Peptides Inducing Short-Lived Antibody Responses against *Plasmodium falciparum* Malaria Have Shorter Structures and Are Read in a Different MHC II Functional Register†

Manuel E. Patarroyo,*,‡,§ Martha P. Alba,§ Luis E. Vargas,§ Yolanda Silva,§ Jaiver Rosas,§ and Raúl Rodríguez§

Fundación Instituto de Inmunología de Colombia and Universidad Nacional de Colombia, Bogotá, Columbia

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ABSTRACT: The search for a rational method of developing an antimalarial vaccine (malaria caused by *Plasmodium falciparum*) consists of blocking receptor−ligand interaction. Conserved peptides derived from proteins involved in invasion and having strong red blood cell binding ability have thus been identified; immunization studies using *Aotus* monkeys revealed that these peptides were neither immunogenic nor protection-inducing. Some of these peptides induced long-lasting and very high antibody titers and protection when their critical red blood cell binding residues were replaced to change their immunological properties. Others induced short-lived antibodies that were not associated with inducing protection. The three-dimensional structure of the short-lived antibody-inducing peptide was determined by ^{1}H NMR. Their HLA-DRβ1* molecule binding ability was also determined to ascertain the relationship among three-dimensional structure, their ability to bind to major histocompatibility complex class II molecules (MHC II), and possible short-lived antibody production. These short-lived antibody-inducing peptides were 6.8 ± 0.5 Å shorter between those residues theoretically coming into contact with pocket 1 and pocket 9 of HLA-DRβ1* molecules to which they bind than immunogenic and protection-inducing peptides. These more compact α-helical structures suggest that these short-lived antibody-inducing peptides could have a structure more similar to those of native peptides than immunogenic and protective ones. Such shortening was associated with a shift in HLA-DRβ1* molecule binding and a consequent shift in functional register reading, mainly by alleles of the same haplotype when compared with immunogenic protection-inducing HABPs, suggesting an imperfect and different conformation of the MHC II peptide−TCR complex.

A protective immune response involves (as one of its main actors) long-lived antibody production, usually having high affinity and being strain-specific (*1*). However, little attention has been paid to the presence of high levels of short-lived antibodies which could be temporally mediating or blocking such protection phenomena. The physicochemical characteristics of peptides inducing these antibodies and the immunogenetic characteristics of individuals developing such antibodies have also received little attention.

In the search for a rational methodology for developing a vaccine against *Plasmodium falciparum* malaria (the most lethal form of the disease attacking ∼200 million people per year and killing ∼2 million of them, mainly children less than 5 years of age, especially in sub-Saharan Africa) (*2*), hundreds of malarial peptides were produced and analyzed in terms of their immunogenic and protection-inducing abilities when faced with experimental challenge in thousands of *Aotus* monkeys, a non-human primate species highly susceptible to the experimental development of this disease.

A method has been developed for recognizing *P. falciparum* protein amino acid sequences involved in very high activity receptor−ligand interactions interacting with red blood cells or hepatocytes involved in invasion, here called high-activity binding peptides (HABPs).[1] Blocking or destroying these receptor−ligand interactions by inducing antibodies or cellular immune responses (*3*, *4*) could lead to protective immune responses against this lethal disease. Peptides were selected which did not present genetic variability (conserved) to prevent the *P. falciparum* parasite's high genetic polymorphism.

However, conserved HABPs are neither immunogenic nor protection-inducing. Modifications were therefore made, replacing critical binding residues (determined by glycine analogue scanning) with others having similar mass but opposite polarity. The majority of these peptides were not immunogenic and non-protection-inducing; others were just immunogenic, and a minority were immunogenic and protec-

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* To whom correspondence should be addressed: Carrera 50, No. 26-00, Fundacion Instituto de Inmunologia de Colombia (FIDIC), Bogotá, Columbia. Phone: (57-1)4815219. Fax: (57-1)4815269. E-mail: mepatarr@mail.com.

‡ Universidad Nacional de Colombia.

§ Fundación Instituto de Inmunología de Colombia.

[1] Abbreviations: HABPs, high-activity binding peptide; RBC, red blood cells; NMR, nuclear magnetic resonance; MHC II, major histocompatibility complex class II; TCR, T-cell receptor; HLA, human leukocyte antigen; FVO, *falciparum* Vietnam Oak Noll, FCB-2, *falciparum* Colombia Bogotá-2; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; TFE, 2,2,2-trifluoroethanol; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy.

