Long synthetic peptides encompassing the *Plasmodium falciparum* LSA3 are the target of human B and T cells and are potent inducers of B helper, T helper and cytolytic T cell responses in mice

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We synthesized 17 long synthetic peptides (LSP) spanning the whole 200-kDa *Plasmodium falciparum* liver stage antigen-3 (LSA3), an antigen that induces protection in chimpanzee, and analyzed their immunogenicity in BALB/c mice and their antigenicity in individuals living in a hyper-endemic malaria area. Our findings show that both specific antibodies and T cell proliferation against most LSA3-LSP develop in malaria-exposed adults. All individuals studied had detectable antibodies against a minimum of 6 and a maximum of 15 polypeptides. It is noteworthy that antibody prevalence and titers were as high against non-repeat as repeat regions. Although the extent of T cell reactivity was lower than that observed for B cells, most of the sequences contained at least one T helper epitope, indicating that the majority of LSA3-LSP contain both B and T cell epitopes within the same sequence. Injection of LSA3-LSP with SBSA2 adjuvant in mice, showed strong immunogenicity for most of them, eliciting both T cell responses and specific antibody production. While all the peptides were immunogenic for B cells, different patterns of T cell responses were induced. These peptides were thus classified in three sets according to the levels of the T cell proliferative and of the IFN-γ-specific responses. Importantly, antibodies and T cells against some of the LSP were able to recognize LSA3 native protein on *P. falciparum* sporozoites. Additionally, some LSP (44–119, 1026–1095, 1601–1712) also contained epitopes recognized by H-2^d class I-restricted T cells. These results led to the identification of numerous domains that are highly antigenic and immunogenic within the LSA3 protein, and underline the value of the LSP approach for vaccine development.

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1 Introduction

Multiple approaches for the development of sub-unit malaria vaccines based on selected antigens have been investigated, including recombinant proteins, small synthetic peptides, live viral or bacterial vectors and DNA [1]. Synthetic peptide-based vaccines provide several practical advantages and have been shown to be efficient in several disease models either in higher primates (HIV, malaria) [2, 3] or in humans (hepatitis B virus, HIV) [4, 5]. Recently, the chemical synthesis of long synthetic peptides (LSP) has steadily improved to accommodate longer sequences. This strategy allows the inclusion in the same peptide of several B helper, T helper and T cytotoxic epitopes capable of binding to different MHC class I and II molecules, thus overcoming the MHC restriction observed previously in response to smaller peptides [6]. Recently, this strategy has contributed to evaluation of the antigenicity [7, 8] and immunogenicity of the N- and C-terminal domains of the circumsporozoite (CS) protein in animal species and humans [9, 10].

The pre-erythrocytic stages of the malaria parasite are known to be the target of strong protective immune

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Abbreviations: LSA3: Liver stage antigen-3 **LSP:** Long synthetic peptides **SI:** Stimulation index **IFAT:** Immunofluorescent antibody test **SFC:** Spot-forming cells **CS:** Circumsporozoite

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responses induced by inoculation of irradiated sporozoites in humans [11, 12]. We have selected, within a subset of *Plasmodium falciparum* pre-erythrocytic antigens [13], a 200-kDa protein denoted as liver stage antigen-3 (LSA3) on the basis of the differential immune responses between protected and non-protected volunteers, both similarly immunized with irradiated sporozoites [11, 14]. This antigen is highly conserved and its protective potential has been demonstrated in non-human primates against challenge with *P. falciparum* sporozoites [11, 14] and in mice against *P. yoelii* sporozoites [15]. The protective efficacy shown by this antigen prompted us to further analyze its antigenicity and immunogenicity.

We synthesized 17 LSP ranging between 44 and 186 amino acids in length, spanning the whole *P. falciparum* LSA3 protein. Immunogenicity studies were undertaken in mice using adjuvant SBAS2, which using the RTS'S formulation, as the LSA3 recombinant protein, proved to be able to promote protective responses against a *P. falciparum* challenge in humans and in higher primates [14, 16, 17]. To assess the relevance of these data in humans, we also undertook preliminary antigenicity studies of the same LSP in human donors living in a hyperendemic area of Africa (Dielmo-Senegal).

