Distorting Malaria Peptide Backbone Structure to Enable Fitting into MHC Class II Molecules Renders Modified Peptides Immunogenic and Protective

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Received October 4, 2002

The conserved, nonantigenic, nonimmunogenic malaria Merozoite Surface Protein-2 peptide **1**, having high affinity for red blood cells, was rendered immunogenic and protective in *Aotus* monkeys by specifically changing some critical residues. The NMR structure revealed a switch from classical type III′ into distorted III′ and III β turns in the protective peptides. These changes may lead to a better fit into the *Aotus* MHC class II human HLA-DRβ1* 12 molecule equivalent, thus activating the immune system.

1. Introduction

The development of malaria vaccines against the most lethal form of this parasite, *Plasmodium falciparum,* is one the main goals of the world's health authorities. This parasite infects more than 250 million people annually, killing around 2.5 million, mainly children below 5 years of age, especially in sub-Saharan Africa.[1]

Although several empirical attempts have been made to develop a fully protective malarial vaccine, including synthetic peptide (such as Spf66 which induced limited protective efficacy in South America and Africa) and recombinant or virally expressed protein or DNA vaccines, most of them have failed.[2]

The identification of those *conserved* High Activity Binding Peptides (HABPs) from the most relevant proteins involved in the invasion process has been proposed to elicit an appropriate immune response able to block those receptor/ligand interactions in the attempt to develop a rational approach for the development of malarial vaccines.[3−5]

Several proteins have been suggested as being excellent candidates for use in a malaria vaccine,[6] one being the Merozoite Surface Protein-2 (MSP-2). The entire MSP-2 protein was synthesized in 20 mer long peptides to identify MSP-2 HABPs.[5] One completely conserved HABP was identified (called **1**), having a 72 nM affinity coefficient (K_d), positive cooperativity for the receptor−ligand interaction, and the ability to block parasite invasion of RBC by 96 ± 12% at 200 μM concentration. The critical binding residues (underlined) were identified by glycine analogue replacement analysis for peptide **1** (K**NE**SK**YS**NTF**IN**NAYNM**S**IR).[5]

As conserved HABPs are not antigenic[7] nor immunogenic (as shown with different proteins in other papers),[8,9] a series of peptide analogues having had some critical binding residues changed for amino acids having similar mass but different charge were synthesized and tested in the *Aotus* monkey for their capacity to induce antibodies and to protect them against experimental challenge with a highly virulent strain of *P. falciparum.* This South American monkey species is extremely susceptible to human malaria parasites,[10] developing severe malaria in a fashion similar to humans, including anemia, but not cerebral malaria.

The three-dimensional structure of the lead **1** and modified protective peptides was determined by ^{1}H nuclear magnetic resonance (NMR) to try to correlate it with their biological functions.

2. Results and Discussion

Peptide Characterization. Each one of the HPLC-purified monomeric peptides used for NMR studies showed one single peak. The experimental masses (followed by theoretical masses in parentheses) shown by mass spectroscopy (MS) were 2315.1 (2313.0) for peptide **1**, 2434.97 (2438.8) for **2**, 2444.80 (2451.1) for **3**, and 2454.2 (2453.0) for peptide **4**.

Immunological Studies. The polymers used for immunization had molecular weights in the 8 kDa to 24 kDa range as assessed by size exclusion chromatography (SEC). Their structural conformation was similar to their monomeric counterparts as determined by circular dichroism (CD) (data not shown).

The immunological and protective efficacy induced by these modifications showed three different groups. The change of polarity in group A of two critical binding residues (I_{11}E plus N_{12}V) having similar mass and volume (as in peptides **2** and **3**) or inverting critical binding residues I_{11}N to N_{12}I in peptide **1**'s sequence (as in peptide **4**) rendered it highly immunogenic (as assessed by the high IFA titers) and protective against experimental challenge in some monkeys. Further changes to critical binding residues N_2, E_3, or Y_6, and S_7 did not improve their immunogenicity or their protective efficacy (as in peptides 5, 6, and 7). Only the S_{18}V change in peptides **3** and **4** improved their efficacy, requiring only two doses to immunize the *Aotus* monkeys. Experiments were repeated twice with peptide **4** with two immunizations, and 1/10 and 1/8 monkeys were fully protected, giving 4/26 or ≈15% overall protective efficacy.

