Shifting the Polarity of some Critical Residues in Malarial Peptides' Binding to Host Cells is a Key Factor in Breaking Conserved Antigens' Code of Silence

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Abstract: As microbes use many mechanisms for avoiding immunological pressure, new strategies must be developed to bypass the immunological code of silence of conserved, functionally-important amino acid sequences, such as those involved in high activity binding peptides' (HABPs) attaching to their host cells.

Hundreds of experiments in large numbers of *Aotus* monkeys revealed that this immunological code of silence could be broken by shifting the polarity of some critical host cell binding residues in these HABPs by substituting F for R and *vice versa,* Y↔W, L↔H, I↔N, P↔D, M↔K or E, C↔T, V↔N or S; there are special rules for A, G and S. ^{1}H-nuclear magnetic resonance of these modified, immunogenic, protection-inducing HABPs and molecular modelling revealed that such modifications induced appropriate fitting into specific HLA-DRβ1* Pockets, suggesting the presence of new pockets and a haplotype- and allele-specific conscious TCR. A highly immunogenic and protection-inducing anti-malarial vaccine can thus be produced.

Key Words: Malaria, *P. falciparum*, ^{1}H-NMR, HLA-DRβ1* molecules, immunogenic peptides.

INTRODUCTION

Micro-organisms use a tremendous array of mechanisms for evading immunological pressure, such as genetic variability [1], interference with antigen presentation [2], immune cell apoptosis [3], "antigenic sin" [4], smokescreen sequences [5] etc., but perhaps the most commonly used is the conserved antigens' code of silence which must be broken for developing vaccines against diseases scourging humankind, such as malaria, tuberculosis, AIDS and hepatitis.

The synthetic SPf66 anti-malarial vaccine which did not induce protection in new born babies (as most vaccines do not) and a batch produced elsewhere, with a different degree of polymerisation, was not protective either, thereby dropping its protective efficacy. A recent meta-analysis [6] however gave 38.8% and 35% protection-induction in Colombia [7], 55% in Venezuela [8], 60.8% in Ecuador [9] and 31% in Tanzania in people aged more than 1 [10] for a period of up to 2 years [7].

SPf66 is a polymerised 45-mer chimeric synthetic hybrid protein composed of 3 merozoite proteins (one derived from MSP-1 and the other two from 55kDa and 35 kDa proteins amino terminal sequences) and one sporozoite protein (the circumsporozoite protein NANP sequence) intercalated twice in this synthetic protein amino acid sequences. The *P. falciparum* merozoite invades red blood cells or RBCs whilst the sporozoite invades the liver or hepatic cells, making this the first multi-antigenic, multistage chemically-synthesised antimalarial vaccine.

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‡ This paper is dedicated to the memory of Prof. Bruce Merrifield who taught us all we know about protein synthesis.

In binding assays performed years ago [11], it was found that 2 out of the 3 merozoite-derived sequences bound to RBCs with high affinity, one of them being semi-conserved. This suggested that the protective efficacy induced by this synthetic vaccine could be a consequence of the immune response elicited against these binding sequences, especially the conserved ones.

Based on these findings, merozoite protein-derived conserved amino acid sequences which bind to RBCs with high affinity (high affinity binding peptides or HABPs) were identified by our Institute in more than 50 proteins involved in merozoite invasion to RBCs as possible targets for inducing an appropriate immune response able to block it [12-16]. These conserved HABPs were synthesised in polymeric form and used to immunise hundreds of *Aotus* monkeys (primates which are highly susceptible to developing human malaria) [17, 18]. However, conserved HABPs were immunologically silent, since no immune responses were elicited against anyone of them nor protection was induced upon immunisation. Furthermore, it has been widely documented that *P. falciparum* protein conserved sequences are poorly antigenic [19-22].

Simultaneously, the extensive field-trials conducted with SPf66 in different parts of South-America have revealed that immune response against this chemically synthesised malarial vaccine was under strong genetic control, associated with major histocompatibility complex (MHC) Class II molecules, since most individuals (~70%) who have <u>not</u> responded to immunisation with this vaccine carry the HLA-DRβ1*04 genetic marker associated with T-lymphocyte receptor (TCR) Vβ3, 10 and 11 preferential usage [23].

This data suggested imperfect peptide interactions in such non-responding individuals when the MHCII-peptide-

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TCR complex was being formed, leading us to suggest that modifying such characteristics allowing these HABPs (modified HABPs) to fit properly into such MHC-peptide-TCR complex could lead to the breaking of the immunological code of silence.

Predicting that this problem lay in their physicochemical structure, critical amino acids (determined by glycine analogue scanning) were replaced by glycine, originally with ambiguous results. Molecular mass and volume were then modified with continuing negative results. This was followed by charge modification, also being accompanied by disappointing negative results. However, some of these modified conserved HABPs were reproducibly able to induce antibody production and protective immune responses, in some monkeys, when polarity was shifted in some of these critical residues, thereby allowing us to break the conserved antigens' code of silence, as shown here and for many individual peptides thoroughly described [24-32].

Simultaneously, binding assays were undertaken to find out whether these native and modified immunogenic and protection-inducing HABPs bound to purified HLA-DRβ*1 molecules, as a possible explanation of this immunological silence and how it might be broken.

Serological studies (confirmed later on by molecular biology) have described 16 large groups of HLA-DRβ1* molecule alleles (denominated HLA-DRβ1*01 to HLA-DRβ1*16 in humans), with more than 250 variants. Five large groups of these HLA-DR haplotypes have also been evolutionarily, serologically and molecularly grouped with the presence of a variable number of expressed HLA-DRβ*, alleles forming each haplotype. Such haplotypes are called HLA-DR1 (including DRβ1*01, 10 and 103 alleles), HLA-DR51 (specifically grouping DRβ1*15 and DRβ1*16), HLA-DR52 (including alleles DRβ1* 03, 11, 12, 13, 1403 and 1404), HLA-DR8 (exclusively including the DRβ1*08 allele) and the HLA-DR53 haplotype (grouping alleles DRβ1*04, 07 and 09) [33].

Cloning and DNA sequencing studies carried out on 110 *Aotus* revealed that Class II molecules encoded by the *Aotus* gene equivalent to human HLA-DRβ generally presented 84% to 100% homology with their human counterpart HLA-DRβ1* 04, 03, 08, 11, 12, 13, 14, 07, 15, 16, 10 and 01 molecules [34]. This homology was particularly high with some HLA-DRβ1* allele pockets, such as HLA- DRβ1* 04 (97 to 100%) and HLA- DRβ1* 03 (93 to 95%), suggesting that peptides accommodating into these *Aotus* monkeys' peptide binding regions (PBR) are very similar to those for humans and that such modified HABPs could be practically used with minor modifications for human use. This therefore makes the *Aotus* monkey an excellent model for developing human anti-malarial vaccines.

HLA-DRβ1*-related molecules' allele frequency in the *Aotus* was ~25% for HLA-DRβ1*0403/0407, HLA-DRβ1* 0422; HLA-DRβ1*0301; HLA-DRβ1*15-16 related alleles, ~10% for HLA-DRβ1*08, 11, 12, 13, 14 group, ~7% for HLA-DRβ1*1001 and HLA-DRβ1*0101 alleles and ~4% for HLA-DRβ1*0701. It is thus not surprising that the maximum percentage of monkeys protected was 25% per individually modified HABP (this being the maximum percentage of that allele to which it can bind).

Trying to find an association between structure and immunological performance, structural analysis by ^{1}H-NMR revealed striking differences between native and immunogenic protection-inducing modified HABPs [24-32].

Based on the information disclosed here, we can state that shifting the polarity of some critical binding residues to RBCs, made them immunogenic and protection-inducing and allow them to form a more appropriate MHC II-peptide-TCR complex. This approach enable us thereby to break the conserved antigens' code of silence and to establish emergent rules for the appropriate design of vaccines for human use such as that against malaria which affects around 500 million people per year, killing ~2.5 million of them [35].

RESULTS

Peptide Analysis

Analytical chromatography results showed that peptide purity after semi preparative RP-HPLC was sufficiently high to be analyzed by 1H-NMR. Peptides are numbered according to our Institute's code where bold is used through the rest of this article to distinguish modified HABPs from native ones (given in brackets following their mention).

The polymers used for immunisation had molecular weights in the 8 kDa to 24 kDa range as assessed by Size Exclusion Chromatography (SEC). Monomer and polymer peptide CD spectra in trifluoroethanol/H_2O solution (30%) (Fig. **1**) showed that both molecules had similar secondary structures, suggesting that the configuration adopted by polymers used for immunising *Aotus* monkeys to render them immunogenic (as assessed by IFA and Western blot) and protection-inducing was quite similar to the structure of monomer analogues used for structural (CD, ^{1}H-NMR) and biological analysis (red blood cell and HLA-DRβ1* binding). Later 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, therefore they can be stabilised into well-ordered conformations [36]. New peptides 6673, **14532, 13830** and **9928** have been included in this manuscript to complete the general overview; they all presented two negative minima centred at around 208nm and 222nm, indicating their tendency to display an α-helical structure.