2 Results

2.1 Each of the 17 LSP covering LSA3 defines at least one B cell epitope in humans

Using 17 LSA3-LSP we performed a preliminary mapping of B cell epitopes using sera from 20 villagers living in Dielmo, an African hyperendemic malaria region (Fig. 1B). Each of the peptides was recognized by serum antibodies from at least one individual, and many by most individual sera. Conversely, control sera from malaria non-exposed individuals were negative in these ELISA assays. The LSP strategy led us to identify several antigenically dominant regions. A first, highly antigenic region lies within the R2 repeats region since sera from almost all of the individuals reacted with the three peptides covering the sequence 501–854. This region includes different repeated octameric motifs which vary in numbers and organization, although the sequence of each motif is highly conserved among various parasite strains, which suggests a conformational conservation of the R2 region [14]. In contrast to the CS protein in which the central repeat region is the only B cell immunodominant region, a high prevalence of antibodies against LSP in the non-repeat (NR)-B region was also detected. Despite the fact that it is a non-repetitive region, the prevalence of specific antibodies was as high as that recorded for anti-R2 antibodies. The sequence 100–222 in the NR-A from N-terminal region includes a fragment that had been previously identified using

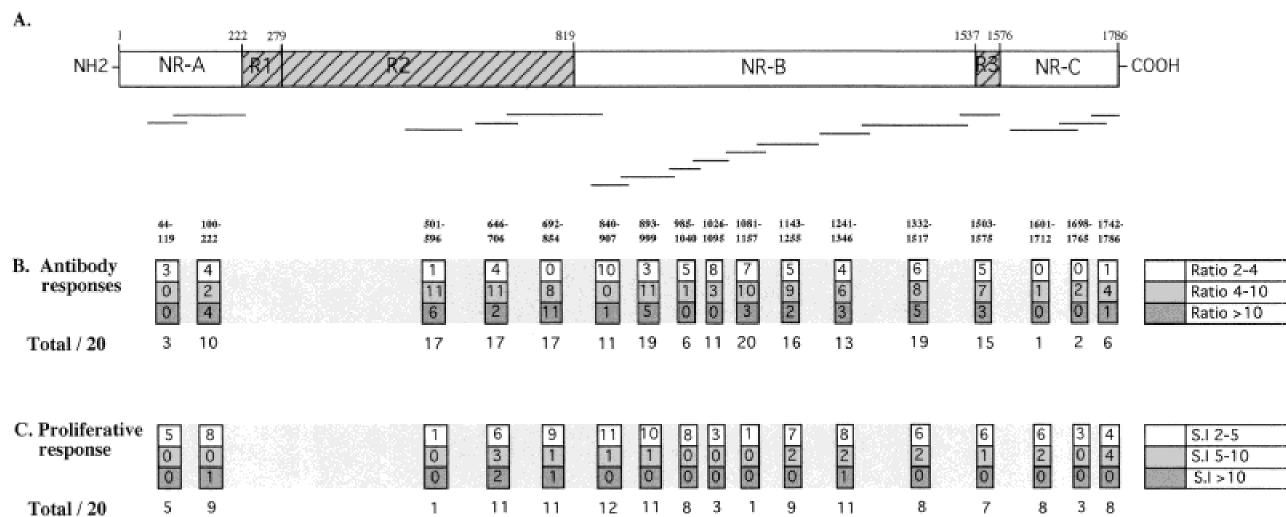

Fig. 1. Antigenicity of *P. falciparum* LSA3-LSP in adults donors from Dielmo. (A) *P. falciparum* LSA-3 antigen, clone K1, showing the non-repeat (NR-A, NR-B and NR-C) and repeat (R1, R2 and R3) fragments and the relative position of the 17 LSP. Antibody (B) and proliferative T cell responses (C) to the 17 LSA3-LSP in 20 individuals from Dielmo. As indicated, the responses were classified in three categories according to the ratio of antibodies or the SI of proliferative response. Shown are the numbers of responders in each category.

shorter peptides as a target of B and T cell responses [18].

2.2 Mapping of human T cell epitopes

PBL from the same 20 donors were used to identify sequences containing LSA3 T cell epitopes. Sixteen donors (80%) showed a specific proliferative T cell response to at least one of the peptides (Fig. 1C), whereas no specific response was recorded using T cells from 5 non-exposed individuals studied in parallel [not shown, stimulation index (SI) <2]. 45% and 55% of individuals recognized sequence 100–222 from N-terminal region, and sequences 646–706 and 692–854 from the R2 repeat region, respectively. However, in contrast to the serological response, only a small percentage of individuals (5%) responded to stimulation with the sequence 501–596. In the NR-B region, similar to the B cell antigenic pattern, almost all peptides (6/8) were targeted by highly prevalent T cell responses (40–60% of individuals studied).

According to these results, although the prevalence was lower than that observed for B cell epitopes, most of the peptide sequences contain at least one T helper epitope recognized by PBL from hyper-immune donors.

2.3 LSA3-LSP are all capable of inducing high antibody levels in mice

To assess the potential of LSP as vaccine candidates, their immunogenicity in mice was investigated using the SBAS2 adjuvant approved for human use. Specific antibody responses were measured 20 days after the second injection.

