in vitro* with LSA3-NRII peptide at the indicated concentrations, the proliferation determined by [^{3}H]dThd incorporation, and results are expressed as the difference in cpm (Δ cpm). The background cpm, in unstimulated cells, were: 856 for C57BL/6, 1786 for SJL/J, 1156 for BALB/c, 685 for DBA/1, and 1254 for C3H/HeJ.

in Ab titers, with the $\log_{10}$ IgG decreasing from 3.5 to 3.1 in BALB/c, from 4.7 to 4.5 in DBA/1, and from 4.5 to 3.5 in C3H/HeJ (means from five mice). This result is remarkable considering that it was obtained when LP was administered without adjuvant.

3.4 T helper cell proliferative responses confirm the greater immunogenicity of the lipopeptide

The proliferative responses of inguinal LN cells from the same five strains of mice showed a similar improvement in responses to LP. Strong proliferative responses were observed in LN cells from SJL/J, DBA/1, and C3H/HeJ mice, following immunization with either NLP in FIA or LP in saline (Fig. 3). Moreover, a proliferative response was obtained after immunization with LP in saline in the remaining two strains of mice, while the immunization of these strains by NLP in FIA induced essentially no response in BALB/c mice or only borderline response in C57BL/6 detectable only at the highest Ag concentration tested: (stimulation index (SI)=2). In contrast with the data obtained with LP, no proliferative response was observed in any strain of mice injected with NLP in saline (data not shown).

When cells were challenged *in vitro* using LP, the SI were very similar to those obtained with NLP and are therefore not shown. The association of the peptide with MHC class II on specific T cells therefore remained related to its primary amino acid sequence, and was not affected by the addition of a lipid-conjugated amino acid. The specificity of the proliferative response detected in mice immunized with LP was ascertained *in vitro* by restimulation of LN cells with control lipopeptide LSA-J, and by NLP restimulation of cells from mice that had been immunized with control lipopeptide LSA-J, both of which failed to induce proliferation (Fig. 3). This also demonstrated that the LSA3-NRII peptide had no mitogenic effect, either in NLP or LP form.

3.5 Immunization by LSA3-NRII lipopeptide in saline also induces strong B and T cell responses in chimpanzees

The previous data demonstrate that the addition of a palmitic acid chain to the LSA3-NRII peptide increased its immunogenicity in all strains of mice. This led us to analyze the responses induced by the LP in nonhuman primates. Two injections of 100 μg LP in saline spaced 3 weeks apart induced significant levels of anti-LSA3-NRII IgG Ab in the serum of chimpanzee Gerda that remained detectable for a further 70 days (longer delays were not tested). After four immunizations, stronger responses were found which remained detectable at high levels for at least 50 days after the last boost (Fig. 4A). A second chimpanzee, Dirk, previously nonresponsive to two immunizations with NLP administered in alum, and also nonresponsive to this peptide after recombinant LSA3 immunization, proved able to develop specific IgG against LSA3-NRII peptide after immunization with LP (ELISA ratio = 3.1; not shown).

