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Changing ABRA protein peptide to fit into the HLA-DRβ1*0301 molecule renders it protection-inducing

Luz M. Salazar[a], Martha P. Alba[a,b], Hernando Curtidor[a,b], Adriana Bermúdez[a], Luis E. Vargas[a], Zuly J. Rivera[a], Manuel E. Patarroyo[a,b,*]

[a] *Fundación Instituto de Inmunología de Colombia (FIDIC), Cra 50 No. 26-00, Bogota, Colombia*
[b] *Universidad Nacional de Colombia, Bogota, Colombia*

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Abstract

The *Plasmodium falciparum* acidic–basic repeat antigen represents a potential malarial vaccine candidate. One of this protein's high activity binding peptides, named **2150** ([161]KMNM**LKE**NV**DY**I**Q**KNQN**LFK**[180]), is conserved, non-immunogenic, and non-protection-inducing. Analogue peptides whose critical binding residues (in bold) were replaced by amino-acids having similar mass but different charge were synthesized and tested to try to modify such immunological properties. These analogues' HLA-DRβ1* molecule binding ability were also studied in an attempt to explain their biological mechanisms and correlate binding capacity and immunological function with their three-dimensional structure determined by ^{1}H NMR. A 3_{10} distorted helical structure was identified in protective and immunogenic peptide **24922** whilst α-helical structure was found for non-immunogenic, non-protective peptides having differences in α-helical position. The changes performed on immunogenic, protection-inducing peptide **24922** allowed it to bind specifically to the HLA-DRβ1*0301 molecule, suggesting that these changes may lead to better interaction with the MHC Class II-peptide-TCR complex rendering it immunogenic and protective, thus suggesting a new way of developing multi-component, sub-unit-based anti-malarial vaccines.

Keywords: ABRA protein; Peptide analogues; Vaccine candidate; Malaria; Conformation; NMR

Despite world-wide efforts made since 1950 at controlling and eradicating *Plasmodium falciparum* malaria, between 200 and 300 million people become infected by this parasite annually and 2–3 million of them die, mainly children aged less than 5, especially in sub-Saharan Africa [1].

A substantial effort is consequently being made to develop an effective vaccine for controlling this deadly disease; this is a difficult task due to the complexity of the malarial parasite's life-cycle and antigenic variation. One approach involves developing a subunit-based multi-antigenic, multistage vaccine; several *P. falciparum* malaria proteins have been proposed as anti-malarial vaccine candidates.

Malaria proteinases seem to function in releasing merozoites from infected erythrocytes and merozoite invasion of erythrocytes, appearing to be excellent vaccine candidates.

The *Plasmodium falciparum* acidic–basic repeat antigen (ABRA) localized in the parasitophorous vacuole and associated with the merozoite surface at the time of schizont rupture is one of the above candidates [2]. Chymostatin, a chymotrypsin-like proteinase inhibitor, inhibits malaria invasion and also inhibits apparent autoproteolysis of the 101 kDa ABRA antigen (p101-ABRA) when localized in the vacuolar space surrounding merozoites in *Plasmodium falciparum*-infected erythrocytes [3]. *Escherichia coli* expressed recombinant

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

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ABRA fragments were used to show that ABRA interacts with red blood cells through its N-terminus. Out of the four human erythrocyte proteins tested (named band 3, glycophorin A and B, and spectrin), ABRA showed dose-dependent and saturable band 3 protein-binding activity [2].

High activity human red blood cell binding peptides have been identified in this protein. One of them (peptide **2150**), for which critical binding residues have already been defined by using a competition binding assay, has no amino-acid genetic variations (i.e., conserved). Peptide **2150** located in the fragment's N-terminus (KMNM**LKE**NV**DYIQ**KNQN**LF**K) has an 80 nM affinity constant (K_d) [4].

Conserved binding peptides do not induce an immune response during natural infection (i.e., non-antigenic) or when used as vaccines (i.e., immunogenic) [5,6].