The results show a strong and rather homogeneous antibody response against each of the 17 LSP studied. Only the very C-terminal end peptide 1742–1786 proved to be less immunogenic than the other LSP (Fig. 2A). Titers, evaluated as a ratio of absorbance values to pre-immunization control sera values, were high for 14 of the 17 peptides studied (ELISA ratio >20). The three C-terminal peptides eliciting the lowest levels of Ab (Fig. 2A) were also those to be rarely found as targets of Ab in humans (Fig. 1B). Mice receiving only adjuvant were seronegative for each peptide.

In conclusion, all peptides proved to define at least one B cell epitope for the BALB/c immune repertoire and many were capable of inducing high levels of antibodies (Fig. 2A).

2.4 *P. falciparum* LSA3-LSP induce various types of T cell responses in mice

Many of the peptides proved to be immunogenic in mice as evaluated by either proliferative or IFN-γ response or both (Fig. 2B, C). The frequency of specific IFN-γ-producing cells was measured by *ex vivo* Elispot on freshly isolated LN cells from each immunized mouse. Despite the fact that inbred mice were used in this study, the variation in the magnitude of the T cell responses among individual mice was more pronounced than that observed for the serological response.

In the N-terminal region, the sequence 44–119 defined a strong T cell epitope since all mice proliferated and showed the highest frequency of IFN-γ-producing T cells. In contrast, no T cell response was detected against sequence 100–222.

In the R2 repeat region, the peptides 692–854 and 646–706 induced proliferative response in 50% of the mice and IFN-γ-production in some of them. In contrast, no response was observed against the sequence 501–596.

The remaining sequences corresponding to NR-B, R3 and NR-C regions induced various patterns of proliferation and IFN-γ-production that can be classified in three different categories as follows: (a) sequences 1081–1255 and 1601–1712, corresponding to three peptides, induced both proliferative response and IFN- γ production in most of the mice; (b) sequences 893–999 and 1698–1786 also corresponding to three peptides, induced moderate to high proliferative responses; however, only one mouse per group produced significant levels of IFN- γ against the immunizing peptide; and (c) sequences 840–907, 985–1095 and 1241–1517, corresponding to five peptides, elicited poor T cell responses: *i.e.* few mice (one or two per group) showed proliferative response in absence of IFN-γ-production. Thus, the combination of proliferation and Elispot assays suggests the induction of T cells with distinct phenotypes. Comparison with the results of T cell studies in humans showed that they were more divergent than for Ab (*e.g.* in mice, but not in humans, poor or no response was found for the three peptides in region 1241–1575. Conversely, high responses to the three C-terminal peptides were found in mice, though rarely in humans).

2.5 H-2^d class I-restricted T cell responses are induced by LSA3-LSP

To investigate class I-restricted responses, *ex vivo* Elispot were performed on fresh cells using P815 cell line as