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10.1021/jm020440w CCC: $25.00 © 2003 American Chemical Society
Published on Web 04/22/2003

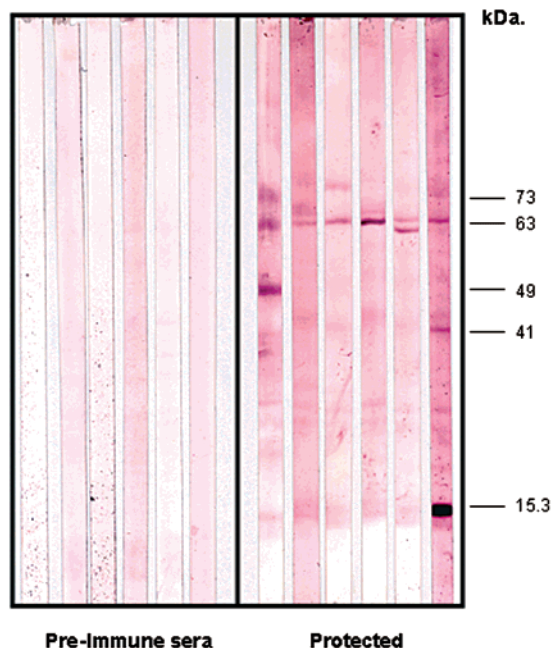

Figure 1. Western blot analysis of protected *Aotus* monkey sera immunized with peptides modified against *P. falciparum* schizont lysates. Sera taken 15 days after the second immunization, used at 1:100 dilution, recognized a 63 kDa band corresponding to MSP-2 molecular weight.

Some additional changes to the immunogenic and protective peptides **2**, **3**, and **4** (for which their NMR structure had been determined) meant that they kept their immunogenicity with two or three doses, but the induced antibodies disappeared with the third dose, perhaps as a consequence of antidiotipic response, different antibody affinity or IgG subclasses, or their capacity to provide enough protection, as can be observed in group B. Changes performed on group C were not immunogenic or protective. Sera from monkeys immunized with peptides **2**, **3**, and **4** (having led to them being protected) recognized a 63 kDa band by Western blot corresponding to MSP-2's molecular weight (Figure 1).

Structural Analysis. All peptide NOESY spectra showed αHi-NHi+1 sequence signals stronger than intraresidue cross-peaks. Medium or weak intensity H−NH cross-peaks were also observed. Some medium range αHi-NHi+2 cross-peaks were found in addition to these sequential cross-peaks. The presence of sequential, short and medium range $d_{NN}(i,i+1)$, $d_{\alpha N}(i,i+2)$ NOE connectivity and low-temperature dependence ($-\Delta\delta HN/\Delta T$) values observed for some of the amino acids revealed the presence of some types of turn. Peptides **1**, **2**, **3**, and **4** sequential medium range NOEs are summarized in the Supporting Information.