ABRA **2150** peptides' critical binding residues determined by glycine analogue scanning (shown in bold) were changed for amino-acids having similar mass but different charge to change such immunological properties and overcome this problem; these changes generated peptide analogues, some of which became immunogenic and protective when used to immunize *Aotus* monkeys. ^{1}H NMR was then similarly used to investigate these substitutions' effect on peptide conformation.

Peptide **2150** and others modified from it were used in this work for studying binding to different HLA-DRβ1* purified molecules since peptides have to be presented by Major Histocompatibility Class-II (or HLA-DRβ1) molecules to be recognized by T-cells. Such biological function was correlated with tridimensional structure data (determined by ^{1}H NMR) and immunological protection results obtained in the *Aotus nancymaae* monkey were used as experimental model in these assays.

Materials and methods

Peptide synthesis. Standard t-Boc solid phase peptide synthesis (SPPS) was used [7]; peptides were numbered according to FIDIC serial numbering, purified by RP-HPLC, and their molecular mass was determined by mass spectroscopy (Bruker Protein MALDI-TOF spectrophotometer). CG was added to the C- and N-termini of each peptide to allow polymerization via cysteine oxidation (oxygen, pH 7.4). This procedure has been carefully standardized to guarantee including high molecular weight polymers (8–24 kDa) for immunization purposes as assessed by size exclusion chromatography.

Animals and immunization. *Aotus* monkeys were immunized with synthesized polymeric peptide analogues (Table 1) to induce humoral immune responses and protection against experimental challenge with the *P. falciparum* malaria parasite [8]. Blood was drawn for immunological analysis on days 0, 15, and 20 days after each immunization.

Challenge and parasitemia assessment. Immunized and control *A. nancymaae* monkeys were infected with 200,000 *P. falciparum* FVO-strain infected RBC via femoral vein for challenge 20 days after the last immunization [9]. Protection was defined as total absence of parasites in blood during the 15 days of the experiment. Non-protected monkeys developed patent parasitemia from day 5 or 6, reaching $\geqslant$6% levels between days 8 and 10. Immunofluorescence was used for daily reading of parasites 5 days after challenge (i.e., percentage of parasitized RBC on a slide following acridine orange staining).

IFA and Western blot. Late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain) were synchronized by Lambros and Vandenberg's method [10]. They were washed and treated [9]. Twenty percent late parasitemia RBCs were washed with PBS (pH 7.2) and lysed with 0.2% saponin (Merck) for Western blotting.

HLA-DR molecule affinity purification. Human molecules were purified from DR1 WT100BIS (DRβ1*0101), DR3 COX (DRβ$_1$*0301), DR4 BSM (DR β$_1$*0401), and DR11 BM21 (DR β$_1$*1101) homozygous EBV-B cell lysates by affinity chromatography [11] using anti-HLA-DR mAb L-243 cross-linked to protein-A–Sepharose CL-4B (Amersham–Pharmacia Biotech AB) as affinity support.

Competition binding assays. Peptide binding competition assays were conducted for measuring unlabeled peptide's ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules [12]. Biotinylated hemagglutinin HA 306–318 (PKYVKQNTLK

Table 1
Humoral immune response and protective efficacy induced by **2150**-derived peptides in *Aotus* monkeys

Polimerized peptide Nr.	Peptide sequence	IFA ≥1:160 Post 2nd	IFA ≥1:160 Post 3rd	Nr. of protected monkeys	Group
2150	K M N M L K E N V D Y I Q K N Q N L F K	0	0	0	
24922	— — — — H L — — — P W — M N K — — — — —	1(320)	ND	2/7	A
24296	— — — — H M — — — A W — M — — — — — — —	1(1280)	1(1280)	0/9	B
25852	— — — — H L — H — P W — M — — — — — — —	1(320)	0/6	0/6	B
25854	— — — — N L — H — P W — M — — — — — — —	2(320)	0/8	0/0	B
27616	— — — — H M — — — P W — V — — — — — — —	0/8	3(320)	0/7	B
23394	K — — — — H M — — — I — — M — — — — — — —	0/9	3 (320)	0/9	B
27614	— — — — H M — — — P — — V — — — — — — —	0/8	2(640)	0/5	B
26020	— — — — H M — — — P — — M — — — — — — —	0/8	0/8	0/8	C
22912	K E — — — H M K — — — — — — — — — —	0/10	0/10	0/10	C
26018	— — — — H M — — — P W — M — — — — — — —	0/8	0/7	0/7	C
Controls		0/50	0/50	0/50	

