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Adjuvant activity of polymer microparticles and Montanide ISA 720 on immune responses to *Plasmodium falciparum* MSP2 long synthetic peptides in mice

E. Mata [a], A.M. Carcaboso [a], R.M. Hernández [a], M. Igartua [a], G. Corradin [b], J.L. Pedraz [a,*]

[a] *Pharmacy and Pharmaceutical Technology Laboratory, Pharmacy Faculty, University of the Basque Country (UPV-EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain*
[b] *Institute of Biochemistry, University of Lausanne, Ch. de Boveresses 155, CH-1066 Epalinges, Switzerland*

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Abstract

The purpose of this work was to test the immunogenicity in C57BL mice of two synthetic peptides derived from the constant region of 3D7 and FC27 *Plasmodium falciparum* MSP2 dimorphic proteins, either microencapsulated into poly-lactide-*co*-glycolide acid microparticles (PLGA MP) or delivered with the human compatible adjuvant Montanide® ISA 720 for comparison. Potent and prolonged antibody responses were obtained for both peptides by using PLGA MP formulations after subcutaneous or intradermal injections. As compared to the subcutaneous route of immunization, the intradermal route induced greater immune responses. Montanide adjuvant was effective in eliciting antibodies against the 3D7 peptide but not against the FC27 peptide. Peptide-specific cytophilic antibodies (IgG2a) were detected after boosting with homologous peptide for all vaccine formulations. MP formulations elicited a lower IgE secretion as compared to that observed for both Montanide formulated vaccines. Our results demonstrate the ability of the polymer microparticles to overcome the lack of immunogenicity of FC27 MSP2 peptide in C57BL mice and their potential to induce desirable immune responses against malaria.

Keywords: Microparticles; Synthetic peptides; Malaria

1. Introduction

Today, near the half world inhabitants are exposed to malaria infectious disease, distributed in the 100 countries of tropical and subtropical zones where it still remains endemic. Malaria causes annually 1 million deaths and 500 million acute clinical cases [1], most of them in developing countries. Its long-term economic burden provokes a reduction of gross national product (GNP) by more than a half as compared to that of non-malarious countries [2].

In the last decade, after a long time of neglect and rampant spreading of the disease, efforts in multiple directions were initiated from rich countries in the race of disease control and eradication, with the clear intention from WHO to reduce malaria burden by 50% by 2010. However, at the halfway point the campaign is not being effective because the statistics reveal an increase on deaths [3].

Vaccination could be a sustainable and cost-effective strategy in this fight against malaria. Hence, recent progress in the understanding of immune protection mechanisms and in the knowledge of the parasite and mosquito genomes offers new tools that could ease the identification of suitable vaccine candidate antigens. Although several malaria vaccine candidates targeting to different life cycle stages have been tested in clinical trials [4–6], only a few have progressed to phase III. The most advanced candidate at the moment, the recombinant RTS,S/AS02A, revealed only a partial protection of 35% in Africa children for a period of 18 months [7].

Reasons for vaccine failure or slow pace of development are multiple and include the complexity of the parasite, extensive polymorphism of candidate vaccines and, last but not

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

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least, availability of appropriate adjuvants to obtain a protective immune response [8].

In order to optimize vaccine immunogenicity several adjuvant formulations have been postulated to reach the adequate T- and B-cell based response needed for protection against malaria [5,8–10]. Among these, taking advantage of the inherent stability of peptide antigens, poly-lactide-*co*-glycolide acid biodegradable polymer microparticles (PLGA MP) seem to represent one of the most attractive tools as vaccine adjuvant/delivery system. Their major contribution to the specific immunostimulation comes from their ability to particulate soluble peptides, allowing the sustained uptake and process of the antigen depot by the APCs. This also allows a single microparticle dose immunization to mimic the repeated injections of the conventional vaccination schedules [11]. MP are able to elicit T helper (Th), cytotoxic T (CTL) and B cell responses [12–17]. Moreover, these microparticulate systems confer antigen stability to low pHs and enzymatic hydrolysis, permitting vaccine administration by alternative routes, including mucosal ones [18]. The use of oily adjuvants (Montanide®) based on dispersions of antigen aqueous droplets (about 1 μm size) in a continuous oily phase, stabilized by surfactants, has also been proposed as immunogenicity enhancers for synthetic antigens [19] and have been approved for human clinical trials [20,21].

The aim of the present study is to elicit and characterize potent and long lasting immune responses in mice with adjuvanted novel malarial long synthetic peptides, corresponding to the C-terminally constant region of the two 3D7 and FC27 dimorphic proteins of the merozoite surface protein 2 (MSP2) which is considered a leading malaria vaccine candidate. In fact, immunization with the 3D7 full length recombinant protein together with two other malaria proteins was accompanied by a reduction of infection by 3D7 parasite [22] and a concomitant increase of infection by FC27 parasite; thus, the need to include both 3D7 and FC27 proteins in a future vaccine was postulated. Due to the fact that the intact proteins contain a highly polymorphic repeat region, the invariant part of the two proteins is an attractive choice for vaccine development. These C-terminal segments are also characterized by an amino acid sequence of low complexity which is associated with intrinsically unstructured domains, thus very susceptable to protease degradation [23,24]. Since synthetic peptide approach is free of contamination by protease traces, it might present some advantages over production of the same segments by recombinant technology. The human antibody response to these two segments has been recently characterized (manuscript in preparation).