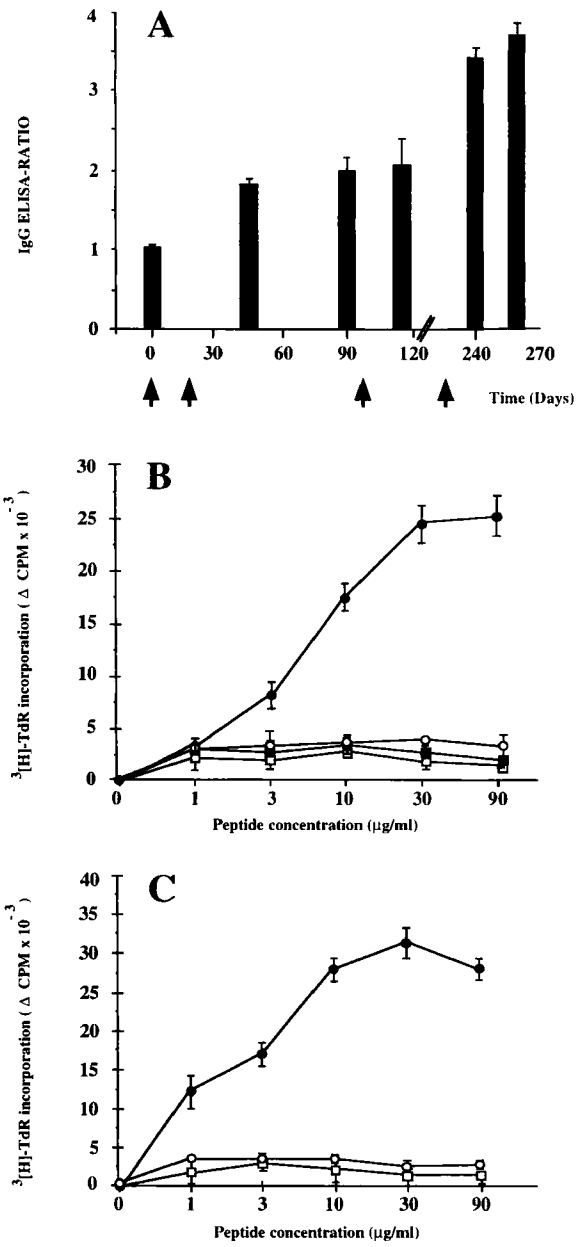

Figure 4. Antibody (A) and lymphoproliferative responses (B and C) induced in chimpanzees by LSA3-NRII LP immunization without adjuvant. (A) Chimpanzee Gerda was injected subcutaneously on day 0, 21, 93, and 217 (arrows) with 100 μg LSA3-NRII LP in saline. Serum samples taken at different times were tested by ELISA. In (B), PBMC were isolated from chimpanzee Gerda 12 weeks after the third subcutaneous injection with LSA3-NRII LP and challenged *in vitro* with either LSA3-NRII peptide (solid circles) or the control peptide LSA-J (open circles) at the indicated concentrations. PBMC from the same chimpanzees before immunization (solid squares) and PBMC from a nonimmunized chimpanzees Bram (open squares) incubated with LSA3-NRII peptide were used as controls. In (C), the PBMC were isolated from chimpanzee Dirk 15 days after the second LSA3-NRII LP immunization and restimulated *in vitro* with LSA3-NRII peptide (solid circles) or with Th2R control peptide (open squares). Results from Dirk's cells, taken before LP injection incubated with the LSA3-NRII peptide are also shown (open circles). Values represent the mean Δ cpm of three wells ± SD. The mean cpm in control cultures, without antigen, ranged between 1250 and 2600 cpm for Gerda's PBMC, and between 2130 and 3220 for Dirk's PBMC.

Table 1. IFN-γ production by PBMC from immunized chimpanzees in response to LSA3-NRII peptide or to the native epitope(s) on *P. falciparum* sporozoites[a)]

	Gerda	Dirk	Bram
LSA3-NRII peptide	55	25	8
P. falciparum sporozoites	46	88	16
Control peptide (RESA)	7	5	2
P. berghei sporozoites	18	16	9
None	5	6	10
PHA	224	164	195

a) Fifteen days after the last immunization with LSA3-NRII LP, PBMC from the immunized chimpanzees Gerda and Dirk or Bram (control) were incubated with either LSA3-NRII peptide, with a control RESA peptide (10 μg/ml), with PHA (5 μg/ml), or with whole *P. falciparum* or control *P. berghei* radiation-sterilized sporozoites (100 sporozoites per 2×10^5 cells). The IFN-γ concentrations in 5-day cultures are expressed in IU. Only one representative experiment out of three performed is shown.

The proliferative responses of PBMC from chimpanzee Gerda immunized with LP in saline was assessed at various time points. Two weeks after the second immunization, a strong lymphoproliferative response to the LSA3-NRII peptide was observed (not shown), and this response remained at similarly high levels when assessed 12 weeks after the third immunization (Fig. 4B). The proliferative response *in vitro* of PBMC from chimpanzee Dirk was also studied at various time points of the immunization. Dirk's PBMC showed a high proliferative response only following boosting with the lipopeptide (Fig. 4C). In both animals, stimulated PBMC secreted substantial levels of IFN-γ (Table 1). No such responses were observed with the control peptides RESA or lipo-LSA-J, or with LSA3-NRII in control chimpanzees (Fig. 4 and Table 1), confirming the specificity of the responses and the lack of mitogenic effect of the lipopeptides.