Immunological Analysis

Some critical residues in RBC binding were replaced by glycine (the smallest amino acid and an α-helix breaker) in the first trials, trying to convert these conserved native HABPs non-immunogenic and non-protection-inducing into immunogenic and protection-inducing ones (Table **1**, Group A).

Their ability to induce antibodies against the *P. falciparum* malaria parasite was assessed by Immunofluorescence (IFA) with air dried unfixed parasite cultures and native proteins as determined by Western blot with merozoite lysates. Their protective capacity was tested when these monkeys

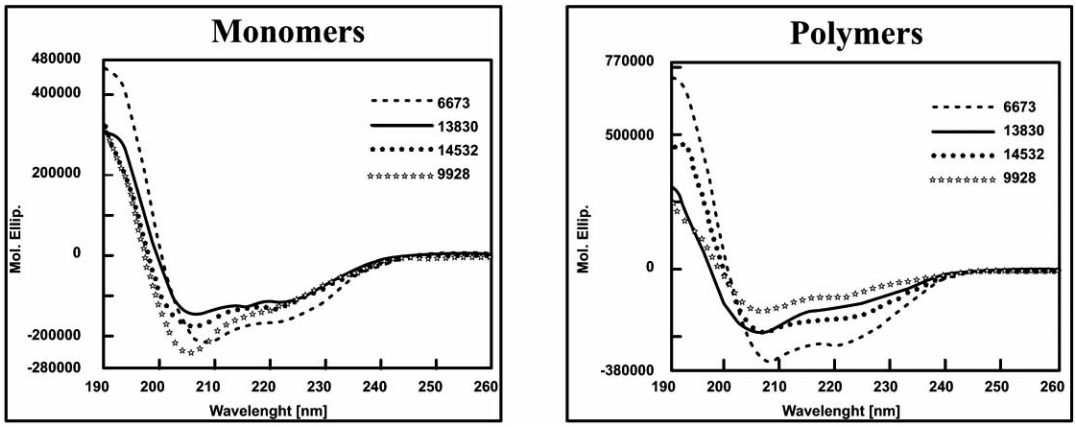

Fig. (1). Circular dichroism results for monomer peptides 6673, **13830, 14532, 9928** and their polymers.

were experimentally challenged with a 100% infective *Aotus*-adapted strain of this parasite (FVO) [17].

It sometimes, but not consistently, induced immunogenicity and protection in some monkeys (Table **1**, Group B) with some peptides [25, 27] but not with others [37] suggesting that extra factors besides changing residues could be involved in breaking these conserved antigens' (HABPs) immunological silence (Table **1**, Group B).

For the sake of brevity, after several different and combined trials (Groups C and D), it was found that the key determining factor was the shift in polarity (maintaining mass and volume) of some of the critical binding residues (Table **1**, group E). These modifications consistently and reproducibly induced immunogenicity in some monkeys since antibodies against the complete parasite (as assessed by IFA) and the corresponding proteins (determined by Western blot) were induced and total protection was ensured against experimental challenge in some *Aotus* monkeys as shown in Table **1**, Group E.

Such experiments with these immunogenic, protection-inducing, modified HABPs were repeated with new groups of *Aotus* monkeys twice to three times with similar results (Table **1**, Group E experiments b with the corresponding peptide). The conserved antigens' code of silence had thus been broken.

The fine tuning of the phenomenon after hundreds of experiments performed on large numbers of *Aotus* monkeys as described in previous papers for individual peptides [24-32] and the new ones shown in Table **1**, led to us ascertain that some critical binding residues had to be replaced by others specifically and reciprocally shifting their polarity but maintaining their other physicochemical characteristics such as mass, volume, surface, etc. (as shown here for the first time). F could thus be replaced by R and *vice versa*; L↔H, P↔D, W↔Y, M↔K and E, I↔N, T↔C and V↔N or S.

Serine, alanine and glycine remained undefined due to their very small mass, similar polarity, small volume and the absence of counterparts with opposite polarity as occurs with the other amino acids. A↔S sometimes worked, suggesting that some undefined rules for these small residues still have to be identified.

Structural Analysis

The three-dimensional structure of native, modified non-immunogenic and modified immunogenic and protection-inducing HABPs was determined by ^{1}H-NMR studies in the search for an association between these chemical modifications and immunological functions. All peptides were dissolved in (TFE-d_3)/H_2O mixture (30/70 v/v). The 3D structures of the following native peptides and analogues have already been reported; **17912** (1783) [27], **24310** (6762) [28], **23790**, **24216** (6746) [29], **10000, 13492** (6671) [30], **24112** (4044) [28], **13768, 10022** (4313) [25], 20034 (4325) [38] and **24166** (1818) [38].

NOESY spectra for HABPs **14532, 13830, 9928** and 6673 (all being first described in this manuscript) and which allowed us to complete the chemical and structural rules to break the immunological code of silence, showed sequential, short and medium range $d_{NN}(i,i+1)$, $d_{\alpha\beta}(i,i+3)$, $d_{\alpha N}(i,i+3)$ and $d_{\alpha N}(i,i+4)$ NOE connectivity, lowered amide proton chemical shift temperature coefficients for some of the amino acids and < 6 Hz $^3J_{HN\text{-}H\alpha}$ coupling constant values, suggesting the presence of an α-helix structure (Fig. **2**).

Families of 25, 22, 26 and 17 low energy conformers, chosen from the 50 resulting ones, were obtained for peptides **14532, 13830**, **9928** and 6673, respectively. The chosen structures had no distance violation larger than 0.30 Å or ω angle greater than 1.5°. Average root mean square deviations (RMSD) for main-chain atoms were 0.30Å for peptides **14532** and 6673, 0.20Å for peptide **13830** and 0.25Å for peptide **9928.** These values were obtained by superimposing the structures between amino-acids G6-M21 in peptide 6673**,** F3-M20 in peptide **14532**, G2-D17 in peptide **13830** and N6-I18 in peptide **9928**. The DSSP programme [39] was used for providing a clearer analysis of how helical fragments could be defined in the peptides.

Changes in the 3D structural characteristics of modified HABPs were observed, such as α-helix shortening as in **9928** (N6-I18) when compared to its parental native HABP 1783 (N6-K20), or displacement of the α-helix as in the non-immunogenic, non-protective analogue **17912** (D2-I13). Shortening was also observed in **13830** (G2-D17) when compared to the non-immunogenic **14532** (F3-M20) 6673 analogue, as well as in **24310** (M2-H15) when compared to

Table 1. New Panel of Native and Modified HABPs' Amino Acid Sequences (Critical Residues are Underlined). Our Institute's Sequential Numbers for Native and Non-Immunogenic, Non-Protection-Inducing Modified HABPs are Shown in Normal Type, whilst Immunogenic and Protection-Inducing HABP Numbers are Shown in Bold. The Table also Shows the Modifications Made to the Amino Sequences.

Amino-acid sequences have been aligned according to the residue corresponding to immunogenic, protection-inducing, modified HABP binding motifs and reading registers binding to HLA-DRβ1* in Pockets 1, 4, 6 and 9. PI, PII_{15}, $PIII_{15}$ are the bleeding days where antibodies titres were determined by IFA corresponding to reciprocal sera dilution and the number of *Aotus* monkeys developing such antibodies. Prot = number of protected monkeys.

