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Immune response after oral administration of the encapsulated malaria synthetic peptide SPf66

A.M. Carcaboso [a], R.M. Hernández [a], M. Igartua [a], A.R. Gascón [a], J.E. Rosas [b], M.E. Patarroyo [b], J.L. Pedraz [a,*]

[a] *Pharmacy and Pharmaceutical Technology Laboratory, Pharmacy Faculty, University of the Basque Country (UPV-EHU), Paseo de la Universidad n° 7, 01006 Vitoria-Gasteiz, Spain*
[b] *Fundación Instituto de Inmunología de Colombia (FIDIC), Universidad Nacional de Colombia, Carrera 50, n° 2600 Santafé de Bogotá, Colombia*

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Abstract

The synthetic peptide SPf66 adsorbed on alum is one of the few *Plasmodium falciparum* vaccines which have been tested in field trials. We previously reported that subcutaneous administration of SPf66 loaded PLGA microparticles (MP) enhances the antibody response to this antigen compared to the conventional alum formulation. We now evaluate the suitability of polymeric formulations to obtain systemic immune responses by gastric intubation of Balb/c mice. Formulations composed of 1:1 mixtures of PLGA 50:50 and 75:25 (lactic:glycolic) microparticles were administered by the oral route, and when animals were boosted 3 weeks later significant systemic IgG antibody responses were elicited, comparable to alum triple shot and superior to the aqueous vaccine given by the oral route. The finding of IgG2a isotype for PLGA-vaccinated mice compared to the absent levels of this isotype for the alum-vaccinated group could be interpreted as a sign of Th1-like immune response and cellular immune response activation. Our results confirm that using the appropriate schedule the oral administration of PLGA particles is suitable to obtain systemic immune responses to the carried antigen.

Keywords: SPf66; Malaria; Vaccination; Microparticle; Poly-D,L-lactide-co-glycolide; Oral administration

1. Introduction

More than one million people, most of them children in developing countries, die every year of malaria in the world (W.H.O., 2003). Although an efficient malaria vaccine has not been yet implemented, more than a dozen subunit vaccines (Doherty et al., 1999; Galindo et al., 2000; Stowers et al., 2001; Tsuji and Zavala, 2001) and DNA vaccines (Wang et al., 1998) are in clinical trials with the hope of obtaining a commercialised formulation. Even under the most optimistic scheme of unlimited resources, for any given clinical grade immunogen, the completion of the stages of clinical trials up to phase 3 is supposed to require not less than 10 years (Richie and Saul, 2002). So it will still take long to develop an efficient vaccine and, once this hypothetical vaccine is applied, problems would appear associated to the practical implementation of vaccination programs to millions of receptors, involving high costs and compliance failures, inherent to the traditional administration, which requires multiple shooting by parenteral routes. An

* Corresponding author. Tel.: +34-945013091; fax: +34-945013040.
E-mail address: knppemuj@vc.ehu.es (J.L. Pedraz).

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ideal vaccine for massive administration in countries without economical resources should be effective and meet the following criteria: inexpensive, safe, capable of inducing lifetime immunity, stable to the environmental conditions in order to avoid the cold chain necessity, easy to administer, preferably by non-invasive routes, given during childhood and, if possible, by a single shot (Bloom, 1989).

Many vaccines fail before or during clinical trials. One of the explanations for the low efficacy of synthetic or biotechnological antigens is the low power of the conventional adjuvants, like aluminum salts, to stimulate an adequate immune response (Gupta, 1998). From the late eighties it has been demonstrated that the microencapsulation of these antigens in biodegradable and biocompatible polymers as the copolymer of lactic and glycolic acids (PLGA) elicits an adjuvant effect. The administration of microencapsulated model antigens often induces a potent and perdurable humoral immune response (Igartua et al., 1998), activation of the T helper type 1 (Th1) dependant cellular immune response (Newman et al., 1998), as well as delayed type hypersensitivity and cytotoxic T cell activity (Maloy et al., 1994). These immunological advantages seem to be due to the particulation of the antigens by the polymer, which allows their uptake by the professional antigen presenting cells (APC) (Scheicher et al., 1995), and also to the longer remaining of the particles in the site of administration. This is based on the slow biodegradation of the PLGA, which forms a depot from the one the antigen is progressively taken, processed and presented to the immune system (Coombes et al., 1996).

