

Protection against *Plasmodium falciparum* challenge induced in *Aotus* monkeys by liver-stage antigen-3-derived long synthetic peptides

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The vaccine potential of *Plasmodium falciparum* liver stage antigen-3 (LSA3) was investigated in *Aotus* monkeys using two long synthetic peptides corresponding respectively to an N-terminal non-repeat peptide (NRP) and repeat 2 (R2) region of the LSA3, adjuvanted by ASO2. Both 100–222 (NRP) and 501–596 repeat peptides induced effector B- and T-cell responses in terms of antigen-driven antibodies and/or specific IFN- γ secretion. Animals challenged with *P. falciparum* sporozoites were protected following immunization with either the NRP region alone or the NRP combined with the R2 repeat region, as compared with controls receiving the adjuvant alone. These results indicate that the NRP may be sufficient to induce full, sterile protection and confirm the vaccine potential of LSA3 previously demonstrated in chimpanzees and in *Aotus*.

Key words: *Aotus* monkeys · Immune response · Long synthetic peptides · Malaria pre-erythrocytic stage · Malaria vaccine

Introduction

A solid and justified hope for an effective pre-erythrocytic malaria vaccine has emerged from the evidence that sterile immunity can be obtained by immunization with irradiated sporozoites both in humans and in animal models [1]. Others and we have brought evidences that the so-called ant sporozoite immunity is in fact liver-stage dependent. There is now convergent data indicating that this protective immunity is induced by the arrested liver trophozoites that persist in the liver and target-infected hepatocytes [2]. This has been the primary driving force behind our

work on the liver stages and has led to identification of new antigens expressed during the pre-erythrocytic phase of the parasite life cycle. The selection of the vaccine candidates relied on experimentally induced protection in humans immunized by irradiated sporozoites. By analyzing the differential immune responses observed between protected and non-protected volunteers upon a viable sporozoite challenge, we selected within a subset of *Plasmodium falciparum* pre-erythrocytic antigens a protein that we named liver stage antigen-3 (LSA3), which was preferentially recognized by sera from protected humans [2, 3]. This antigen is highly conserved, is expressed in sporozoite and liver stages, and its protective potential against challenge with *P. falciparum* sporozoites has been demonstrated in non-human primates using a large set of antigen-presentation systems [3, 4].

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The protective efficacy shown by this antigen prompted us to further analyze its antigenicity and immunogenicity using 17 long synthetic peptides (LSP) ranging between 44 and 186 amino acids in length, spanning the whole *P. falciparum* LSA3 protein. Most of the peptides were shown to be highly immunogenic in mice and antigenic in humans exposed to malaria in a hyperendemic area of Africa [5]. The chemical synthesis of LSP provides several practical advantages and has contributed to the evaluation of the antigenicity and immunogenicity of both *P. falciparum* and *P. vivax* proteins in animals and humans [6]. We selected two LSA3-LSP from the N-terminal non-repeated (100–222) and the repeat region R2 (501–596), which cover the sequence of the LSA3-729 subunit protein used in previous protective experiments [3, 4] and which is a target of Th1 protective responses in primates. In order to determine the region of greatest importance for the induction of protective mechanisms, we immunized *Aotus* monkeys with either the non-repeat region peptide alone, or mixed with the repeat region peptide and evaluated the efficacy of the immune responses induced in this manner against challenge by *P. falciparum* sporozoites.

Results and discussion

General remarks

The present study was performed in view of the protective effect induced previously by immunization with different formulations of LSA3 (Fig. 1) against a *P. falciparum* sporozoite challenge in chimpanzees and in *Aotus* monkeys [3, 4]. Four formulations have been found able to induce protection: the LSA3-729 recombinant protein either adsorbed to microparticles without adjuvant or adjuvanted by ASO2, lipopeptides formulations of the NRI and NR2 peptides with the non-lipid-tailed R peptide [3, 4], and genetic immunization using the nearly full-length gene [7] (Daubersies *et al.*, in preparation). Here, we perform a preliminary evaluation of the immunogenicity and protective

efficacy in *A. l. griseimembra* of two LSP covering either the non-repeat (100–222) or the repeat (501–596) sequences derived from the *P. falciparum* LSA3-729 protein (Fig. 1). In view of the strong Th1 responses obtained against the NRI and NR2 peptides in previous studies relying on lipopeptide or protein-adjuvanted formulations, the importance of the non-repeated region for protection was evaluated by immunizing with the non-repeat peptide (NRP) (including NRI and NR2 sequences) either alone or combined with the RP.