PEPT	SEQUENCE	PI	II_{15}	III_{15}	Prot	Group
	P1 P4 P6 P9					
EBA						
1783	**HRNKKNDKLYRDEWWKVIKK**	0	0/5	0/5	0/5	A
7570	**G**RNKKNDK**L**YR**D**E**G**WK**V**IKK	0	0/5	0/5	0/4	B
7382	HRNKKNDK**L**YR**D**E**G**WK**G**IKK	0	0/5	0/5	ND	B
17912	NDK**L**YR**D**E**K**WK**D**IKK	0	0/5	0/5	0/5	C
9928a	HRNKKNDK**L**YR**D**E**Y**WK**N**IKK	0	0/5	1(2560)	1/3	E
9928b	HRNKKNDK**L**YR**D**E**Y**WK**N**IKK	0	1(2560)	2(2560)	2/8	E
RESA						
6673	**YLGRSGGDIIKKMQTLWDEIM**	0	0/5	0/5	ND	A
9268	YLG**G**SGGDIIKKMQTLWDEIM	0	0/5	0/5	0/5	B
9270	YLGR**G**GGDIIKKMQTLWDEIM	0	0/5	0/5	0/5	B
9276	YLGRSGGDIIKKMQ**G**LWDEIM	0	0/5	0/5	0/5	B
9278	YLGRSGGDIIKKMQT**G**WDEIM	0	0/5	0/5	0/5	B
9280	YLGRSGGDIIKKMQTLWDE**G**M	0	0/5	0/5	0/5	B
16010	LG**F**SGGDIIKKMQ**VH**WDE**NE**	0	0/5	0/5	0/5	C
16012	LG**FA**GGDIIKKMQ**VH**WDE**DE**	0	0/5	0/5	0/5	C
13826	LGRSGGDIIKKMQ**AH**WDE**N**M	0	0/5	0/5	0/5	C
14532	LG**FV**GGDI**N**KKMQ**VH**WD**MN**M	0	0/5	1(640)	0/5	D
13830a	LG**FV**GGDIIKKMQ**AH**WDE**N**M	0	0	1(160)	1/5	E
13830b	LG**FV**GGDIIKKMQ**AH**WDE**N**M	0	ND	2(160)	1/5	E
SERA						
6762	**NEVSERVHVYHILKHIKDGK**	0	0/5	0/5	0/5	A
12756	**IM**SERVHVYHILKHIKDGK	0	0/4	0/4	0/4	C
12758	**IN**SERVHVYHILKHI**M**DGK	0	0/4	0/4	0/4	C
13502	**EN**SERVHVYHILKHIKDGK	0	0/6	0/6	0/6	C
13504	**EN**SERVHVYHILKHI**M**DGK	0	0/6	0/6	0/6	C
14102	**M**VSERVHVYHILKHI**M**DGK	0	0/6	0/6	0/6	D
14106	**MS**SERVHVYHILKHI**M**DGK	0	0/6	0/6	0/6	D
14108	**MT**SERVHVYHILKHI**M**DGK	0	0/6	0/6	0/6	D
24310a	N**M**VSERVHVYH**N**L**M**HIKDGK	0	1(320)	ND	1/6	E
24310b	N**M**VSERVHVYH**N**L**M**HIKDGK	0	1(640)	ND	1/6	E
SERA						
6746	**DQGNCDTSWIFASKYHLETI**	0	0/5	0/5	0/5	A
20468	DQG**IS**DTSW**N**FAAKY**L**LETI	0	0/7	0/7	0/5	D
20470	D**M**G**IS**DTSW**N**FAAKY**L**LETI	0	0/7	0/7	0/6	D
24998	DQGN**T**I**T**AW**N**R**A**AK**W**HLETI	0	0/6	0/6	0/6	D
24214	DQGN**S**I**T**AWIR**A**AK**F**HLETI	0	0/10	ND	0/9	D
23790	DQGN**S**I**T**AW**N**R**AM**YHLETI	0	0/10	ND	0/8	D
24216	DQGN**T**I**T**AW**N**R**A**AK**F**HLETI	0	5(320)	ND	2/9	E
RESA						
6671	**MTDVNRYRYSNNYEAIPHIS**	0	0/5	0/5	0/5	A
15534	MTDV**I**RYRYSNNYE**SN**PHIS	0	0/5	0/5	0/5	D
10002	MTDVNRYRYSNNYE**ST**PHIS	0	0/5	0/3	0/2	D
13494	MTDV**I**RYRYSNNYEA**ES**HIS	0	4(5120)	1(320)	0/2	E
13492a	MTDV**I**RYRYSNNYEA**SD**HIS	0	2(5120)	ND	1/6	E
13492b	MTDV**I**RYRYSNNYEA**SD**HIS	0	2(5120)	0/6	1/6	E
MSP-2						
4044	**KNESKYSNTFINNAYNMSIR**	0	0/5	0/5	0/5	A
9936	KNESKYSNTF**ER**NAYNMSIR	0	0/5	0/5	0/5	C
9938	KNESKYSNTF**EM**NAYNMSIR	0	0/5	0/5	0/5	C
9940	KNESKYSNTF**QR**NAYNMSIR	0	0/5	0/5	0/4	C
11330	K**MI**SK**F**SNTF**EV**NAYNMSIR	0	0/5	0/4	0/4	D

(Table 1. Contd….)

11332	**MI**SK**R**SNTF**EV**NAYNMSIR	0	0/5	0/4	0/3	D
11868	**VI**SKYSNTF**EV**NAYNMSIR	0	0/4	0/4	0/4	D
11870	**VI**SK**W**SNTF**EV**NAYNMSIR	0	0/6	0/6	0/6	D
13744	**IM**SKYSNTF**EV**NAYNMS**EM**	0	0/5	0/4	0/4	D
15986	**IM**SK**WG**NTF**DD**NAYNMSIR	0	0/4	0/4	0/3	D
15988	**IM**SK**WG**NTF**NI**NAYNMSIR	0	0/6	0/6	0/6	D
15992	**IM**SK**WG**NTFI**D**NAYNMSIR	0	0/5	0/4	0/4	D
15498	**IM**SK**WA**NTF**NI**NAYNMSIR	0	0/5	1(5120)	1/3	E
24112a	SKYSNTF**NI**NAYNM**V**IRRSM	0	1(1280)	ND	1/8	E
24112b	SKYSNTF**NI**NAYNM**V**IRRSM	0	2(1280)	ND	2/10	E
AMA-1						
4313	**DAEVAGTQYRLPSGKCPVF**	0	0/5	0/5	0/5	A
15996	DAEVAGTQY**F**LPSGK**T**PVFG	0	0/6	0/6	0/6	D
15994	DAEVAGTQ**WF**LPSGK**T**PVFG	0	0/6	0/6	0/6	D
10024	DAEVAGTQY**FD**PSGK**S**PVFG	0	0/4	0/4	0/4	C
10026	DAEVAGTQY**FN**PSGK**S**PVFG	0	0/5	0/5	0/5	D
15998	DAEVAGTQ**W**RLPSGK**T**PVFG	0	0/6	0/6	0/6	C
14024	DAEVAGTQ**WFH**PSGK**S**PVFG	0	0/5	0/4	0/3	D
13768	DAEVAGTQ**WFN**PSGK**S**PVFG	0	0/6	0/6	0/6	D
10022	DAEVAGTQY**FH**PSGK**S**PVFG	0	0/5	1(5120)	1/5	E
AMA-1						
4325	**MIKSAFLPTGAFKADRYKSH**	0	0/5	0/5	0/5	A
9196	MIKSAFL**V**TGAFK**G**DRYKSH	0	0/5	0/5	0/5	B
13484	MIKA**S**F**H**PTGAFK**S**PRYKSH	0	0/6	0/5	0/5	E
16002	MIKS**S**F**H**PTGAFKADRYKSH	0	0/6	0/5	0/5	E
16008	MIKSAF**H**PTGAFKA**P**RYKSH	0	0/5	0/5	0/5	E
16004	MIKS**S**F**H**PTGAFKA**P**RYKSH	0	0/5	0/5	0/5	E
20034	MIKAAFLPTGAF**M**ADRYKSH	0	2(320)	1(160)	2/8	E
EBA						
1818	NNNFNNI PSRYNLYDKKLDL	0	0/5	0/5	0/5	A
24166	FNNIPSRYNLYDKMLPLDD	0	1(320)	2(640)	2/5	E

Grouping was as follows: A = native, B = glycine replacement, C = mass difference replacement, D = charge difference changes, E = replaced by an amino acid having opposite polarity, but similar mass and charge.

its parental 6762 (E2-K17) or **24216** (W9-H16) when compared to non-immunogenic **23790** (N4-L17).

Inducing short α-helical regions in peptides having β-turn tendency such as 6671 (as determined by CD analysis), in **13492** (V4-R8), or in the non-immunogenic, non-protection-inducer **10000** (T2- Y9) was also detected. Formation of distorted β-turns was also observed in **24112** (Y3-T6) *cf* 4044**,** (S7-F10) and in **10022** (T7-F10) *cf* 4313 having random structure. Peptide **24166** adopted a classical type III β turn (S9-N12) followed by a distorted type III β turn (Y14-M17) whilst parental native 1818 had an undefined structure by CD analysis.

This manuscript compares native or non-immunogenic, non-protection-inducing HABPs with immunogenic protection inducing modified HABPs for which the 3D structure was determined, seeking to establish an association between structure and immunological performance.

Class II Molecules' Binding

Promiscuous binding to some HLA-DRβ1* alleles was observed in non-modified native HABPs such as 1783, 6746, 6671 and 4325. The lack of binding to any of the alleles studied here (i.e. 6762, 4044, 4313 and 1818) or to a single allele (such as 6673 which binds to HLA-DRB1*11) was also observed in some of these native HABPs.

Structural Modifications Leading to Immunogenicity and Protection

When modifications were performed to render these conserved HABPs immunogenic and protection-inducing, it was observed that these modified HABPs' binding had become dramatically limited to a single specific allele as **9928** (1783) bound exclusively and particularly to HLA-DRβ1*0301 just like **24216** (6746) to HLA-DRβ1* 1101 and **13492** (6671) to HLA-DRβ1* 0401, whilst others which previously had not bound to any HLA-DRβ1* acquired the ability to bind specifically to a determined allele, such as **24310** (6762) to HLA-DRβ1* 0301; **24112** (4044) to HLA-DRβ1* 0401; **10022** (4313) to HLA-DRβ1* 0701 and **24116** to HLA-DRβ1*0101 and HLA-DRβ1*1101. Others bound specifically to two alleles from the same haplotype, such as **13830** to HLA-DRβ1* 0301 and HLA-DRβ1* 1101; and **20034** (4325) which changes its ability to bind to HLA-DR52 alleles to binding to alleles from HLA-DR53 (Table **2**).

