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Protection against malaria is conferred by passive transferring rabbit F(ab)$_2'$ antibody fragments, induced by *Plasmodium falciparum* MSP-1 site-directed designed pseudopeptide-BSA conjugates assessed in a rodent model

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ABSTRACT

F(ab)$_2'$-immunoglobulin (Ig) fragments induced by site-directed designed immunogens are emerging as novel tools of potential utility in the treatment of clinical episodes of transmissible diseases such as malaria. Immunogens based on reduced amide pseudopeptides based on site-directed molecular modifications represent structural probes that could be considered as novel vaccine candidates, as we have previously demonstrated.

We have obtained F(ab)$_2'$-Ig rabbit antibodies induced against the *N*-terminal sequence of the native Merozoite Surface Protein-1 (MSP-1) of *Plasmodium falciparum* and a set of five MSP-1-derived reduced amide pseudopeptides. Pseudopeptides were designed for inducing functional neutralizing monospecific polyclonal antibodies with potential applications in the control of malaria. Following a classical enzyme immunoglobulin fractionation, F(ab)$_2'$-Ig fragments were tested for their ability to suppress blood-stage parasitemia by passive immunization in malaria-infected mice. Some of these fragments proved totally effective in suppressing a lethal blood-stage challenge infection and others reduced malarial parasitemia.

These data suggest that protection against *Plasmodium yoelii* malaria following passive transfer of structurally well-defined β-strand F(ab)$_2'$-Ig fragments can be associated with specific immunoglobulins induced by site-directed designed MSP-1 reduced amide pseudopeptides.

1. Introduction

A peptide derived from the *N*-terminal portion of the *Plasmodium falciparum* Merozoite Surface Protein-1 (MSP-1) designated as 1513 (^{42}GYSLFQKEKMVLNEGTSGTA^{61}) has been extensively consider as a candidate component to be included in a multicomponent subunit-based chemically synthesized antimalarial vaccine, in spite of being poorly immunogenic and non-protection inducer against malaria. As has been demonstrated in the research performed in our Institute, critical binding residues of high activity binding peptides (HABPs), has to be identified and strategically substituted. Once the critical binding residues of peptide 1513 were identified by specific receptor-ligand experiments, a rational design allowed us to strategically include site-directed peptide bond isosters, in order to produce a novel class of structural probes (Lozano et al., 1998).

Thus, each peptide bond associated to the ^{48}K-KMV^{52} Red Blood Cell (RBC) binding motif was systematically replaced by a methylene amine ψ-[CH_2NH] isoster bond to generate a family of malarial pseudopeptides.

Pseudopeptide identity and purity were analyzed by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry by the

Abbreviations: F(ab)$'_2$, antibody fragment for antigen binding; HABP, high activity binding peptide; ψ-[CH_2NH], reduced amide isoster bond; MHC, major histocompatibility complex; TCR, T-cell receptor; TCC, T-cell clone; APC, antigen presenting cell; CDR, complimentarily determinant region; HLA, human leukocyte antigens; IL-4, interleulin-4; INF-γ, gamma interferon; mAb, monoclonal antibody; MSA-1 or MSP-1, Merozoite Surface Antigen-1; MSA-2 or MSP-2, Merozoite Surface Antigen-2; RBCs, red blood cells; iRBC, infected red blood cell; CSP, circumsporozoite surface protein; IgM, immunoglobulin-M isotype; IgG, immunoglobulin-G isotype; F(ab)$_2'$, immunoglobulins antigen binding fragment-2; $NaBH(OAc)_3$, triacetoxyborohydride; DMF, *N*,*N*′-dimethylformamide; DCE, dichloroethane; THF, tetrahydrofuran; $NaCNBH_3$, sodium cyanoborohydride; Pd/C, palladium over charcoal; 4-MBHA, 4-methylbenzhydrilamine; *t*-Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; m-*R*,*S*-TMD, 2,2,5-trimethyl-1,3-dioxane-4,6-dione; RP-HPLC, reverse-phase high-performance liquid chromatography; [D6]DMSO, deuterated-dimethylsulfoxide; ^{1}H-NMR, proton-nuclear magnetic resonance; CD, Circular Dichroism; NOE, Nuclear Overhauser effect; SPf-66, synthetic *Plasmodium falciparum*-66 vaccine.

