Protection against experimental malaria associated with AMA-1 peptide analogue structures

Luz M. Salazar, Martha P. Alba, Maria H. Torres, Martha Pinto, Ximena Cortes, Libardo Torres, Manuel E. Patarroyo*

Fundación Instituto de Inmunología de Colombia (FIDIC), Universidad Nacional de Colombia, Carrera 50 No. 26-00 Bogotá, Colombia

Received 16 May 2002; revised 20 June 2002; accepted 31 July 2002

First published online 8 August 2002

Edited by Thomas L. James

Abstract One *Plasmodium falciparum* malaria antigen is an integral membrane protein called apical membrane antigen-1. High activity binding peptides to human red blood cells have been identified in this protein. 4337 is a conserved, non-immunogenic peptide with high activity red blood cell binding and its critical residues have already been identified. Peptide analogues (with amino acids having the same mass but different charge) were generated to change their immunogenic and protective characteristics. Three analogues having positive or negative immunological results were studied by nuclear magnetic resonance. The studied peptides all had an α-helix fragment, but in different peptide regions and extensions, except for randomly structured 4337. We show that altering a few amino acids induced immunogenicity and protectivity against experimental malaria and changed their three-dimensional structure, suggesting a better fit with immune system molecules and that modified peptides having better immunological properties can be included in the design of new malaria multi-component subunit-based vaccine.

Key words: Apical membrane antigen-1 protein; Peptide analogues; Vaccine candidate; Nuclear magnetic resonance; *Plasmodium falciparum*

1. Introduction

Malaria continues to be one of the great public health problems throughout the world, causing 300 million cases of infection and more than 2.5 million deaths annually, mainly children less than 5 years old in Africa. The search for cheap, reliable control methods for this disease (such as anti-malarial insecticides and vaccines) has thus become a target for the main health providing entities throughout the world [1,2].

We thus attempted to block the *Plasmodium falciparum* merozoite red blood cell (RBC) recepter–ligand interaction when developing such immune–prophylactic methods [3,4]. Various proteins are excellent targets since they seem to be involved in this interaction [5], apical membrane antigen-1 (AMA)-1 (also named Pf83/AMA-1) being one of them. This molecule has been associated with the invasion interface and may be involved in the merozoite's reorientation via a mechanism which is inverse to that of the membrane transport [6,7].

The *P. falciparum* Pf83/AMA-1 protein has 622 amino acids with a 71 929 Da mass [8]. It shows integral membrane protein characteristics, being located in the neck of the rhoptria and is transported to the merozoite membrane during maturation. It has high homology among the different *P. falciparum* strains [9,10].

20-mer, radio-labeled, non-overlapping, synthetic peptide binding assays (covering the protein's entire length) showed that the conserved peptide coded 4337 (**WGEEK**-RAS**H**TTPVLM**E**K**P**YY), located in the protein's C-terminal region, bound to RBC with a 120 ± 12 nm affinity constant (peptide numbers given here correspond to those serial numbers used in our institute) [11]. The critical residues for binding to RBCs were determined by glycine substitution analogues and were identified as W1, E4, H9, M15 and K17, underlined above [11].

Since conserved sequences are poorly antigenic [12] and poorly or non-immunogenic [13], peptides were synthesized, critical amino acids for binding to RBCs being replaced by amino acids having the same mass but different charge to improve these molecules' biological activity.

Here we present the three-dimensional structure for peptides 14044, 14048 and 13814 (4337 peptide analogues) determined by ^{1}H nuclear magnetic resonance (NMR), with positive and negative results in immunological assays. Our purpose was to try to find correlation between these peptides' structure and immunological function to adopt a rational approach towards synthetic, multi-component subunit-based, malaria vaccine development.

2. Materials and methods

2.1. Animals and immunization

Groups of five to eight *P. falciparum* indirect immunofluorescence assay (IFA) negative *Aotus* monkeys, [14] kept in Leticia (the Amazon Department) according to National Institute of Health guidelines for animal handling, were subcutaneously immunized with 125 μg polymerized peptide (on days 1, 20 and 40) which had been previously homogenized with complete Freund's Adjuvant for the first dose and incomplete Freund's Adjuvant for the second and third doses. Controls received only Freund's Adjuvant and saline solution on the same days. Blood samples were drawn pre-immunization and 20 days after the second and third doses.