Antibody titers are shown in parentheses; the number of monkeys having such titers is shown before the parenthesis. ND, non-determined.

LAT)-labeled peptide was used as control peptide for DRβ_1*0101, DR β_1*0301, DRβ_1*0401, and Gly-Phe-Lys-$(Ala)_7$ ($GFKA_7$) for DR β_1*1101. Both peptides were +N-terminally labeled with sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, III). The amount of bound peptide was normalized to maximum observed binding.

Relative binding affinities were determined for other peptides by competition assay; a good competitor was a peptide which was capable of inhibiting indicator peptide's binding to the HLA molecule being tested by more than 50%.

NMR analysis. NMR experiment samples were prepared by dissolving 7–10 mg peptide in 500 μL TFE-d3 (Cambridge Isotope, 99.94%)/H_2O mixture (30/70 v/v). Studies have shown that nascent structures have an inherent propensity for native structure formation; in the presence of solvents such as 2,2,2-trifluoroethanol (TFE) they can be stabilized into well-ordered conformations [13].

All NMR spectra were recorded on a BRUKER DRX-600 spectrometer at 295 K. Basic NMR structure determination protocol for all peptides was as follows: proton spectra were assigned by DQF-COSY [14], TOCSY [15], and NOESY [16]; TOCSY and DQF-COSY spectra were then used for identifying individual spin systems (amino-acids) and NOESY (350 ms mixing time) for stretches of amino-acids within a given primary structure (sequential assignment). Three-dimensional structure was assigned by Wüthrich's method [17]. 2D NMR data were processed with XWIN-NMR software. TOCSY spectra, recorded at different temperatures (285–315 K), were used for obtaining amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T$ppb/K). DQF-COSY spectra spin coupling constants ($^3J_{HN\text{-}H\alpha}$) were measured.

Structure calculations. Peptide structure was determined by Accelrys software. NOESY peak signals were classified as strong, medium, and weak according to their relative intensity; these corresponded to 1.8–2.5, 2.5–3.5, and 3.5–5.0 Å interproton distances, respectively. Hydrogen bond constraints were introduced for low amide temperature coefficients; only <4 and $-\Delta\delta H^N/\Delta T$ppb/K were used in structure calculations. Distance ranges involving these likely NH$\cdots$O hydrogen bonds were set at 1.8–2.5Å, between residue acceptor oxygen ($i-4$) and residue donor amide hydrogen (i). The ϕ angle constraints derived from $^3J_{HN\text{-}H\alpha}$ were restricted to $-70°$ 30° if $^3J_{HN\text{-}H\alpha}$ <6 Hz. All peptide bonds were forced to *trans* and Cα chirality to L during calculations.

Distance Geometry (DGII) software was used for generating a family of 50 structures. These structures were refined by using simulated annealing protocol (DISCOVER software).

The calculations were repeated until a structure having a minimum of distance and angle restraint violations and the least root-mean-square deviation (RMSD), respecting consensus least energy structure, was obtained. Structures having reasonable geometry and few violations were then selected.

Structural analysis by molecular modeling. The HLA-DRB1*0301 human molecule crystal structure (Protein Data Bank access code 1A6A) [18] was used for modeling peptide **24922** docking with this Class-II molecule. Insight II (2000) Biopolymer module software (Accelrys Software, USA) run on an Indigo 2 Station (Silicon Graphics) was used for superimposing the backbone of the CLIP peptide crystallized with HLA-DRB1*0301 and peptide **24922** allowing RMSD to be determined. This peptide's fitting into empty HLADRβ1* molecules was determined without any further refinement.