As expected, these lymphoproliferative responses concerned mostly the $CD4^+$ subset, as shown by the blocking effect against the responses from Gerda, of an mAb against HLA-DR, though not of an mAb against HLA-A-B and C (Table 2).

3.6 Induction of CTL by the lipopeptide without adjuvant

In preliminary experiments, lymphocytes from a group of three C3H/HeJ mice immunized by two subcutaneous injections of LP in saline (50 μg at 15 day intervals) were stimulated by LSA3-NRII peptide *in vitro*. These stimulated cells induced between 30 % and 40 % specific lysis at an effector:target ratio of 40:1, using peptide-pulsed RDM4 cells as target cells (not shown). Further studies on the induction of CTL were focused on the development of the response in chimpanzees.

PBMC from chimpanzee Gerda that had been stimulated on day 0 and day 7 of culture *in vitro* with LSA3-NRII, lysed peptide-pulsed autologous target PHA-blast cells, but not target cells pulsed with the control peptide LSA-J (Fig. 5A). MHC-mismatched PHA blasts from chimpan-

Table 2. CD4-dependent and DR-restricted chimpanzee lymphoproliferative responses against LSA3-NRII peptide and *P. falciparum* sporozoites[a)]

Chimpanzee Gerda

Ab added	LSA3-NRII peptide	Sporozoite
None	25 000 (22.2)	68 480 (61.0)
Anti-CD4	1 780 (1.6)	10 461 (9.3)
Anti-CD8	19 800 (17.6)	45 576 (40.6)
Anti-HLA-DR	5 340 (4.7)	34 583 (30.8)
Anti-HLA-A-B-C	21 800 (19.4)	63 710 (56.7)
Control mouse IgG2a	21 450 (19.1)	67 500 (60.1)

Control chimpanzee Bram

Ab added	LSA3-NRII peptide	Sporozoite
None	3 500 (1.9)	3 740 (2.0)
Anti-CD4	2 500 (1.3)	4 012 (2.1)
Anti-CD8	2 641 (1.4)	2 601 (1.4)
Anti-HLA-DR	750 (0.4)	1 250 (0.6)
Anti-HLA-A-B-C	250 (0.6)	5 680 (3.1)
Control mouse IgG2a	2 050 (1.0)	1 570 (0.8)

a) Fifteen days after the third immunization by LSA3-NRII LP, PBMC from Gerda or Bram (control) were incubated with 10 μg/ml recall peptide or with 100 *P. falciparum* sporozoites per well in the presence or absence of anti-CD4, anti-CD8, anti-HLA-DR, anti-HLA-A, B and C mAb, or of an irrelavant IgG2a mAb (Sect. 2.6). Results are expressed both in Δ cpm and, between parenthesis, in stimulation indices. A representative experiment, among three performed, is shown.

zee Bram that had been pulsed with the NLP were not lysed by effector cells from chimpanzee Gerda. Results were found to be reproducible in experiments performed with lymphocytes from Gerda taken 2, 4, and 12 weeks after the third immunization (Fig. 5A).

We evaluated the CTL activity to peptide LSA3-NRII in Dirk after immunization with the recombinant DG729 protein, after immunization with the NLP adsorbed on alum, and after LP immunization in PBS. Only LP immunization was found to induce a cytolytic activity which was reproducible in assays performed with lymphocyte samples taken 2, 6, and 18 weeks after the last immunization. This cytolytic activity was abrogated after the addition of anti-class I, but not anti-class II mAb to the culture system during the 5-h CTL assay, demonstrating that the cytolytic activity was restricted by class I MHC molecules. Depletion of $CD8^+$ T cells, but not of $CD4^+$ T cells, significantly reduced cytolytic activity (Fig. 5B).