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tion-inducing (*5*−*8*). However, a few peptides induced short-lived high antibody titers in some *Aotus* monkeys which disappeared rapidly and permanently; their short-lived presence was not associated with protection induction.

Malarial parasite immunity is associated with major histocompatibility complex (MHC) class II proteins (*9*); it was thus suggested that modifications of these HABPs could allow them to fit better into the MHC II−peptide−TCR complex (*10*, *11*). Imperfect or inappropriate fitting into this complex could therefore alter the immune response, leading to a lack of response or short-lived antibodies being produced.

The *raison d'être* for this study was to identify the relationship among their three-dimensional structure, class II molecule binding capacity (to conform the MHC II−peptide−TCR complex), their ability to induce a protective immune response against the *P. falciparum* parasite, and their ability to induce these short-lived antibody responses. Peptide three-dimensional structure was therefore determined by ^{1}H NMR; their ability to bind to some of the HLA-DRβ1*-purified molecules has thus been analyzed.

MATERIALS AND METHODS

Synthetic Peptides. Synthetic peptides included in this study were synthesized by the Multiple Solid-Phase technique, using the previously described *tert*-butyloxycarbonyl (t-Boc) strategy (*12*). They were then purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 LiChrospher column. The products were assessed by analytical RP-HPLC and mass spectrometry (MALDI-TOF). Peptide polymers were obtained for immunization studies after Cys and Gly had been added at the N- and C-termini, respectively. Briefly, each peptide was dissolved in water (4 mg/mL); the pH was adjusted to 7.4, and the peptide solution was then shaken and oxygen-blown (medicinal oxygen) for 12 h to obtain a final product. Dialysis against distilled water through 6−8 kDa pore membranes was used to remove most of the monomers and very low molecular mass contaminants. Cysteine oxidation yielded high-molecular mass polymers for immunization purposes, usually in the 8−24 kDa range as assessed by size exclusion chromatography.

The modified peptides were synthesized (Table 1) and numbered according to our laboratory's sequential numbering system.

Animals. Spleen-intact *Aotus nancymaae* monkeys, captured in the Amazon jungle and kept in our monkey colony in Leticia, Colombia, were used for this trial. This species proved to be highly susceptible to experimental infection with *P. falciparum* using different FVO (*falciparum* Vietnam Oak Noll) strain inoculums (*13*). All monkeys included in this report were phenotypically characterized, having an average weight of 700 g and hematocrite higher than 45%. Members of the same family were randomly distributed into different experimental groups to prevent immunological phenomena that can be attributed to immune response genes. Animal handling was performed according to U.S. National Institutes of Health animal handling criteria.

Immunizations. Groups of 5−10 *Aotus* monkeys each received three 125 μg subcutaneous injections of the polymerized peptide on days 1, 20, and 40, homogenized in the first dose with Freund's complete adjuvant; Freund's incomplete adjuvant was used in the second and third doses. Controls received only Freund's adjuvant on the same days.

Challenge. The *P. falciparum* (*Aotus*-adapted) FVO strain was kept by successive passages in two or three nonimmune, spleen-intact owl monkeys prior to challenge to have a fresh, stable, and quantifiable accurate infection dose (*13*).

Aotus monkeys were given an intravenous inoculation of 200 000 infected RBC from the *P. falciparum* FVO strain 20 days after the last immunization (day 60 of the whole experiment). Protection was defined as the complete absence of parasites in immunized monkey blood throughout the 15 days following challenge (i.e., up to day 75) with a dose which was 100% infective for *Aotus* monkeys. Nonprotected as well as control monkeys developing parasitemia of ≥6% by 8−10 days after the challenge were treated with pediatric doses of quinine and kept in quarantine to ensure cure (*13*). Some of the vaccinated and challenged monkeys were kept in our monkey facility in Leticia for three more months to perform a second challenge, after which they were treated and liberated in the surrounding jungle.

Parasitemia Assessment. Fresh blood samples (0.05 mL/monkey), obtained from saphenous veins, were used; parasitemia was measured daily for each monkey starting on day 5 after challenge (day 65 of the whole experiment). Twenty-five microliters of each sample was diluted in 25−50 μL of a 0.06% acridine orange solution in a 0.86% saline solution. Parasites were visualized by reading the percentage of parasitized RBC on the slide by fluorescence.

