Systemic immune responses induced by mucosal administration of lipopeptides without adjuvant

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We recently reported that parenteral injection of malaria palmitoyl-tailed peptides without adjuvant efficiently induces B, Th and CTL responses. We now show that intranasal (IN) or sub-lingual (SL) delivery of such lipopeptides induces strong systemic immune responses, as demonstrated by specific Th cell responses from the spleen as well as inguinal lymph nodes, and by the production of high levels of serum antibodies. Overall, both types of responses were significantly higher than in parallel experiments in which the same lipopeptides were delivered by the subcutaneous (s.c.) route. Moreover, the mucosal route resulted in the preferential induction of IFN-γ producing T cells and of IgG2a antibody production, as compared to the dominant IL-4 and IgG1 responses obtained by the s.c. route, thus bringing a distinct advantage in the field of many infectious diseases and allergy. Possibly related to this Th1 response, we found that dendritic cells, the principal immune-competent cells to encounter antigens within mucosal membranes, take up lipopeptide antigens more efficiently than macrophages. Mucosal immunization by lipidated peptides appears therefore as a novel, noninvasive vaccine approach that does not require the use of extraneous adjuvant and which, besides cost-effectiveness, has attractive practical and immunological features.

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1 Introduction

Human vaccines are traditionally administered intramuscularly by needle inoculation and most are given to children less than 2 years old. Immunization via the mucosal surfaces, without adjuvant and without the use of needles, would greatly increase the ease of vaccination. However, mucosal immunization has proven to be a major challenge [1]. It has been hampered in practical terms by poor peptide adsorption to mucosal membranes and by poor immunogenicity, coupled with a paucity of sufficiently potent adjuvants that can be tolerated by humans [1]. The cholera toxin (CT) produced by the bacterium *Vibrio cholerae* and the closely related heat-labile enterotoxin (LT) of *Escherichia coli* are amongst the sole mucosal adjuvants that indeed promote local and systemic immune responses to peptide or protein Ag [2, 3]. However, despite attempts aimed at reducing their side effects, their severe diarrheagenic property when ingested in amounts as low as 0.5 μg, make them unacceptable for human use.

We and others have recently established that parenteral injections of soluble lipopeptides induce, without adjuvant, strong systemic B, Th cell and CTL responses [4–8]. Although the mechanisms by which lipopeptides induce B and T cell responses *in vivo* are not yet fully understood [4–6, 9, 10], we hypothesized that the palmitic tail of lipopeptides may be able to fuse to the lipidic component of cell membranes, and thereby deliver the peptide into the cytoplasm [4, 9, 10]. We have reasoned that if this hypothesis is correct, then the lipopeptides should be able to bind and pass trough mucosal membranes, allowing transit of functional epitopes to the central lymphoid system. This would also imply differences in the APC involved in the mucosal route as compared to the parenteral route.

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Abbreviations: CT: Cholera toxin **IN:** Intranasal **SL:** Sublingual **CFSE:** Carboxyfluorescein diacetate succinimidyl ester **Tp cell responses:** T cell proliferative responses

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In this study, synthetic lipopeptides, derived from the *Plasmodium falciparum* LSA-1 and LSA-3 Ag [7, 11] which B and T cell immunogenicity by s.c. route has been well established [4, 5, 7, 11], were administered sub-lingually (SL) and intranasally (IN) in mice. Systemic B and T cell responses were determined to probe the transmucosal delivery, together with experiments aimed at assessing the relative contribution of dendritic cells (DC) and macrophages (Mϕ) in the uptake of lipopeptide Ag.

2 Results

2.1 Intranasal and sub-lingual delivery of LSA3-NRII lipopeptide induce circulating specific antibodies

The LSA3-NRII lipopeptide was administrated either IN or SL in BALB/c and C3H/HeJ mice, which were previously found to respond strongly to the epitopes of LSA3-NRII [4]. The C57BL/6, a poorly responding strain [4], was also included. As positive and negative controls, strain- and age-matched mice received, respectively, the same lipopeptide injected s.c., or the non-lipidated peptide administrated by mucosal routes without adjuvant.