*Corresponding author. Fax: (57)-1-4815269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

Abbreviations: AMA-1, apical membrane antigen-1; NMR, nuclear magnetic resonance; RBC, red blood cell; IFA, indirect immunofluorescence assay; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, NOE, nuclear Overhauser effect spectroscopy or signal

0014-5793/02/$22.00
PII: S0014-5793(02)03174-5

2.2. Challenge and infection assessment

Immunized and control monkeys received an intravenous injection of 200 000 fresh *P. falciparum* FVO strain infected RBC 20 days after the third dose (this being an infective dose for 100% of *Aotus* monkeys [14]). Infection was assessed after day 5 by immunofluorescence with Acridine Orange staining. The percentage of infected RBC was read [14]. Protection was defined as being the complete absence of parasites in the blood during the 15 days of the trial. Control and non-protected monkeys developed patent parasitemia by day 5, reaching $\geq 6\%$ by days 8–10; they were then treated with pediatric doses of antimalarial drugs so as to be returned to the jungle 4 weeks later.

2.3. IFA and Western blot

The *P. falciparum* FCB-2 strain, kept in culture (O^+ RBC) according to Lambros and Vanderberg's method, was used [15]. 20% late parasitemia cultures were air dried, fixed on slides, blocked with 1% non-fat milk, incubated with two-fold sera dilutions (starting at 1:40), washed and stained with anti-*Aotus* IgG/FITC conjugate at 1:100 dilution and then read by fluorescence microscope.

The 20% late parasitemia saponine pellet, dissolved 1:5 in Laemmli's buffer, was electrophoretically separated, transferred to nitrocellulose paper and incubated with 1:100 diluted pre-immune or immune sera for Western blot analysis. The reaction was revealed with affinity purified goat anti-*Aotus* IgG-alkaline phosphatase conjugate.

2.4. Peptide synthesis

20 amino acid long peptides were synthesized by standard Solid Phase Peptide Synthesis technique [16], purified by HPLC and characterized by RP-HPLC and mass spectrometry, the expected theoretical mass being found. CG was added at the peptides' N- and C-termini to allow polymerization by oxidation for use in immunization studies. This procedure was carefully standardized to guarantee the inclusion of high molecular weight polymers; some peptides were also synthesized as dendrimers [17].

2.5. NMR

All NMR experiment samples were prepared by dissolving 10 mg peptide in 500 μl 2,2,2,-trifluoroethanol–d_3/H_2O mixture (30/70 v/v) [18]. ^1H spectra were acquired on a BRUKER DRX-500 spectrometer at 295 K. Proton spectra were assigned by double quantum filtered correlation spectroscopy (DQF-COSY) [19] and total correlation spectroscopy (TOCSY) [20]. Inter-proton nuclear Overhauser effects (NOEs) were determined by phase sensitive NOESY (NOE spectroscopy) [21]. Assignments were done according to Wüthrich's standard procedure [22]. TOCSY spectra recorded at different temperatures (285–315 K) were used to obtain amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T < 4$). DQF-COSY spectra spin coupling constants ($^3J_{HNH\alpha}$) were measured.

2.6. Structure calculations

Structure was determined by using a combination of distance geometry and simulated annealing (Molecular Simulations Inc., DGII program and Discover programs, respectively). NOE peaks, selected from 400 ms NOESY data sets, were integrated and converted into distance restraints. These restraints were grouped as strong, medium and weak corresponding to 1.8–2.5 Å; 2.5–3.5 Å; and 3.5–5.0 Å distance restraints respectively. Strong NOE intensities between the two Pro18 H^δ protons (1.79 Å) set the standard for relative intensity calibration. Hydrogen bond constraints were introduced for low amide temperature coefficients; only $< 4 - \Delta\delta H^N/\Delta T$ ppb/K were used in structure calculations.

Distance ranges involving these likely NH...O hydrogen bonds were set at 1.8–2.5 Å between the residue acceptor oxygen (i-4) and the residue donor amide hydrogen (i). The ϕ angle constraints derived from $^3J_{HNH\alpha}$ were restricted to $-70 \pm 30°$ if $^3J_{HNH\alpha} < 6$ Hz.