In essence, reducing the number of alleles to which an unmodified HABP could bind (promiscuity) or inducing the ability to bind to a determined and specific allele to which it did not bind before or changing binding to a different haplotype seemed to be associated with the ability to induce a protection-inducing immune response.

Binding Motifs

All modified HABPs binding to HLA-DRβ1* 0301 displayed the binding motifs and reading-register characteristic for these alleles; [40] therefore peptides **9928, 13830** and **24310,** displayed L, V and V to fit into Pocket 1; D, D, and N into Pocket 4; Y, I, M into Pocket 6 and N, M, K into Pocket 9. Something similar occurred with modified HABP **24216** binding to HLA-DRβ1* 1101 with residues W, A, K, L fitting into Pockets 1, 4, 6 and 9 respectively, these being

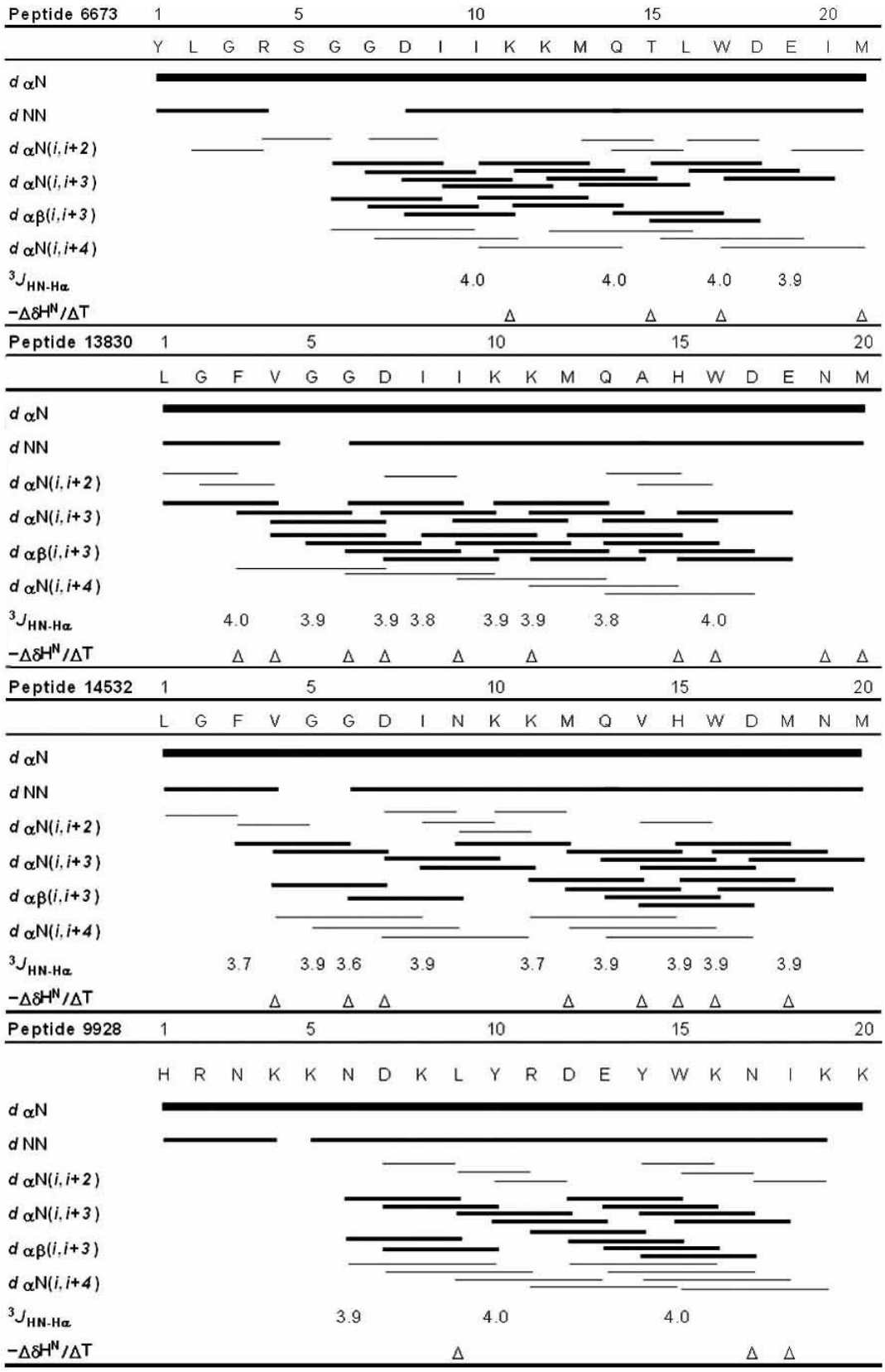

Fig. (2). Summary of sequential medium range NOE connectivity (NOE intensities are represented by different line thickness). $^{3}J_{HN\text{-}H\alpha}$ are the coupling constants. Low HN amide temperatures coefficients are indicated by their value.

Table 2. Conserved HABP Structural Features and those of their Analogues as Determined by ^{1}H-NMR; Biological Activity as Assessed by the Percentage of their Ability to Bind to Purified HLA-DRβ1* Molecules (Affinity ≥ 50% is Shaded) Grouped According to their Corresponding Haplotypes. Inter-Atomic Distance is Given in Å between the most Distant Hydrogen of Residues Fitting into Pocket 1 to Pocket 9 of Corresponding HLA-DRβ1* Alleles. TCR Putative Contacting Residues are also Indicated.

Protein	Peptide	Structural features	Distance (Å)	DR1	DR52		DR53		TCR con-tact.	Ref. for 3D Struct.
				% Binding			HLA-DRB1			
					alleles					
				0101	0301	1101	0401	0701		
EBA-175	1783	α-helix N6-K20	18.7	3	**49**	**65**	-2	7		27
	17912	α-helix D2-I13	17.5	4	15	17	4	33		27
	9928	α-helix N6-I18	17.8	4	**51**	22	4	ND	3, 7	New
RESA-155	6673	α-helix G5-M20	16	-2	-10	**48**	20	35		New
	14532	α-helix F3-M20	15.5	-15	32	1	**83**	30		New
	13830	α-helix G2-D17	18.9	6	**85**	**52**	1	30	3, 7	New
SERA	6762	α-helix E2-K17	19.3	2	-5	3	3	28		28
	24310	α–helix M2-H15	19.8	-1	**60**	35	17	26	3, 7	28
SERA	6746	helix tendency (CD)	–	-15	**88**	**86**	-10	42		29
	23790	α-helix N4-L17	15.3	-6	**55**	40	10	ND		29
	24216	α-helix W9-H16	22.4	-27	37	**44**	5	ND	3, 7	29
RESA-155	6671	β-turn tendency (CD)	–	24	45	16	**61**	**55**		30
	10000	α-helix T2-Y9	18.4	3	17	**73**	11	**72**		30
	13492	α-helix V4-R8	24.7	-5	33	16	**50**	16	2	30
MSP-2	4044	Classical type III′ β–turn S7-F10	19	5	0	-60	38	39		25
	24112	Distorted type III′ β–turn Y3-T6 and	26.5	9	1	0	**53**	46		28
		Classical type III β–turn A11-M14							2	
AMA-1	4313	Random	–	0	26	26	9	23		25
	13768	Classical type III β–turn T7-F10	19.3	0	8	**44**	1	15		25
	10022	Distorted type III′ β–turn T7-F10	25	9	7	4	2	**42**	2, 8	25
AMA-1	4325	α-helix K13-R16	21.5	18	**83**	**74**	28	48		38
	20034	α-helix K3-F6 and A11-R16	25.5	40	34	7	**69**	**64**	2, 8	38

(Table 2. Contd….)

Protein	Peptide	Structural features	Distance (Å)	DR1	DR52		DR53		TCR con-tact.	Ref. for 3D Struct.
				% Binding			HLA-DRB1			
					alleles					
				0101	0301	1101	0401	0701		
EBA-175	1818	Random	–	2	33	6	11	36		44
	24166	Classical type III β–turn S9-N12 and	24.9	**53**	5	**69**	18	38	5, 8	44
		distorted type III β–turn Y14-N17								

characteristic binding motifs for these alleles which belong to the HLA-DR52 haplotype [33, 40] (Table **1**).

Immunogenic, protection-inducing HAPB binding motifs, characteristic for HLA-DRβ1* 0401 observed in peptides **13492** and **24112,** were Y and Y for Pocket 1; N and V for Pocket 4; E and R for Pocket 6 and D and M for Pocket 9. By the same token, modified HABP **10022** and **20034** residues F and F will fit into Pocket 1 for HLA-DRβ1* 0701; S and T into Pocket 4; K and A into Pocket 6 and V and A into Pocket 9. These amino acids were characteristic binding motifs for these alleles, being members of the HLA-DR53 haplotype.