The LSP formulations were safe, since neither local nor generalized reactions were detected and hematological parameters, liver and kidney function remained within normal values in *Aotus* following subcutaneous immunizations with the LSA3-LSP in ASO2 adjuvant.

LSA3-LSP induce high IFN- γ response

At the T-cell level, the production of IFN- γ is of particular interest, since this cytokine is known to efficiently stimulate the elimination of *P. falciparum* liver forms even at low concentrations [8]. Its role in protection has been observed *in vitro* in *P. falciparum*-infected human hepatocytes, at concentrations ranging from 1 to 101 U [8] as well as *in vivo* in mice immunized by irradiated sporozoites [9] and is supported by studies in chimpanzees immunized either with irradiated sporozoites or with LSA3-derived antigens [2, 10], or in *Aotus* immunized with particulate formulations [4]. Because peptides NRP and RP contain epitopes, which are main targets of Th1 responses and the protection is associated with strong Th1 responses [4], analysis of T-cell responses in immunized *Aotus* was limited to the study of IFN- γ production by specific T cells. PBMC from immunized monkeys produced substantial amounts of IFN- γ as determined by ELISPOT upon stimulation with at least one of the short or long synthetic peptides, or in one animal in culture supernatants. The frequency of specific IFN- γ -producing cells detected by the *ex vivo* ELISPOT assay using cells from

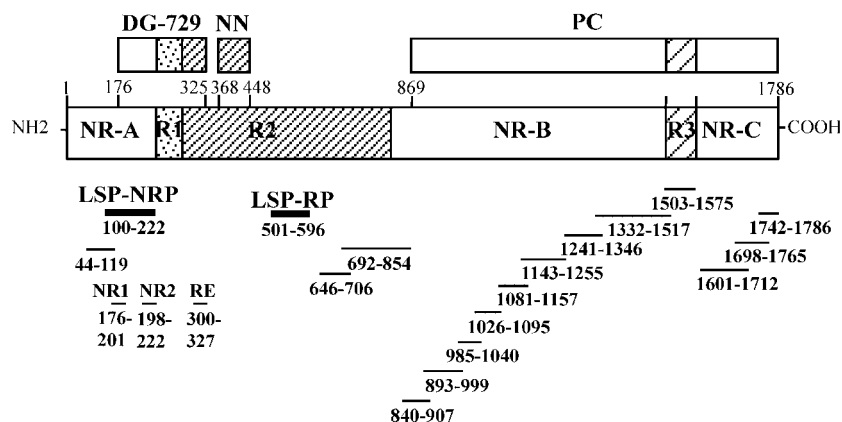


Figure 1. Schematic representation of *P. falciparum* LSA3 antigen. Recombinant proteins DG729, NN and PC are shown in the upper part and synthetic peptides in the lower part. The latter were used for antigenicity studies [5]. Immunogenicity and protective efficacy were evaluated previously in chimpanzee and *Aotus* monkeys using either DG 729 or peptides derived from the latter or a mix of 729, NN and PC [3,4]. Here, we employed in *Aotus* the LSP-NRP (100–222) and LSP-RP (501–596) regions.

monkeys immunized with either NRP or the mixture NRP+RP was highest in response to *in vitro* stimulation with the NRP (Fig. 2A). Lymphocytes from *Aotus* V114 and M88 from NRP and NRP+RP groups, respectively, also showed high IFN- γ response (determined either by ELISPOT or by ELISA) when challenged *in vitro* by the shorter peptides NRI and NRII contained in the NRP sequence. Responses were actually higher than those recorded previously by immunization with the DG 729 recombinant protein [4]. The specificity of the response was demonstrated by negative results obtained using lymphocytes from either pre-immunization samples or control monkeys. The high and specific IFN- γ secretion against the LSP-NRP and LSA3-729 shorter peptides is in agreement with previous results obtained in *Aotus*, mice, and chimpanzee immunized with LSA3-729-derived lipopeptides without adjuvant [10, 11] and recombinant proteins [4]. It indicates that the immunization with LSP-NRP is an efficient means of induction of strong T-cell responses.