Both IN and SL administrations induced high titers of peptide-specific antibody responses in BALB/c and C3H/HeJ strains (Fig. 1A). The mean titers of IgG antibodies induced by both mucosal routes were significantly higher than those recorded by s.c. route ($p < 0.05$). Peptide-specific antibody titers were significantly higher in SL immunized groups than in IN groups ($p < 0.05$ in BALB/c and $p < 0.01$ in C3H/HeJ). The antibody titers could be enhanced by further mucosal administrations of the lipopeptide (data not shown). In contrast, using the non-lipidated peptide, results were negative (ELISA-RATIO = 0.9 in C3H/HeJ, 0.8 in BALB/c). No antibody response was found in C57BL/6 mice after either mucosal or subcutaneous administration of the LSA3-NRII lipopeptide. Both IN and SL administrations were well tolerated, as following close examination, particularly of the sub-lingual mucosa; no local reaction could be detected.

The Ab produced by mucosally immunized BALB/c and C3H/HeJ reacted with the intact parasite, both at sporozoite and liver stages, thus demonstrating the biological relevance of this means of immunization (Fig. 2). These Ab did not react with infected RBC at various steps of intra-erythrocytic maturation (data not shown). These results demonstrate that administration of a lipopeptide by the mucosal route effectively delivers the Ag to the central lymphoid system and shows that the lipid moiety is absolutely required.

2.2 Mucosal administration of lipopeptides is effective in stimulating both systemic and local T cell responses

As seen in Fig. 3A, both SL and IN administrations of LSA3-NRII lipid tailed peptide, without a mucosal adju-

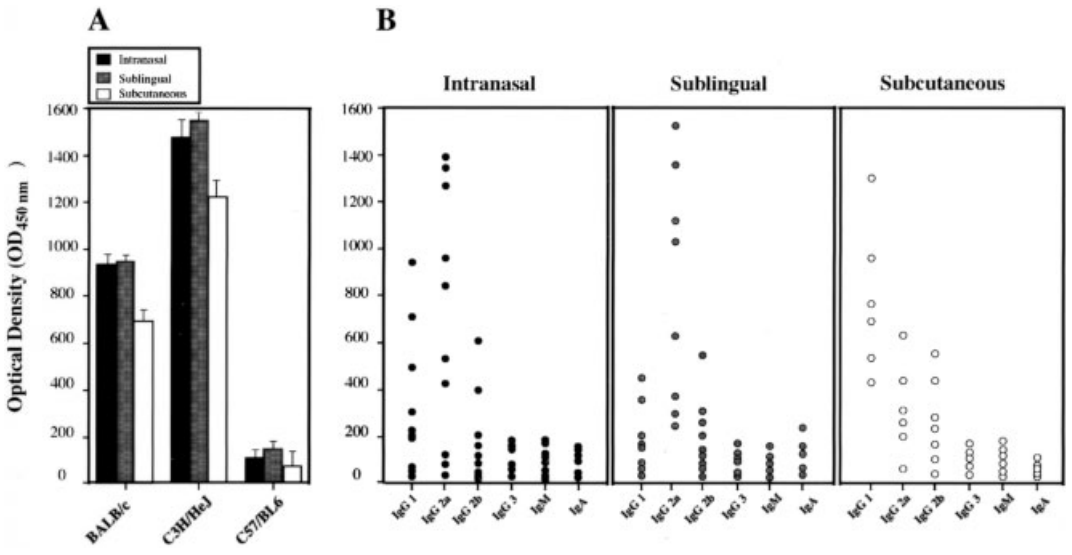

Fig. 1. Peptide-specific antibody responses induced in the serum following mucosal administration of LSA3-NRII lipopeptide without adjuvant. (A) Results obtained by either intranasal (black bars), sub-lingual (grey bars) or subcutaneous route (open bars), are expressed as the geometric mean of five sera in $OD_{450\ nm}$ ±SD. (B) Profile of isotypes induced. Results are expressed as individual $OD_{450\ nm}$ of sera from five to nine mice in each group and are representative of three separate experiments.