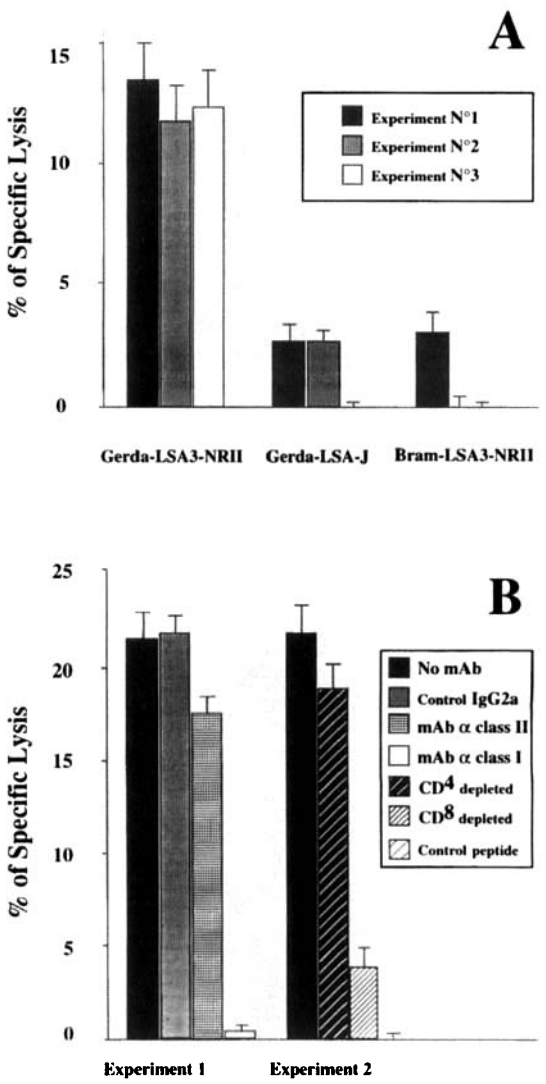

Figure 5. $CD8^+$ CTL induction by LSA3-NRII LP in chimpanzees: (A) effector cells from chimpanzee Gerda were generated from samples taken 2 (plain bars), 4 (grey bars) and 12 (open bars) weeks after the third immunization, by restimulation *in vitro* with 10 μg/ml LSA3-NRII peptide in IL-2-containing medium. After 7 days, the effector cells were tested against autologous or heterologous (from chimpanzee Bram) PHA blasts pulsed with the same peptide or with the control peptide LSA-J (20 μg/ml) in a 5-h ^{51}Cr-release assay at an E:T ratio of 40:1. (B) Results from CTL assays performed 7 days after a single restimulation *in vitro* of PBMC recovered from chimpanzee Dirk 2 weeks after the second subcutaneous immunization with LSA3-NRII LP. Specific lysis recorded after addition *in vitro* of either anti-HLA-A, B, and C (W6/32) or anti-HLA-DR mAb, or after the depletion of either $CD8^+$ T cells or $CD4^+$ T cells subsets are also shown.

3.7 Lipopeptide-induced B- and T-dependent immune responses are relevant to the native malaria protein

After LP immunization, sera from both C3H/HeJ mice and from chimpanzee Gerda were found to react with both *P. falciparum* sporozoites and *P. falciparum*-infected hepatocytes, thus demonstrating the biological relevance of lipopeptide-based immunization (Fig. 6). These Ab did not react with infected RBC at different steps of the intraerythrocytic development (not shown).