Indirect Immunofluorescence Assays (IFAs). IFA was performed 10 and 15 days after the second (II_{10} and II_{15}, respectively) and 15 days after the third immunization (III_{15}) to asses humoral immune response.

Late-stage schizonts from a continuous *P. falciparum* culture FCB-2 strain (*falciparum* Colombia Bogotá-2), presenting a 10% parasitemia, synchronized according to Lambros and Vandenberg's (*14*) method, collected and washed in sterile PBS [phosphate buffer, containing 0.015 M NaCl (pH 7.2)], were used. The infected cell pellet was suspended in fetal bovine serum (FBS) and PBS (1:1, v/v) and left to dry on the slide. The parasites were blocked for 10 min with 1% skim milk and incubated for 30 min with preimmune or immune sera. Reactivity was visualized by fluorescence microscopy using purified goat anti-purified monkey IgG−FITC conjugate $F(ab)_2$ fragment at a 1:100 dilution. Preimmune *Aotus* sera were used as negative controls.

Parasites. Trager and Jensen's (*15*) culture method was modified (*16*) for culturing the *P. falciparum* FCB-2 strain from Bogotá, Colombia, in vitro with human group O+ erythrocytes.

Parasite Lysate for Western Blotting. Late-stage schizonts from continuous *P. falciparum* cultures, exhibiting 20% parasitemia, were collected, washed in sterile PBS, and lysed in a 0.2% saponine solution with vigorous vortexing for 20 s. The pellet was washed twice with large volumes of PBS to remove hemoglobin and erythrocyte debris. Seven volumes of lysis buffer (5% SDS, 1 mM EDTA, and 1 mM PMSF) was added to the pellet and vortexed for 10 min; the supernatant was spun again at 22500*g* for 30 min. This lysate was kept frozen in liquid nitrogen until it was used. Noninfected RBC lysate was prepared in the same way.

Table 1: Conserved HAPB Amino Acid Sequences and Those of Their Analogues Used for Immunizing *Aotus* Monkeys (numbered according to our institute's serial system)[a]