Two hundred and fifty one NOEs, three angular constraints, and one hydrogen bond constraint between residue *i* main chain C=O and residue *i*+3 NH were used in peptide **1** structure calculations. NOE signal NH*i*+2 N_8−F_{10} was concordant with the hydrogen bond corresponding to F_{10} NH−S_7 C=O and the distance between C_i^{α} and C^{α}_{i+3} was less than 7 Å corresponding to the β-turn structure. The sets of 50 generated structures obtained satisfied the experimental constraints. A family of 25 low energy conformers (having root-mean-square deviation (RMSD) equal to 0.25 Å) superimposed backbone atoms from residues S_7 to F_{10} were used to analyze the consensus structure. These structures had no angle-constraint violation larger than 1.20° (degrees) nor distance constraint violation larger than 0.40 Å. These data shows that peptide **1** (neither immunogenic nor protective) had a *classical type III′ β-turn* structure between S_7 to F_{10} amino acids according to ϕ and ψ angle values; the rest of the peptide was highly flexible. Peptide **2** (immunogenic and protective) showed two distorted β-turns (distorted type III′ between Y_6 to T_9 and distorted type III between A_{14} to M_{17} amino acids). A flexible region from F_{10} to N_{13} connected these two β-turns. The values observed for these turns were $\phi_{i+1} = 47°$, $\psi_{i+1} = 55°$, $\phi_{i+2} = 49°$, $\psi_{i+2} = 29°$ (the ideal values for a type III′ turn being $\phi_{i+1} = 60°$, $\psi_{i+1} = 30°$, $\phi_{i+2} = 60$ $\psi_{i+2} = 30°$), showing distortion in the ψ_{i+1} angle, and $\phi_{i+1} = -52°$, $\psi_{i+1} = -47°$, $\phi_{i+2} = -66°$, $\psi_{i+2} = -25°$. (the ideal values for a type III turn being $\phi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\phi_{i+2} = -60$ $\psi_{i+2} = -30°$), showing distortion in the ψ_{i+1} angle. The Supporting Information shows the structure calculations and results for peptides **3** and **4**.

Although side chain conformation reliability was limited by relatively high conformational freedom, dramatic changes in the relative spatial arrangements were observed when comparing the original **1**, nonimmunogenic, nonprotective peptide versus the **2**, **4**, and **3**, highly immunogenic and protective peptides. It can be suggested that these protective modified peptides could be fitting into the human HLA-DRβ_1*12 allele equivalent of the *Aotus* monkey based on peptide binding motifs to the human major histocompatibity complex (MHC) class II molecules.[11] This class II molecule uses (among other residues) F for pocket 1, I or V for pocket 3, Y for pocket 6, and V or S for pocket 9,[11] the same peptide motifs found in the immunogenic and protective modified peptides.

The *Aotus* counterpart to this human HLA-DRβ_1*12 allele has recently been identified and named *Aona* DRβ_1*0301. As the α chain is identical, the amino acid sequence was practically the same in the β chain,[12] having only four residue differences (F_9E, S_{13}G, R56P, and S57V, the last two in pocket 9). The rest of the residues composing the other pockets were identical to HLA-DRβ_1*12. These modified peptides could thus be fitting well into *Aotus Aona* DRβ_1*0301 allele pockets 1, 3, 6, and 9.[12] This genetic marker has an allelic frequency of ~15% in the *Aotus* monkey. When analyzing the immunogenic and protective **2**, **4**, and **3** peptides' structures in the area where changes were made starting with amino acids F10 in **2** and **3** or F7 in **4,** these protective peptides could theoretically have been fitting into Pocket 1 (fuchsia). V12 in **2** and **3** as well as I9 in **4** fit into pocket 3 (pale blue). Y_{15} in **2** and **3** or Y12 in **4** (brown) fitted into pocket 6. S18 in **2** or V18 in **3** or V15 in **4** fitted into pocket 9. All pointed downward to fit properly into the theoretical pockets of HLA-DRβ_1*12. The only two residues that seemed to be properly oriented in **1** were those that could theoretically have fit into pockets 1 and 9 (Figure 2).