Results and discussion

Immunological and protection studies

Table 1 shows amino-acid sequences for peptide **2150** and its analogues which have had some of their critical binding residues modified. It also shows parasite antibody titers, determined by immunofluorescence antibody assay (IFA) and complete or total protection of *Aotus* following experimental challenge.

Three groups can be clearly seen. Group A consisted of a single peptide **24922** (for which the tri-dimensional structure was then determined) that became highly immunogenic (as assessed by IFA antibody titers) and became protection-inducing since it induced full protection in 2 out of 7 monkeys immunized. Modified peptides in group B that induced antibodies (as assessed by IFA) were not protection-inducers, suggesting that such antibodies could have been from a different subclass, or had different affinity or specificity for the native molecule; peptide **24296** (whose 3D structure was then determined) together with peptide **25852** (random structure) were two of them. The peptides presented in group C showed that their modifications did not render them immunogenic or protection-inducing.

Fig. 1 shows Western blotting for the two protected *Aotus* monkeys' sera following a second immunization with **24922**; native protein ABRA (101 kDa) and its 74, 55, 46, and 14 kDa cleavage products were specifically recognized. These data suggest that modifications performed on peptide **2150** rendered it immunogenic (as antibodies recognized denatured protein by Western blot and native molecule by IFA) and protection-inducing, since 2 out of 7 monkeys (~28%) were protected against experimental challenge with a highly infective *P. falciparum* strain.

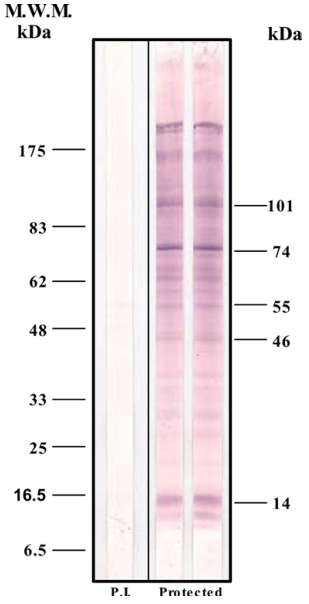

Fig. 1. Western blot analysis. Western blot analysis of solubilized antigens obtained from late *P. falciparum* schizont lysate showing protected *Aotus* sera reactivity with ABRA native protein (101 kDa) or 74, 55, 46, and 14 kDa cleavage products.

NMR analysis

All peptides' NOESY spectra showed αN(i,i + 1) sequence signals to be stronger than intra-residue cross-peaks. The presence of d_{NN} cross-peaks indicated a significant population of conformations in the α region of $\varphi\psi$ the space.

Some medium-range $d_{NN}(i, i+1)$, $d_{\alpha\beta}(i, i+3)$, $d_{\alpha N}(i, i{+}3)$, and $d_{\alpha N}(i, i+4)$ cross-peaks, lowered amide proton chemical shift temperature coefficients, and <6 Hz spin coupling constants were also found for some amino-acids indicating the presence of typical helical fragments in native **2150** and in solely immunogenic **24296** peptides included in this study. However, differences were observed in helical length, since native peptide **2150** was more structured, having two α helical fragments between residues N8–Y11 and Q13–N17, whilst solely immunogenic **24296** presented a shorter α helical fragment between residues N8 and N15. The immunogenic and protection-inducing peptide **24922** presented a very short 3_{10} helix fragment between residues L6 and V9 having a small distortion in residue V9 ϕ and ψ angle values, possibly due to the presence of P10 in the next residue.

The NOEs between P10 δ protons with V9 α proton and V9 H^N proton for peptide **24922** indicated that this peptide was a *trans* isomer. The only immunogenic **25852** peptide had a totally extended form due to the absence of medium range signals; 3D structure could thus not be determined.