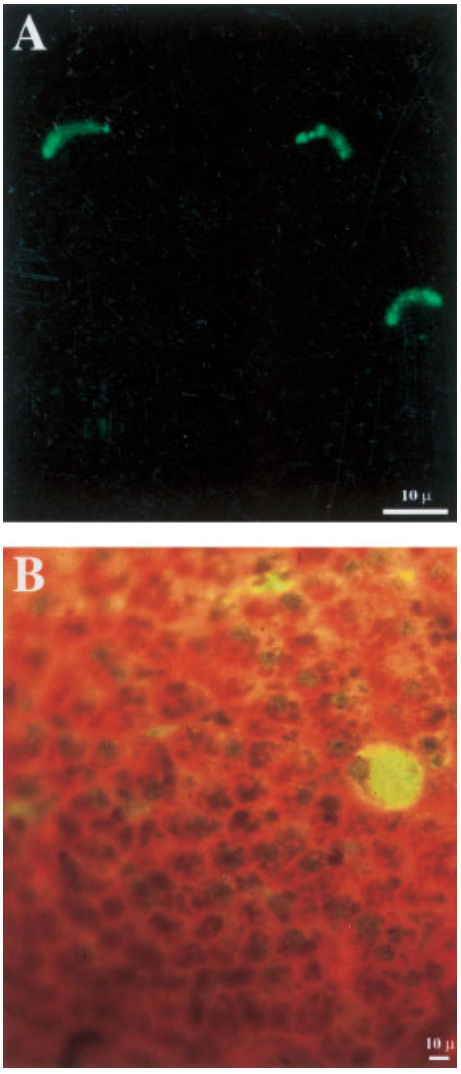

Fig. 2. Parasite-specific antibody responses elicited by mucosal delivery of lipopeptides. Sera obtained from C3H/HeJ mice 2 weeks post- sub-lingual immunization were assayed for recognition of (A) *P. falciparum* sporozoites and (B) hepatic schizonts in an IFI assay. Similar results were obtained following intra-nasal delivery of lipopeptides.

vant, induced strong T cell proliferative responses (Tp cell responses) in spleen cells. Peptide-specific Tp cell responses were also detected in the inguinal LN cells (maximal Δ cpm =12,525 cpm in IN and 16,189 cpm in SL groups), *i.e.* in T cells taken in secondary lymphoid organs at a remote distance from the Ag delivery site. Surprisingly, using the same dose of LSA3-NRII lipopeptide, Tp cell responses of mucosally immunized animals were at higher levels compared to those observed in subcutaneously immunized animals ($p < 0.05$). Tp cell responses were Ag-specific, as indicated by the lack of response against an irrelevant peptide (peptide LSA1-J) (data not shown). It should be emphasized that control animals that received an equimolar amount of the non-lipidated LSA3-NRII peptide without adjuvant show insignificant Tp cell responses (Fig. 3A). Tp cell responses were found to be of the CD4 phenotype, as the responses were abrogated by anti-CD4 but not by anti-CD8 antibodies (data not shown).

After SL administration of LSA3-NRII lipopeptide in mice, significant peptide-specific Tp cell responses were also recorded in the local draining submandibular LN cells following a prime-boost immunization with the lipopeptide (Fig. 3B). The peptide-specific Tp cell division involved $CD3^+/CD4^+$ (Fig. 3B) but not $CD3^+/CD8^+$ T cells (data not shown). The Tp cell responses induced were strong as shown by a major decrease in carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, reflecting the number of T cell divisions, and were Ag-dose dependent: 31% of cells divided at a peptide concentration of 50 µg/ml, 12% at a concentration of 20 µg/ml and 3% at a concentration of 5 µg/ml (Fig. 3B) as compared to 1.1% in unstimulated control cells and 55% in ConA-stimulated cells.