Interestingly, all the other residues were pointing upward in the immunogenic and protectogenic peptides, suggesting that this orientation (better observed in peptide **3)** was directed toward the T-cell receptor (TCR)

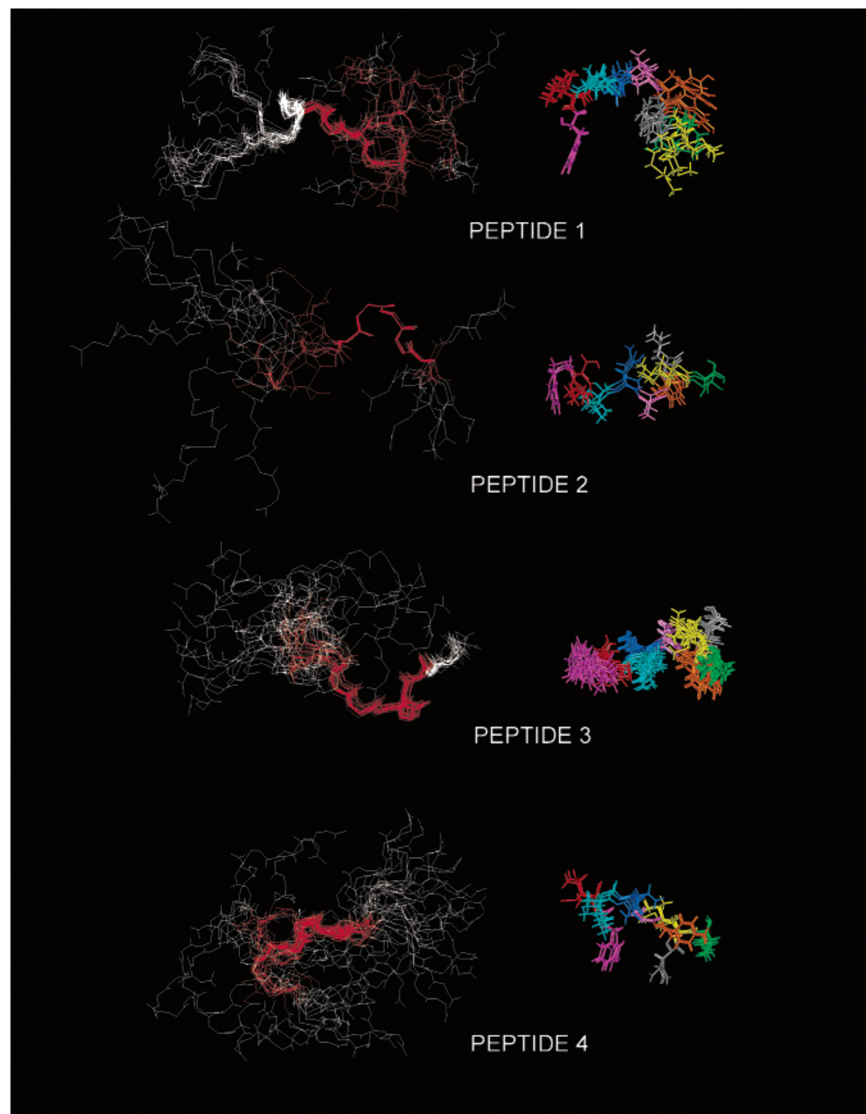

Figure 2. Structures of nonprotective peptide **1** as well as protective peptides **2**, **3**, and **4**. Left: Modified amino acids' backbones are shown in red. Right: modified section amino acids are represented by thicker lines color coded: F10 (1, 2, and 3), F7 (4) fuchsia; I11 (1), E11 (2 and 3), N8 (4) red; N12 (1), V12 (2 and 3), I9 (4) pale blue; N13 (1, 2, 3), N10 (4) dark blue; A14 (1, 2, 3), A11(4) pink; Y15 (1, 2, 3), Y12 (4) brown; N16 (1, 2, 3), N13 (4) gray; M17 (1, 2, 3), M14 (4) yellow and S18 (1 and 2), V18 (3), V15 (4) green.