Responses are relevant to the parasite protein

The relevance to the native LSA3 protein of T-cell responses induced by immunization with LSP was demonstrated by high specific responses to *P. falciparum* sporozoite. All immunized *Aotus* showed significant amounts of IFN- γ detected specifically by ELISPOT, or in culture supernatants, in response to *in vitro*

exposure of lymphocytes by a *P. falciparum* sporozoite extract, at levels similar to those produced using LSP. Results indirectly confirm the antigenicity of the molecule and indicate that substantial amounts of LSA3 are expressed at the sporozoite stage. In contrast, no IFN- γ response was detected in control animals or in lymphocytes from immunized animals in response to non-infected *Anopheles* salivary glands, used as controls. Based on previous studies [2, 3, 10], strong IFN- γ responses to the native protein are considered to be of good predictive value for protection against pre-erythrocytic stages of *P. falciparum*.

Antibody responses target the NRP region

Antibodies determined by ELISA showed titers ranging between 900 and 27 000 (Fig. 2B). Monkeys immunized with either NRP or the mixture NRP+RP produced high levels of antibodies to both NRP and the 729 recombinant proteins, indicating that B-cell epitopes contained in the NRP alone are strongly immunogenic in *Aotus*. Antibody titers were lower in the RP region, in contrast to results obtained in exposed populations, and non-significant in the group immunized with NRP alone, as expected. These results are similar to those observed in immunized mice [5] and chimpanzees [10], confirming that the sequences from the N-terminal region of the protein contain potent B-cell epitopes.