2.3 Mucosal route results in preferential Th1 immune response

The mucosal administration of LSA3-NRII lipopeptide resulted in a preferential IgG2a serum antibody response, whereas the s.c. route was associated with a dominant IgG1 isotype response, and only a modest increase in total serum IgG2a (Fig. 1B). Anti-peptide IgA, IgM and IgG3 were detected at low and similar levels in all groups. In agreement with these findings, IN administration induced mainly IFN-γ-producing $CD4^+$ T cell, whereas s.c. injection induced preferentially IL-4-producing $CD4^+$ T cells (Fig. 3C). Cytokine productions were Ag specific, as indicated by the lack of response against an irrelevant peptide (Fig. 3C). These results indicate that the transmucosal route preferentially triggers the $CD4^+$ Th1 lymphocyte subset.

2.4 Mucosal immunization extends to other antigens

To test if the concept was generally applicable, the LSA1-J lipopeptide, selected from LSA-1 Ag, [11] was delivered to BALB/c mice via mucosal route. Both IN and SL administrations of LSA1-J lipopeptide were found to induce serum IgG responses (ELISA-RATIO range from 3.9 to 6.9), whereas mucosal administration of an equimolar amount of the non-lipidated peptide analog, without adjuvant, failed to induce any responses (ELISA-

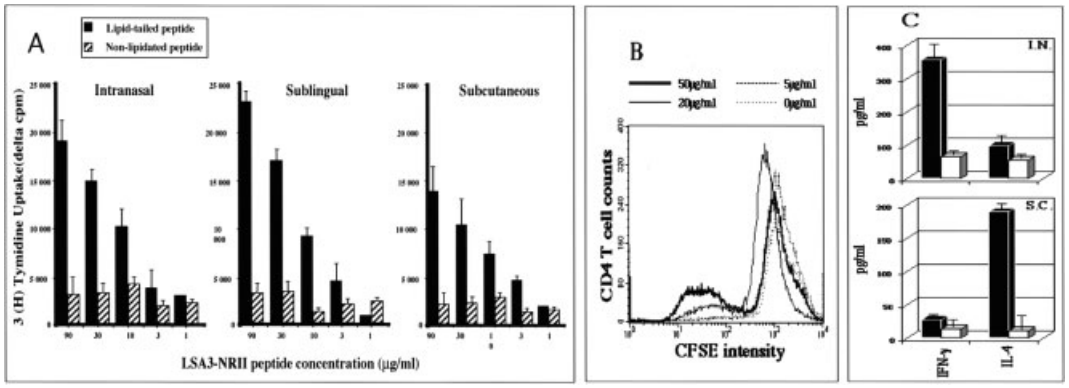

Fig. 3. Systemic and local cellular immune responses elicited by mucosal delivery of lipopeptides. (A) Spleen cell responses to LSA3-NRII lipopeptide (black bars) or its non-lipidated analog (hatched bars) from groups of five C3H/HeJ mice immunized intranasally, sub-lingually, or subcutaneously (mean Δ cpm ± SD in each group 2 weeks after the second administration). The background cpm in unstimulated cells were 1,548 for IN, 2,356 for SL and 1965 for s.c. routes. (B) Cells recovered from submandibular lymph nodes from mice administered twice (SL) the LSA3-NRII lipopeptide show a strong CFSE decrease, *i.e.* proliferation to the recall peptide in a dose-dependant manner at 50 μg/ml (thick line), 20 μg/ml (thin line), and 5 μg/ml (dotted line) and compared to the unstimulated cells (thin dotted line). (C) Preferential induction of specific Th1-type $CD4^+$ responses following IN administration of lipopeptides as compared to s.c. administration. IFN-γ and IL-4 cytokines induced by recall peptide (dark columns) and by a control peptide (clear columns). Mean ± SEM from five mice in each experimental group.