Modified HABP **24166,** the only one binding with high affinity to HLA-DRβ1* 0101 (this being a member of the HLA-DR1 haplotype) and HLA-DRβ1*1101, displayed Y, M, P and D for Pockets 1, 4, 6 and 9 respectively. The presence of P fitting into Pocket 6 determined the preference of this peptide for HLA-DRβ1*0101 since HLA-DRβ1*1101 prefers large positively-charged residues (R, K, H) for this Pocket whilst HLA-DRβ1*0101 prefers small apolar residues (A, G, S, T, P). This binding motif is characteristic of this allele [40] (Table **1**).

Distance Between Residues Fitting into Pockets 1 to 9

Distance measurements between the furthest H atoms of those residues fitting into Pockets 1 to 9 showed clear differences between modified, immunogenic, protection-inducing HABPs binding to alleles from different haplotypes. Distances were 17.8 Å, 18.9 Å and 19.8 Å for peptides **9928, 13830** and **24310,** respectively, binding to HLA-DRβ1* 0301 and 22.4 Å for peptide **24216** binding to HLA-DRβ1* 1101, both being members of the HLA-DR52 haplotype (Fig. **3a**, left-hand panel).

Modified HABPs **13492** and **24112** binding to HLA-DRβ1* 0401 had 24.7 Å and 26.5 Å distances, respectively, and **10022** and **20034** binding to HLA-DRβ1* 0701 had correspondingly 25.0 Å and 25.5 Å distances. All these peptides bound to allele molecules belonging to the HLA-DR53 haplotype (Fig. **3a**, left-hand panel).

The only peptide binding to HLA-DRβ1* 0101 (haplotype HLA-DR1), **24166,** had a 24.9 Å distance between the furthest atoms fitting into Pockets 1 to 9 [38].

In essence, there is a 19.2 Å ± 2.5 Å distance between the furthest H atoms of residues fitting into Pockets 1 to 9 of immunogenic, protection-inducing, modified HABPs binding to HLA-DR52 alleles whilst the measurement of the same distance was 25.4 Å ± 1.5 Å for those binding to HLA-DR53 alleles and 24.9 Å for the only one binding to the HLA-DR1 allele. These distances were 2.5 ± 1.0 Å and 4.5 ± 1.5 Å shorter in native and non-immunogenic, non-protection-inducing native or modified HABPs binding to HLA-DR52 and HLA-DR53 allelic molecules, respectively.

Residue Orientation

Although side-chain conformation reliability is limited by relatively high conformational freedom, molecular modelling of peptides whose structure was determined by ^{1}H-NMR clearly showed that when frontally viewing peptides binding to HLA-DR52 haplotype alleles HLA-DRβ1* 0301 and HLA-DRβ1* 1101, residue corresponding to P7 (shown in grey) was upwardly orientated, towards the centre or the left hand-side of the molecule in modified, immunogenic, protection-inducing HABPs **9928, 13830, 24310** and **24216**; whilst in non-immunogenic, non-protection-inducing **17912, 14532** or **23790,** as well in native 6762 HABP, this residue was orientated towards the right in the molecule's frontal view (Fig. **3b**). This suggested that a different spatial orientation for this P7 residue enables TCR contact interaction.

Similarly, in all the above-mentioned peptides binding to HLA-DR52 haplotype alleles, residue corresponding to P2 (red) was localised in the same plane for residues fitting into Pocket 4 (dark blue) and Pocket 6 (brown) of this Class II molecule's groove, or below, suggesting the existence of a Pocket 2 in these molecules.

Amino acids corresponding to P7 in peptides **13492** and **24112** binding to HLA-DRβ1*0401 were downwardly orientated and to the right in the frontal view of these modified HABPs and downwardly orientated but to the left in peptides **10022** and **20034** binding to HLA-DRβ1*0701, suggesting a Pocket 7 in these HLA-DR53-related molecules.

Similarly, when frontally were viewing immunogenic, protection-inducing, modified HABPs **13492** and **24112** binding to HLA-DRβ1*0401, residue P2(red) was orientated up and to the left whilst in the non-immunogenic, non-protection-inducing

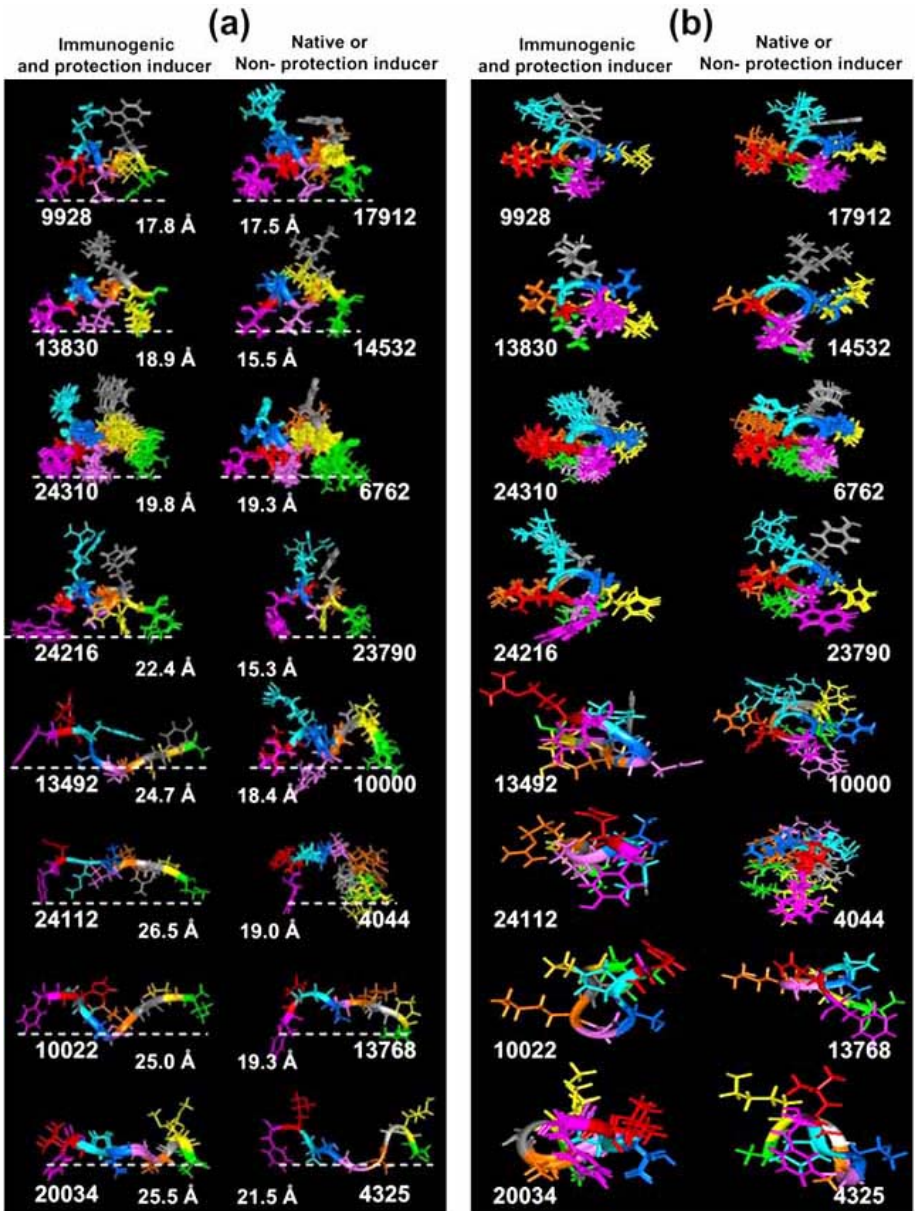

Fig. (3). Lateral (a) and frontal (b) view of immunogenic, protection inducing (left-hand panels) and native or non-immunogenic modified HABPs (right-hand panels) amino acid side chains as determined by ^{1}H-NMR. Residues were aligned according to the corresponding HLA-DRβ1* molecule's binding motif for the immunogenic protection-inducing modified HABP which binds to it. In all peptides shown, fuchsia corresponds to Pocket 1, red (P2), turquoise (P3), dark blue (Pocket 4), rose (P5) orange (Pocket 6), grey (P7), yellow (P8), green (Pocket 9). The lateral view (a) shows the 19.1 ±2.5Å distance between the most distant atoms of residues fitting into Pocket 1 and Pocket 9 in HLA-DR52 haplotype binding immunogenic protection-inducing HABPs. This distance was 25.4 ±1.5Å between the same atoms in the HLA-DR53 haplotype-binding immunogenic protection-inducing HABPs. This distance was 2.5Å shorter for native and non-immunogenic, non-protective HLA-DR52-associated peptides and 4.5±1.5Å shorter for native non-immunogenic, non-protective HLA-DR53 associated peptides. The front view shows the 3D structure of the immunogenic protection-inducing modified HABPs belonging to the HLA-DR52 haplotype. The orientation of P2 (red) in the same plane as the canonical P4 (dark blue) and P6 (orange) HLA-DRβ1* binding residues suggests the existence of an extra P2 pocket in these alleles. The same holds true for P7 (grey) in the HLA-DR53-associated protection-inducing modified HABPs, suggesting the existence of an extra Pocket 7 in these alleles.