(Figure 2). Such orientation was not observed in native peptide **1**. Crystallographic analysis of peptides found in the Class II molecule grooves[13] showed that they had a polyprolinyl type II structure[14] with a lateral chain configuration similar to the one described here. So far we have not found such a structure in our modified peptides, perhaps due to technical and methodological differences. We have described that 4313 modified, immunogenic, and protective peptides acquired distorted β-turn type III or III′ structures in another malaria protein (the Apical Merozoite Antigen-1, AMA-1), while the lead peptide had a *classical* type III β-turn.[9] In some other papers we have also recognized a shortening of their α-helical region leading to an increase in the modified and protective 1585,[8] 1522[16] Merozoite Surface Protein-1 (MSP-1), and 4337 AMA-1[15] peptides' random structures. It can thus be suggested that in malarial peptides, distorted β-turn type conformations[9] or short helical fragments associated with random configurations[8,15,16] may adopt more favorable conformations in solution to properly fit into and activate the MHC−peptide−TCR complex conformation.

On the basis of the previously described work, it is tempting to suggest that modifications made to conserved HABPs to a make them (a) fit perfectly well into *Aotus* MHC Class II molecules pockets and (b) properly orientate their contacting residues toward the T-cell receptor activate the immune system to produce very high antibody levels against *P. falciparum* and to protect *Aotus* monkeys against experimental challenge with this parasite. This may represent a novel way to develop multistage, multicomponent synthetic vaccines against malaria.

3. Experimental Section

Synthetic Peptides. The peptides were synthesized by the multiple solid-phase peptide synthesis method,[12] using *tert*-butoxycarbonyl strategy (Supporting Information, Table 1) and purified and characterized by RP-HPLC and mass spectrometry (MS). Peptide polymers for immunization studies were obtained after CG addition at the N and C termini. Our

Institute's carefully defined cysteine oxidation methodology was used to obtain high molecular weight polymers (with oxygen at pH 7.4). Thirty peptides, numbered according to our laboratory's sequential numbering with modifications shown in bold, were synthesized; critical residues were replaced by amino acids with different charge but similar mass to induce a protective immune response. Peptide **4** was made three residues shorter at the N terminus and three longer at the C terminus.

NMR Analysis. NMR experiment samples were prepared by dissolving 10 mg of peptide in 500 μL of DMSO-d_6 [17] for the structure analysis. ^{1}H spectra were run in a Bruker DRX-500 spectrometer. Proton spectra were assigned by using double quantum filter correlation spectroscopY (DQF−COSY),[18] total correlation spectroscopy (TOCSY),[19] and nuclear Overhauser enhancement spectroscopy (NOESY) experiments.[20] The 2D NMR data were processed with XWIN NMR software. The NOESY spectra recorded at different temperatures (285−315 K) were used to obtain amide temperature coefficients (−DdHN/DT). Spin coupling constants ($^3J_{NH-CaH}$) on DQF−COSY spectra were measured.

Structure Calculations. Peptide structure was determined by using Molecular Simulations Inc. (MSI) software. The NOEs were grouped into three categories (strong, medium, and weak) and then converted into distance restraints (1.8−2.7 Å, 2.7−3.5 Å, and 3.5−5.0 Å). Hydrogen bond constraints were introduced for the slow exchange rate peptide NH; distance ranges involving these likely NH···O hydrogen bonds were set at 1.8−2.5 Å. The *f* angle constraints derived from $^3J_{NH-CaH}$ were restricted to $-70° \pm 30°$ if $^3J_{NH-CaH} < 6$ Hz and to $-120° \pm 30°$ if 9 Hz $> {}^3J_{NH-CaH} >$ 8 Hz. The Distance Geometry (DGII) program was used to generate 50 starting structures. These structures were refined using a restrained simulated annealing protocol. Iterative calculation (with violated constraint correction) was performed from original data, and the final representative family of low energy conformers (satisfying the set of experimental distance restraints) was determined by energy and root-mean-square deviation (rmsd).

Acknowledgment. This research has been supported by the Colombian President of the Republic's Office and the Ministry of Health.

Supporting Information Available: Complete experimental details, all biological test protocol and data, summary of sequential range NOE connectivity, and summary of structure calculations for peptide **1** and its analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM020440W