Herein we describe a series of studies on the immunogenicity of two different vaccine formulations, PLGA MP and Montanide® ISA 720 (M-ISA720), an experimental adjuvant approved for human clinical trials, both given by subcutaneous (s.c.) route [19,20]. Furthermore, we evaluated the influence of the administration route by comparing the antibody response obtained by s.c. and intradermal (i.d.) immunization with the two MP formulations.

2. Material and methods

2.1. Antigens

The two long synthetic MSP2 peptides corresponding to the dimorphic region of 3D7 and FC27 alleles where synthesized. The peptide sequences are shown in Fig. 1. Peptide MR141 (3D7-MSP2) includes 88 amino acids of the non-repetitive semi-conserved part of the 3D7 molecule and 40 amino acids of the C-terminal conserved part. Peptide MR144 (FC27-MSP2) represents the other allelic family (48 amino acids of the non-repetitive dimorphic part) plus the 40 amino acids of the C-terminal conserved part [25]. Peptides were chemically synthesized using solid-phase F-moc methodology as described by Atherton et al. [26] and purified to homogeneity by reverse phase HPLC. Purity (>80%) was assessed by analytical HPLC and mass spectrometry.

2.2. Particle preparation and characterization

PLGA microparticles were formulated using a w/o/w double emulsion solvent extraction technique [27] under aseptic conditions. The microparticle formulations containing 3D7 or FC27 peptides were prepared using Resomer® RG 503 (MW 40,600; viscosity 0.41 dL/g in chloroform 0.1%, 25 °C) with a copolymer ratio of 50:50 lactic/glycolic (%) (Boehringer Ingelheim G.K., Ingelheim, Germany). Briefly, 30 μL of a 100 mg/mL peptide solution in distilled water was

SEQUENCE OF 3D7 AND FC27 PEPTIDES

AEASTSTSSENPNHKNAETNPKGKGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQ

ADTPTATESISPSPPITTTESSSSGNAPNKTDGKGAESEKQNELNESTEEGPKAPQEPQTAENENPA

PEQAENSAPTAEQTESPELQSAPENKGTGQHGHMHGSRNNHPQNTSDSQKECTDGNKENCG

.....APENKGTGQHGHMHGSRNNHPQNTSDSQKECTDGNKENCG

Fig. 1. Amino acid sequence of the two long synthetic peptides. Sequences represent peptides MR141 (aa 111-238 3D7, in italics) and MR144 (aa 124-230 FC27) where the last 40 C-terminal amino acids are similar (underlined). Position of the gap in the sequence is arbitrary.

emulsified in 1.2 mL methylene chloride containing 60 mg of polymer, using a microtip sonicator for 30 s at 50 W (Branson® 250 Sonifier, CT, USA). The resulting primary emulsion (w/o) was added into 6 mL of 8% (w/v) polyvinyl alcohol (PVA; MW 30–70,000; Sigma Chemicals, St. Louis, MO, USA) and was further homogenized for 5 min with a turbine homogenizer (Ultra-Turrax® T 25, IKA-Labortechnik, Staufen, Germany) at 9500 rpm to perform the secondary emulsion (w/o/w). This emulsion was poured into 12 mL of 2% (v/v) isopropanol and stirred for 2 h to favour the extraction of the organic solvent and complete microparticles hardening. The produced microparticles were then collected by centrifugation (10,000 × *g* for 10 min at 4 °C), washed three times with distilled water to remove residual PVA, suspended in ultra-purified water, and freeze-dried for 24 h.

Particle size distribution was determined by laser diffractometry (Coulter Counter® LS130 particle size analyzer, Amherst, MA, USA). Zeta potential was measured by laser doppler velocimetry (Malvern® Zetasizer 3000, Southborough, MA, USA). Total loaded peptide and surface associated peptide (S.A.P.) were determined using the bicinchoninic acid assay (micro-BCA, Pierce Co., Rockford, IL, USA) [28] in a linear working range for peptide concentrations of 5–20 μg/mL. Total peptide entrapped into microspheres was evaluated after their disruption in 0.2 M NaOH. Surface associated peptide was measured in the supernatant after centrifuging a microparticle suspension in 20 mM PBS maintained under orbital rotation at 37 °C for 30 min [29].

2.3. SDS-PAGE

Peptide integrity was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) in tris-glicine buffer. Stacking and separating gels were prepared with 4 and 12.5% polyacrylamide, respectively. The stability of the microencapsulated peptide was determined after dissolving the microparticles in methylene chloride and extracting with distilled water. All the samples were freeze-dried in order to concentrate the peptide, and dissolved in sample buffer. Electropherograms were performed at a constant voltage of 150 mV in tris-glicine-SDS pH 8.3 running buffer, using an electrophoresis system (Bio-Rad Mini-Protean II). After migration, gels were stained with Coomassie blue R 250.