SPf66 was the first synthetic vaccine able to induce a partial protective immune response to malaria when it was formulated with alum as adjuvant in several pre-clinical and clinical trials (Alonso et al., 1994; Noya et al., 1994; Galindo et al., 2000; Patarroyo et al., 1987). Recent investigations in animal models have demonstrated the applicability of the PLGA microencapsulation to this antigen in order to improve the immune responses and protection induced by alum, both in mice and monkeys (Rosas et al., 2001, 2002). The humoral immune response induced by the SPf66 antigen was clearly increased when encapsulated in PLGA MP, even with a single immunization by subcutaneous route, suggesting the possibility of simplifying immunization protocols.

By the oral route, PLGA particles protect the antigens from degradation into the gastrointestinal tract, and direct them to be captured by the gut associated lymphoid tissues (GALT), mainly represented by the Peyer's patches (O'Hagan, 1994). The search for ideal vaccination routes prompted us to test the oral immunogenicity of these microparticle formulations in Balb/c mice. Systemic IgG response to the administration of an oral formulation was assessed. IgG isotype (IgG1, IgG2a) was also characterized in order to indirectly evaluate the activation of a Th1-like response.

2. Materials and methods

2.1. Antigen

The malaria synthetic peptide SPf66 (lot 15) was produced under GMP conditions and supplied by the Fundación Instituto de Inmunología de Colombia. The molecule was synthesized using the solid-phase synthesis methodology (Merrifield, 1963).

2.2. Preparation and characterization of the PLGA particles

PLGA particles were formulated by a modification of the solvent extraction technique using double emulsion (Rosas et al., 2001). Two SPf66 loaded microparticle formulations (MP) were prepared using Resomer® RG 506 (M_w 102,900), with a copolymer ratio of 50:50 lactic/glycolic (%), or Resomer® RG 756 (M_w 92,000), with a copolymer ratio of 75:25 lactic/glycolic (%), both supplied by Boehringer Ingelheim K.G. (Ingelheim, Germany). Briefly, 250 mg PLGA were dissolved in 5 ml dichloromethane and emulsified with 250 μl of a 10% w/v SPf66 aqueous solution by probe sonication for 30 s at 50 W (Branson® sonifier 250). The resulting emulsion (w/o) was poured into 25 ml 8% polyvinylalcohol (PVA, average M_w 30,000–70,000, from Sigma Chemical Co., Madrid, Spain) and emulsified for 5 min at 9500 rpm using a turbine homogeniser (Ultraturrax® T-25) in order to obtain the double emulsion (w/o/w). Finally, to favor the removal of the organic solvent from the microparticles to the external phase, 50 ml of 2% aqueous isopropanol solution was added and stirred for 1 h. The

Table 1
Immunization protocols in groups of mice ($n = 10$)

Group	Day of immunization	Route	SPf66 (μg/dose)	Formulation
Sol	0, 1, 2; 21, 22, 23	Oral	500	Solution
MP	0, 1, 2	Oral	500	PLGA MP
MPboost	0, 1, 2; 21, 22, 23	Oral	500	PLGA MP
Alum	0, 21, 42	Subcutaneous	100	Alum gel

resulting suspension was separated by centrifugation, washed three times in distilled water and freeze-dried.

Microparticle size distribution was determined by laser diffractometry using a Coulter Counter® LS130 particle size analyzer. Sphere morphology and surface appearance was examined by scanning electron microscopy (SEM; Jeol® JSM-35 CF). Total loaded peptide and surface adsorbed peptide were estimated using the micro-BCA assay kit (linear working range for diluted peptide concentrations of 0.5–20 μg/ml) from Pierce, Teknovas (Bilbao, Spain) as previously described (Rosas et al., 2001).