PEPTIDE	SEQUENCE (P1 P4 P6 P9)	PI	II_{10}	II_{15}	III_{15}	Prot
1522	**QIPYNLKIRANELDVLKKLV**	0	0	0	0	0/5
▲15474	QIPYNLKIFAIMLDTHKMLV	0	3(1280)	6(320)	0	0/4
15476	QIPYNLKIFAIMLDTHKMLT	0	0	2(320)	0	0/3
17898	QIPYNLKIFANMLDTHKMG	0	0	3(320)	0	0/6
15480	QIPYNLKIFAIELDTLKKLT	0	0	1(320)	0	0/4
• 9782	QIPYNLKIRAGGLDGGKKLV	0	2(2560)	1(2560)	ND	1/3
1585	**EVLYLKPLAGVYRSLKKQLE**	0	0	0	0	0/5
▲15484	EVLYHMPLAGVYRALKKQLE	0	3(320)	1(320)	0	0/5
15482	EVLYHKPLAGVYRALKKQLE	0	3(640)	3(320)	0	0/6
19992	VLYHKPLAGVYRALKKQLG	0	0	1(320)	0	0/6
19994	VIYHKPLAGVYRALKKQIG	0	0	1(320)	0	0/7
•13450	EVLYLLDLAGVYRSLKKQLE	0	1(5120)	1(2560)	1(5120)	2/4
1758	**KSYGTPDNIDKNMSLIHKHN**	0	0	0	0	0/5
12902	KSYGSDDNDDKNKSLIHKHN	0	2(320)	2(640)	0	0/4
13790	MAYGSDDNDDKNKSLDHKHN	0	1(320)	1(320)	2(320)	2/4
6725	**YLKETNNAISFESNSGSLEKK**	0	0	0	0/5	0/5
23008	LKMTNNAISFMSISASLEKK	0	3(320)	1(640)	ND	0/10
24056	KETNNSISFMSNAGSLEKK	0	0	3(320)	ND	0/6
23422	KETNNAISFMSNAGSLEKK	0	0	1(640)	ND	1/8
6737	**DNILVKMFKTNENNDKSELI**	0	0	0	0	0/5
▲14096	DNIHVKMRKVIMNNDKSELI	0	2(320)	3(640)	0	0/6
17974	DNILVKMFKVIMNNDKSELI	0	1(320)	1(320)	0	0/6
•22834	DNIHVKMFKVIENNDKSELI	0	0	1(2560)	ND	2/9
6746	**DQGNCDTSWIFASKYHLETI**	0	0	0	0	0/5
▲21742	DQGNSDTSYIFASKYHH	0	0	2(320)	0	0/8
20466	DQGNSDTSWNFAAKYLLETI	0	0	3(320)	0	0/5
•23230	GNSITAWIRASKYLLET	0	0	1(320)	ND	1/9
1513	**GYSLFQKEKMVLNEGTSGTA**	0	0	0	0	0/5
12886	GYSLFQKEMETLNEGTSGTA	0	1(1280)	1(320)	0	0/4
13942	GYSLFQMEMKTLNEGTSGTA	0	0	2(320)	0	0/5
13946	GYSLFQKEMKTLNEGTSGTA	0	2(320)	2(320)	0	2/12
4325	**MIKSAFLPTGAFKADRYKSH**	0		0	0	0/6
14518	MIKVGFHPTGAFKSPRWKSH	0	1(320)	0	0	0/3
14520	MIKVGFHPTGAFMSPRWKSH	0	2(640)	0	0	0/3
17934	KASFHVTGAFMSDRWKS	0	3(320)	0	0	0/7
20034	MIKAAFLPTGAFMADRYKSH	0	0	2(320)	1(160)	2/8
4044	**KNESKYSNTFINNAYNMSIR**	0	0	0	0	0/5
14502	KIMSKWGNTFNINAYNMSNF	0	1(320)	0	0	0/3
14504	KIMSKWVNTFNINAYIMSNF	0	2(320)	0	0	0/3
14508	KIMSKWVNVFNINAYIMSNF	0	1(320)	0	0	0/4
24112	SKYSNTFNINAYNMVIRRSM	0	1(5120)		ND	1/8
6671	**MTDVNRYRYSNNYEAIPHIS**	0	0	0	0	0/5
▲10000	MTDVNRYRYSNNYERPHIS	0	1(160)	0	0	0/4
17964	VIRYRYSNDYDANDHIS	0	2(320)	0	0	0/8
12684	MTDVVFYRYSNNYEGQPHIS	0	1(320)	0	0	0/5
22826	MTDVIRYRYSNNYESSDK	0	2(640)	0	0	0/10
•13492	MTDVIRYRYSNNYEASDHIS	0	2(5120)	1(1280)	0	1/6
5501	**MLNISQHQCVKKQCPQNSY**	0	0	0	0	0/5
13466	MLNISQMQSVKKQSDQNS	0	2(2560)	3(1280)	0	0/6
13978	MLNISQHQSVMKMSDQNS	0	3(640)	0	0	0/5
15968	MHNISQLQTVKKQTDQNS	0	1(320)	0	0	0/5
24148	MLNISMLQTVMMMTPQK	0	3(2560)	3(2560)	ND	3/16

[a] These peptides were aligned according to HLA-DRB1 molecule pockets 1, 4, 6, and 9 to which immunogenic and protection-inducing peptides marked with a circle bound. Their three-dimensional structure was determined by ^{1}H NMR for peptides presenting short-lived, non-protection-inducing antibodies marked with a triangle. PI, II_{15}, and III_{20} are the days when monkeys were bled and antibody titers determined by IFA (shown in brackets). The prefix corresponds to the total number of *Aotus* monkeys presenting these antibody titers. Prot. corresponds to the total number of *Aotus* monkeys protected against experimental challenge with a 100% effective *P. falciparum* strain for these monkeys.

Immunoblotting. The proteins were electrophoretically separated, transferred to nitrocellulose paper, and incubated with preimmune serum at a 1:100 dilution. The reaction was revealed with affinity-purified goat anti-*Aotus* IgG alkaline phosphatase conjugate; nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-infonilphosphate (BCI) substrate were used as developing reagents, according to the method of Blake et al. (*17*).