2.4. Immunization protocols and sampling schedule

For antibody induction against malarial peptides, 48 female C57BL/6j mice, aged 7 weeks (Harlan Interfauna Iberica S.L., Barcelona, Spain), were randomly divided into six groups of 8 and immunized with one of the antigens, either microencapsulated in PLGA or emulsified in M-ISA720, kindly provided by SEPPIC, Paris, France. All mice were immunized with an initial peptide dose of 20 and a 2 μg boosting dose at 20 weeks based on preliminary results. The first two groups, called 3D7MPsc and FC27MPsc, were subcutaneously injected in the nape of the neck with 3D7 or FC27 peptide loaded microparticles suspended in 100 μL of PBS buffer. Another two groups (named 3D7MPid and FC27MPid) received an encapsulated peptide suspension (50 μL) by intradermal route divided into 4–5 injections on the shaved back skin.

The last two groups (3D7Mont and FC27Mont groups) received the same dose of peptide emulsified in Montanide. M-ISA720 is made of a natural metabolizable non-mineral oil and a highly refined emulsifier from the mannide mono-oleate family [30], that provides an (w/o) emulsion after mixing with an aqueous phase. Immediately before the administration, an aqueous peptide solution was emulsified with M-ISA720 (3:7, v/v). The emulsion was performed by mixing the two phases in a 5 mL glass syringe fitted with a 21 G needle (diameter 0.8 mm–length 25 mm), followed by 10 up and down strokes. The final peptide concentration in the w/o emulsions was 200 μg/mL. After injecting mice s.c. as in microparticles groups, a slight massage was given to avoid leakages from the administration site due to the high surface tension of the emulsion.

Blood samples were collected from the retroorbital plexus of mice, under anaesthesia with ether, prior to immunization (preimmune sera) and periodically until 30 weeks. Samples were centrifuged and serum was collected and stored frozen at −80 °C until assayed by ELISA (anti-3D7 and anti-FC27 IgG, IgG1, IgG2a, IgE).

2.5. Antibody response evaluation

A conventional ELISA was used to determine anti-3D7 and anti-FC27 antibodies. Briefly, microtitre plates (Nunc-Immuno Plate® FP 96 Maxisorp NUNC) were coated with 50 μL per well of 1 μg/mL peptide in 0.1 M carbonate coating buffer (pH 9.5) and incubated overnight at 4 °C. Plates were washed three times with PBS-Tween 20 (0.05%) (PBST) and blocked for 1 h at room temperature (RT) with 200 μL of PBST containing 2% non-fat dry milk (PBSTM). Serum samples were serially diluted starting from 1:50 dilution in blocking buffer and 100 μL of each sample was added to each coated ELISA plate well and incubated for 1 h at RT. After washing three times with PBST, 100 μL of HRP-conjugated goat anti-mouse IgG (Sigma), diluted 1:1000 in PBSTM, were added to the wells. After incubation at RT for 1 h, washing was repeated, and 100 μL of ABTS substrate (0.2 mg/mL solution in 50 mM citrate-phosphate buffer pH 5.0 containing 0.25 μL of 30% hydrogen peroxide per mL), was added to each well, incubated at 37 °C for 20 min for colour development and measured at 405 nm with an automatic microplate reader (Multiscan EX, Labsystems, Helsinki, Finland). The end-point titers were expressed as the log 10 of the last dilution reciprocal that gave an OD405 above the mean OD405 of the pre-immune sera plus two standard deviations (S.D.).

The protocol was similar for the IgG subtype determination, using HRP-conjugated goat anti mouse IgG1 and IgG2a, respectively, diluted 1:4000, for detection (Southern Biotechnology Associates Inc., Birmingham, AL, USA).

In the case of specific IgE levels, HRP-conjugated goat anti-mouse IgE was used, 1:2000 (Nordic Immunological Laboratories, Tilburg, The Netherlands) and modified ABTS substrate (0.3 mg/mL in 0.1 M citric acid, pH 4.3, containing 1 μL of 30% H_2O_2/mL).

For total IgE levels, plates were coated with 100 μL of anti-mouse IgE mAb as capture antibody (Pharmingen, Becton Dickinson, San Diego, CA, USA) at 2 μg/mL in PBS buffer. After washing and blocking, plasma samples were coincubated with mouse IgE standard dilutions (Pharmingen). Next steps were performed as for specific IgE. For non-specific IgE, plasma samples were added in peptide-coated plates and, after incubation overnight at 4 °C, they were transferred to anti-mouse IgE mAb coated plates, then continuing as for total IgE. The amount of total or non-specific IgE was determined with the standard curve of IgE, linear from 0.24 to 15.63 ng/mL.

2.6. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.) for each group. Normal distribution of samples was evaluated by Shapiro–Wilk trial, and differences among groups of animals at significance levels of 95% were calculated by the non-parametric Mann–Whitney *U*-test. Pairwise Sperman rank correlation was used to assess relation between variables. Statistical analysis was completed with the SPSS 11.0 program (SPSS®, Chicago, USA).