2.3. SDS–PAGE

Peptide structure was analyzed by polyacrylamide gel electrophoresis (SDS–PAGE) in tris–tricine buffer. Stacking and separating gels were prepared with 4 and 10.4% polyacrylamide, respectively. The stability of the microencapsulated peptide was determined after dissolving MP in methylene chloride and extracting with distilled water. Peptide released from the MP was also evaluated by incubating in phosphate buffered saline pH 7.4 at 37 °C for 1 h and collecting supernatant by centrifugation. All the samples were freeze-dried in order to concentrate the peptide, and dissolved in tricine sample buffer. Electropherograms of samples were performed at a constant voltage of 150 mV in tris–tricine–SDS buffer (cathode) and tris–Cl pH 8.9 buffer (anode), using a Bio-Rad Mini-Protean II electrophoresis system. After migration, gel sheets were stained with Coomassie R.

2.4. Immunization protocols

For antibody induction against SPf66, 40 female Balb/c mice, aged 7 weeks (Harlan Interfauna Iberica S.L., Barcelona, Spain), were randomly divided into four groups of 10 and were immunized with the antigen, either microencapsulated, free or adsorbed on alum. Procedures are described in Table 1. Briefly, two groups of 10 mice were orally immunized at days 0, 1 and 2 with 500 μg of the antigen each day, with SPf66-loaded MP, in 0.3% sodium bicarbonate by intragastric intubation. The employed formulation was a 1:1 mixture of PLGA 50:50 and 75:25 (lactic:glycolic) microparticles. One of these groups was boosted with the same doses at days 21, 22 and 23. The doses were administered on three consecutive days to avoid the variability associated with the oral canulation of the animals (Maloy et al., 1994; Partidos et al., 1999; Felder et al., 2001). One group received 500 μg of free peptide at days 0, 1, 2, 21, 22 and 23. One more group of 10 mice was subcutaneously immunized in the nape of the neck with SPf66 adsorbed onto aluminum hydroxide, alum (100 μg SPf66 at days 0, 21 and 42).

Blood samples were collected from the retroorbital plexus of the mice, under anesthesia with ether, at weeks 3, 6, 9 and 12. Samples were centrifuged and serum was collected and stored frozen at −30 °C until assayed by ELISA (anti-SPf66 IgG, IgG1, IgG2a).

2.5. Determination of SPf66-specific antibody responses

A conventional ELISA was used to determine anti-SPf66 antibodies (Rosas et al., 2001). An affinity-purified goat anti mouse IgG (peroxidase conjugate, from Sigma) was used and total IgG titers were measured at all time points, while IgG1 and IgG2a isotypes were assessed with goat anti mouse IgG1 and goat anti mouse IgG2a (peroxidase conjugates, purchased from Southern Technology) at 6 weeks. The end-point titers were expressed as the $\log_{10}$ of the last dilution reciprocal which gave an OD405 above the mean OD405 of the preimmune sera plus 2 standard deviations.

2.6. Statistical analysis

The results were expressed as mean ± S.D. for each group. Normal distribution of samples was assessed by Shapiro-Wilk trial using the SPSS 10.1 program (SPSS®, Chicago, USA). Differences among groups of animals at significance levels of 95% were calculated by the non-parametric Mann–Whitney *U*-test.

3. Results

3.1. Particle characterization

Freeze-dried, sphere-shaped microparticles were obtained. The mean particle size for RG 506 and RG 756 MP was 1.40 and 1.33 μm, respectively, with a narrow size distribution (Fig. 1). Table 2 shows the results for peptide loadings and surface-associated peptide for both formulations.

3.2. Acrylamide gel electrophoresis

Acrylamide gel electrophoresis showed that the initial structure of the native peptide was not altered by the encapsulation procedure (Fig. 2), as we found in previous studies using size exclusion chromatography, capillary electrophoresis and western blotting (Rosas et al., 2001, 2002). Extracted and released peptide electropherograms presented the same bands as the native peptide, irrespective of the formulated batch. There were no new bands indicating partial degradation or aggregation of the protein.