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Time Of Flight technique (MALDI-TOF) and analytical Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC), respectively. Previously reported proton-Nuclear Magnetic Resonance (^{1}H NMR) data demonstrated how the secondary structure of this protein fragment was specifically modulated by each isoster bond (Lozano et al., 1998). The immunological properties of each component of this structural family were determined by inducing monoclonal antibodies, which were shown to possess neutralizing properties against *in vitro* malarial infection. In a recent study, we reported that the passive transfer of immunoglobulins directed against reduced amide pseudopeptides derived from a highly conserved sequence of MSP-2, another relevant target for an antimalarial vaccine component. Such experiment demonstrated that passively transferred immunoglobulins protected and delayed onset of malarial infection in BALB/c mice after being experimentally challenged with the *Plasmodium berghei* ANKA strain.

In the present work, we have examined the potential of passively transferring F(ab)$_2'$ antibody fragments induced by MSP1-pseudopeptides. F(ab)$_2'$ fragments were obtained by papain treatment of Ig from rabbit polyclonal mono-specific antibodies induced by pseudopeptide-bovine serum albumin (BSA) conjugates.

The results of the present study demonstrate that F(ab)$_2'$ fragments possess well defined secondary structure patterns, as indicated by Circular Dichroism (CD) experiments, and that such patterns could be associated with a protective effect against malarial infection, as assessed in a rodent animal model challenged with a lethal dose of a *Plasmodium yoelii* 17XL strain.

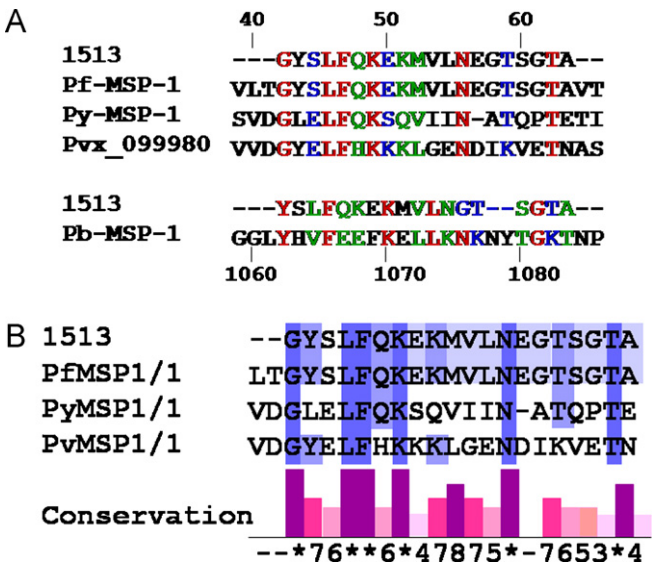

Fig. 1. Structural characteristics of the MSP-1-derived peptide 1513. (A) Alignment of the 1513 peptide sequence (MSP-1$^{42-61}$) and the MSP-1 homolog sequences in *Pf* (*Plasmodium falciparum*), *Py* (*Plasmodium yoelli*) and *Pv* (*Plasmodium vivax*). (B) Degree of sequence identify is represented according to the following color code: 80%, mild blue; higher than 60%, light blue; higher than 40%, light gray and lower than 40%, white. The degree of sequence conservation was analyzed using Jalview-EBI, where the 11* score represents identical amino acids, 8 and 7 conserved amino acids, and 6 and 5 semi-conserved amino acids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2. Material and methods