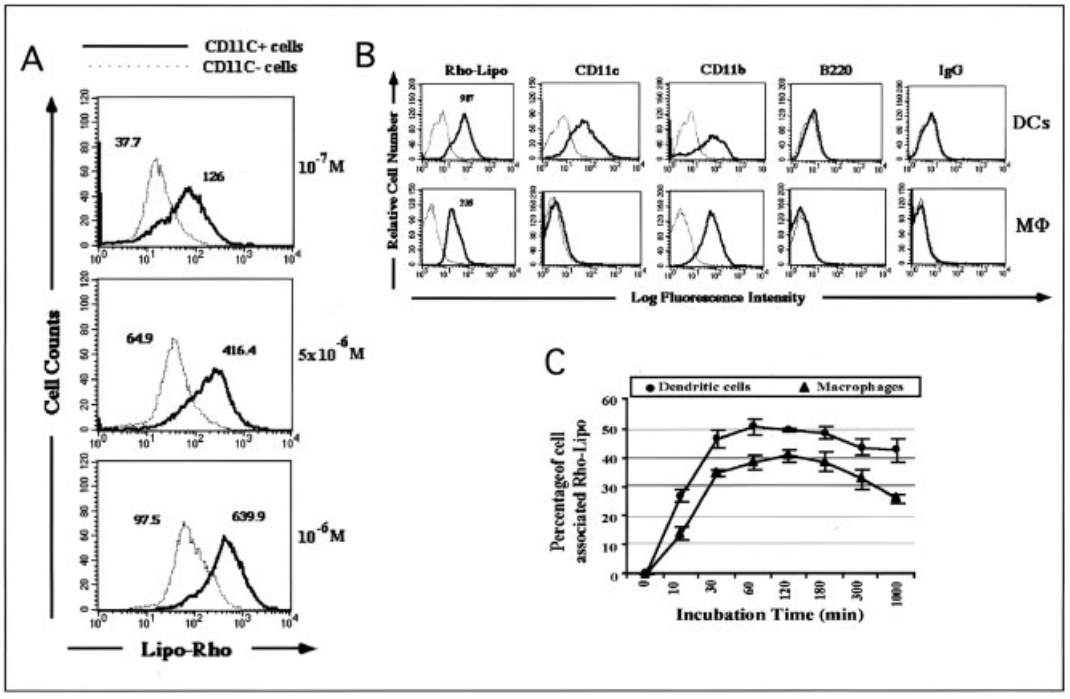

Fig. 4. Relative uptake of a lipopeptide Ag by DC and Mϕ populations. (A) Primary cultures of DC population were incubated for 30 min at 37°C with 10^{-6} M, 5×10^{-6} M or 10^{-7} M of a rhodamine-labeled lipopeptide. The histograms show loading of the lipopeptide on $CD11c^+$ (thick line) and $CD11c^-$ (thin doted line) gated cells. (B) Single-cell suspensions of DC (upper row) or Mϕ (lower row) population were incubated for 30 min at 37°C with 10^{-6} M of the rhodamine-labeled lipopeptide. The forward and side scatter properties of each population are depicted. To ascertain the cell lineage composition of each population, cells were stained with anti-CD11c, anti-Mac-1 (CD11b), anti-B220 or a control anti-IgG. The dotted line in each histogram represents the isotype-matched Ab control. Values of the rhodamine fluorescence intensity in DC and Mϕ cells are indicated. (C) Quantification of lipopeptide uptake by DC versus Mϕ was done by measuring the percentage of cell associated-fluorescence after incubation with the rhodamine-labeled lipopeptide.