3.3. Immunogenicity of SPf66 encapsulated in PLGA formulations

The development of serum anti-SPf66 IgG antibody responses was investigated. As presented in Fig. 3, none of the animals immunized by the oral route with the peptide solution secreted antibody levels above

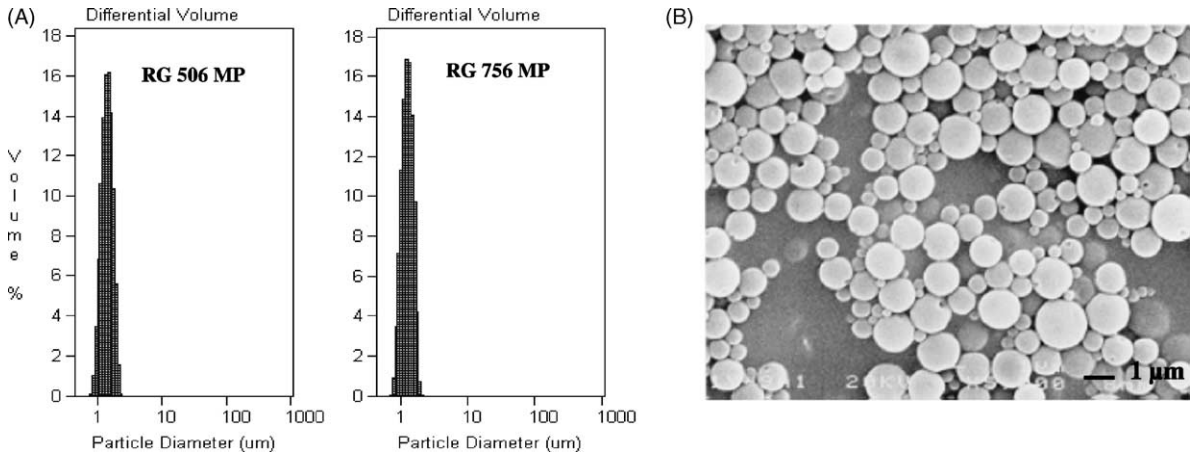

Fig. 1. Morphological characterization of the microparticles: (A) size distribution of the PLGA formulations; (B) scanning electron photograph (5000×) of one of the SPf66-loaded PLGA batches.

Table 2
Microparticle characterization ($n = 3$ batches)

Formulation	Size (μm)	Encapsulation efficiency (%)	Peptide loading (%)	S.A.P.[a] (%)
RG 506	1.40 (95% 0.82–1.96)	84.0 ± 6.1	8.4 ± 0.6	14.8 ± 6.7
RG 756	1.33 (95% 0.79–1.74)	77.3 ± 6.4	7.7 ± 0.6	17.3 ± 2.3

[a] S.A.P.: surface-associated peptide.