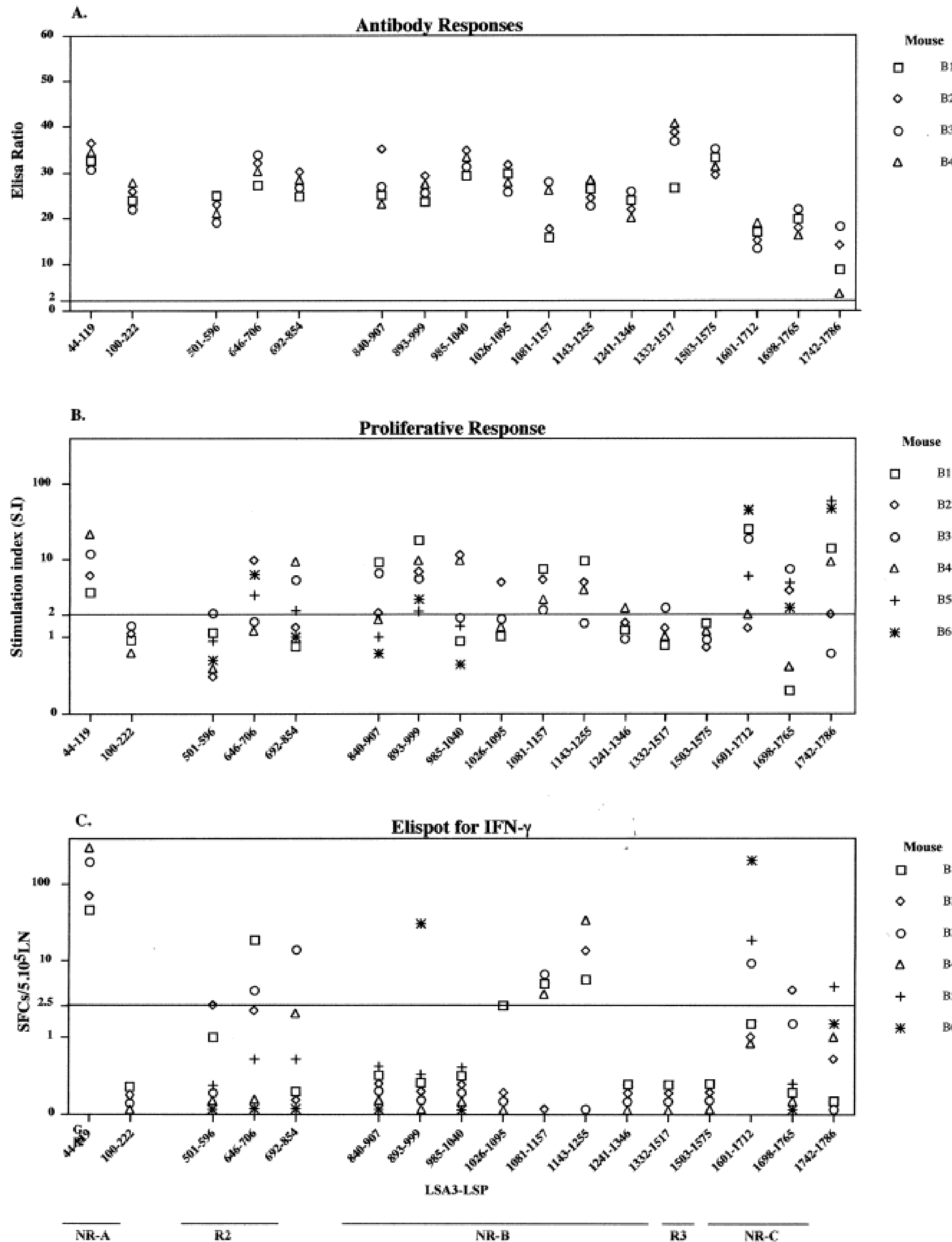

Fig. 2. Immune responses in BALB/c mice immunized with LSA3-LSP. (A) ELISA ratio of mouse (B1, B2, B3, B4) sera tested in a single dilution (1:100) against the corresponding LSP and read at an absorbance of 450 nm. Ratio >2 are considered positive. (B) Lymphocyte proliferation measured as [^{3}H]thymidine incorporation after 4 days in culture in the absence of peptide or in presence of each corresponding LSP. Value are expressed as SI and values >2 are considered positive. (C) Ex *vivo* Elispot assay was performed with freshly isolated LN cells cultured during 40 h in absence of peptide or in the presence of the corresponding LSA3-LSP. LN cells from mice immunized with adjuvant SBSBA2 alone tested in parallel against each LSP were used as control. The values are expressed as the mean of IFN-γ SFC in 5×10^5 LN cell of immune mice. Values are considered positive above 2.5 corresponding to the mean + 3SD of IFN-γ SCF of control mice tested in parallel against all the LSP.

APC-expressing class I (H-2^d) but not class II molecules [19]. The response to each long peptide was evaluated after 40 h of *in vitro* culture and expressed as a frequency of spot-forming cells (SFC), allowing to evaluate the proportion of LSP able to elicit class I-restricted T cell responses.

A significant proportion of the response against the two peptides that induced highest IFN-γ-production (*i.e.* peptides 44–119 and 1601–1712) was found to be class I restricted. Indeed, all mice immunized with sequence 44–119 presented between 16–32% of class I-restricted IFN-γ SFC. The C-terminal peptide 1601–1712 induced between 33% and 51% of class I-restricted IFN-γ-producing cells in 75% of mice (Fig. 3A, B).

In parallel, specific CTL activity to each LSP was also evaluated by a ^{51}Cr-release assay using primed LN from the same immunized mice restimulated twice *in vitro* with the homologous LSP. As seen in Fig. 3C, a cell line derived from restimulation with peptide 1026–1095 from the NR-B region displayed a H-2^d-restricted cytolytic activity of 60% at effector to target ratio of 33:1. A limited activity of 11% and 8% was seen using peptides 44–119 and 1601–1712, respectively (data not shown). The remaining cell lines did not exhibit any detectable cytolytic activity. The cytolytic activity was not generated by *in vitro* stimulation since LN cells from control mice receiving SBSA2 alone and restimulated *in vitro* with this peptide did not display any CTL activity under the same conditions (data not shown). Typing of the 1026–1095 CTL line showed that 98% of the stimulated population corresponded to CD8$^+$ T cell. Although a class I MHC-restricted T cell response was not detected by Elispot in the primed cultures using fresh cells stimulated with peptide 1026–1095, a high number of IFN-γ-producing cells were observed after two *in vitro* restimulations. This would indicate that this long peptide is less efficiently processed than the other two peptides (44–119 and 1601–1712), resulting in slow generation (or very low quantities) of the optimal nonapeptide and/or a low frequency of CTL precursors requiring a higher number of cell divisions to attain enough expansion for detection.