The orientation of residues pointing upwards and hypothetically making contact with the TCR is worth noting in the front view (b). The P7 (grey) residue has left-hand orientation in the immunogenic protection-inducing HABPs belonging to HLA-DR52 whilst the same P7 (grey) residue is orientated towards the right in native or non-protective peptides associated with the same haplotype. It can be observed that the P2 (red) residue has left-hand orientation in HLA-DRβ1*0401 binding immunogenic protection inducing modified HABPs whilst being orientated towards the right in the HLA-DRβ1*0701 immunogenic protection-inducing peptides. All this structural information predicts an allele and haplotype-conscious TCR.

10000 peptide and native 4044 this residue was orientated on the same plane of Pockets 4 and 6 and towards the left.

There was also a striking change in immunogenic, modified peptides **10022** and **20034** HLA-DRβ1* 0701 binding in residue P2 (red) which was upwardly orientated and towards the right whilst in non-protection-inducing **13768** and native 4325 it was centrally orientated or towards the left. All this data suggests the existence of a Pocket 7 in HLA-DR53 allele molecules and a differential orientation of P2 residues to contact TCR, to the left in HLA-DRβ1* 0401 and to the right in HLA-DRβ1* 0701 in immunogenic, protection-inducing modified HABPs.

Molecular Modelling

Docking studies between peptide **24112** and the HLA-DRβ1* 0401 molecule showed that this modified peptide had an optimum fit (1.86Å RMSD between this peptide's backbones) into this Class II molecule's groove when overlapped with Col II peptide backbone with which HLA- DRβ1* 0401 was crystallised. Similarly, docking between peptide **24166** and the HLA-DRβ1* 0101 molecule showed a good fit in this molecule's groove (3.26Å RMSD between this peptide's backbone and haemagglutinin HA) with which these molecules' three-dimensional structure was determined, by x-ray crystallography.

DISCUSSION

Previous studies performed with dozens of native malarial HABPs in hundreds of *Aotus* monkeys have revealed their absence of immunogenicity and their inability to induce a protective immune response against experimental challenge with a highly infective *Aotus*-adapted *P. falciparum* strain (FVO).

Elongating these native HABPs by adding more amino acids to their N or C terminal regions, acting as T-helper epitopes, induced antibodies against the added amino acid sequences, but not against native conserved HABPs nor protection against experimental challenge [32], providing more evidence that conserved HABPs were not suppressor epitopes but immunologically silent.

This work, plus further experimental evidence [24-32], allow us state that after a very careful fine-tuning with hundreds of peptides assayed in large numbers of *Aotus* monkeys, the shifting of the polarity in some of the critical binding residues can break the immunological code of silence of conserved HABPs rendering them immunogenic and protection-inducing.

Based on this, we can suggest that critical binding residues can thus be shifted in both directions, where F can replace R or *vice versa*; W↔Y, L↔H, I↔N, P↔D, M↔K or E, T↔C, V↔N or S. Serine, alanine and glycine may act as "jokers" during the molecule's structural organisation due to their very small size, similar polarity and absence of counterparts having opposite polarity. Shifts in critical binding residues' polarity are therefore able to break the immunological code of silence.

Such shifts must by performed properly and carefully since such modifications may also induce very short-lived, antibody-inducing, non-protective immune responses [30], non-protection-inducing long-lived antibody responses [38] or protection-inducing cellular immune ones without antibody production [28] (Table **1**). More rules, such as amino acid side-chain orientation (gauche +, gauche-, trans, etc) in modified HABPs, have still to be identified; however, shifting polarity provides the background for breaking such immunological code of silence.

Specific 3 dimensional rules must thus be followed so that modified HABPs fit appropriately into the peptide binding region (PBR) and therefore allow the formation of an appropriate MHCII-peptide-TCR complex since particular and specific MHCII molecule characteristics for each haplotype and each allele have to be taken into account.

The data gathered from purified HLA-DRβ1* molecule binding studies, complemented by recognition of binding motifs and their corresponding binding registers, as well as ^{1}H-NMR three-dimensional structure studies of native and modified HABPs, showed a 19.2 ±2.5 Å distance between the furthest atoms of amino acids fitting into Pockets 1 to 9 of molecules corresponding to HLA-DR52 haplotype alleles (HLA-DRβ1*0301 and HLA-DRβ1*1101). This experimental data also predicted the existence of a Pocket 2 in HLA-DR52 haplotype molecules as well as a vertical position or one directed towards the left of residue P7, which could be making contact with the TCR in these peptides (Fig. **3**).

These and previous data [25, 30] have also shown the existence of a 25.5 ± 1.5Å distance between the furthest atoms of amino acids fitting into Pockets 1 to 9 of molecules corresponding to the HLA-DR53 haplotype (HLA-DRβ1* 0401, HLA-DRβ1*0701). The residues' orientation predicted the existence of a Pocket 7 in these HLA-DR53 haplotype alleles, a finding corroborated by the existence of binding motifs specific for Pocket 7 in the HLA-DRβ1*04 molecules [40, 41] and the presence of this P7 in the Col II- HLA-DRβ1*0401 complex [42]. Residue P2 in these modified HABPs was orientated upwards in HLA-DRβ1*0401 towards the left and in HLA-DRβ1*0701 towards the right, in these peptides suggesting an allele-specific conscious TCR (Fig. **3**).

Equally important, these structural and HLA-DRβ1* molecule binding studies have suggested a ~2.5 Å difference between Pockets 1 to 9 of molecules belonging to haplotype HLA-DR53 when comparing the same distance between these pockets in alleles belonging to the HLA-DR52 haplotype as well as differences in the residues' organisation and orientation in different alleles, suggesting a haplotype- and allele-conscious TCR.

Favouring the above, it is well-known that the peptide binding groove of HLA-DRβ1*0401 bound to the Collagen II (Col II) peptide is 2 Å smaller than the HLA-DRβ1*0401 binding groove presenting the Hemagglutinin A (HA) peptide, showing that the width of the peptide-binding groove is determined by the bound peptide's amino acid sequence [42]. The existence of flexibility in the peptide binding region, depending on the peptide, gives more support to the "induced fit" theory [43] and grounds for our findings.

Furthermore, when comparing the 3D structure of HLA-DRβ1*0301 bound to CLIP *versus* HLA-DRβ1*0101 bound

to the HA peptide, the former's β-chain seemed to have collapsed slightly (1.5Å) towards the peptide, perhaps due to the CLIP peptide's lateral chain fitting into HLA- DRβ1*0301, where residue fitting into Pocket 4 is much smaller [37].

This data also shows that there is a certain degree of freedom (~2.0 Å) in the displacement of these HLA-DRβ1* molecules' α and β chains which, according to their segmental mobility, could allow the conformation of other pockets in different alleles in the shallow PBR areas. By analogy, such displacement freedom could also explain the ~2.5 Å difference in distance between Pockets 1 to 9 of molecules corresponding to HLA-DR52 and HLA-DR53 haplotypes. These structural differences could have been the results of these HLA-DR haplotypes' evolutionary divergence more than 60 million years ago, in trying to cope with the very aggressive and plastic microbial world.

Molecular modelling studies by docking modified HABP **24112** (immunogenic and protection-inducing) into the HLA-DRβ1* 0401 molecule to which it binds with high affinity, presented optimum fit into this molecule's PBR, (Fig. **4a**), spontaneously forming seven of the nine H-bonds between the peptide backbone and the HLA-DRβ1*0401 residues anchoring the peptide to such molecule. When the backbone of **24112** was superimposed on the Col II peptide's backbone it presented a 1.86 RMSD.

Fig. **4b** shows the spontaneously formed H-bonds between Oδ1 from Asn 82β with the peptide's N from N13 (atoms are underlined for clarifying the interactions), Nδ2 from Asn82β with O from N13, Oε1 from Gln 9α with N from V15, Oδ1 from Asn62α with O from V15, Nz from Lys 71β with O from I16, Oδ1 from Asn 69α with N from M20 and Nδ2 from Asn69α with O from R18. These H-bonds between the peptide and the lateral chains from the previously mentioned amino acids stabilised peptide binding to these Class II molecules. Distances > 3.5 Å were manually established between Oδ1 from Asn 62α with N from Arg 17 and Nε1 from Trp 61β with O from S19 from peptide **24112** backbone. The formation of H-bonds between αS53 and βH81 was not analysed as residues –P1 and –P2 are outside HLA-DRβ1*0401 pockets. These two completed the 11 H-bonds established between these amino acids and the peptide backbone stabilising peptide binding to MHC II molecules. It should be stressed that the *Aotus* HLA-DRβ1* 0403/0407 like molecules exhibited the greatest Class-II molecular homology with that of humans (97%) in a recent study regarding HLA-DRβ1* allele molecular biology characterisation in *Aotus* monkeys [34].