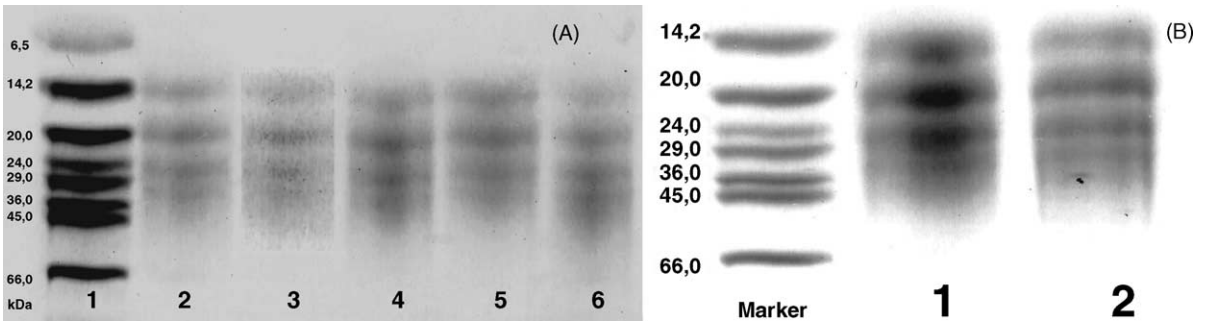

Fig. 2. Acrylamide gel electrophoresis of SPf66 peptide. (A) SPf66 extracted from MP (lane 1: molecular weight marker; lane 2: native peptide; lanes 3 and 4: peptide extracted from two batches of Resomer RG 506 MP; lanes 5 and 6: peptide extracted from two batches of Resomer RG 756 MP). (B) SPf66 released from MP at first day (lane 1: released from Resomer RG 506; lane 2: released from Resomer RG 756).

background values. The administration of three consecutive oral doses of 500 μg of SPf66 peptide encapsulated into MP elicited detectable antibody levels in only 10% (week 3) to 30% (weeks 9 and 12) of the mice, and the difference was not statistically significant with the group immunized with the free peptide. Only when the animals were orally boosted at week 3 with MP the number of responders increases up to 80% at week 9, and the difference among this group and free antigen group was statistically significant

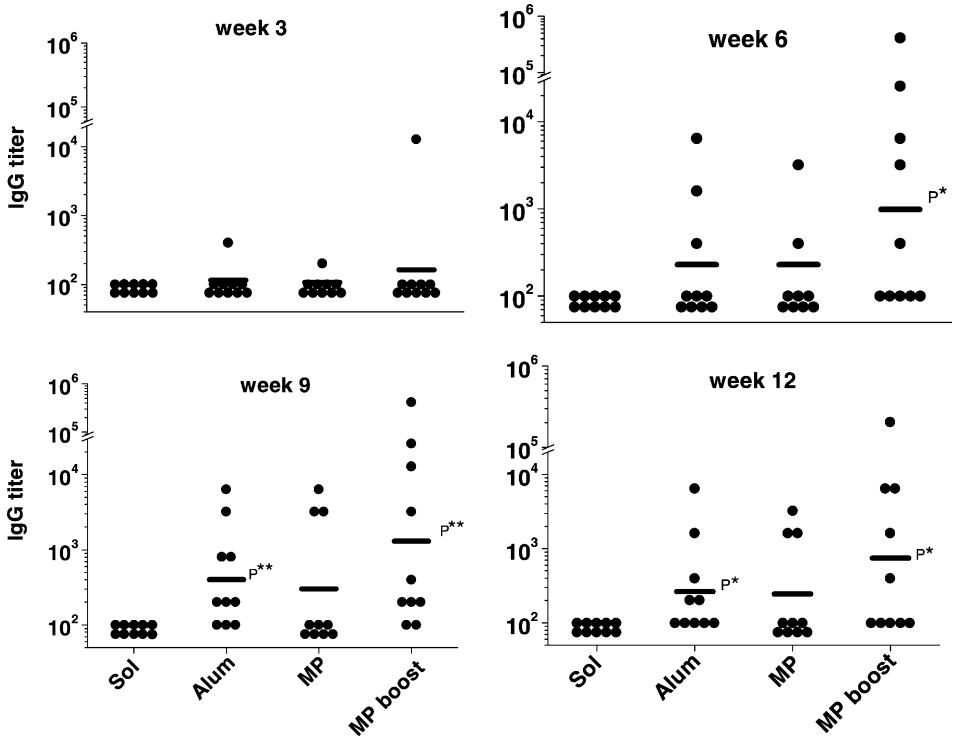

Fig. 3. Anti-SPf66 antibody responses (individual (●) or mean (▬)) in sera of Balb/c mice immunized orally using free peptide (Sol), SPf66 loaded microparticles (MP), SPf66 loaded microparticles with boosting (MPboost) compared to subcutaneous SPf66 adsorbed on $Al(OH)_3$ gel (Alum). P^*: $P < 0.05$; P^{**}: $P < 0.01$ (significantly different compared to Sol group).