Peptides' **2150**, **24296**, and **24922** sequential medium range NOEs, $^3J_{HN\text{-}H\alpha}$, and $\Delta\delta H^N/\Delta T$ are summarized in Fig. 2.

Structure calculations

A set of 50 independently generated structures were obtained for peptide **2150**, satisfying the experimental constraints when using 208 distance restraints (including 60 inter-residue constraints) derived from the NOEs

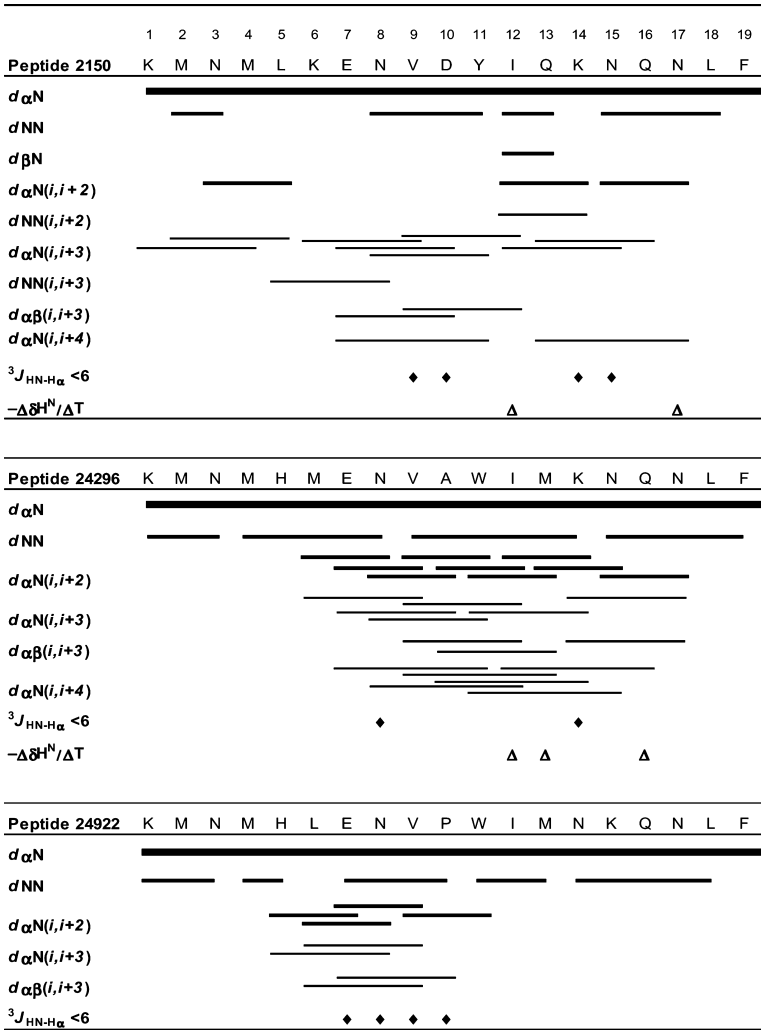

Fig. 2. Summary of sequential NOEs observed for peptides **2150**, **24296**, and **24922**. The bars connect residues between which sequential NOEs were observed. The thickness of the bars indicates the NOE intensity. (♦) $^3J_{HN\text{-}H\alpha} < 6$; (△) indicate H^N protons in H bond.

previously classified by signal-strength. 19 ω dihedral angle restraints were used. Four ϕ angular restraints were restricted to $-70°\pm30°$ with $^{3}J_{\mathrm{HN\text{-}H\alpha}} < 6$ Hz for residues V9, D10, K14, and N15. Two hydrogen bond constraints were introduced for the HN of residues I12 and N17 having <4 ppb/K temperature coefficients C═O of corresponding $i-4$ residues. A family of 21 low-energy conformers, which did not have a distance violation larger than 0.3 Å or dihedral angle violation greater than 2°, was accepted. These structures had a 0.2 Å RMSD superimposition value for the backbone atoms. Structures were helical between residues N8–Y11 and Q13–N17 for peptide **2150**. According to Kabasch and Sander [19], **2150** and **24296** have well-defined helical structures. Table 2 shows the structure calculations for peptides **24296** and **24922**.