3. Results

3.1. Microspheres characterization

The 3D7 and FC27 MSP2 peptides were successfully entrapped into PLGA microparticles by the w/o/w emulsion solvent extraction process and their formulations were almost identical for parameters such as mean particle size, 1 μm, or zeta potential, around −16 mV (Table 1). Final total peptide loadings were 4.6% (3D7) and 2.9% (FC27), while the initial theoretical load was 5% for both antigens. The encapsulation efficiency (the percentage of initial peptide load entrapped into microparticles) was thus higher for 3D7 peptide than for FC27, 93 and 58%, respectively. Both formulations revealed a high percentage of microencapsulated peptide adsorbed to the particle surface, near 45%.

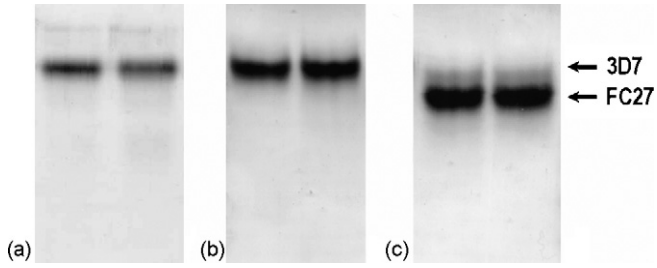

Fig. 2. SDS-PAGE of 3D7 and FC27 peptides. Left lanes: native peptides; right lanes: peptide extracted from PLGA MP (a), or sonicated (b and c).

3.2. Acrylamide gel electrophoresis

The performed acrylamide electrophoresis gels showed that the structure of both native peptides was not altered by the encapsulation procedure (Fig. 2). The electropherograms obtained for the peptides extracted from microparticles presented the same band pattern as native peptides, as well as for the peptides exposed to ultrasonication, which represents the most aggressive step in the microencapsulation procedure. Since no new bands were detected, degradation or aggregation of peptides was excluded.

3.3. Antibody response

The specific antibody response was followed up over a period of 30 weeks. The antibody production profiles for each group are shown in Fig. 3. Immunization with 3D7 peptide elicited a similar IgG production for both Montanide and MP formulations when the subcutaneous route was chosen (Fig. 3A). Significant differences were found between the 3D7MPsc and the 3D7MPid groups, with a higher anti-3D7 secretion for the i.d. group ($P < 0.05$). The 3D7MPid group also showed significantly higher IgG levels than the 3D7Mont group at some time points ($P < 0.01$). The Montanide immunized mice presented a wide variability in the antibody levels.

Regarding the immune responses to the FC27 peptide, the microparticles induced a greater IgG secretion than Montanide at all time points following i.d. ($P < 0.01$), or s.c. ($P < 0.05$) administration (Fig. 3B), except for the first weeks after boosting (22, 24 weeks). Similar to the 3D7 peptide, the response to the FC27 antigen was immunization route-dependent. The antibody response was greater in the FC27MPid group as compared to that obtained in the FC27MPsc group at most of the points analyzed ($P < 0.05$).

Table 1
Microparticle formulations characterization

Formulation	Encapsulated antigen	Size (μm)	Zeta potential (mV)	Encapsulation efficiency (E.E.%)	Surface adsorbed antigen (S.A.P.%)	Ag-loading (μg/mg MP)
BMP	–	1.01 (95% 0.67–1.34)	−16.9	–		–
3D7MP	3D7	1.08 (95% 0.67–1.49)	−17.5	92.5	43.2	46.3
FC27MP	FC27	1.02 (95% 0.65–1.41)	−14.9	57.8	55.0	28.9

Two batches were prepared for each peptide with similar results. Results shown are from the batches used *in vivo*.

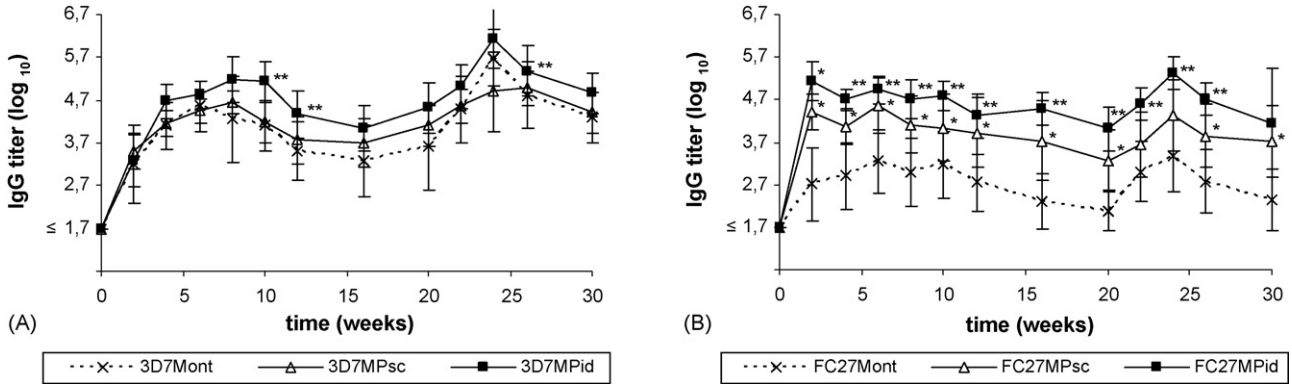

Fig. 3. Specific antibody response profiles from 2 up to 30 weeks, for mice immunized with 3D7 peptide (A) or FC27 (B). Data are presented as mean ± S.D. $^{*}P<0.05$, $^{**}P<0.01$ significant against M-ISA720 group.