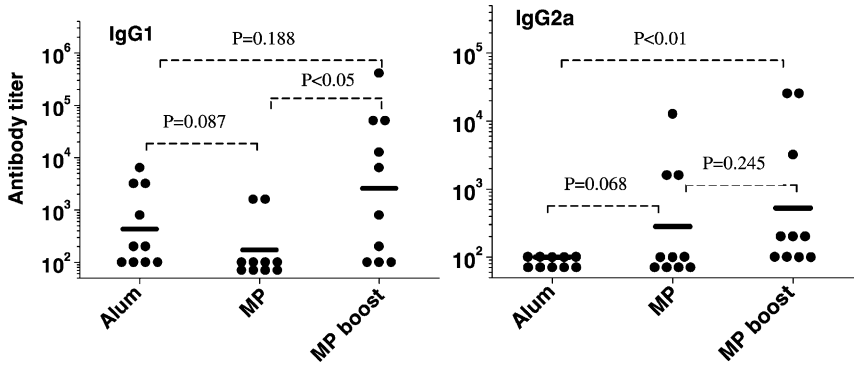

Fig. 4. Anti-SPf66 antibody IgG isotypes at 6 weeks in sera of Balb/c mice (groups of Fig. 3).

from week 6. The subcutaneous administration of three doses of 100 μg of free antigen in alum at days 0, 21 and 42 elicited responses in 70% of mice at week 9, and no differences with the orally boosted group could be found.

We next determined whether differences on the isotype profiles existed, measuring SPf66-specific IgG1 and IgG2a levels. None of the mice immunized with free peptide elicited either IgG1 or IgG2a antibodies. Compared to the free peptide group, the alum group presented significantly higher ($P < 0.01$) IgG1 levels, and similar IgG2a profile. Concerning to oral MP, only the boosted animals presented differences ($P < 0.01$) with the free peptide group. Fig. 4 shows that the IgG responses in mice vaccinated with alum formulation were biased towards IgG1 and, moreover, no IgG2a antibodies could be detected in any of the animals. On the other hand, significantly higher IgG2a titers, with 60% of responders, were present in the sera of the orally boosted mice, and also a 30% of the non-boosted animals were positive for IgG2a isotype. IgG1 titer for the MP groups was significantly higher for the boosted group compared to the non-boosted one.

4. Discussion

The need for making oral vaccines for developing countries prompted us to test the suitability of the oral route to induce systemic immune responses to an encapsulated malaria synthetic vaccine. The results showed that in mice, an appropriate oral administration schedule with SPf66-loaded PLGA MP induced significant IgG titers, superior to the vaccine solution by the oral route and similar to the alum parenteral vaccination, with presence of IgG2a isotype, the humoral marker of Th1-type immune response.

According to our recent results the most proper formulation to immunize was a 1:1 mixture of PLGA 50:50 and 75:25 (lactic:glycolic) MP, probably because the first follows a fast to moderate release profile and the second provides a more sustained, slow release pattern of the antigen (Rosas et al., 2002). The particle size is thought to be a critical factor for the oral administration. It seems to be clear that only less than 1 μm sized particles are able to reach the gut associated lymphoid tissues (GALT), mainly represented by the Peyer's patches (Tabata et al., 1996). Matsunaga reported that small particles (3–4 μm) can systemically prime the mice and are distributed to the spleen, while 7–10 μm sized induced tolerance and larger than 10 μm failed to induce an immune response (Matsunaga et al., 2001). Other studies reveal that sizes smaller than 1 μm (nanoparticles) enhance the uptake by the Peyer's patches (Desai et al., 1996), the translocation to the spleen (Seo et al., 2002), and stimulates the immune system against peptides and DNA vaccines (Jung et al., 2001; Singh et al., 2000), but we recently found that antibody response to BSA-loaded PLGA MP decreases with particle size in the nanometer range and optimal responses were obtained for 1 μm size (Gutierro et al., 2002), employed in this study. Both PLGA 50:50 and PLGA 75:25 MP formulations were characterized for antigen loading and surface-associated peptide, with no important differences among them. Previous stud-