Similarly, we studied the responses of PBMC from LP-immunized chimpanzees to the whole sporozoite. PBMC from both Gerda and Dirk showed a strong induction of IFN-γ *in vitro* in response to stimulation with *P. falciparum* sporozoites. Under the same conditions, PBMC from the nonimmunized chimpanzee Bram were not stimulated (Table 1). T cell proliferation in Gerda was also specifically induced *in vitro* by exposure to *P. falciparum* sporozoites, whereas lymphocytes from Bram were again nonresponsive (Table 2). Thus, the response was antigen specific and was not due to mitogenic effect of sporozoites *in*

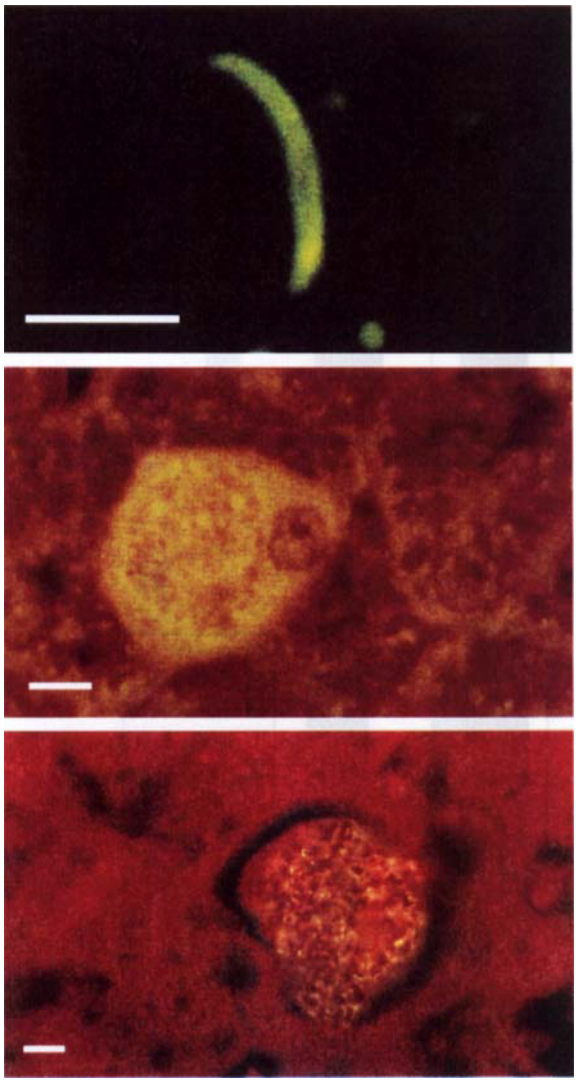

Figure 6. Reactivity of antibodies induced by LSA3-NRII LP in mouse (upper and middle pictures) and chimpanzee (lower picture) with the native parasite LSA3 protein. Sera were tested by IFA on *P. falciparum* sporozoites (upper picture) or on sections of 5-day-old *P. falciparum* liver stages obtained in *Cebus apella* [42] (middle picture) or 6.5-day-old *P. falciparum* liver stages obtained in a chimpanzee [43] (lower picture). The mouse serum was from a C3H/HeJ mouse and the chimpanzee serum was from Gerda taken 1 month after the second injection with LSA3-NRII LP in saline.

vitro. The sporozoite-induced proliferative response was strongly reduced following either anti-CD4 or anti-HLA-DR mAb treatment, but not by anti-HLA-A-B-C Ab, and was only partially reduced by anti-CD8 Ab treatment (Table 2).

3.8 Sequence analysis reveals that the LSA3-NR-II region is highly conserved

Having demonstrated the very valuable immunological properties in the LSA3-NRII peptide, we were then concerned with the evolutionary conservation of those epitopes. Bearing in mind that many malarial antigens exhibit considerable sequence polymorphism (for a review, see [51]) and that the relevance of this polymorphism to escape from host defense mechanisms is supported by preferential diversity within both Th and CTL epitopes [52–55], we sequenced the NRII region in 27 *P. falciparum* DNA samples, including 4 laboratory strains, 18 isolates (7 from Africa, 3 from Madagascar, 3 from Burma and 5 from Brazil), and 5 Thai parasite clones [56]. Not only the amino acid, but also the nucleotide sequences of LSA3-NRII were found to be fully conserved in all DNA samples. These results are in clear contrast to those obtained with immunodominant regions of other Ag expressed in pre-erythrocytic stages, notably the CS protein and thrombospondin-related anonymous protein (TRAP) [54, 55, 57], since LSA3-NRII, despite the fact that it contains dominant Th and CTL epitopes, proved to be perfectly conserved among isolates of diverse geographic origin. This further strengthens the potential applicability of this particular T cell epitope.