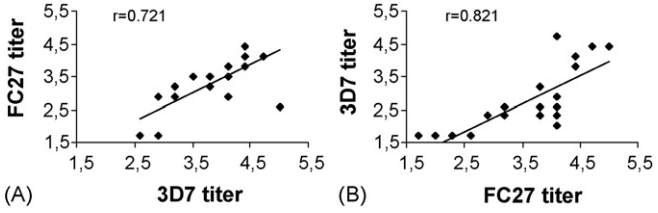

Fig. 4. Cross-reactivity between 3D7 and FC27 peptide. Correlation between 3D7 titer vs. FC27 titer and vice versa, at 12 weeks, in mice immunized with 3D7 (A) or FC27 (B). Correlation is expressed by *r* (rho, Spearman rank correlator).

An immunofluorescent assay (IFA) confirmed the presence of antibodies able to recognize the parasite in infected erythrocytes (data not shown). For both peptides, the immunization with MP formulations generated positive antibodies earlier than Montanide, which required a boosting dose to induce IFA responses. For the 3D7 MP immunized mice, an earlier recognition was evidenced in the 3D7MPid group when compared to the 3D7MPsc group (data not shown).

3.4. Cross-reactivity between antigens

Since the 3D7 and FC27 antigens share a common 40 amino acid constant domain (Fig. 1), potential cross-reaction was evaluated at 12 weeks by measuring the anti-FC27 IgG titer that 3D7 immunized mice elicited, and vice versa. The cross-reacting antibody levels were then compared to their respective specific antibody titer (Fig. 4). All mice that showed significant IgG levels against the vaccinating peptide also presented detectable antibody levels against the other one. Moreover, there was a good correlation of cross-reacting antibodies with the anti-peptide IgG titers. Statistical analysis showed a significant correlation between anti-3D7 and anti-FC27 IgG titers in 3D7 and FC27 vaccinated mice with an *r* coefficient of 0.721 and 0.821, respectively ($P<0.01$).

3.5. IgG subclass response

The IgG subclasses secretion was measured at 8 and 24 weeks in order to indirectly evaluate the type of T helper cells (Th1 versus Th2) elicited. As can be seen in Fig. 5, although a mixed IgG1/IgG2a response was elicited, IgG1 subtype predominated after the first dose while IgG2a levels were low. In the case of the 3D7 peptide, all groups incremented significantly both IgG subtypes after the booster dose ($P<0.01$). There were few differences among groups at the same time point for either IgG1 or IgG2a subclasses (Fig. 5a).

As displayed in Fig. 5b for the FC27 antigen, MP formulations evoked superior IgG1 titers than Montanide at

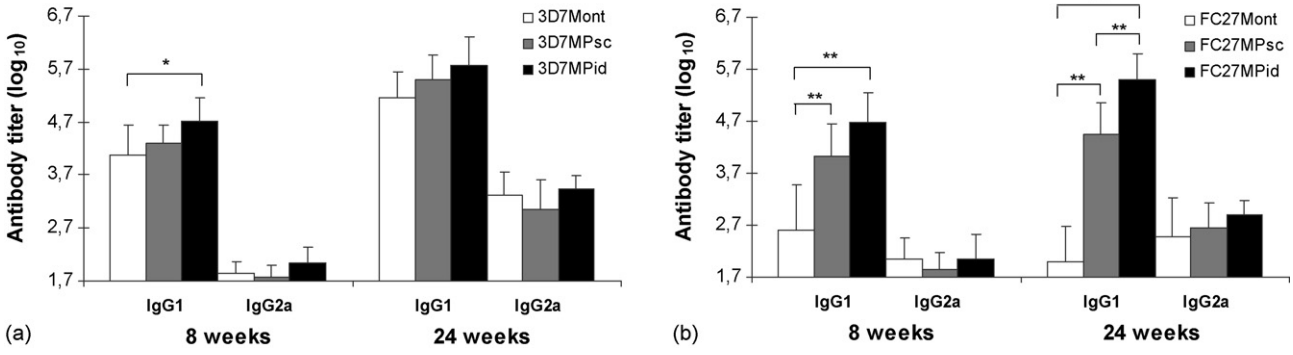

Fig. 5. Specific IgG1 and IgG2a antibody subclasses after initial dose (8 weeks) and postboosting (24 weeks), for 3D7 (a) or FC27 (b) immunized groups. Values are mean ± S.D.; $^{*}P<0.05$, $^{**}P<0.01$.

all time points ($P < 0.01$). The antibody levels were similar between FC27MPsc and FC27MPid groups at 8 weeks, but not at 24 weeks, when i.d. route resulted in an improved IgG1 response ($P < 0.01$) (Fig. 5b). Surprisingly, the IgG1 titers for the FC27Mont group decreased after the boosting. Although the IgG2a secretion was similar for the three FC27 peptide immunized groups, microparticles incremented it significantly after boosting ($P < 0.01$), independently of the administration route.