2.6 Relevance to native parasite proteins

Synthetic peptides may not always properly mimic the conformation of epitopes contained in the native parasite protein. Since this can affect both the protective efficiency of the immune response and the ability of parasite-derived antigen to boost it, this question was addressed both at the B and the T cell levels by testing the reactivity with *P. falciparum* sporozoites of antibodies and LN cells induced by LSP in mice.

Antibodies raised against 15 out of 17 peptides were also able to recognize the LSA3 native protein on *P. falciparum* sporozoite surface as assessed by immunofluorescent antibody test (IFAT). The IFAT antibody titers

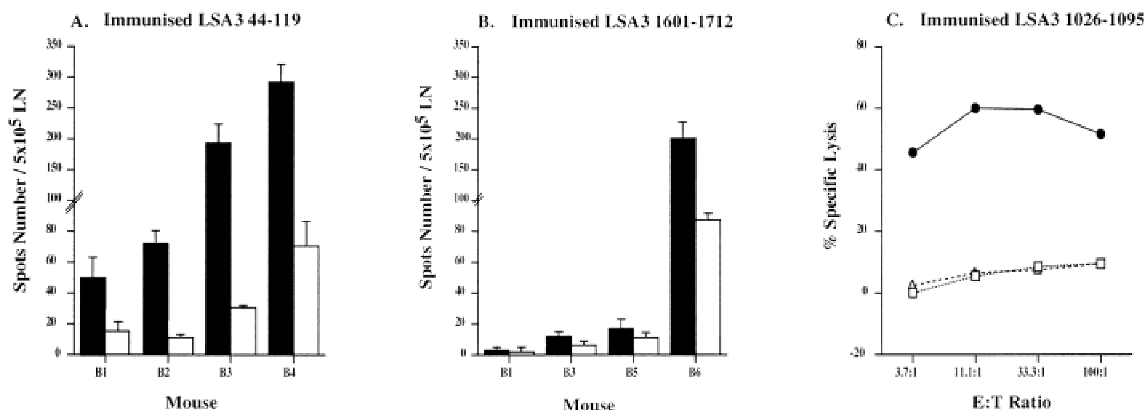

Fig. 3. H-2^d class I-restricted T cell responses induced *in vivo* in BALB/c mice immunized with LSA3-LSP. The number of IFN-γ SFC were evaluated individually in four to six mice previously immunized with N-terminal LSP (44–119) or C-terminal LSP (1601–1712) (A and B, respectively). Freshly isolated LN cells from each mice were tested in the absence of peptide and in the presence of free peptide (black bars) or P815 cells (white bars) pulsed with the respective LSP 44–119 (A) or LSP 1601–1712 (B). (C) CTL response induced by *in vivo* priming of BALB/c mice with LSA3-LSP 1026–1095. A CTL line was derived from a pool of LN cells by restimulation *in vitro* for 1 week with autologous spleen cells pulsed with the same LSP 1026–1095 and assayed at day 14 for CTL activity in a standard ^{51}Cr-release assay. Effector T cells were tested at various effector to target cell ratios. H-2^d P815 targets cells were ^{51}Cr-labeled in the presence of 10 μg/ml LSA3-LSP 1026–1095 (close circles), HIV-Nef irrelevant peptide (open triangles) or medium (open squares).

ranged between 1:100 to 1:48,600. The highest IFAT titers were those induced by immunization with peptides 1241–1346 and 1143–1255 (1:48,600 and 1:5,400, respectively). In addition, serum to three of five peptides tested (840–907, 1026–1095 and 1143–1255) showed reactivity against *P. falciparum* liver schizonts. These results suggest that LSA3-LSP and the native protein share common antigenic determinants.

Moreover, T cells from the mice immunized with LSP were able to recognize the native protein, *i.e.* proliferate specifically to stimulation by the parasite. Proliferative T cell responses to sporozoite-derived antigen were obtained from mice immunized with 13 of the 17 LSP with SI ranging from 2.1 to 12. However, as for peptide-specific proliferative T cell responses, a degree of heterogeneity was observed among inbred animals. The proliferative response to sporozoites was specific, since no significant proliferation was observed with cells from each of the control mice receiving only adjuvant, or among cells from mice immunized with peptide but challenged with *P. berghei* sporozoites.