RATIO range from 0.3 to 0.7). Similarly, both SL and IN administration of LSA1-J lipopeptide induced significant Tp cell responses in spleen and inguinal lymph node cells (Δ cpm range from 7,698 to 10,503), whereas the homologous non-lipidated peptide, without adjuvant, was inefficient (Δ cpm range from 1,632 to 2,698). Both local and systemic immune responses were also induced by lipopeptides derived from herpes and cytomegalovirus showing that this mode of mucosal immunization extends to other antigens (data not shown).

2.5 Relative role of DC and Mϕ in the uptake of lipopeptides

The $CD11c^+$ cells from primary cultures of DC showed intense staining, while the intensity was lower in the $CD11c^-$ cells, indicating that a major proportion of the lipopeptide was taken up by DC population (Fig. 4A). The lipopeptide was taken up less by the non-DC population even when an increasing dose was used (Fig. 4A). To explore this issue more directly, primary cultures of bone-marrow-derived DC and Mϕ cells were similarly incubated at 37°C with 10^{-6}M of the rhodamine-labeled lipopeptide for 30 min. Interestingly, under identical conditions, we found significantly higher uptake of the lipopeptide in DC ($CD11c^+$, $Mac1^-$, $B220^-$) compared to Mϕ population ($CD11c^-$, Mac1+, B220–) *i.e.* intensity of 765 to 987 and 128 to 235 in DC and Mϕ, respectively (Fig. 4B).

Kinetic analysis of lipopeptide uptake by DC versus Mϕ showed that the proportion of DC-associated lipopeptide was consistently higher than of Mϕ: *e.g.* after 30 min of incubation 47% DC versus only 35% Mϕ cells uptake the lipopeptide. A gradual increase in the percentage of cell-associated fluorescence was found over time, reaching a plateau of 40–55% after 60 min (Fig. 4C). After only 10 min of loading, significantly higher amount of lipopeptide Ag was taken up by DC as compared to Mϕ cells (Fig. 4C). An optimal percentage of fluorescent cells were found upon further incubation for 30 to 60 min, with a plateau being reached at 60 min. The fluorescence intensity decreased gradually in both DC and Mϕ cells over time, indicating that the HLA-peptide complex had a halftime of approximately 60 min. Fluorescent confocal microscopy of double-stained cultures revealed lipopeptide Ag associating with the cell membrane and cytosolic delivery in both DC and Mϕ (not shown). A significant amount of lipopeptide was visualized in the cytosol of DC as early as 10 min. After 30-min incubation, the lipopeptide seemed to accumulate in vesicular compartments under the plasma membrane, suggesting internalization into early endosomes. In contrast to DC, no diffuse staining was observed after internalization of lipopeptide by Mϕ cells, indicating that cytosolic transport of exogenous Ag did not occur in Mϕ cells. Both were inhibited at 4°C, suggesting an active mechanism during the intracellular delivery of lipopeptide Ag (data not shown).

3 Discussion

In the present work, we bring evidence that simple application of lipopeptides to the nasal and buccal cavities, without a mucosal adjuvant, results in efficient delivery to the central lymphoid system, as evidenced by specific antibody and T cell responses in distant lymphoid organs. The immune responses induced were strong and entirely dependent upon the presence of the lipid moiety. Interestingly, this Ag delivery method resulted in the preferential induction of Th1 type of immune responses. In addition, we show that DC, which are responsible for the priming of immune responses at the systemic as well as at the mucosal level, are the predominant APC involved in the uptake of lipopeptide Ag.