4 Discussion

Our data extend previous findings about the unique improvement in immunogenicity obtained when proteins or peptides are linked to a lipidic component, and demonstrate the value of simple lipopeptides made by incorporating a fatty acid-conjugated amino acid during synthesis. Using a *P. falciparum*-specific peptide which is being investigated for malaria vaccine development, several advantages in favor of the lipidic lipopeptide (LP) compared to its nonlipidic (NLP) homolog were found. Our data confirm that a lipopeptide alone can induce cytolytic T cells, show in addition that the same lipopeptide containing also B and T helper epitopes can induce the corresponding immune responses at high level, and demonstrate that such an immunogen bypasses the requirement for adjuvant. The improved LP immunogenicity also overcomes an apparent genetic restriction, since responses were found in otherwise nonresponsive strains of mice. Finally in the animal whose immune system is the closest to that of humans, the chimpanzee, the simple type of LP formulation employed here, which can be easily produced under GMP conditions, is well tolerated and strongly immunogenic.

Although it has previously been reported for a single mouse strain that B and T helper responses could be induced by a lipid-modified peptide [21], previous studies have mainly focused on improvements in CTL induction through lipid modification [21–23, 38]. The results we report demonstrate that, in addition to CTL induction, the simple lipopeptide modification used here provides wide-ranging immunological improvements. This was obvious at Ab level, since LP without adjuvant induced high titers of Ab, whereas no Ab was detected in any strain of mice immunized with NLP alone Even when using FIA, Ab responses were greater with LP than NLP (*e.g.* Ab titers were up to 150 times higher in SJL/J mice). Advantages of the LP formulation are evident comparing LP injected in saline, without adjuvant, with NLP in FIA: titers were similar in three of four mouse strains, and only slightly higher with FIA in SJL/J mice. A marked improvement of Ab responses was reported in BALB/c mice when the *P. falciparum* CS repetitive epitope was covalently coupled to tripalmitoyl-S-glycerylcysteinyl-serine (P_3CS) [58]. Our data extend these findings and contradict the widespread

belief that high titers and long-lasting antibody responses unavoidably require strong adjuvants.

T helper cell induction results complement the Ab data. When comparing immunization with LP in saline with NLP in FIA, the SI improved, sometimes dramatically (DBA/1, BALB/c, and C57BL/6) or were at least equivalent (C3H/HeJ and SJL/J). All five haplotypes responded to LP, only three of five to NLP in Freund's, and none to NLP without adjuvant. Since the ability of the peptide to associate with MHC class II is related to its peptide sequence (identical in both formulations), these results indicate that enhanced immunogenicity of LP improved T helper cell responses from levels that were undetectable or borderline to levels that were clearly positive. An apparent class II restriction barrier was overcome by employing an antigen presentation method that greatly increased peptide immunogenicity. This is important for vaccine development since the genetic restriction of many malaria parasite epitopes has long been thought to be a critical limitation [5–7].

Similarly, the immunization of outbred chimpanzees led to strong B and T helper responses, the latter evaluated by [^{3}H]thymidine incorporation and IFN-γ production. Secretion of IFN-γ is of particular importance in view of the strong inhibitory effect of this cytokine upon development of the LS [59]. Taken together, results from studies in chimpanzees and mice suggest that the peptide selected in LSA3 contains either a series of overlapping Th epitopes restricted by distinct class II alleles, or one multirestricted Th epitope.

Immunological responses induced in this way proved relevant to native parasite B and T cell epitopes. This observation has two major implications: that Ab- and T cell-dependent effector mechanisms induced by the simple synthetic LP immunization procedure can be directed at accessible native structures, and that following this artificial immunization, boosting of immune responses will occur at the time of challenge. This has indeed been observed recently in LSA3-NRII LP-immunized chimpanzees following sporozoite challenge (Luty et al., in preparation).