Regarding the IgG1/IgG2a ratio, the booster dose was able to reduce it notably in all 3D7 immunized groups ($P < 0.01$) and in the FC27Mont and FC27MPsc groups ($P < 0.05$). On the contrary, the decrease in the IgG1/IgG2a ratio of the FC27MPid group was not significant. With regard to the influence of the administrated formulation (MP or Montanide) on the IgG subclass ratio, no difference was found between all 3D7 vaccinated groups. However, Montanide elicited a lower IgG1/IgG2a ratio than MP formulations (s.c. or i.d.) for the FC27 peptide ($P < 0.01$).

3.6. IgE response

IgE antibody production was studied at 8 and 24 weeks, as a component of the immune response associated with a Th2 subset activation. It was evaluated not only the specific IgE secretion against 3D7 and FC27 antigens but also the total and non-specific production of this antibody. Fig. 6 shows that all groups secreted detectable levels of specific IgE. Whereas the specific anti-3D7 IgE titer was similar for both Montanide emulsified and microencapsulated antigen, the anti-FC27 IgE

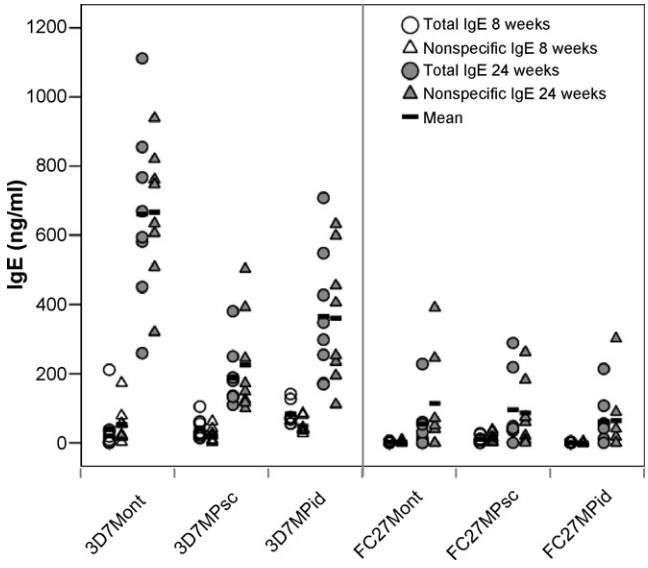

Fig. 7. Total and non-specific IgE levels at 8 and 24 weeks. Individual values and means are represented. The total IgE levels from preimmunized mice were 1.3–7.2 ng/mL (3D7) and 0.4–1.4 ng/mL (FC27).

was higher for the FC27MPid group as compared to the FC27MPsc and the FC27Mont groups ($P < 0.05$). The correlation between the specific IgE and IgG1 secretions was also studied. All groups showed a good correlation, with similar IgE/IgG1 ratios (around 0.7), except for the FC27Mont group that yet at 8 weeks presented a higher ratio than MP formulations and at 24 weeks was greatly superior (1.5; $P < 0.001$). Total and non-specific IgE were similar in all cases, with a