Fig. 3 shows that changes in critical amino-acids led to conformational changes respecting helical native peptide being the area where modified critical residues were found. Our results also show that the presence of a short 3_{10} helical region between L6 and V9 amino acids and greater flexibility in the rest of the molecule were associated with antibody production and protection against *P. falciparum*. This was not seen in peptide **2150** as its α-helical motif was six residues larger, limiting the flexibility of the rest of the molecule. If the molecule were totally random (e.g., peptide **25852)** then high flexibility seems to cause the same impediment in activating the immune system.

Purified HLA-DRβ$_1$ molecule binding studies*

Native peptide **2150** did not bind to any of the studied HLA-DR β1* molecules (Table 3), suggesting that α-helical configurations were not associated with binding to the molecules being studied here.

Peptide **24922** bound to the HLA-DRB1*0301 molecule, suggesting that modifications made to this peptide may allow it to fit better into HLA-DRB1*0301 molecules.

The other immunogenic peptides being studied belonging to group B (Table 3) (e.g., peptide **24296**), some with antibody titers up to 1:1280, were non-protection-inducing, among them immunogenic, non-protection-inducing peptide **25852** (random structure) also did not bind to HLA-DRB1* molecules, suggesting that they could be binding to *Aotus* specific HLA-DRB1*-like molecules [20] or to some other HLA-DRB1* molecules not included in the present study. Some other modified peptides bound to HLA-DRβ1*0301 and were solely immunogenic, suggesting that modifications made were not enough to render them protection-inducing and that further modifications, perhaps to TCR contacting residues, could be required like those made to **27616** and **27614**.

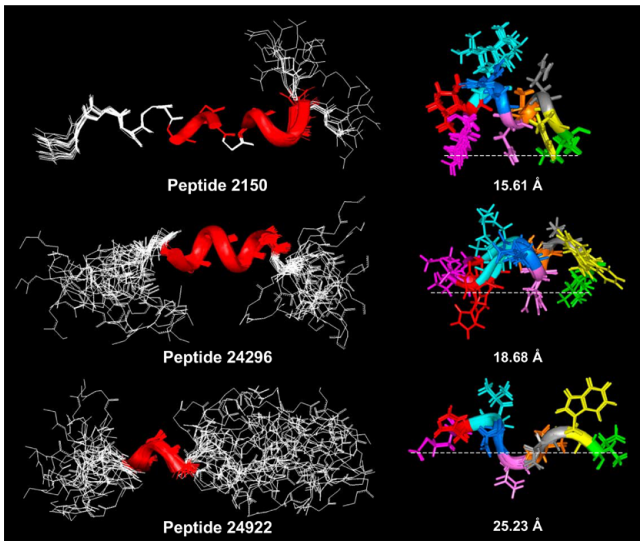

Fig. 3. Families of structures selected from non-immunogenic, non-protective **2150**, immunogenic, non-protective **24296**, and immunogenic, protective **24922** peptides. (Left) Backbone representation of analogue peptides; the core where the main modifications were made and the structured part of the peptides is shown in red. (Right) Ribbon representation of the 4–12 fragment. Color code: fuchsia—**M4**; red—**L5** (**2150**) and **H5** (**24922**, **24296**); turquoise—**K6** (**2150**), **L6** (**24922**), and **M6** (**24296**); blue—**E7**; pink—**N8**; light brown—**V9**; grey—**D10** (**2150**), P10 (**24922**), and A10 (**24296**); yellow—**Y11** (**2150**), **W11** (**24922**, **24296**); and green—**I12**.

Peptide **27614** also bound to HLA-DRB1*1101 molecules, suggesting that modifications had an effect on another functional part of the peptide, probably in TCR-contacting residues.