All peptide bonds were forced to trans and Cα chirality to L during calculations. A non-bonded 8 Å cut-off distance was used; no charges nor cross terms were included. Only those structures having reasonable geometry and low constraint violation were retained. The restrained simulated annealing protocol (Discover 2.98) consisted of eight phases. These included raising the initial temperature to 1000 K for 12 ps in three phases. The system was then cooled to 300 K. Four energy minimization phases then followed: 100 steepest descent cycles, 1000 conjugate gradient cycles (until maximum derivative was less than 0.1 for both of them), 100 steepest descent cycles (until maximum derivative was less than 0.01) and 2000 conjugate gradient cycles (until maximum derivative was less than 0.0001).

3. Results and discussion

3.1. Immunological and protectivity studies

Critical residues from conserved, high-activity, binding peptides were replaced by amino acids with similar mass but different charge to render these peptides immunogenic and protective since conserved sequences are not antigenic, nor immunogenic. This paper tries to correlate these immunological results with their three-dimensional structure.

Amino acid changes made to 4337, their capacity to induce

Table 1
Humoral immune response and protective efficacy induced by 4337 and derived peptides in *Aotus* monkeys

Polimerized Peptide No.	Peptide Sequence	IFA ≥1:160 Post 2nd	IFA ≥1:160 Post 3rd	No. of protected monkeys	
4337	**W** G E **E** K R A S **H** T T P V L **M** E **K** P **Y** Y	0/5	0/5	0/5	
(P)14044	Y S — M — — — — L — — — — — K — — — — —	1/5(1:160)	1/5(1:320)	1/5	
(D)22158	Y S — M — — — — L — — — — — K — — — — —	4/10(1:160)	-	5/10	GROUP A
22822 H T T Y S — M — — — — L — — — — — K — — — — —		1/8(1:1280)	-	I/7	
14048	Y S — M — — — — L — — — — — K — M — W —	0/6	2/6(1:320)	0/5	GROUP B
23776	Y S — M — — — — L — — — — — M M — — —	0/6	1/6(1:320)	0/6	
9200	G — — — — — — — — — — — — — — — — —	0/6	0/6	0/5	
9202	W — — G — — — — — — — — — — — — — —	0/5	0/5	0/5	
9204	W — — — — — — G — — — — — — — — — —	0/5	0/5	0/5	
20472	W — — M — — — — — — — — — — — — — —	0/6	0/6	0/5	
13814	Y S — M — — — — M — — — — — — — — — —	0/6	0/6	0/5	
13816	Y S — M — — — — L — — — — — — — — — —	0/6	0/4	0/4	
14050	Y S — M — — — — L — — — — — — — — W Y	0/4	0/3	0/3	GROUP C
24416	Y S — M — — — — L A — — — — M M — — —	0/7	0/7	0/7	
23408	Y S — E M — — — H — — — — — K — M — —	0/9	0/9	0/9	
23182	Y S — M — F — A H — — — — — K — M P	0/8	0/8	0/8	
22820 H T T Y S — E — — — — H — — — — — — — K		0/8	0/8	0/8	
22436 H T T W G — E — — — — L — — — — — K — M		0/8	0/8	0/8	
CONTROLS		0/50	0/50	0/50	

14044 and 22158 have the same sequence and similar activity; this is due to 14044 being synthesized as a polymer (P) and 22158 synthesized as a dendrimer (D). Peptide numbers given here correspond to those serial numbers used in our institute.

antibodies as determined by IFA and their ability/inability to induce protection are shown in Table 1.

Three peptides having a common amino acid sequence and different forms of presentation were able to provide protection: 14044 (for which the three-dimensional structure was determined) protected one out of five monkeys, with a 1:320 IFA titer after three doses. The same sequence in peptide 22158 synthesized as dendrimer fully protected five out of 10 monkeys with 1:160 antibody titer and with only two doses. The same amino acid sequence, to which HTT was added to the N-terminal sequence, in peptide 22822 protected one monkey out of seven with a 1:1280 IFA titer with only two immunizations. The specific W1Y, G2S, E4M, H9L and M15K modifications (with or without extensions in the N-termini, polymerized with cysteine or synthesized as dendrimer) were thus able to protect seven out of 22 monkeys in different assays. Assays included two peptides having different formulations, but the same amino acid sequence. A third peptide had the same amino sequence, elongated at the N-terminal. 50 *Aotus* used as controls in the different experiments were never protected.