3 Discussion

Solid-phase peptide synthesis represents an attractive alternative method to recombinant DNA technology for the obtention of reagents representing large protein domains. Indeed, this technique has been successfully used to produce long polypeptides covering the sequences of different antigens such as HIV-1 [20], bovine thymopoietin II (bTP-II) [21] *P. falciparum* CS protein [6, 22], and the precursor molecule of the *Aequorea* green fluorescent protein (GFP) [23]. The difficulties initially encountered in the synthesis of long polypeptides have progressively been solved and further progresses can be expected, so that the routine chemical synthesis of molecules in the range of 200 amino acids can now be envisaged. A previous study using 102-mer and 104-mer LSP had shown that such a strategy could be applied to epitope mapping in proteins such as the CS, which is 412 amino acids long, and that it can be successfully employed in phase I clinical vaccine studies [9]. We now extend these results by showing: (1) the synthesis of LSP of up to 186 amino acids, (2) the epitope mapping of a protein as large as LSA3, which is 1786 amino acids long, using not more than 17 LSP, (3) a detailed analysis of the B and T cell reactivity to the protein, in particular to the NR-B, R3 and NR-C regions which had been poorly explored thus far, (4) the remarkable immunogenicity of these constructions, and (5) the possibility to generate *in vivo* and restimulate *in vitro* class I-restricted CTL and IFN-γ responses without nonamers, using 113-amino acids LSP.

In the present study, using long synthetic peptides, we describe the antigenicity of the whole LSA3 molecule in individuals from a malaria-endemic area in Africa and their immunogenic potential in mice. This analysis was particularly needed in view of the protective effect induced against a *P. falciparum* challenge, by adjuvated LSA3 recombinants or lipopeptides in chimpanzee [11, 14] as well as microparticulate formulations in *Aotus* monkeys (Perlaza et al., submitted).

Our findings show that in Dielmo, an area with high malaria transmission, both specific B and T cell epitopes were detected in most of the 17 LSA3-LSP each defining distinct non-cross-reactive regions of the molecule. At least one individual was positive for each of the 17 peptides. Moreover, each individual studied had detectable antibodies against a minimum of 6 and a maximum of 15 of the 17 polypeptides studied. The prevalences and levels of antibodies to both repeat and non-repeat regions were high and of comparable magnitude. This observation does not support the concept that dominant B cell epitopes segregate in repetitive regions [24, 25].

At the T cell level, although the prevalence of LSA3-specific responses was lower compared with B cell responses, 16 of the 20 donors examined showed lymphoproliferative responses against at least 2, and up to 12 of the 17 peptides analyzed. Except for peptide 1081–1157 from the NR-B region, which did not stimulate T cell responses, the pattern of proliferative responses roughly paralleled that of antibody responses. The T cell response observed against peptide 100–222 from N-terminal region extends previous studies on the recognition of T cell epitopes defined by smaller peptides within the same sequence, such as peptide NRII. NRII has been shown to contain a strong Th epitope in individuals from the same endemic area [2] and is at the origin of the selection of the LSA3 gene [11, 14] by differentiating immune responses in protected versus non-protected immunized volunteers.

Taken together, our immunoepidemiological data indicate that the majority of LSA3-LSP contains both B and T cell epitopes within the same sequence. Our results are similar to those found in Gambian adults for PfTRAP. Using 50 overlapping PfTRAP peptides, 26 Th cell epitopes were found along the entire protein [26]. However, in contrast to PfTRAP in which only 10 out of 26 epitopes were found to be conserved, to date the available data for LSA3 show conservation of all epitopes identified so far [14].

Most of LSA3-LSP were found to be strongly immunogenic in mice, eliciting both T cell responses and specific antibody production. All of the peptides were immuno-

genic in terms of antibody production. All but one (1742–1786) induced high antibody levels, with mean ELISA ratio >10 for each of them, and 14 inducing ELISA ratios >20. Different patterns of T cell responses were induced by these peptides and were classified in three sets according to the combined pattern of T cell proliferation and IFN-γ response. This suggests the elicitation of T cells with distinct phenotypes and hence paves the way to studying the protective effect of distinct regions of the molecule inducing different patterns of response (*e.g*. high IFN- γ responses which are associated with protection), so as to guide future vaccine constructions based on the pattern of response given by each LSP. Remarkably, these high antibody and cellular responses were observed after only two injections of peptides. Moreover, antibodies and T cells elicited by most LSP were able to specifically react with LSA3 on *P. falciparum* sporozoites, suggesting that the immune responses induced by immunization with synthetic polypeptides, are relevant to the native protein and hence could be boosted upon challenge.