Mucosal surfaces constitute an impressive first-line defense system that is frequently exposed to an array of exogenous Ag and pathogens. Mucosal membranes contain a number of APC types and are particularly well equipped in immature DC, which have properties to optimize Ag uptake, processing, and T cell stimulation [12]. Eriksson et al. [13] demonstrated that mucosal DC take up Ag delivered with CTB and prompt a strong immune response after migrating to nearby lymph nodes. The above mentioned studies along with our findings may suggest that DC are the principal APC responsible to uptake and facilitate tracking of lipopeptide Ag to cross the mucosal barrier. It is noteworthy that mucosal-resident DC differ from DC derived from central lymphoid organs (*e.g*. spleen-resident DC) in that they express higher levels of MHC molecules and are much more potent in stimulating T cells [12].

In the present study, quantitative and qualitative features of mucosal immunization by lipopeptides have been identified. Not only the Ab and the T cell responses were of higher intensity after IN or SL administration with lipopeptides; in addition they preferentially promoted Th1 cytokines and IgG2a antibody production, as compared to the dominant Th2 cytokines and IgG1 production obtained by the s.c. route. The bias towards Th1-immune response obtained by transmucosal route is of particular interest since IFN-γ, as well as IgG2a, are critical in defense mechanisms against a wide array of pathogens. Hence, the method is not only an exciting alternative to parenteral delivery of immunogens, but may also be used to preferentially channel the type of immune

response, a goal of recognized importance in many infectious diseases and allergy.

Most human vaccines are currently administered by intramuscular injections, which carry the risk of transmitting blood-borne infectious pathogens such as HIV and hepatitis viruses [14], and mucosal delivery of pediatric vaccines has become an explicit goal of the NIH as well as of WHO [14]. Furthermore, the cost of equipment and well-trained personnel for delivering vaccines by parenteral routes is, for GPVI vaccines, several times higher than the vaccines themselves [14]. In many populations, immunization through mucosal surfaces would be more readily acceptable [1, 14], so that a move from needle injection to mucosal application, would be very positive from economical, logistical and safety standpoints [1].

Mucosal immunization by monopalmitoylated peptides represents a step forward as compared to previous attempts using peptides or proteins Ag incorporated into liposomes [15], proteosomes [16], virosomes [17], immunostimulating complexes [18], or PLG microspheres [2] or using larger constructs such as multiple antigenic peptides [19]. Although a lipidated multimeric peptide has been shown to induce moderate systemic immune responses it relied on a relatively complex tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P_3CSS) chain [2], which is mitogenic and somewhat toxic [20]. IN immunization with plasmid DNA-lipid complexes has been also reported recently to induce mucosal and systemic B- and T cell responses [21]. We show here the efficacy of a simple to produce and purify lipidic peptide. Moreover, both IN and SL administrations were explored and were found to be well tolerated. The SL route has been far less explored for immunization, although it is known to be efficient in bloodstream-delivery of drugs.

The results of the present study should stimulate attempts to extend this mode of mucosal immunization to larger, synthetic or recombinant subunit vaccines. This approach would benefit from the development of chemical methods that direct regiospecific monoacylation of recombinant proteins. The prospect of vaccination protocols relying on spray, drops, aerosol, gels or sweets formulations is particularly attractive as a novel, noninvasive vaccine approach that does not require the use of extraneous adjuvant and, besides cost-effectiveness, has attractive practical and immunological features.

4 Materials and methods

4.1 Immunization of mice

The LSA3-NRII (Ac-LEESQVNDDIFNSLVKSVQQEQQHNVK (Pam)NH_2) and the LSA1-J (Ac-ERRAKEKLQEQQS-DLEQRKADTKKK (Pam)NH_2) peptides and lipopeptides used in this study have been described previously [4, 9–11].

Groups of three to six C57BL/6 (H-2^b), BALB/c (H-2^d) or C3H/HeJ (H-2^k) mice, obtained from the Institut Pasteur (Paris, France), were given lipopeptides on days 0 and 14. For IN administration, drops of 5 μl/nostril were gently delivered into the nasal cavities for 20 to 30 min from a stock of 30 μl of sterile PBS containing 100 μg of lipopeptide, to avoid swallowing and oral delivery of lipopeptides. For SL administration, 30 μL of sterile PBS containing 100 μg of a lipopeptide was soaked into a cotton wool, and then applied in the SL location. Positive control mice were immunized s.c. with the lipid-tailed peptide. Negative control mice were immunized IN, SL or s.c. with an equimolar amount of non-lipidated analogous peptides. Sera were obtained via the retro-orbital plexus 14 days post-immunization.