ies using size exclusion chromatography, capillary electrophoresis and western blotting showed that the integrity of the loaded peptide was not significantly affected by the encapsulation procedure (Rosas et al., 2001, 2002). In the new study, we confirm again the chemical integrity of the microencapsulated peptide by acrylamide electrophoresis. Often protein instability has been reported after PLGA microencapsulation, related to the organic solvents used to form the MP or to the acidic environment inside them during polymer degradation (Weert van de et al., 2000). The synthetic nature of the SPf66 antigen, a short and chemically defined peptide, could be a reason for a better stability compared to the protein antigens.

Our first main goal was to demonstrate that particulate formulation is able to induce a humoral immune response by the oral route. We verified that oral immunization with soluble peptide SPf66 failed to elicit detectable IgG titers in any mouse. The results for the groups orally immunized with peptide-loaded PLGA MP overcame the absence of response to the free peptide, but only the boosted animals elicited significant IgG titers. It has already been shown that schedules without secondary immunization cycles are not sufficient to stimulate successful immunization by the oral route (Doherty et al., 2002; Kende et al., 2002). The oral dose was chosen as 5–10 times the subcutaneous (3×100 μg, Alum group), taking into account the previous reports showing that less than 1% of an oral dose of PLGA MP reached the Peyer's patches (Eldridge et al., 1989). Other immunization studies, which compared the oral route with subcutaneous, used high oral doses of microencapsulated antigens to obtain significant immune responses in mice, even when potent immunogens as BSA or OVA were used (Gutierro et al., 2002; Flanagan et al., 1994). In our study, not statistically different humoral immune responses against the SPf66 malaria synthetic peptide were induced by either oral administration of SPf66 loaded PLGA MP or conventional subcutaneous alum formulations, in terms of mean IgG titers and number of responder mice. Thus, we could infer that the PLGA immunization by the oral route would be able to reach similar results to the ones obtained in several clinical trials, which used alum as an adjuvant (Alonso et al., 1994; Galindo et al., 2000). Some mice did not produce antibody titers against SPf66 neither after the third dose of subcutaneous alum vehicle nor after the boosting with oral microparticles. This could be related to an ineffective presentation of the synthetic peptide associated to certain HLA molecules or could indicate a defective recognition at the T cell level. Human trials with alum in triple administration followed a similar pattern, showing a fraction of non-responder volunteers even after the third dose (Salcedo et al., 1991).

In animal models good immune response activations and protection against infectious agents like *Bordetella pertussis*, *Streptococcus pneumoniae* or *Vibrio cholerae* have been obtained after the oral administration of less than 10 μm sized particles of PLGA or other polymers (Tabata et al., 1996; Conway et al., 2001; Seo et al., 2002; Yeh et al., 2002). Protection is a consequence of the local and systemic immune response activation, even producing cytotoxic T cell responses (Partidos et al., 1999). Nevertheless, the lack of efficacy of the microparticulate systems to reach immune responses by the oral route have been frequently reported, although successful by parenteral route (Allaoui-Attarki et al., 1998; Barackman et al., 1998; Chandrashekhar et al., 1994; Felder et al., 2001; Smith et al., 2000), maybe as a consequence of poor particle uptake (Eldridge et al., 1989) or induction of oral tolerance (Matsunaga et al., 2001; Pecqet et al., 2000). In the present experiment, the oral tolerance phenomenon could be considered as a reason for the lack of efficacy in some mice. Several studies have considered the role of the dose on the oral tolerance occurrence. In contrast with the oral feeding of soluble antigens, in which high doses in multiple feedings are required to induce a tolerogenic effect (Friedman and Weiner, 1994), with microparticulated antigens given by the oral route the lowest doses seem to be more tolerogenic than the highest ones (Pecqet et al., 2000). In our study, the oral antigen dose was considerably increased compared to the subcutaneous to avoid this possible tolerogenic effect of the MP formulations.