Our study confirms previous observations on the ability of lipopeptides to induce cytolytic T cells in mice [21–23] and lower primates [38]. We show in addition that the same activity can be induced in chimpanzees. This is the first report of CTL induced by a lipopeptide in the animal whose immune system is the closest to that of humans. In contrast to peptide linked with P_3CSS which was both mitogenic and to some extent toxic [19, 20], the LSA3-NRII LP proved to be well tolerated in chimpanzees. No *local* or general reaction was observed in these animals. No toxic or mitogenic activity was observed in cultured cells. Results obtained in chimpanzee Gerda show that the lipopeptide construct alone can induce $CD8^+$-dependent MHC class I restricted CTL; this result has been confirmed more recently in three additional chimpanzees bearing distinct class I alleles (BenMohamed et al., in preparation). Even more interesting were the results obtained in chimpanzee Dirk, who did not produce a CTL response in response to either recombinant immunization or NLP immunization. The poor immunogenicity of recombinant proteins with regard to CTL induction is well established. Our initial impression that class I restriction of LSA3-NRII epitopes was responsible for the nonreactivity of Dirk's lymphocytes was contradicted by positive CTL induction following re-immunization with the LSA3-NRII in a LP form. The precise reasons for the improved induction of CTL due to the lipid component are not yet fully understood. Possibilities include interchain interaction via fatty acids resulting in increased half-life *in vivo,* or a lipid tail-mediated increase in the ability of the peptide to be diverted from the phagosome to the cytosol and thus directed into the endogenous processing pathway [60].

For vaccine development, it has frequently proven necessary to associate various epitopes specific for either B, T helper, or CTL [11, 61] together with powerful adjuvants or to employ live vectors, *e.g.* virus presentation, to induce a wide range of distinct effector mechanisms. In this study, we have deliberately chosen to study responses to a relatively large peptide, rather than first to identify and then combine a series of discrete epitopes each known to associate with a given MHC class I or class II molecule. This choice was made for several reasons. First, epitopes corresponding to distinct class I or class II molecules are sometimes clustered within a limited region of a protein [24, 25, 48]. These epitopes may be processed in a manner similar to that of whole proteins when large synthetic peptides are channeled properly through the endogenous pathway. The 26 amino acids of the LSA3-NRII peptide lie within a less organized, flexible area of the molecule that may be prone (based on previous experience with LSA1, [33]), to be of greater immunological relevance than other regions of the molecule. The induction [62] and especially the long-term persistence [22, 23] of $CD8^+$ CTL cells often requires $CD4^+$ T helper activity, hence the need to associate T helper and CTL epitopes within the same immunogen [61]. Finally, a wide range of effectors, such as Ab able to inhibit sporozoite invasion or act in ADCC, as well as cytokines produced by CD4 or CD8 T lymphocytes and CTL activity, might all be needed to achieve effective immunity to malaria pre-erythrocytic stage (reviewed in [63, 64]). A molecule combining various and universal B, Th, and CTL epitopes could therefore have a number of distinct advantages. The results obtained with LSA3-NRII LP support our strategy, since a single relatively short peptide proved to combine these advantages.

Of particular importance for vaccine development is the fact that immune responses induced by the LP proved to be long lasting. Ab were detected at high titers up to 8 months after immunization in mice and in chimpanzees. T helper cells and CTL were detected after 1 and 3 months in immunized chimpanzees. This contrasts with previous reports with HIV or influenza peptides, whose endogenous Th epitopes proved poorly efficient [21, 22] and for which the addition of strong Th epitopes was necessary to stimulate long-lasting CTL responses. These results suggest that this LP formulation provided an efficient helper effect sufficient to promote CTL responses. This conclusion is in agreement with our observation of a strong T helper cell effect in various strains of mice, suggesting that CD4 memory T cells may be long-lasting following LP immunization.

On the basis of the above findings, the LP formulation described in this study is of interest from a pure immunological viewpoint, but may also be of great relevance in the search for effective methods of immunization against malaria. Given its outstanding immunological properties, an attractive feature is the ease with which such constructs can be synthesized in pure form, providing the ability to develop rapidly this type of vaccine at low cost under GMP grade formulation.

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