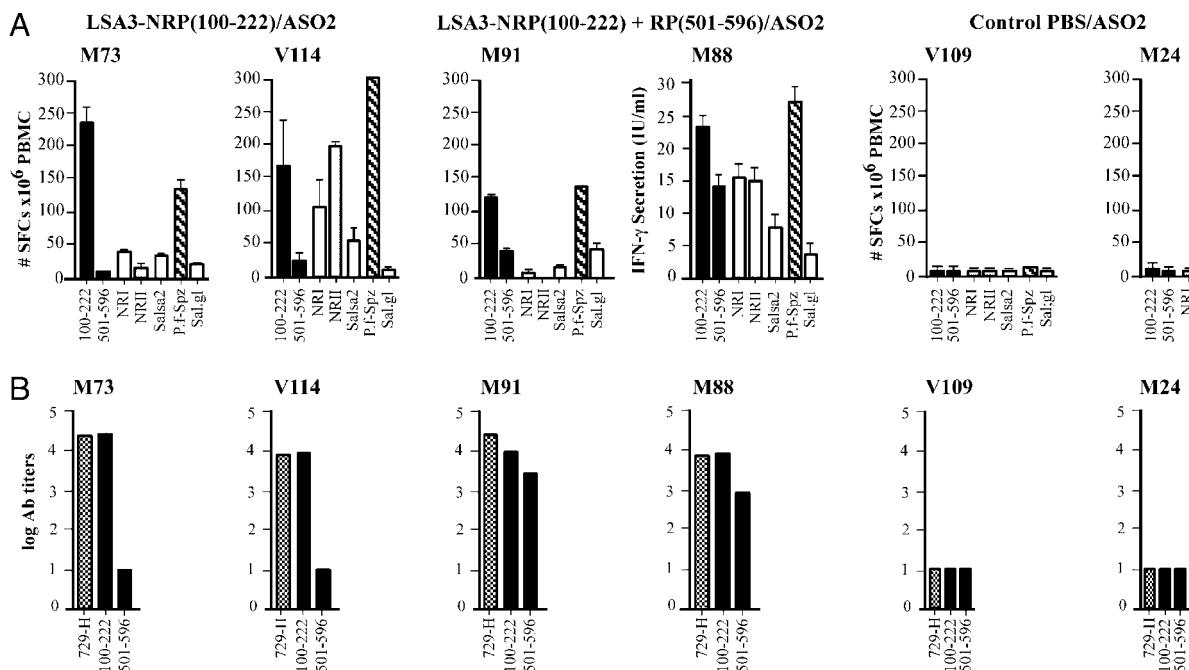


Figure 2. T- and B-cell responses in immunized and control *Aotus*. Two groups of *Aotus* monkeys were immunized with either NRP (100–222) or with the mix of NRP+RP (501–596) peptides adjuvanted in ASO2 as described in *Materials and methods*. A control group was immunized with PBS mixed with the same adjuvant. (A) IFN- γ production was evaluated on PBMC 15 days after the last immunization. Cell suspensions were mixed with the synthetic peptides LSA3-NRP, -RP, -NRI, -NRII, and with *P. falciparum* sporozoites as a native protein. Peptide SALSA-2 and salivary glands from non-infected *An. albimanus* were used as negative controls. Results are expressed as the mean+SD of the triplicate determination of IFN- γ spot forming cells per 10^6 PBMC. For *Aotus* M88, IFN- γ was titrated in culture supernatants by ELISA and expressed in IU/mL. (B) Specific antibody responses were assessed by ELISA against both peptides NRP and RP, and the LSA3-729 recombinant protein. Results are expressed as log titers.

LSA3-LSP induce protection against *P. falciparum* sporozoite challenge

The challenge was performed 60 days after the last immunization in order to avoid a possible nonspecific effect upon liver stages development of pro-inflammatory mediators, such as IFN- α , TNF- α , free oxygen and nitrogen radicals, induced by the adjuvant [2]. As shown in Fig. 3, the three challenge control monkeys, two immunized with PBS in ASO2 (M24, V109) and one naive animal (V111) developed a *P. falciparum* infection detected by the parasite antigen lactate dehydrogenase (pLDH) assay from day 14 to 28. Although there were some variations from one animal to the other, results are in agreement with previous data showing that *Aotus* monkeys can be successfully infected with sporozoites derived from the *P. falciparum* Santa Lucia strain in a reproducible manner [4, 12]. In contrast, pLDH was negative in all LSA3-immunized animals in all of the 120 samples collected during the 60 days of follow-up, indicating the induction of a sterile protection.

Although the number of animals was limited in this preliminary study, the fact that the *Aotus* immunized with the NRP alone or the mixture NRP+RP were fully protected suggests that epitopes contained within the NRP region alone are sufficient to induce protection against *P. falciparum*-sporozoites challenge, *i.e.* induce critical mechanisms. Therefore, further analysis of responses elicited by NRP in models and in humans may contribute to elucidation of these mechanisms. Although, the number of monkeys does not allow to reach statistical significance, these results are consistent with previous data obtained with *P. falciparum* LSA3-DG729 region [3, 4], particularly with the protection induced by lipopeptide formulations of the peptides NRI and NRII contained in the NRP employed in the present work [3].

It is noteworthy that the vaccination with LSA3-LSP induced immune responses that protected against a challenge with a strain heterologous to that used to design the vaccine. This is in agreement both with previous challenge data also performed using heterologous *P. falciparum* NF54 parasites [3] and with the high degree of LSA3-sequence conservation [3].

Concluding remarks

The present study confirms the high immunogenicity of LSA3 and particularly the induction of IFN- γ -secreting T cells; it suggests that responses elicited by the NRP region of the molecule are of particular significance, as NRP defines a minimal region associated with protection against *P. falciparum* heterologous challenge. It lends support to the LSP strategy as means to readily investigate the interest of diverse regions from large molecules and provides yet another vaccine formulation to the list of four LSA3-derived antigen presentations that successfully induced protection against *P. falciparum* challenge in a strain-independent manner. It also supports the use of *Aotus* monkey as a valuable alternative to chimpanzees for the discovery and pre-clinical development of *P. falciparum* pre-erythrocytic vaccines.