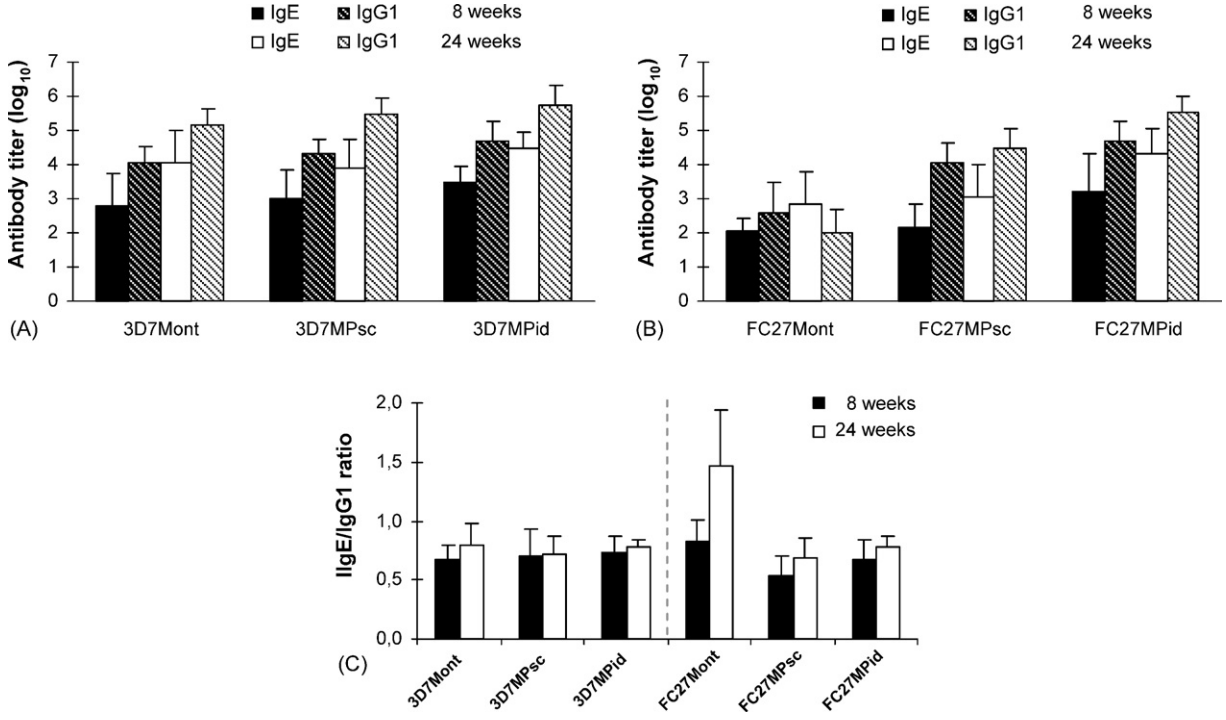

Fig. 6. Specific IgE antibody in comparison to specific IgG1 secretion for 3D7 (A) or FC27 (B) immunized groups, at 8 weeks and 24 weeks. The ratio IgE/IgG1 (C) is expressed as the mean ± S.D. of the individual ratios in each group.

notable increase from 8 to 24 weeks for the 3D7 immunized mice ($P < 0.01$; Fig. 7). The total IgE amounts were low for all groups at 8 weeks post-immunization. The values at 24 weeks were in the range of 55–100 ng/mL for the FC27 immunized groups and 200–350 ng/mL for the 3D7MPsc and 3D7MPid groups. Remarkably, the 3D7Mont group showed an important IgE secretion up to 660 ng/mL ($P < 0.02$ and $P < 0.01$ against 3D7MPid and 3D7MPsc, respectively; Fig. 7).

4. Discussion

Immunoepidemiological studies have demonstrated a correlation between anti-MSP2 antibodies and protection against malaria [31,32]. From these observations it was inferred that the MSP2 antigen was a candidate malaria vaccine and subsequent clinical trials were carried out combining 3D7 MSP2 recombinant protein with other blood-stage *Plasmodium* antigens [22].

In the present work, a synthetic strategy was chosen to produce the candidate vaccines MR141 and MR144 corresponding to 3D7 and FC27 MSP2 constant C-terminal alleles, respectively. Protein or peptide based vaccines need to be formulated in appropriate adjuvants and administered through an adequate route to elicit the desired type of immune response. The main goal of our work was thus to study the adjuvant activity of both a polymer (PLGA MP) and an oily emulsion (Montanide) on the selected 3D7 and FC27 MSP2 long synthetic peptides. As a second objective, we tested the hypothesis that a targeted administration to the dermal tissue would enhance or modulate the antigen specific immune response. In the case of the *Plasmodium* parasite, both antibody and cellular responses seem to be required for a protective immunity. Specifically, a response against blood stage antigens as MSP2 is thought to require potent and subclass-specific antibodies, in order to eliminate the infected red blood cells by the antibody-dependent cellular inhibition (ADCI) [22] or by direct parasite growth inhibition.

The microencapsulation w/o/w solvent extraction technique used to entrap the 3D7 and FC27 peptides resulted in satisfactory and appropriate products for our requirements. A medium molecular weight PLGA 50:50 polymer was chosen because of its adequate degradation rate (6–12 weeks) and encapsulation efficiency for our long-term vaccination studies. The lower encapsulation efficiency obtained for the FC27 peptide as compared to the 3D7 peptide could be a result of its lower molecular weight favouring the release to the outer water phase during the encapsulation [33]. As previous works showed for other synthetic peptides, the chemical structure of the MSP2 antigens was conserved during the encapsulation process [27], which requires sonication and organic solvents.

The *in vivo* studies show that potent and long-lasting IgG antibody responses were obtained for both antigens when they were entrapped in PLGA MP and administrated by a subcutaneous route. Interestingly, Montanide adjuvant worked in a similar fashion as PLGA MP for one of the peptides (3D7), but was unable to induce the desired IgG response when combined with the FC27 peptide, even after a boosting dose. The reason for this is not known and may be related to the strain of mice used since CB6F1 mice are good responders (manuscript in preparation). Although a previous work showed a greater adjuvant activity of Montanide as compared to PLGA MP using HIV short synthetic peptides, this difference could be a consequence of the low antigen load into the performed MP (around 0.05%), insufficient to trigger immune responses after being uptaken by the APCs [34]. The MSP2 peptide loads into the MP we used in the present study, 4.6% for 3D7 and 2.9% for FC27, would overcome the required threshold for the immune activation of the APC. This threshold appeared to be lower when the intradermal route was tested, since the mice vaccinated with MP into the dermal tissue presented a higher IgG antibody titer as compared to the subcutaneous groups. Unfortunately, the viscosity and surface tension physico-chemical properties of the Montanide adjuvant did not allow the i.d. administration of the emulsified peptides in C57B mice.