Group B peptides (including peptide 14048 for which the three-dimensional structure was determined) induced antibodies with three doses, in 1:320 titers but did not protect monkeys against experimental challenge, suggesting differences in their IgG subclass or antibody affinity. Changes made to 4337 peptide analogues from group C were not immunogenic, nor protective.

The antibodies induced by the 14044, 22158 and 22822 peptides in the protected monkeys recognized either the whole 116 kDa precursor, the 83/90 kDa doublet and its 36 and 22 kDa cleavage product range, or just some of them by Western blot analysis (Fig. 1).

It can be concluded from these experiments that antibodies elicited by immunization with 14044, 22158 and 22822 peptides recognized functional, native and secondary structures in the AMA-1 protein, as assessed by protection, IFA and Western blot analysis respectively.

3.2. NMR

The presence of typical α-helix fragments in peptides 14044, 14048 and 13814 is supported by $d_{NN}(i,i+1)$, $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha N}(i,i+4)$ NOE connectivity and low amide proton temperature coefficients, as indicated by ▲ in Fig. 2. The latter data was used to determine hydrogen bond presence: for peptide 14044 between K5 and L9 and A6 and T10; for

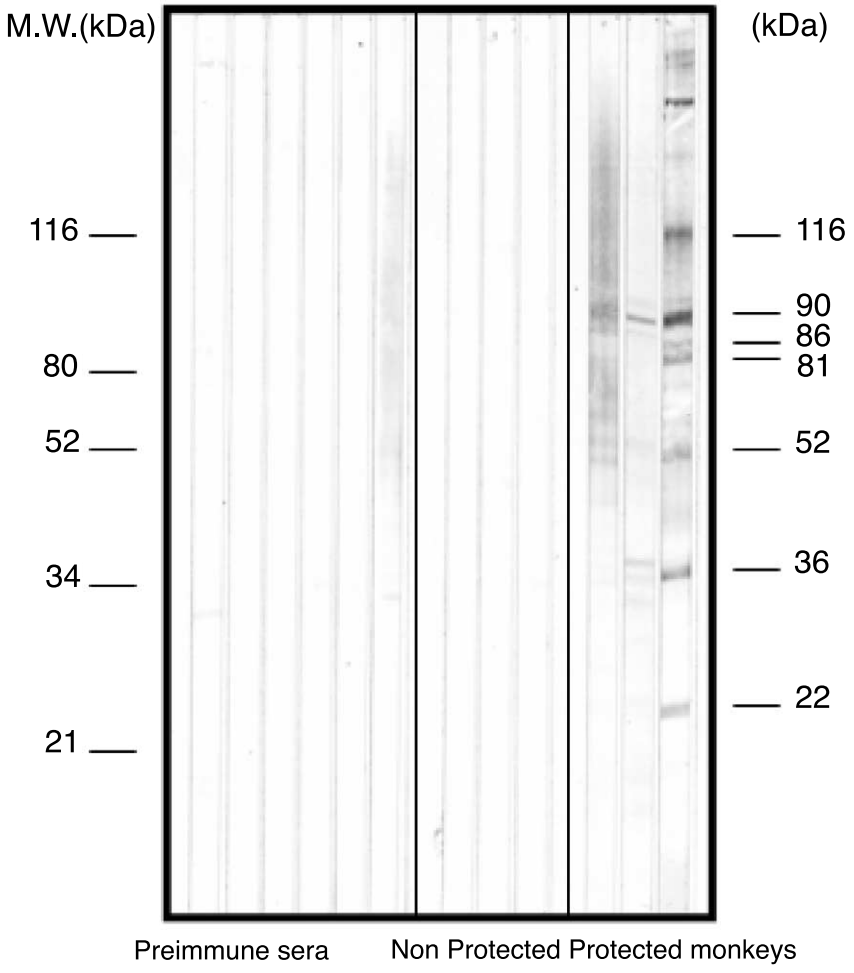

Fig. 1. Western blot analysis of *P. falciparum* schizont solubilized antigens showing protected *Aotus* sera reactivity with either the whole 116 kDa precursor, the 83/90 kDa doublet and its 36 and 22 kDa cleavage product range or just some of them. Pre-immune as well as non-protected sera are also shown.