However, data obtained in pre-clinical models can only be taken as indicative, even when they are convergent and promising. Results indicate that immunization by full-length LSA3 may not be required. This and earlier studies rather suggest that formulations based on the DG 729 protein and/or the NRP, which can be easily produced under GMP conditions, should now be evaluated for safety and protective efficacy in clinical trials.

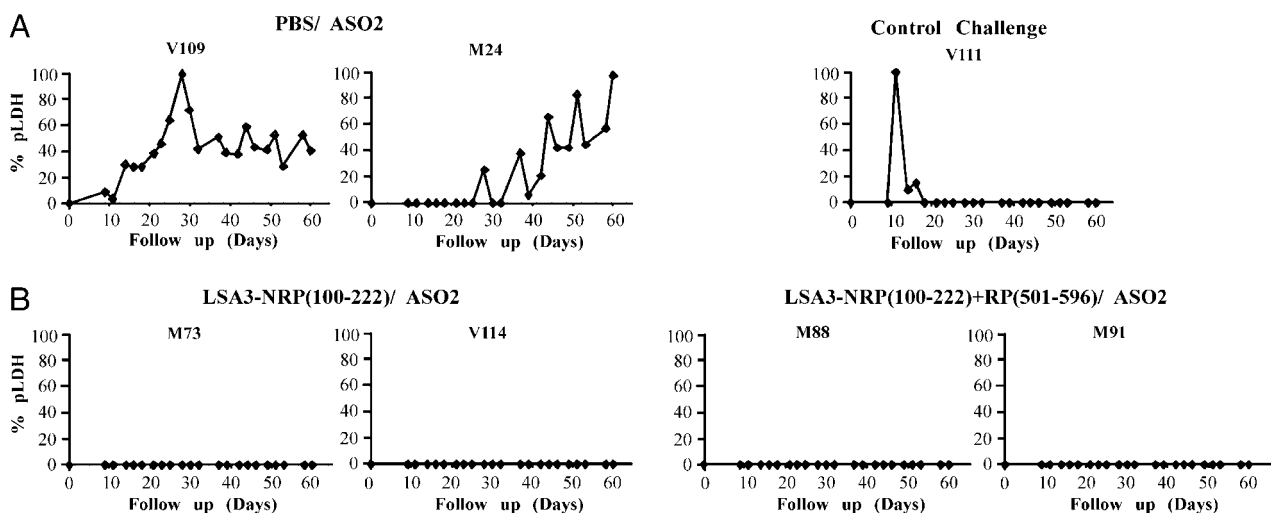


Figure 3. Parasitemia follow-up after challenge with *P. falciparum* sporozoites. Immunized and control *Aotus* as described in Fig. 2 were challenged 2 months after the third immunization by intravenous inoculation of 10^5 sporozoites from *P. falciparum* Santa Lucia strain. (A) The negative controls are two monkeys (V109, M24) that received the adjuvant ASO2 alone with PBS and one naive monkey (V111). (B) *Aotus* immunized with either NRP (100–222) or with the mix of NRP+RP (501–596) peptides. Parasitemia was evaluated by measuring circulating parasite pLDH in 30 blood samples from each animal collected every 2 days during 60 days. The results are expressed as percentages of maximal OD value observed in challenged control *Aotus*, as described in the *Materials and methods*.

Materials and methods

Long synthetic peptides

Two peptides covering the DG729 protein employed in previous studies [3,4] were synthesized and selected from a panel of 17 LSP based on previous studies, which showed strong antigenicity in human donors and strong immunogenicity in mice [5]. Peptide 100–222 (NRP) represents most of non-repeat region A from the N-terminal part of the protein. Peptide 501–596 (RP) is representative of the repeat region 2 (R2).