2.1. Bioinformatics analysis

The sequence of *P. falciparum* MSP-1 (accession number Pfi1475w) previously reported by Gilson et al. was used as bait for obtaining information about orthologous sequences in other *Plasmodium* species from the *Plasmodb* database (http://plasmodb.org/plasmo/), which were confirmed in the NCBI database (http://www.ncbi.nlm.nih.gov). Such screening allowed us to obtain the codes of the followings orthologous sequences: *Plasmodium vivax* MSP-1 Pvx_099980, *Plasmodium berghei* MSP-1 Pb000172.01.0, *Plasmodium yoelii* MSP-1 Py05748, *Plasmodium chabaudi* MSP-1 Pc000255.03.0 and *Plasmodium knowlesi* pkh_072850.

The MSP-1$^{42-61}$ sequence was aligned using ClustalW available at http://personal.rhul.ac.uk/ujba/110/bioinfo/clustalE.html, to obtain the degree of homology between these sequences. The degree identity and conservation of each residue was then determined using the Jalview Java Alignment Editor (Clamp et al., 2004).

2.2. Synthesis of peptide bond isosters and conjugation to BSA

Based on the primary structure of MSP-1$^{42-61}$ (peptide 1513) shown in Fig. 1A, a set of five reduced amide oligomer pseudopeptides was designed and synthesized. In brief, all molecules were manually synthesized through *tert*-butyloxycarbonyl (*t*-Boc)-based solid-phase peptide synthesis (SPPS), following a protocol first reported by Merrifield (1963) and later modified for multiple peptide synthesis (Houghten, 1985). Each amino acid residue was placed on the pseudopeptide backbone as described elsewhere (Cushman and Oh, 1991; Ho et al., 1993; Sasaki et al., 1987). The ψ-[CH_2NH] surrogate was introduced by deprotected N^{α}-amino group's resin-bound reductive alkylation with the *t*-Boc-protected amino acid aldehyde (0.576 mmol) in DMF containing 0.5% acetic acid (HOAc), followed by portion-wise addition of $NaBH_3CN$ (0.67 mmol) for 40–60 min. Total coupling was checked by the Ninhydrin test and, repeating the coupling reaction when necessary. Coupling was allowed to proceed for 5 h under constant shaking, followed by washes with *N*,*N*′-dimethylformamide (DMF), isopropanol and dichloromethane. Standard solid phase peptide synthesis was carried out to introduce the remaining *t*-Boc amino acids to the last *N*-terminal residue. Protected pseudopeptide-resin batches were treated with trifluoroacetic acid (TFA) and cleaved from the resin by treatment with low concentrations of anhydrous hydrogen fluoride (HF) containing 10% anisole at 0 °C for 60 min. After HF evaporation in an N_2 stream, each pseudopeptide-resin product was washed with cold diethyl ether, then extracted with 5% HOAc and lyophilized. The crude products obtained for each ψ-[CH_2NH] surrogate were further analyzed by analytical RP-HPLC, purified by preparative RP-HPLC and identified by MALDI-TOF mass spectrometry. A full procedure for peptide-based polymer production is described elsewhere (Lozano et al., 1998; Lioy et al., 2001).

Coupling of pseudopeptides to a bovine serum albumin (BSA) was performed by a classical glutaraldehyde treatment. Briefly, equal amounts of each pseudopeptide and BSA were mixed to obtain a 2 mg/mL preparation. Subsequently, small amounts of 0.38% glutaraldehyde dissolved in phosphate buffered saline (PBS) were added to the protein preparation. Protein-peptides were cross-linked to BSA through bonds established between primary amine groups with the aldehyde groups of glutaraldehyde, letting the reaction to elapsed overnight at room temperature under constant shaking to obtain all possible pseudopeptide-BSA conjugates. Then, all conjugates were dialyzed against saline solution to eliminate non-desirable small size complexes, such as pseudopeptide-glutaraldehyde-pseudopeptide.

2.3. Rabbit immunization to obtain polyclonal antibodies

Polyclonal antibodies against each 1513-derived pseudopeptide conjugated to BSA, were employed for by immunizing New Zealand