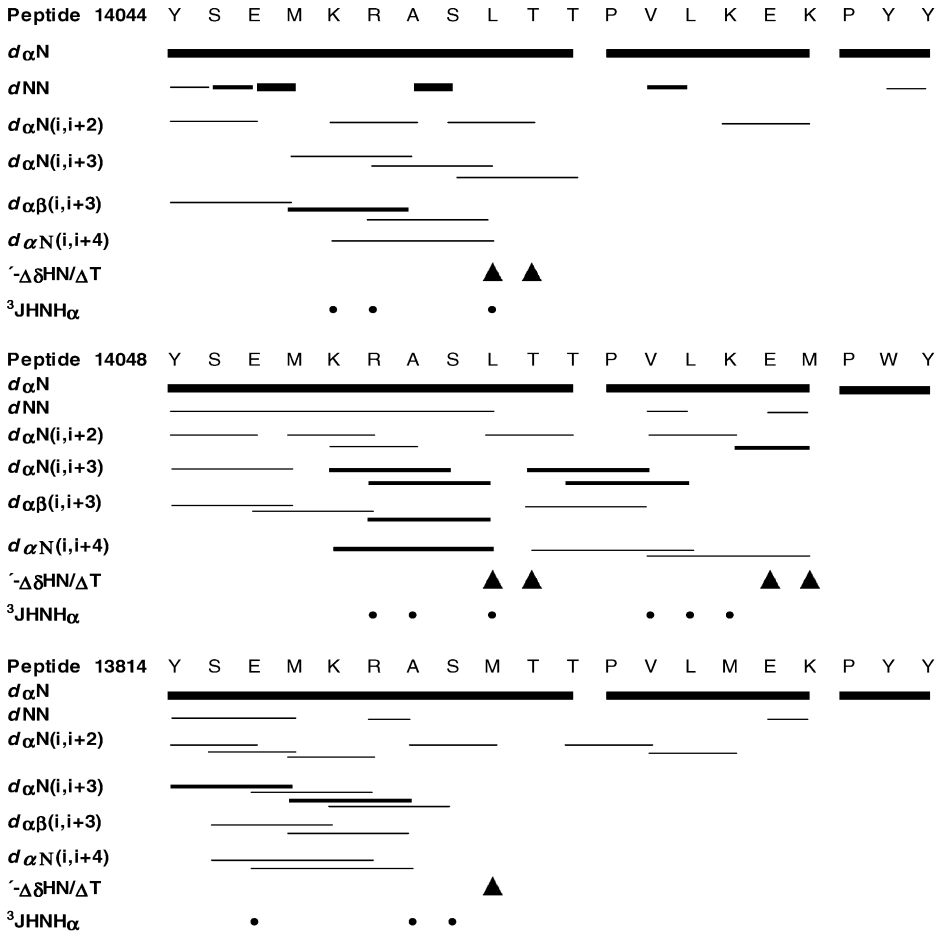

Fig. 2. Summary of sequential and medium range NOEs represented by lines whose thickness is proportional to their NOE intensity. Low H^N amide temperature coefficients are indicated by ▲ and $^3J_{HNH\alpha}$ indicated by • for: peptide 14044, peptide 14048, and peptide 13814.

peptide 14048 between K5 and L9, R6 and T10, P12 and E16 and V13 and M17; and for peptide 13814 between K5 and M9. Some long-range NOEs could be present (or not) in peptide 13814 7–9 region, as a NOESY spectrum region was found where there was signal overlap. It was decided to use only that hydrogen bond defined by the coefficient of temperature as very similar structures were defined in calculations with and without these NOEs and with and without a hydrogen bond. Lead peptide 4337 is not shown in Fig. 2 as it did not show any long-range connectivity, displaying all the sequential signals characterizing a lineal or random peptide.