Peptide **26020** bound to HLA-DRB1*0301 molecules without inducing antibody production or being protection-inducing, suggesting that binding to this molecule is not enough and further modifications are needed for

Table 2
Summary of structure calculation results

Peptide Nr.		Peptide sequence No. of structures superimposed / 50 (structured segments shaded)	NOEs used	Rmsd Å	Maximum NOE violations Å	Maximum angular violations°	Immuno-genecity	Protection
2150	21	K M N M L K E N V D Y I Q K N Q N L F K	208(60)	0.2	0.3	2.0	–	–
24296	25	– – – –H M – – – A W – M – – – – – – –	233(94)	0.3	0.1	2.0	+	–
25852		– – – –H L – H – P W – M – – – – – – –	None				+	–
24922	23	– – – –H L – – – P W – M N K – – – – –	190(59)	0.5	0.1	2.0	+	+

Inter-residue NOEs are shown in parentheses.

Table 3
2150 peptide analogue HLA-DRβ1* allele binding activity

Peptide Nr.	Peptide Sequence	Percentage of binding to HLA-DRB1* molecules				Group
		DRB1*0101	DRB1*0301	DRB1*0401	DRB1*1101	
2150	K M N M L K E N V D Y I Q K N Q N L F K	6	14	4	10	
24922	– – – – H L – – – P W – M N K – – – – –	–5	**51**	–9	13	A
24296	– – – – H M – – – A W – M – – – – – – –	6	23	–10	30	B
25852	– – – – H L – H – P W – M – – – – – – –	–6	32	–1	34	B
27616	– – – – H M – – – P W – V – – – – – – –	0	**67**	28	20	B
27614	– – – – H M – – – P – – V – – – – – – –	–2	**82**	12	**84**	B
26020	– – – – H M – – – P – – M – – – – – – –	4	**64**	25	8	C
22912	K E – – – H M K – – – – – – – – – –	–23	44	12	1	C

These peptides having ⩾50% binding activity to a specific MHC-II molecule were considered to be specific binders. Peptides **24922**, **27616**, and **26020** were HLA-DR β1*0301, specific binders while **27616** bound promiscuously to HLA-DRβ1*0301 and HLA-DRβ1*1101.

Class-II molecule binding (perhaps for T-cell receptor interaction) for suitable immune system activation. Changes made to peptide **22912** produced a molecule which was not immunogenic or protection-inducing and did not bind to HLA-DRB1* molecules.

It can be clearly observed that peptide **24922,** which was rendered immunogenic and protection-inducing, bound specifically to HLA-DRB1*0301 molecules for which an equivalent molecule has been found in about 20% of the *Aotus* monkey population [20] and E. Trujillo et al., unpublished data.

As peptide **24922** is immunogenic and protection-inducing, this suggests that the residue in this peptide's amino-acid sequence binding to HLA-DRB1*0301 which fits into Pocket 1 could be M4, E7 in Pocket 4, V9 in Pocket 6, and I12 in Pocket 9. Amino-acids constituting specific motifs allowing binding to HLA-DRB1*0301 have been reported for Pocket 1 (L, I, M, F, V), Pocket 4 (D and E), Pocket 6 (K, R, E, Q, N, V), and Pocket 9 (L, Y, I, F) [21–23]. The residues present in these positions in peptide **24922** are underlined above.

Structural analysis by molecular modeling

Immunogenic, protection-inducing **24922** peptide docking studies have shown that there is an excellent, but not perfect, fit, between this peptide and the empty HLAβ1*0301 molecule [18] to which it binds. There is also a 2.7 Å RMSD between CLIP peptide backbone crystallized with HLA-DRβ1*0301 and peptide **24922** backbone when these two peptides are superimposed. This difference could have been due to both structures being determined by different methods (X-ray crystallography and ^{1}H NMR) or that **24922** structure was determined in solution whilst CLIP structure was determined by complexing it with the HLA-DRβ1*0301 molecule. These docking studies (Fig. 4) showed that residues M4, E7, V9, P10, and I12 fitted perfectly well and were buried inside Pockets 1, 4, 6, 7, and 9 leaving residues H5, L6, and W11 solvent-exposed. This led us to suggesting that when peptide **24922** bound to this allele the last mentioned residues (corresponding to positions P2, P3, and P8 in the peptide functional register) would be those which would be making contact with the TCR.