4.2 Peptide- and parasite-specific antibody responses

Anti-LSA3-NRII or LSA1-J peptide antibodies were determined by ELISA as described [22, 23], using sera diluted at 1/100 and revealed by peroxidase-conjugated goat anti-mouse IgG (Biosys, Compiègne, France). Results were expressed either as optical density ($OD_{450\ nm}$) or as ELISA-RATIO calculated as previously described [22, 23]. Isotype analysis was carried out using class-specific alkaline phosphatase-conjugated Goat anti-Mouse IgA, IgM, IgG1, IgG2a, IgG2b or IgG3 HRP-labeled as second antibody (Southern Biotechnology Associates, Birmingham, USA).

The reactivity of sera against native epitopes was analyzed by IFA as previously described [23], using *P. falciparum* sporozoites or liver sections from day 5 *P. falciparum* schizonts (NF54 strain), and IFAT-labeled anti-mouse Ab (Cappel, Wester Chester, PA).

4.3 Th cell assays

Peptide-specific Th cell responses were determined, using standard methods [4, 24], from spleen and lymph node cells (3×10^5 cells/well) collected 14 days post-immunization, in the presence of recall or control peptides (90, 30, 10, 3, or 1 μg/ml). The results were expressed as Δ cpm (average of triplicates), and considered positive when the Δ cpm was $>$1,000 cpm and stimulation index was $>$2 [24].

Cell division in submandibular draining LN cells was assessed by flow cytometry using CFSE (Molecular Probe, Eugene, OR) as previously described [12]. Lymphocyte division estimated by decrease of the CFSE-labeling was measured with a FACSCaliber® on $CD3^+/CD4^+$ or $CD3^+/CD8^+$ gated cells and analyzed using the CellQuest® software.

For cytokine analysis, $CD4^+$ spleen T cells (1×10^6 cell/ml) were isolated as above and cultured with untreated DC or

DC pulsed with recall peptide or control peptide (10 μg/ml) for 72 h. ConA was used as positive control at 0.5 μg/ml. Culture media were then harvested and IFN-γ or IL-4 concentrations determined by specific sandwich ELISA following the manufacturer's instructions (PharMingen, San Diego, CA).

4.4 Analysis of uptake by dendritic cells and macrophages

DC and Mϕ were generated as described [12]. Differentiation of bone marrow cells into DC and Mϕ was followed by flow cytometry analysis of different surface markers.

DC or Mϕ cells (1×10^6) were suspended in 37°C pre-warmed RPMI-1640 medium containing 5% FCS and incubated with 10^{-7}M, 5×10^{-6} M or 10^{-6} M of the rhodamine-labeled lipopeptide for 10 min, 30 min, 1 h, 2 h, 6 h and 18 h. Cells were washed in cold FACS® buffer and stained with 1 mg/ml of FITC-labeled anti-CD11c, anti-Mac-1 (CD11b), anti-G4/80 or anti-B220 (PharMingen). For each sample, 20,000 cells were acquired using a FACSCaliber® with two excitation laser sources and analyzed with a CellQuest® software (Becton Dickinson, San Jose, CA).

Confocal microscopy was used to follow the intracellular delivery of the rhodamine-labeled lipopeptide into DC and Mϕ cells as described [4, 9, 10].

4.5 Statistical analysis

Figures represent data from at least two independent experiments. The data are expressed as the mean ± SEM and compared by using Student's *t* test using the STATVIEW II statistical program (Abacus Concepts, Berkeley, CA).

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