A set of 50 structures was calculated for the 14044 peptide using 163 distance restraints, two hydrogen bond restraints and 19 ω dihedral angle restraints. 11 of the 50 structures which did not have distance violation larger than 0.37 Å nor dihedral angle violation greater than 1° were accepted. These structures had 0.154 Å root mean square deviation superimposition value for the backbone atoms (in red, Fig. 3, left panel). Six structures were then selected on the basis of lowest total energy and agreement with experimental data (Fig. 3, right panel). Kabash and Sander's program [23] was used to provide clearer analysis of how helical fragments were defined in the peptides. Structures were helical for peptide 14044 between residues 5 and 10 (corresponding to shaded area in Table 2).

Results of structure calculations for peptides 14048 and 13814 are also shown in Table 2. If those structures defined

Table 2
Summary of structure calculation results

Peptide No.		Peptide sequence No. of structures superimposed / 50 (helical segments shaded)	NOEs used	Rmsd Å	Maximum NOE ViolationsÅ	Maximum Angular Violations°	Immuno-genecity	Protection
4337		W G E E K R A S H T T P V L M E K P Y Y					-	-
14044	11	Y S E M K R A S L T T P V L K E K P Y Y	163	0,154	0,37	1	+	+
14048	27	Y S E M K R A S L T T P V L K E M P W Y	197	0,156	0,35	1,6	+	-
13814	16	Y S E M K R A S M T T P V L M E K P Y Y	218	0,439	0,40	2,9	-	-