Immunization of monkeys

Two groups of two adult, naive, male and female *Aotus lemurinus griseimembra* monkeys, originating from the northern forests of Colombia, were immunized three times subcutaneously at 3-wk intervals with the two peptides mixed with the adjuvant ASO2. *Aotus* of group I received 50 µg of NRP alone, whereas *Aotus* of group II received a mix of 50 µg of NRP and 50 µg of RP. A control group of two monkeys was immunized with PBS mixed with the ASO2 adjuvant. In order to check the safety of the vaccine candidate, animals were inspected daily by a veterinary doctor performing physical examinations throughout the experimental period. Hematological parameters including Hemoglobin and Hematocrit, and liver and kidney biochemical parameters (BUN, Creatinine ASAT, ALAT) were evaluated before and during the immunization and challenge process. The experimental protocol was reviewed and approved by the animal ethical committee of Universidad del Valle and it was conducted following the US National Institutes of Health guidelines for the care and use of laboratory animals.

B- and T-cell responses

Immune responses in immunized *Aotus* were assessed to the two NRP and RP used as immunogens, to the recombinant protein LSA3-729 overlapping part of both regions, and the peptides LSA3-NRI, LSA3-NRII covering the short non-repeated sequence found in the NRP and in the LSA3-729 recombinant protein.

Serum samples collected from immunized and control monkeys before and after immunization were used for evaluation of specific antibody responses to the peptides by ELISA as described elsewhere [4].

T-cell assays were performed using *Aotus* PBMC from blood taken by femoral venipuncture at day 0 (pre-immunization) and 15 days after the third immunization. Cell suspensions were mixed with the corresponding LSA3 synthetic peptides described above or with a sonicated *P. falciparum* sporozoites. An irrelevant peptide from the SALSA protein [13] and salivary glands obtained from non-infected *Anopheles albimanus* mosquitoes were used as negative controls. IFN- γ production was evaluated

here because high levels of this cytokine were shown to be associated with protection against sporozoite challenge and were found to-date the best surrogate marker of protection in chimpanzees and *Aotus* [3, 4, 10]. The frequency of PBMC producing IFN- γ *ex vivo* was evaluated using a commercial kit for human IFN- γ ELISPOT (MABTECH, Stockholm, Sweden) as described elsewhere [4]. Results are expressed as the mean number of IFN- γ spot forming cells per 10^6 PBMC. Since numbers of PBMC recovered from one of the studied *Aotus* were insufficient, in this case we performed a more classical determination of IFN- γ in supernatants collected on day 5 from *in vitro* antigenic stimulation of lymphocyte cultures. IFN- γ was titrated by a two-site capture ELISA as described previously [10]. Negative and positive controls (unstimulated cells and cells stimulated with PHA) were included in each assay. The specificity was verified by the absence of signal in control supernatants.

Challenge and parasitemia follow-up

P. falciparum Santa Lucia strain previously adapted by serial passages in *Aotus* monkeys and able to produce infective gametocytes [14] was used to infect laboratory reared *An. albimanus* mosquitoes by artificial membrane feeding. Mature infective sporozoites collected on day 15 post-feeding were used to challenge monkeys as described previously [4]. The challenge was performed 2 months after the last immunization. Blood from all challenged animals was sampled every 2 days during 60 days. Parasitemia was monitored by the detection of the pLDH using a double-site enzyme-linked lactate dehydrogenase immunodetection assay [15]. The OD values recorded with this double-site antigen-capture assay have been found to be a direct function of parasite densities both for *P. falciparum* and for *P. vivax* [15, 16]. The assay proved to be more sensitive for the detection of *P. falciparum* and *P. vivax* than thick blood smears and was able to detect a parasitemia as low as 1 infected RBC in 10^8 RBC [15, 16]. The method was further validated by assessment of pLDH in samples from previous challenge studies in test and control animals [4]. The baseline absorbance value, above which a sample was considered positive, was the mean value plus 3 SD of the OD values obtained using blood samples collected before challenge. Positive parasitemia were expressed as the percentage of the highest OD value obtained from each of the three control *Aotus*. Negative parasitemia were expressed as 0%. At the end of the follow-up period, the animals were treated with the combination Sulfadoxine–Pyrimethamine, as in previous studies [4].

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: LSA3: liver stage antigen-3 · LSP: long synthetic peptide · NRP: non-repeat peptide · pLDH: parasite lactate dehydrogenase · RP: repeat peptide

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