Interestingly, the most antigenic and immunogenic LSP (100–222, 692–854, 893–999, 1143–1255) were among the longest (ranging from 100 to 180 amino acids), suggesting that the likely association of several B and T cell epitopes in the same construction may be beneficial in terms of overall immune reactivity. This also indicates that these LSP can be processed and presented in association with MHC molecules. Exogenous antigens are presumably processed in specialized endosomal compartments yielding peptides that bind to MHC class II molecules. Additionally, some LSP from the N-terminal and C-terminal regions (44–119, 1026–1095, 1601–1712) contained epitopes that appeared to be recognized by H-2^d class I-restricted T cells. Although the experimental conditions in which we searched for CTL activity may have led to a severe underestimation of the real number of CTL epitopes, our results show that an LSP as large as 113 amino acids can still be processed to yield MHC class I responses. Indeed, evidence accumulated over the last few years indicate that processing of exogenous antigens can lead to MHC class I-restricted presentation [27, 28]. We did not find a close correlation between the Elispot-determined frequencies of IFN- γ-producing cells in *ex vivo* cells to the peptides 44–119 and 1601–1712 and the level of cytolytic activity observed after 2 restimulations by the traditional method of ^{51}Cr release. This could suggest that there are different subsets within the memory CD8$^+$ T cell pool and that many of the IFN-γ-producing cells do not differentiate along the CTL pathway, or alternatively, that some CTL lines did not expand sufficiently upon antigenic stimulation *in vitro* to be detected as cytolytic. Similar results have been found using an influenza molecule, where the frequency of antigen-specific T cell enumerated by *ex vivo* Elispot was several fold higher than that calculated from limiting dilution analysis [29]. These results at least confirm previous reports on the ability of LSP in inducing CTL responses [10, 30]. In addition, in a recent phase I clinical trial it was also showed that a LSP derived from *P. falciparum* CS protein delivered in Montanide ISA 720 adjuvant or alum, able to prime CTL in mice, also induced CTL responses in humans [9]. In the present study, we used SBAS2 which has previously been shown to be a potent adjuvant, acceptable for human use and which, as shown here, proved to be very efficient at inducing B and T cell responses against LSP in mice.

These results further document the immunological characterization of LSA3 and provide tools for future studies. Taking into account the antigenicity and immunogenicity of these LSP, several sequences appear to have greater vaccinal potential. Sequences such as 646–706, 692–854 from the R2 repeat region and 893–999, 1143–1255 and 1241–1346 from the NR-B region, which had not been investigated previously, were efficiently recognized by antibodies and T cell from hyperimmune donors, and proved also to be strongly immunogenic in mice. In addition, our study shows that LSP selected on the basis of immunoepidemiological studies can readily be used for immunogenicity studies, and are capable of inducing humoral and cellular responses when formulated with a clinically approved adjuvant such as SBAS2. As compared to shorter peptides, the LSP strategy required far less synthesis, fewer assays, and fewer mice, and led to focus down rapidly on immunologically important regions in the molecule. As compared to recombinants, which cover diverse regions of a large molecule, LSP did not require the fastidious and often incomplete steps of protein purification. This strategy led to the rapid production of products that are stable, not degraded by contaminating proteases, and not contaminated by lipopolysaccharides, leading to more reliable immunological results. Previous experiments with CS-derived LSP have shown that this strategy could be scaled-up at good laboratory practice/good manufacturing practice level to obtain a fast proof of concept in clinical trials [9]. In the case of LSA3, this strategy allowed the identification of many different domains within LSA3 that are highly antigenic and immunogenic, thus fulfilling the required characteristics for vaccine development.

4 Materials and methods

4.1 Peptide synthesis and purification

Seventeen LSP encompassing the full length of the LSA3 protein of *P. falciparum* K1 isolate were chemically synthesized by solid-phase Fmoc chemistry as described else-

where [6, 22]. Two peptides 44–119 and 100–222, represent most of non-repeat region A (NR-A) from the N-terminal flank of the protein (Fig. 1A). Three peptides 501–596, 646–706 and 692–854 contain sequences representative of the repeat region R2. Of the remaining 12 peptides, 9 represented sequences in the region 840–1575, or non-repeat region B (NR-B), one (1503–1575) covered the block repeat R3, and the last three peptides spanning residues 1601–1786 represented the non-repeat C-terminal region C (NR-C). All the peptides overlapped on 15 amino acids, except the sequences 1503–1575 and 1601–1712 missing 26 amino acids in repeat R3 (Fig. 1A). Purity (70–80%) was determined by HPLC and mass spectrometry. Impurities consisted of either smaller fragments or full-length peptides derivatized by one or two *t*-butyl groups [-C-$(CH_3)_3$].

4.2 Subjects and samples

Twenty adults (>20 years), 10 women and 10 men, from Dielmo, a village situated in a holoendemic area of Senegal, West Africa, where malaria transmission is intense and perennial [31], were selected for analysis in this study. The villagers were under active and daily medical surveillance by a medical team. After informed consent, blood samples were collected in November 1998. During the previous 12 months, they were potentially exposed to a total of 346.15 infected *Anopheles gambiae sl*. bites. At the time of the blood sampling, each individual appeared healthy and asymptomatic, *i.e.* with no detectable or recent history of malaria-related disease, and no particular clinical sign of sickness was recorded. Sera and lymphocytes from five healthy European adults with no history of travel in malaria prone areas were used as negative controls.