Studies of molecular modelling by docking modified HABP **24166** (which was immunogenic and reproducibly induced protection in experimental challenge in some *Aotus*

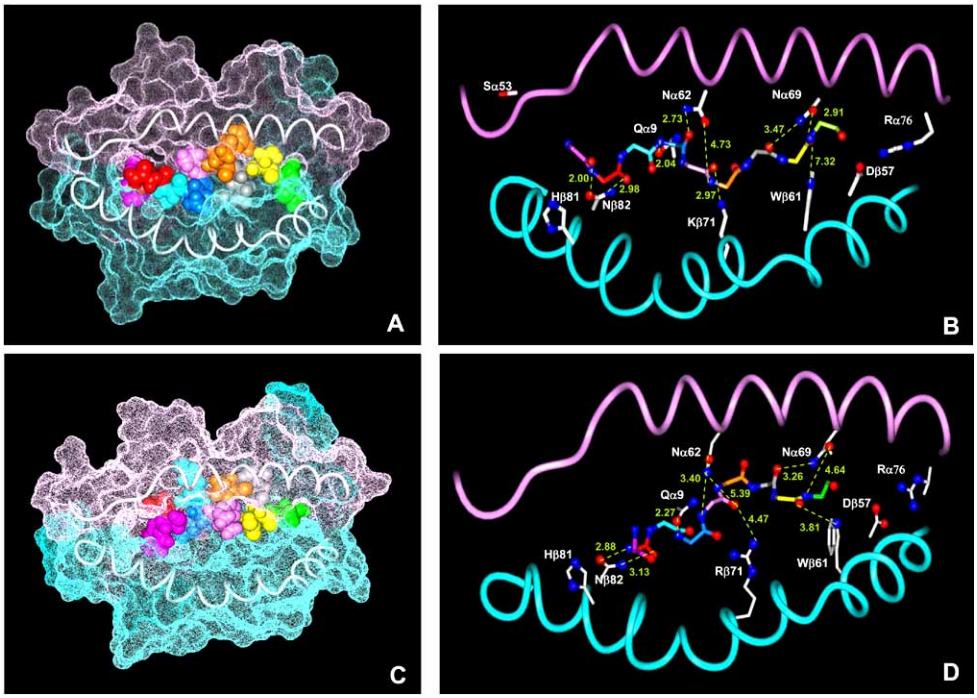

Fig. (4). (a) and (c), Modified immunogenic and protection inducing **24112** and **24166** peptides docking into HLA-DRβ1*0401 and HLA-DRβ1*0101 respectively. HLA-DRα chain Connolly surfaces are shown in pink while HLA-DRβ chains are depicted in pale blue. Colour code for amino acids fitting into these Class II molecules are fuchsia (P1), red (P2), turquoise (P3), dark blue (P4), rose (P5) orange (P6), grey (P7), yellow (P8) and green (P9). Note the buried amino acids corresponding to P1, P4, P6, P7, P9 in the **24112** modified HABP docking into the HLA- DRβ1*0401 molecule, and the P1, P2, P3, P4, P6 and P9 residues of the immunogenic protection inducing **24166** peptide buried into the HLA-DRβ1*0101 molecule. (b) and (d), the inter-atomic distances existing between the amino acids of the α (pink) and β (pale blue) chains of the HLA-DRβ1*0401 molecule and **24112** immunogenic protection inducing peptide backbone residues and between HLA-DRβ1*0101 amino acids and the backbone of the **24166** immunogenic modified HABP are measured in these figures. 7 out of 9 H-bonds were spontaneously formed (distances ≤ 3.5 Å) in the former while there were 5 out of 9 in the latter. The other distances between their corresponding atoms and molecules were manually established.

and bound with high affinity to HLA-DRβ1* 0101 molecules [44], being the only one identified to date presenting such affinity) showed that, when the HA peptide was replaced by **24166** in HLA-DRβ1* 0101- HA complex, it fitted well into this molecule's PBR (Fig. **4c**) and five of the nine hydrogen bonds became spontaneously established (Fig. **4d**). H-bonds were thus spontaneously formed between Oδ1 from Asn82β with N from D12, Nδ2 from Asn 82β with O from D12, Oε1 from Gln9α with N from M14, Oδ1 from Asn62α with N from P16 and Nδ2 from Asn69α with O from L17. All these bonds stabilised binding. The other four distances between Nε1 from Trp 61β with O from D18, Nδ2 from Asn62α with O from M14, NH2 from Arg 71β with O from L15 and Oδ1 from Asn 69α with N from D19 from the backbone of peptide **24166** were manually established since they were greater than 3.5 Å. As before, the formation of H-bonds with residues lying outside the PBR was not analysed. A 3.16 RMSD was found when HA peptide was superimposed on **24166**.

It is worth stressing that these molecules' three-dimensional structures here displayed were established by totally different methods: X-ray crystallography for HLA-DRβ1* 0401, crystallised together with COL II peptide [45] and HLA-DRβ1* 0101 crystallised with HA peptide (306-318) [46] whilst the three-dimensional structures of modified HABPs **24112** and **24166** were determined by ^{1}HNMR in solution [28, 44]. It is thus not strange that slight differences could have been due to the different methodologies used for their study (crystallography *cf* ^{1}H-NMR).

Another alternative is that such differences in some interatomic distances, and therefore in spontaneously forming H-bonds in modelling peptide **24112** with HLA-DRβ1* 0401 structure, could also have been due to the few variations found between some amino acids forming the pockets of *Aotus* HLA-DRβ1*0403/0407-like molecules when compared to those in human HLA-DRβ1* 0401. However, our analysis by quantum chemistry of multi-polar moments' and electrostatic properties of HLA-DR molecules shows that these variations did not have a great impact on these pockets' electrostatic landscape [47]. Therefore, modified HABPs fitting into HLA-DRβ1* 0403/0407 molecules could be used almost immediately for vaccinations in humans, no posterior modification being needed. It should be stressed that residues α9Q, α62N and α69N establishing H-bonds between HLA-DRβ1* alpha chain amino acids and the backbone of peptides did not vary between the two species.

Similar to COL II peptide in HLA-DRβ1*0401, modified HABP **24112** residues corresponding to binding motifs and reading registers fitting into Pocket 1 (Y) and Pocket 9 (M) were directed downwards and were deeply embedded in these pockets. Residues fitting into Pocket 4 (V) and Pocket 6 (R) were not as deeply embedded as the previous ones. Residue P7 (R) was localised in a shallow pocket along the β-chain helix the same as in COL II peptide. Such agreement supports the predicted existence of Pocket 7 in this allele (Fig. **3b**).

Fig. **3a** (left-hand panel) shows that P2 residues (S) from **13492** and (N) **24112** (in red) were orientated upwards (as in COL II peptide in HLA-DRβ1*0401) the same as those from P8 (S) suggesting that they could be making contact with the TCR.

The same as happened with the HA peptide within the HLA-DRβ1* 0101 PBR groove, residues corresponding to modified **24166** HABP's motifs and reading registers fitting into Pockets 1 (Y) and 9 (D) were deeply embedded within this molecule. The residue fitting into Pocket 4 (M) was relatively voluminous whilst that fitting into Pocket 6 (P) was extremely small, this being a characteristic of HLA-DRβ1* 0101 molecules, in which only small apolar amino acids such as A, G, S, P and T fit into this Pocket. All these amino acids represent classical binding motifs for this pocket in this Class II molecule.

Generally, the TCR is diagonally orientated relative to the MHC peptide binding groove's long axis. Peptide contacts are made primarily through CDR3 loops whilst contacts with MHC helices are through CDR 1 and CDR 2 in the canonical system [48].

However, variations in orientation with the pep-MHCII complex ranged from 24º to 80º; their interactions could thus twist, tilt or shift. All these variations suggested that as well as class-conscious TCR [50], haplotype- and allele-conscious TCRs could be suggested from immunogenic and protection-inducing modified HABPs three-dimensional structure, showing different places for TCR contact with the peptide and with MHC II α- and β-chains in HLA-DR52 and HLA-DRβ1*0401 and HLA-DRβ1*0701 (HLA-DR53) binding peptides.

These findings regarding molecular design, complemented with binding to HLA-DRβ1* molecules and the peptides' three-dimensional structure, clearly suggested that shifting critical amino acids' polarity in binding to RBC could break conserved HABPs' code of silence, making them become immunogenic and protection-inducing.

CONCLUSION

These rules (shown here for the first time), tested with hundreds of peptides in large numbers of *Aotus* monkeys immunised with modified HABPs [24-32] show that these modifications could lead to the MHC-II-peptide-TCR complex being appropriately formed to elicit the appropriate protection-inducing immune response and thereby totally breaking conserved HABPs' code of silence. This methodology leads to our final goal of finding a logical and rational methodology for developing multi-antigenic, multi-stage, subunit-based synthetic vaccines, malaria being one of them.