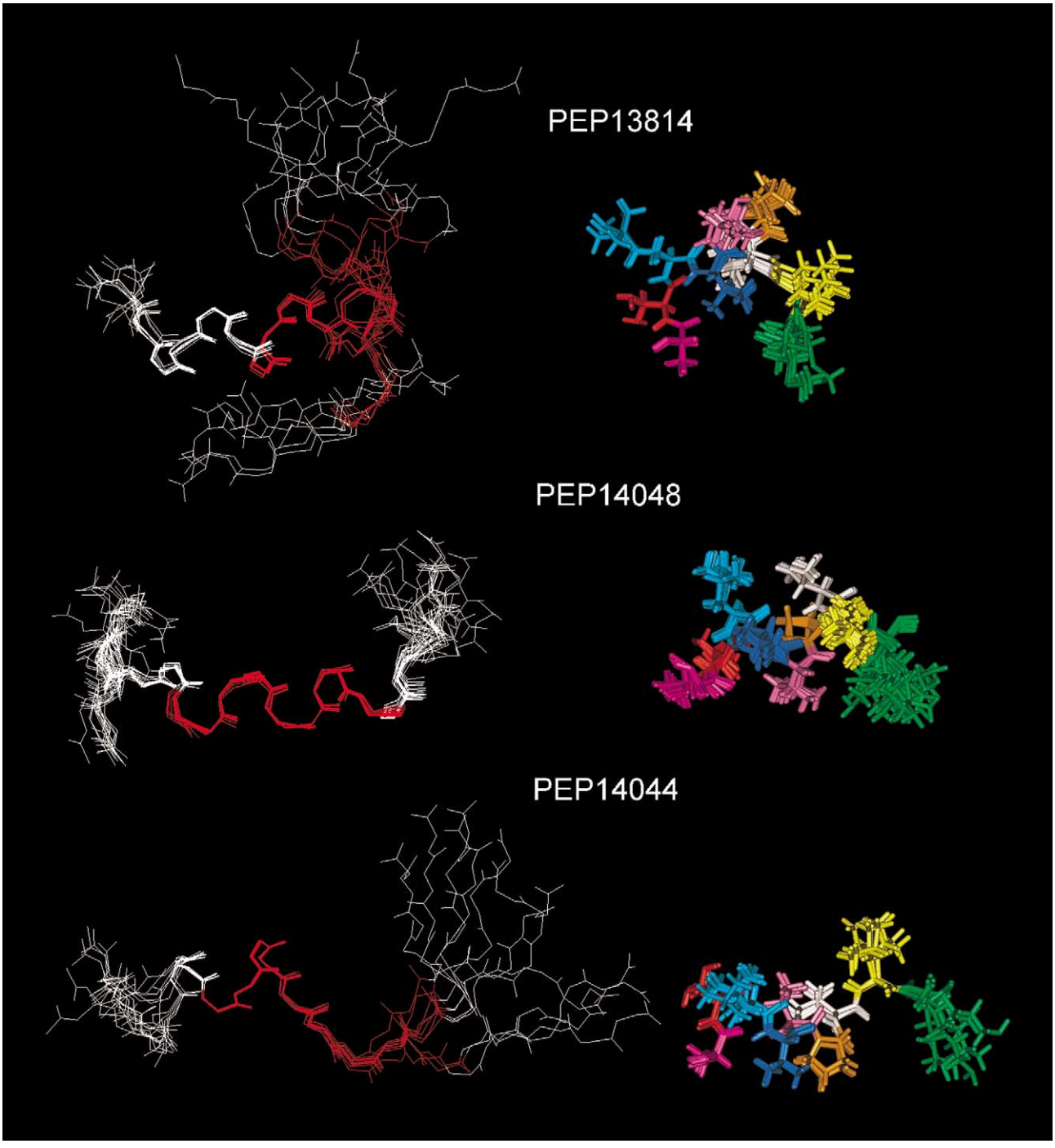

Fig. 3. Peptide 13814 (non-protective, non-immunogenic), 14048 (immunogenic, but non-protective) and 14044 (immunogenic and fully protective) structure. Left: Backbone representation of the same peptides. The core where the main modifications were made is in red. Right: Ribbon representation of the same fragment. Color code: A7 fuchsia; S8 red; L9 (14044, 14048), M9 (13814) pale blue; T10 dark blue; T11 pink; P12 brown; V13 white; L14 yellow; and K15 (14044, 14048), M15 (13814) green.

for peptides 14044 and 14048 are compared, a helical fragment similar to residues 6–9 can be seen. Amino acid replacements in peptide 14048 K17M and Y19W residue C-terminal sequences helped to define a second helical fragment corresponding to residues 13–16. A helical tendency appeared in this peptide; but, the presence of proline in position 12 in the middle of the helix produced a kink, causing a small turn structure in residues 10–12.

It can be observed (Fig. 3, right panel) that in the 14044 immunogenic and protective peptide (W1Y, G2S, E4M, H9L and M15K modifications) the α-helix extends from K5 to T10, maintaining high flexibility for the rest of the molecule. In the immunogenic, but non-protective 14048 peptide (W1Y, G2S, E4M, H9L, M15K and K17M modifications) there are two helical portions (R6–M9 and V13–E16), dramatically reducing the peptide's flexibility. In the non-protective, non-antigenic 13814 peptide (W1Y, G2S, E4M and H9M modifications) the α-helix goes from E3 to M9.

We have tried to correlate those differences observed in peptides' three-dimensional structure with immune response. It can be observed that the changes in critical amino acids led to conformational changes respecting native peptide possessing random structure whilst helical fragments could be seen in modified peptide. Our data suggests that a small helix in peptide 14044 central region simultaneously produces antibodies and also induces protection against *P. falciparum* experimental challenge of *Aotus* monkeys. In turn, this suggests a certain degree of flexibility in the peptide thus enabling it to bind to major histocompatibility complex–T-cell receptor (MHC–TCR) immune system molecules. We have also observed that this protection is lost when a peptide is less flexible (14048) or when it has a helix in another region in the sequence (13814).

Although side chain conformation reliability is limited by a relatively high conformational freedom, differences in the residues' relative spatial assignment are clearly seen. Our data shows clear differences between peptides beginning in residue A7 through residue 15, where the main changes were made in the core of all peptides (Fig. 3, right panel). The modification site and the concomitant change of configuration thus become critical steps in the process of inducing appropriate immune responses.

This data strongly suggests that amino acid changes, as well as the relative residue orientation, may account for a better interaction with immune system molecules possibly repairing MHC–TCR interface defects.

Supporting our findings, it has recently been found that: modifications to an antagonist peptide were able to convert it into an agonist one, repairing the defect in the TCR–peptide–MHC-interface [24]; that positive selection of $CD4^+$ T-cells was induced in vivo by agonist peptides whilst inhibited by antagonist ones [25]; and that some modifications are more potent than the wild type peptide [26], as we have shown here and in a previous paper [13]. It has also been shown that this type of modification is able to induce improved anti-tumor immunity against AH1 colon–rectal carcinoma, stabilizing this interface [27].