The findings of failures to induce significant immune responses after oral administration of PLGA MP were especially relevant in large animal models such as pigs under real field conditions (Felder et al., 2001). The authors of the last study differenced between peroral administration and oral canulation and suggest that the administration mode is a crucial question on successful oral immunization with biodegradable MP. Strategies to enhance the immune response to oral vaccines use the oral route as booster vaccination

for systemically primed animals with PLGA MP (Chattaraj et al., 1999; Marx et al., 1993) or with subunit vaccines in combination with monophosphoryl lipid A adjuvant (Doherty et al., 2002). The few clinical trials (Phase I) in which human volunteers were immunized orally with PLGA failed to elicit consistent responses to encapsulated antigens. Lambert et al. (2001) administered 3 mg of a microencapsulated anti-HIV peptide to healthy seronegative adults, showing no response to the antigen. Katz et al. (2003) obtained low responses, not statistically different from the elicited by non-encapsulated antigen, after immunizing humans with 3–15 mg of an encapsulated enterotoxigenic *Escherichia coli* subunit vaccine.

The major difference between the immune response elicited by conventional alum formulation and PLGA particles is the induction of Th1-type responses to the PLGA particles (Moore et al., 1995; Vordermeier et al., 1995; Newman et al., 1998). A marked shift was noted in the quality of the antibody responses in our study, characterized by significant levels of IgG2a antibodies by the oral PLGA formulation, compared to the absent levels of this isotype elicited by the alum adjuvant or by the free oral peptide. This IgG2a response for oral SPf66-loaded MP presents a similar trend with MP administered by the subcutaneous route (unpublished data). Since IgG2a is the isotype associated to the stimulation of B lymphocytes by the T helper type 1 (Th1) lymphocytes, capable of activating the cellular immune response, which is crucial for malaria (Good et al., 1992; Nardin and Nussenzweig, 1993), we hypothesize that several immunological advantages could be obtained by the oral PLGA vaccination. Kende et al. (2002) correlated IgG2a levels with protection against aerosol challenge in mice orally immunized with ricin toxoid loaded PLGA MP. Nevertheless, some authors consider that the expression of subclasses of immunoglobulins, without measuring cytokines, cannot be seen as a reliable indication of Th1- or Th2-type reaction (Pertmer et al., 1996). A complete immunological study should also include the determination of cytotoxic T lymphocyte (CTL) responses. CTL responses to murine malaria synthetic peptides encapsulated into PLGA MP have already been found (Nixon et al., 1996; Men et al., 1997). This should be the main advantage at the immunological level, since it is a mechanism of protection against intracellular pathogens, recognizing and eliminating the sporozoite-infected hepatocytes.

We recognize that *Plasmodium falciparum* does not infect through a mucosal surface; nonetheless, our findings are significant in that they demonstrate that oral immunization with SPf66 loaded PLGA particles can elicit an immune response comparable to subcutaneous alum in total IgG titer and superior in IgG2a isotype. It should be taken into account that, to our knowledge, this has been the first attempt to obtain systemic immune responses to a malaria antigen by the oral route. However, although the results obtained by the oral route overcome those found for the alum formulation employed for clinical trials in humans, they do not increase the immune response elicited with the subcutaneous MP (Rosas et al., 2001). We are still far away from an efficient oral microparticle-based vaccine, but our study demonstrates that by optimizing formulations and administration protocols significant enhancements in antibody response could be achieved by the oral route.

5. Conclusion

Our research aims to test a new vaccination protocol to the encapsulated SPf66 malaria synthetic vaccine. This report demonstrates that SPf66 loaded PLGA MP are capable of inducing systemic immune responses after oral administration, comparable to the subcutaneous alum schedule. Furthermore, our results confirm an IgG isotype profile suggestive of a Th1 response for oral MP, and Th2 for alum formulation. These findings prove the suitability of the PLGA based systems to obtain immune responses by the oral route, although this immunization scheme needs to be optimized to achieve the efficacy of the parenteral administration.

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