HLA-DR Molecule Affinity Purification. Human molecules were purified from DR1 WT100BIS (DRB1*0101), DR3 COX (DRB1*0301), DR4 BSM (DRB1*0401), and DR11 BM21 (DRB1*1101) homozygous EBV-B cell lysates by affinity chromatography (*18*) using anti-HLA-DR mAb L-243 cross-linked to protein A−Sepharose CL-4B beads as affinity support. Cells were lysed at a density of 10^8 cells/mL on ice for 60 min in 1% (v/v) Nonidet P-40, 25 mM

iodoacetamide, 1 mM phenylmethanesulfonyl fluoride (PMSF), and each of the following at 10 μg/mL: soybean trypsin inhibitor, antipain, pepstatin, leupeptin, and chymostatin in 0.05 M sodium phosphate buffer and 0.15 M NaCl (pH 7.5). Lysates were cleared of nucleic acids and debris by being spun at 27000*g* for 30 min. After 0.2 volume of 5% sodium deoxycholate (DOC) had been added to the supernatant and mixed for 10 min, the lysate was spun at 100000*g* for 2 h and subsequently filtered through a 0.45 μm membrane. Lysates were first passed over a Sepharose CL-4B precolumn, and subsequently over an A Sepharose-mAb L243 protein column for class II molecule affinity purification. The affinity column was then washed with (i) 20 column volumes of 50 mM Tris-HCl (pH 8), 0.15 M NaCl, 0.5% NP-40, and 0.5% DOC, (ii) 5 column volumes of 50 mM Tris-HCl (pH 9), 0.5 M NaCl, 0.5% NP-40, and 0.5% DOC, and (iii) 5 column volumes of 2 mM Tris-HCl (pH 8) and 1% octyl β-D-glucopyranoside. HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl (pH 11.5) containing 1% octyl β-D-glucopyranoside and 1 mM EDTA. The eluate was immediately neutralized with 1/20 volume of 1 M Tris-HCl (pH 6.8). Preparations were kept in aliquots at −70 °C until they were used.

HLA-DRβ1*-like molecules have been identified in *Aotus* monkeys by cloning and sequencing this gene's exon 2 in 115 animals. This study showed 98−86% sequence homology with their human counterparts, especially with HLA-DRβ1*04, -03, -08, -07, and -10 and the complex formed by HLA-DRβ1*11, -12, -13, and -14 (E. Trujillo et al., manuscript submitted to *J. Immunol*.).

Peptide Binding Competition Assays. Peptide binding competition assays were conducted to measure the unlabeled peptide's ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules as described in ref *19*. Biotinylated labeled hemagglutinin HA 306−318 (PKYVKQNTLKLAT) peptide was used as control peptide for DRB1*0101, DRB1*0301, and DRB1*0401 and Gly-Phe-Lys-(Ala7) (GFKA7) for DRB1*1101. Both peptides were N-terminally labeled with sulfo-NHS-LC-biotin. Purified HLA-DR molecules were diluted in freshly prepared binding buffer containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF, and each of the following at 40 μg/mL: soybean trypsin inhibitor, antipain, leupeptin, and chymostatin. Ninety microliters of HLA-DR molecules (0.1 μM) was added to Eppendorf tubes together with 30 μL of biotinylated labeled peptide (5 μM) in DMSO and PBS (1:4) for the direct binding assay; 30 μL (250 μM) was also added to competition assays. After incubation for 24 h at room temperature, the peptide−class II complexes were transferred to ELISA well plates which had been coated with a 10 μg/mL anti-HLA-DRmAb-L-243 solution and subsequently blocked with PBS containing 5% bovine serum albumin. After incubation for 2 h at room temperature, plates were washed with PBS and 0.05% Tween 20. After incubation with alkaline phosphatase-labeled streptavidin, labeled peptide−HLA-DR complexes were revealed with the 4-nitrophenyl phosphate substrate. A Titertek MC Multiscan ELISA reader with a 405 nm filter was used to assess peptide binding to HLA-DR molecules by measuring the optical density (OD). The amount of bound peptide was normalized to the maximum observed binding.

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 the indicator peptide's binding to the HLA molecule being tested by more than 50%.

NMR Spectroscopy. Ten milligrams of each peptide (**13450**, **15484**, **21742**, **15474**, and **10000**) was dissolved in 0.5 mL of TFE and water (30:70, v/v) (*20*) for NMR measurements. A Bruker DRX-600 instrument was used to obtain spectra. COSY (*21*) and TOCSY experiments (*22*) were used for assigning proton resonance; NOE determination between protons was carried out with phase sensitive NOESY (*23*) experiments.