Our previous work and this paper strongly suggest that a way to trigger an appropriate immune response is to specifically modify conserved, non-immunogenic (antagonist) peptides in specific places (critical binding residues) to render them immunogenic and protective (full agonist). This in turn would facilitate the development of rational methodology for obtaining multi-component subunit-based synthetic malarial vaccines.

References

[1] Marsh, K. (1992) Parasitology 104, 553–559.
[2] Sachs, J. and Malaney, P. (2002) Nature 415, 680–685.
[3] Miller, L.H. and Hoffman, S.L. (1998) Nat. Med. Vaccine Suppl. 4, 520–524.
[4] Miller, L.H., Baruch, D.I., Marsh, K. and Doumbo, O.K. (2002) Nature 415, 673–679.
[5] Richie, T.L. and Saul, A. (2002) Nature 415, 694–701.
[6] Peterson, M.G., Marshall, V.M., Smythe, J.A., Crewther, P.E., Lew, A., Silva, A., Anders, R.F. and Kemp, D.J. (1989) Mol. Cell. Biol. 9, 3151–3154.
[7] Narum, D.L. and Thomas, A.W. (1994) Mol. Biochem. Parasitol. 67, 59–68.
[8] Crewther, P.E., Culvenor, J.G., Silva, A., Cooper, J.A. and Anders, R.F. (1990) Exp. Parasitol. 70, 193–206.
[9] Marshall, D.L., Zhang, L., Anders, R.F. and Coppel, R.L. (1996) Mol. Biochem. Parasitol. 77, 109–113.
[10] Hodder, A.N., Crewther, P.E. and Matthew, M.L. (1996) J. Biol. Chem. 271, 29446–29452.
[11] Urquiza, M., Suarez, J.E., Cardenas, C., Lopez, R., Puentes, A., Chavez, F., Calvo, J.C. and Patarroyo, M.E. (2000) Vaccine 19, 508–513.
[12] Good, M.F., Pombo, D., Quakyi, I.A., Riley, E.M., Houghten, R.A., Menon, A., Alling, D.W., Berzofsky, J.A. and Miller, L.H. (1988) Proc. Natl. Acad. Sci. USA 85, 1199–1203.
[13] Espejo, F., Cubillos, M., Salazar, L.M., Guzman, F., Urquiza, M., Ocampo, M., Silva, Y., Rodriguez, R., Lioy, E. and Patarroyo, M.E. (2001) Angew. Chem. Int. Ed. 40, 4654–4657.
[14] Rodríguez, R., Moreno, A., Guzmán, F., Calvo, M. and Patarroyo, M.E. (1990) Am. J. Trop. Med. Hyg. 43, 339–354.
[15] Lambros, C. and Vanderberg, J.P. (1979) J. Parasitol. 65, 418–420.
[16] Houghten, R.A. (1985) Proc. Natl. Acad. Sci. USA 82, 5131–5135.
[17] Rivera, Z., Granados, G., Pinto, M., Varon, D., Carvajal, C., Chaves, F., Clavo, J., Rodriguez, R., Guzman, F. and Patarroyo, M.E. (2002) J. Pept. Res. 59, 62–70.
[18] Waterhouse, V. and Johnson, C. (1994) Biochemistry 33, 2121–2128.
[19] Rance, M., Sorensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[20] Bax, A. and Davis, D.G. (1985) J. Magn. Res. 65, 355–360.
[21] Jeener, J., Meier, B.H., Backman, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[22] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids. John Wiley and Sons, New York.
[23] Kabsch, W. and Sander, C. (1983) Biopolymers 22, 2577–2637.
[24] Baker, B.M., Gagnon, S.J., Biddison, W.E. and Wiley, D.C. (2000) Immunity 13, 475–484.
[25] Tangri, S., Ishioka, G.Y., Huang, X., Sidney, J., Southwood, S., Fikes, J. and Sette, A. (2001) J. Exp. Med. 194, 833–846.
[26] Piotr, K., Pacholczyk, R., Ignatowicz, H., Kisielow, P., Jensen, P. and Ignatowicz, L. (2001) J. Exp. Med. 194, 407–416.
[27] Slansky, J.E., Rattis, F.M., Boyd, L.F., Fahmy, T., Jaffee, E.M., Schneck, J.P., Margulies, D.H. and Pardoll, D.M. (2000) Immunity 13, 529–538.