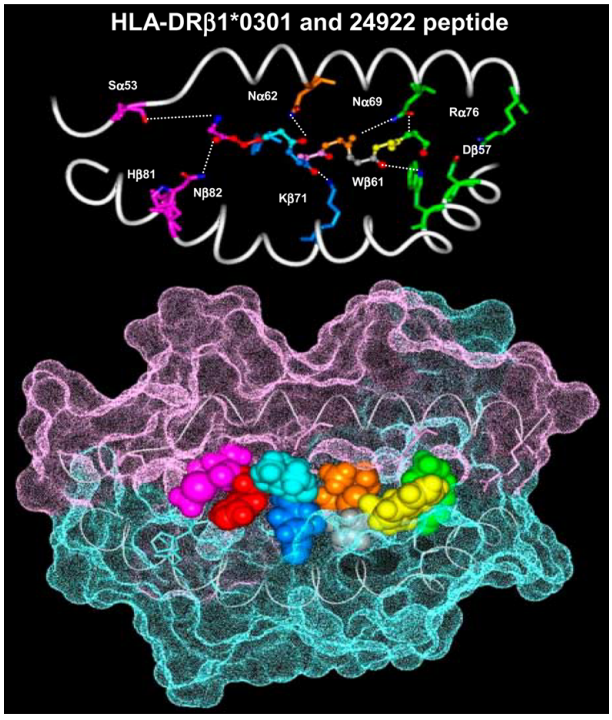

Fig. 4. Modified immunogenic and protection-inducing **24922** docking into HLA-DRβ1* binding groove. Ribbon representation of this peptide binding to its corresponding HLA-DRβ1*0301 molecule. The upper part of the figure shows putative H bonds in dotted white lines; distances were 2.0–5.0 Å between the **24922** peptide backbone atoms and atoms in the lateral chains of those residues making contact with the peptide. The lower part shows Connolly surfaces for HLA-DR α chain in pink and HLA-DR β chain in turquoise. Van der Waals surface for peptide **24922**. Color code for amino acids fitting into Class-II molecules: Fuchsia (Pocket 1), red (Pocket 2), turquoise (Pocket 3), blue (Pocket 4), rose (Pocket 5), light brown (Pocket 6), grey (Pocket 7), yellow (Pocket 8), and green (Pocket 9). Note the buried corresponding residues in Pockets P1, P4, P6, P7, and P9.

The distance between those residues making contact with Pockets 1 and 9 of the respective HLA-DR1*0301 to which protective and immunogenic peptide **24922** bind is 25.23 Å in Fig. 3, quite similar (25 Å ± 3) to that determined for peptide binding to Class-II molecules. Based on **24922** peptide tridimensional structure and peptide docking with the HLA-DRβ_1*0301 molecule without any further refinements (Fig. 4), we suggest that the hypothetical bonds (having distances between 2 and 5 Å) shown in the present work (some of them having characteristic H-bond distances and angles) are those established between the α chain and β-chain residue atoms making contact with those of the peptide backbone. It has been shown that peptides fitting properly into MHC Class-II molecule grooves have a polyprolinyl type II conformation [24]. Therefore, helical peptides may not fit well into these grooves due to their very compact structure. Reduction in a peptide's helical proportion, distorted β turn or 3_{10} helix formation may thus allow for a better fit with MHC Class-II molecules (as shown here), thereby activating the immune system, producing antibodies and inducing protection. These modifications make them excellent candidates for being included in a multi-component, subunit-based malarial vaccine.

Acknowledgments

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