The NOESY spectra recorded at different mixing times (150−600 ms) were used to ensure that peaks were not due to the effect of spin diffusion.

A 4.75 ppm water signal was used as a reference for proton chemical displacements. All spectra (except for experiments aimed at determining temperature coefficients) were recorded at 295 K. The TOCSY spectra recorded at different temperatures (285−315 K) were used for obtaining amide temperature coefficients for predicting hydrogen bonds ($-\Delta\delta H^N/\Delta T$)

Structural Ccalculation. The restraints were derived from the NOESY spectra run with mixing times of 300 ms and a temperature of 295 K to obtain structural models for each of the peptides. The structure was determined by using a combination of distance geometry (DGII) with simulated annealing (Discover), both being commercially available from Accelrys Inc. (San Diego, CA). The NOEs were partitioned into three categories (strong, medium, and weak) and then converted into distance restraints (1.8−2.8, 2.8−3.5, and 3.5−5.0 Å, respectively).

Hydrogen bond constraints were introduced for low amide temperature coefficients; only $-\Delta\delta H^N/\Delta T$ values of <4.0 ppb/K were used in structure calculations. Distance ranges involving these likely NH···O hydrogen bonds were set at 1.8−2.5 Å between the residue acceptor oxygen ($i-4$) and the residue donor amide hydrogen (i).

The chirality of all Cα atoms (except for glycine residues) was fixed to L, and the geometry of the peptide bonds was fixed to trans according to NMR data calculations.

RESULTS

Peptide Analysis. HPLC analysis revealed just one peak after purification; these peptides' molecular mass, experimentally obtained by mass spectrometry (MS), agreed with expected theoretical values (data not shown). Peptide purity is crucial for ensuring that NMR experiments' structural conclusions correspond to the peptides' most probable structure. The polymers used for immunization had molecular masses in the 8−24 kDa range, as assessed by size exclusion chromatography (SEC) (data not shown).

Immunological Aspects. High antibody titers detected by IFA (some up to 1:2560) were induced by some modified HABPs in some monkeys (**1522**, **1585**, **1758**, **6725**, **6737**, **6746**, and **1513** analogues) and detected 10 (II_{10}) or 15 (II_{15}) days after the second (II) immunization. Such antibodies disappeared and did not reappear 15 days after the third dose (III_{15}), when their levels could have been expected to have increased following the third immunization.

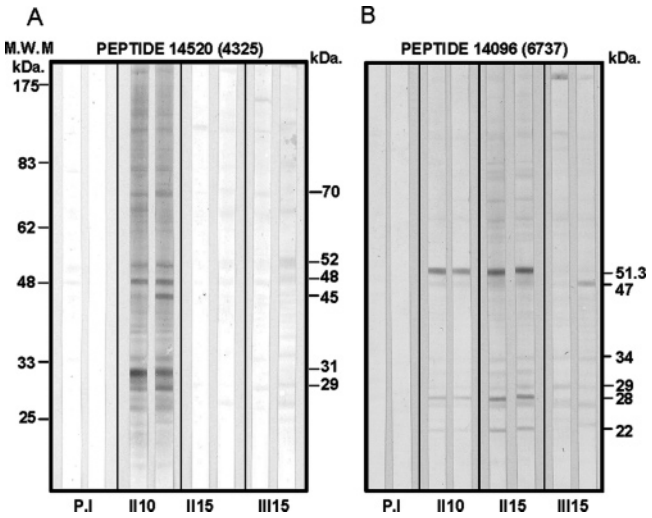

FIGURE 1: Western blot analysis of *Aotus* monkey sera immunized with modified short-lived antibody response-inducing HABP peptides. Peptide **14520** (derived from AMA protein HABP **4325)** induced antibodies that appeared only 10 days after the second dose (II_{10}) and then disappeared; peptide **14096** (derived from SERA protein HABP **6737**) induced short-lived antibodies that lasted slightly longer (II_{10} and II_{15}) and then disappeared.

Some other modified HABPs (**4325**, **4044**, **6671**, and **5501** analogues) induced high antibody titers which could be detected only in II_{10} bleeding but not in II_{15}, suggesting that they were much more short-lived and that their disappearance was not a consequence of the third immunization (Table 1).