Plasma was separated and kept at –20°C until used. Blood cells were diluted in culture medium RPMI 1640 and layered onto Ficoll-Hypaque (Pharmacia) prior to density gradient centrifugation 350×g for 30 min at room temperature. PBL were then washed twice and adjusted to 1×10^6 cells/ml immediately before use.

4.3 Mice and immunization

BALB/c mice were purchased from CER Janvier-France. Groups of four to six mice were immunized with each of the corresponding 17 LSP. Mice were injected subcutaneously at the base of the tail with 100 μl containing 100 μg of peptide dissolved in water and emulsified in SBSA2 adjuvant. Animals received two doses of peptide at 30-day intervals. Sera were collected 2 weeks after the last injection and stored at –20°C until used. Pre-immunization samples from the same mice were used as negative controls.

4.4 ELISA

Peptide-specific antibodies were analyzed in ELISA assay. Microtiter plates (Nunc-Immunoplate, Nunc, Denmark) were coated with 10 μg/ml of LSA3 polypeptide in 100 μl of 50% acetic acid in H_2O and incubated overnight in a chemical hood at room temperature. The plates were then treated as described previously [2]. A pool of normal human sera (Institut Jacques Boys S.A., France) or of ten pre-immune mouse sera were tested in duplicate in the same plates as controls. Results are expressed as the ratio of the mean absorbance from test sera to the mean absorbance of controls. Results are taken as positives for ratios >2.

4.5 Immunofluorescent antibody test

The reactivity of immune mouse sera against the native parasite proteins from various stages of the parasite were analyzed by IFAT using *P. falciparum* sporozoites, strain NF54, as described elsewhere [32].

4.6 Lymphocyte proliferation

Proliferation of human PBL was evaluated in triplicate cultures against each LSA3-LSP. A mix of 3×10^5 cells and 10 μg/ml peptide was resuspended in 200 μl RPMI 1640 medium supplemented with 1% nonessential amino acids (Gibco), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM Hepes, 10,000 U/ml penicillin and 10 μg /ml streptomycin and 10% human AB serum (Institut Jacques Boy S.A.). Cultures were harvested on day 6 after a 16-h pulse with 1 μCi [^{3}H]thymidine; incorporation of the radiochemical was measured by liquid scintillation analysis. In positive control wells, mitogen (PHA-P) was added for the last 3 days of culture only.

Four to six mice from each group immunized with the 17 LSP were used to study the T cell response 15 days after last immunization. Individual mice were analyzed for the proliferative response, frequency of IFN-γ-secreting cells and CTL response against each homologous LSA3 long peptide as described [2, 10, 15] except that, for proliferative responses, serum-free HL-1 medium (Bio-Whittaker, USA) was used instead of RPMI 1640 medium. When *P. falciparum* sporozoites were used, they were adjusted to a concentration of 50,000 sporozoites/ml and 0.1 ml was added to culture. *P. berghei* sporozoites were used as a control. Results are expressed as SI, defined as the mean triplicate cpm of cells recovered from wells containing antigen divided by the mean triplicate cpm of cells recovered from well without antigen. Proliferation was considered positive when SI values were higher than 2, and Δcpm >1,000**.**

4.7 Elispot for mouse IFN-γ

The number of IFN-γ-producing cells was determined in freshly isolated LN cells 40 h after incubation with the corresponding peptides. Nitrocellulose-lined microtiter plates (MultiScreen-HA, sterile plate. Millipore) were coated overnight at 4°C with 50 μl carbonate buffer pH 9.6 containing 5 μg/ml mouse anti-IFN-γ antibody (18181D, Becton Dickinson). Plates were then treated as described [10], using biotinylated anti-mouse IFN-γ (biotin rat anti-mouse 554410, Becton Dickinson) diluted 1:2,000 in PBS as a detection antibody, and streptavidin-phosphatase alkaline conjugate (Boehringer Mannheim, Germany), followed by the BCIP/NBT reagents (Promega, Madison, WI) for the development of spots. In some assays, irradiated P815 mouse mastocytoma cell line (expressing only H-2^d class I molecules on the surface) [19] that were previously pulsed overnight with the corresponding peptide were used as APC. The number of spots present in the duplicate wells, with and without peptide were counted by two different readers in a stereomicroscope. Results are expressed as the number of IFN-γ SFC/ 5×10^5 LN cells.

4.8 CTL cultures and cytolytic assay

Briefly, LN cells from mice immunized with each LSP were pooled and 5×10^6 were cultured for 7 days with 10 μg/ml of the corresponding LSP in 24-well plates as described [10].

Seven days after *in vitro* stimulation, the CTL activity was tested in a standard ^{51}Cr-release assay using P815 cells (10^6) labeled with 100 μCi sodium [^{51}Cr]chromate (DuPont de Nemours, Boston, MA) [33]. The % specific lysis was calculated as 100×(experimental release–spontaneous release)/(total release–spontaneous release).

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