The highly immunogenic effect of the i.d. route as compared to the s.c. one was in concordance with previous works in our laboratory, in which malaria antigen loaded PLGA MP given i.d. elicited comparable antibody responses to those obtained via s.c. route with a 10 times higher dose [35]. The skin-associated lymphoid tissue contains specialized cells that enhance immune responses [36], such as dendritic cells (Langerhans cells) at the epidermis layer that cover the 20% of surface in horizontal disposition and are able to uptake antigens, migrate to regional lymph nodes and spleen and present antigens to T cells [37]. The PLGA MP uptake by dendritic cells and their posterior migration from skin to draining lymph nodes after i.d. route has been recently reported [38].

The ability of the adjuvanted peptides to elicit parasite-specific antibodies was confirmed by IFA. All the formulations tested produced positive IFA results after-boosting doses, but only the MP immunized groups were positive after the priming dose. The fast and long lasting immunostimulation induced by the PLGA MP could be explained by an efficient targeting of the APC via phagocytosis. Recent studies have established that the culture of effector immune cells with microparticulate antigen-charged APCs has led to prolonged presentation of peptides (up to 9 days), as compared to the culture with soluble peptide-loaded APCs [39]. Montanide adjuvant emulsions carry the antigen in micrometer-sized droplets and they could be considered particulate adjuvants, as polymer MP [40]. However, it is more conceivable that their main mechanism of action is related to the immunostimulation produced by their components (oils and surfactants), recognized as foreign material by the immune cells [41]. As shown in our study, this stimulation appeared to be more effective after the boosting doses.

The finding of cross-reactivity between both peptides confirmed the production of specific antibodies against the shared amino acid sequences in the conserved MSP2 protein region. The combination of both antigens in one vaccine

formulation could cover both 3D7 and FC27 *Plasmodium* variants in the field [42–44]. This approach has been substantiated by the results obtained in a phase IIb clinical trial in which the 3D7 MSP2 recombinant protein was tested in combination with two other blood stage antigens. It was observed that the infection by the 3D7 strain was diminished as compared to the FC27 strain infection [22,44].

There were noticeable changes in the IgG subclass profile of our study groups after the reimmunization with microparticles or Montanide. IgG2a isotype raised substantially in all groups and IgG1/IgG2a ratios decreased. This could be expected because it is known that repeated exposures to an antigen can elicit class switchings. The change in the antibody secretion towards a cytophilic isotype (IgG2a but not IgG1 in mice; IgG1 or IgG3 but not IgG2 in humans) could be of clinical relevance for protection, since the cytophilic antibodies have been associated with antibody-mediated blockade of the *Plasmodium in vitro* [45] and aquired protective immunity to malaria [31,46].

As shown in Fig. 6, we observed peptide-specific IgE secretion in all groups. Among the PLGA immunized mice, the highest specific IgE levels corresponded to an i.d. administrated group (FC27MPid). This finding is in accordance with previously reported data that indicated a predominant Th2 subset and high IgE levels during repeated epicutaneous exposure to antigenic proteins [47]. In our experiment, this specific IgE raise was well correlated with higher specific IgG1 levels, which are thought to act as blocking antibodies against a hypothetical IgE-mediated allergic reaction [48]. In the case of the Montanide immunized groups, two findings were specially remarkable. First, in the FC27Mont group we detected a high specific IgE/IgG1 ratio after the priming dose and this ratio increased after the boosting (Fig. 6C). Secondly, the 3D7Mont group presented a higher amount of total and non-specific IgE secretion as compared to the 3D7 MP immunized groups (Fig. 7). Although there is scant information regarding IgE responses following Montanide adjuvanted vaccinations, a recent study evidenced high specific IgE levels in parasite-challenged mice previously vaccinated with a Montanide adjuvanted parasite antigen [49]. The antigen nature by itself may also influence the serum IgE response [50], thus in our study the disparity between both Montanide formulated peptides in inducing IgE could be due to slight chemical differences among them [50]. Our findings on IgE secretion could be ascribed to a Th2-biased reaction to the M-ISA720 adjuvant. In fact, a Th2-like predominant pattern was revealed in a previous study in mice immunized with the Montanide-adjuvanted recombinant *P. vivax* region II (PvRII). M-ISA720 evoked a Th2 activation even higher than the alum adjuvant, which is known to promote Th2 immune responses [51,52].

Based on the above data, our study demonstrates the potent adjuvant effect of the PLGA MP when applied to synthetic peptides encoding MSP2 epitopes of the 3D7 and FC27 *Plasmodium* strains. PLGA MP overcame the inability of the Montanide adjuvant to induce responses against one of the studied peptides and the intradermal administration enhanced the immune response as compared to the subcutaneous one. Most interestingly, the IgG2a cytophilic antibody secretion was elicited by our vaccination schedule. The ability of the peptide entrapment into PLGA MP versus the emulsification in Montanide to reduce the IgE response was also demonstrated. Taken together, our findings are encouraging demonstrations of the efficacy of combining synthetic peptides and polymer microparticles that could provide successful and cost effective malaria vaccines in the future.

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