EXPERIMENTAL SECTION

Peptide Synthesis and Purification

20-residue-long native peptides and their analogues (as well as their corresponding polymers) (Table **1**) were synthesised by using standard t-Boc solid-phase peptide synthesis (SPPS), previously described by Merrifield [49] and modified by Houghten [50].

Cysteine–glycine (CG) was added to each peptide's C- and N-termini during synthesis to allow polymerisation fol-

lowing oxidation. Each peptide was thus dissolved in water (4 mg/mL) and peptide's solution pH was adjusted to 7.4 and subsequently air-blown (medicinal oxygen) for 24 h to obtain a final product consisting of 3–8 individual peptides joined by disulphide bridges. This procedure has been carefully standardised to guarantee including high molecular weight polymers (usually in the 8–24 kDa range), as assessed by size exclusion chromatography (SEC), to be used for immunisation purposes.

Peptides were purified by RP-HPLC for ^{1}H-NMR studies in a Vydac preparative C-18 column by linear-gradient elution from 0% to 100% B using the following solvent system: A, H_2O and 0.05% trifluoroacetic acid; and B, CH_3CN and 0.05% trifluoroacetic acid for 45 min (45–60min for preparative process). All peptides were lyophilised and numbered according to our institute's sequential numbering system. Peptide purity was verified by analytic RP-HPLC; their molecular mass was determined by mass spectrometry (Bruker Protein MALDITOF spectrometry).

Circular Dichroism (CD) Analysis

The CD spectra of the peptides in 50 mM phosphate buffer at pH 7.0 and trifluoroethanol/H_2O solution (30/70) were measured using a JASCO J810 spectropolarimeter with 1mm path-length cell. The peptides' CD spectra were recorded at 20°C in the 190-260 nm range using a 0.2 nm spectra bandwidth and 10 nm/min scan speed. Peptide concentration was 0.2 mM. CD data were expressed as mean residue ellipticity [θ] given in deg cm^2 $dmol^{-1}$.

Animals

Spleen-intact *A. nancymaae* monkeys from the Amazon jungle were used for these trials; they were kept in individual stainless-steel cages in our monkey colony in Leticia, Amazonas, Colombia and handled according to the NIH guidelines for animal handling. This species has proved to be highly susceptible to experimental infection with *P. falciparum* when using different FVO strain inoculates [17].

This monkey's immune system has been shown to be almost identical to that of humans [34]. Their sera were analysed by IFA for the presence of *P. falciparum* parasite antibodies at 1:20 dilution to rule out the possibility of previous infection with monkey plasmodia which would then have biased our results. Monkeys proving positive were immediately returned to the jungle. Members of the same family were randomly allocated into different experimental groups to avoid immunological bias attributable to genetic characteristics that might have affected their immunological response.

Immunisation

Each monkey received three 125 μg polymerised peptide subcutaneous injections on days 1, 20 and 40 homogenised in the first dose with Freund's Complete Adjuvant, whilst Freund's Incomplete Adjuvant was used in the second and third doses. Controls followed the same adjuvant regimen, but in saline solution. Blood samples obtained from the femoral vein 15 days after the second and 15 days after the third dose were used for immunological testing to perform a longitudinal analysis of the antibody response.

Challenge

Immunised and control *A. nancymaae* monkeys were infected, *via* femoral vein, with 200,000 *P. falciparum* FVO strain-infected RBCs, a parasite dose known to be infectious for 100% of *Aotus* monkeys [17]. Parasitaemia was measured daily for each monkey starting on day 5 after challenge. Parasites were counted by staining fluorescence with Acridine orange (AO) using a fluorescence microscope for reading the proportion (%) of parasitised red blood cells. Protection was defined as being the total absence of parasites in the challenged monkeys' blood during the 15 days which the experiment lasted. All control and non-protected monkeys showed parasites in their blood by day 5 and reached 6% parasitaemia by days 8–10, thus requiring treatment. Monkeys with parasitaemia higher than 6% were treated with paediatric doses of anti-malarial drugs and, after 20 days more in quarantine, they were then returned to the jungle.

Parasites

The FCB-2 *P. falciparum* strain from Bogotá, Colombia, was cultured *in vitro* with human group ORh + erythrocytes, using a modification of the Trager and Jensen culture method.

Immunofluorescence Antibody (IFA) Testing and Western Blotting

The *P. falciparum* FCB-2 strain kept in culture (ORh + RBCs) was used according to Lambros and Vandenberg's method [51]. The 20% late parasitaemia cultures were air-dried, fixed on slides, blocked with 1% skimmed milk, incubated with twofold sera dilutions (starting at 1:40), washed and stained with anti-*Aotus* IgG/FITC conjugate at 1:100 dilution, and read by fluorescence microscopy.

NMR Spectroscopy

Lyophilised peptide (10–12 mg) was dissolved in 500 μL of 2,2,2-trifluoroethanol-d_3 (TFE-d_3)/H_2O mixture (30/70 v/v). All NMR spectra were run on a 500-MHz Bruker DRX spectrometer and processed in an Indy computer equipped with updated XWINNMR software (Bruker). ^{1}H assignments were made according to the standard procedure described by Wüthrich, [52] DQF-COSY double-quantum-filtered correlation spectroscopy [53] and Hartman-Hahn homonuclear transfer experiments using Mlev17TOCSY [54] pulse sequences were used for assigning spin systems. Sequential assignment was performed by using nuclear Overhauser effect spectroscopy NOESY [55]. Proton water signals were suppressed by using pulse sequence with field gradients. Quadrature detection in W1 for all two-dimensional experiments was conducted by using TPPI time proportional phase increase. Water signal (4.75 ppm) was used as proton chemical displacement reference.

Temperature coefficients were determined from TOCSY experiments using a 30 K temperature range (285–315 K) as the slope of the chemical displacement graph against temperature for each one of the amide protons ($-\Delta\delta H^N$ (ppb)/ΔT (K)). Except for those experiments depending on temperature, all experiments were conducted at 295 K. The $^3J_{HN\text{-}H\alpha}$

coupling constants were measured by separating multiplet lines in DQF-COSY spectra cross peaks.

Structure Calculations

Once peptides had been analysed by NMR, the data so obtained became the basis for structure calculations conducted using Molecular Simulation Inc software (San Diego) [56] run on an Indigo workstation (Silicon Graphics). Cross-peak volume was obtained by integration using the FELIX programme on the NOESY spectrum from which inter-proton distances (constraints) were derived by using the P3 H2-H1 cross-peak (equal to 1.79 Å) to calibrate them. Cross-peak intensity was classified as being strong, medium and weak corresponding to 1.8–2.8 Å, 2.8–3.5 Å, and 3.5–5.0 Å ranges, respectively.

The Φ angle constraints derived from $^3J_{HN\text{-}H\alpha}$ were restricted to -70° ± 30° if $^3J_{HN\text{-}H\alpha} < 6$Hz and to -120° ± 30° if 9 Hz > $^3J_{HN\text{-}H\alpha} > 8$ Hz. Hydrogen bond constraints were also used corresponding to lower values of amide temperature coefficients $-\Delta\delta H^N$ (ppb)/ΔT (K) in which *i* residue carboxylic oxygen hydrogen binding to *i* - 4 residue amide proton was defined. Distances between these atoms were adjusted in the 1.8–2.5 Å range.

Distance geometry (DGII) was used for producing a 50-structure family. These structures were refined by using simulated annealing (SA) protocol with the Discover software.

The above calculations were repeated several times until obtaining one having the minimum of violations in distance and angle restraints and the least root-mean-square deviation (RMSD) respecting consensus lesser-energy structure. PROCHECK [57] was used for checking refined structure geometry, especially omega dihedral angle and the most probable zone within the Ramachandran plot. Structures having reasonable geometry and few violations were then selected.

Molecular Modelling

The coordinates were extracted from PDB records for HLA-DRβ1*0101-HA (code 1DLH in PDB) [46] and HLA-DRβ1*0401-HA (code 1J8H in PDB) [45] crystallised complexes and Insight II software (Biopolymers) 2000 [56] was used for building the model and measuring H-bonds and distances between HLA-DRβ1* and bound HA peptide's main chain atoms.

HLA-DR Molecule Affinity Purification

Human molecules were purified from DR1, WT100BIS DRβ1*0101), DR3, COX (DRβ1*0301), DR4, BSM (DRβ1*0401), DR7, EKR (DRβ1*0701), and DR11 BM21 (DRβ1*1101) homozygous EBV-B cell lysates by affinity chromatography [58] 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 to measure unlabelled peptide's ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules, as previously described [59]. Biotinylated-labelled haemagglutinin HA 306-318 (PKYVKQNTLKLAT) peptide was used as control peptide for DRβ1*0101, DRβ1*0301, DRβ1*0401 and Gly-Phe-Lys-(Ala)7 (GFKA7) for DRβ1*1101. All peptides were +N-terminally labelled with sulpho-NHS-LC-Biotin (Pierce Chemical, Rockford, III).

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

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