The phenomenon of short-lived antibodies was confirmed by Western blotting where sera from some *Aotus* monkeys immunized with Apical Merozoite Antigen 1 (AMA-1) HABP **14520** (peptide **4325** analogue) were analyzed. These sera in panel A reacted with 70, 52, and 48 kDa and 45, 32, and 29 kDa molecules, corresponding to the molecular masses of the AMA-1 native protein and its cleavage products, respectively, on day 10 (II_{10}) following the second immunization and then completely absent by day 15 (II_{15}) (Figure 1). Immunization with the third dose did not induce their reappearance, confirming by Western blot that their disappearance is long-lived (or permanent) and independent of boosting.

Another example confirming this observation, sustained by antibodies visualized by IFA and confirmed by Western blotting, was observed with sera from panel B where some monkeys immunized with modified peptide **14096** (derived from serine repeat antigen HABP **6737**) (panel B) recognized 53.1, 28, and 22 kDa molecules on days 10 (II_{10}) and 15 (II_{15}) after the second immunization to completely disappear after the third dose (III_{15}). These are well-known cleavage products from the SERA native molecule. None of these peptides inducing short-lived antibodies were able to induce protective immunity against experimental challenge with a *P. falciparum* FVO (*Aotus*-adapted) strain dose which was 100% infective for these monkeys. Furthermore, when some of these *Aotus* monkeys were challenged for the second time (3 months later), none of them were protected against experimental infection, suggesting that this loss of immunity was permanent or at least long-lasting. As a reference, modified HABPs inducing long-lived, protective, high antibody titers and protection after experimental challenge are shown at the end of each group of peptide analogues showing the feasibility of inducing them (Table 1); those inducing short-lived antibodies associated with nonprotection are also shown in the same table.

STRUCTURAL ANALYSIS

Assignment. Cross-peaks between NH and CHα protons were identified by analyzing COSY spectra acquired in a 30% TFE-d_3/H_2O mixture. TOCSY spectra were used for correlating side chain spin systems with NH−CHα cross-peaks. Wüthrich's methodology was used for assignments (*24*).

Structure Determination. NOESY spectra for peptides **13450**, **15484**, **21742**, **15474**, and **10000** showed sequential, short-range, 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, suggesting the presence of a partial α-helical structure. The possibility of a 3_{10} helix was ruled out because of the presence of $d_{\alpha N}(i,i+4)$ NOEs, as well as the presence of $d_{\alpha\beta}(i,i+3)$ NOEs having medium-intensity α-helix characteristics.

The presence of sequential, short-range, and medium-range NOE connectivity and low temperature dependence ($-\Delta\delta H^N/\Delta T$) values observed for some of the amino acids revealed the presence of a secondary α-helix structure extending from residue V2 to Q18 in peptide **13450**, A9 to K17 in peptide **15484**, N4 to A12 in peptide **21742**, K7 to K17 in peptide **15474**, and T2 to Y9 in peptide **10000** (Figure 2). The structures for peptides **23230** (*25*), **9782** (*6*), **13492** (*10*), and **14096** and **22834** (*26*) have already been reported.

Of the 50 resulting structures, 30, 17, 20, 21, and 23 were obtained for peptides **13450**, **15484**, **21742**, **15474**, and **10000**, respectively. The chosen structures had no distance violation larger than 0.30 Å or ω angle greater than 2.0°. Average root-mean-square deviations (rmsds) for main chain atoms were 0.15 Å for peptide **13450**, 0.32 Å for peptide **15484**, 0.2 Å for peptide **21742**, 0.17 Å for peptide **15474**, and 0.14 Å for peptide **10000**; these values were obtained by superimposing the structures between amino acids V2 and Q18 in peptide **13450**, A9 and K17 in peptide **15484**, N4 and A12 in peptide **21742**, K7 and K17 in peptide **15474**, and T2 and Y9 in peptide **10000** and having a consensus structure (that having the lowest total energy). Kabsh and Sander's (*27*) software was used for providing a clearer analysis of how helical fragments could be defined in the peptides. These peptides showed α-helical regions between the previously mentioned residues; amino and carboxy termini were flexible.

Amino acid side chain orientation and superimposition resulted from superimposing main chain atoms between residues implicated in each peptide's structure.

Class II Molecule Binding. An interesting phenomenon observed in this study was the shift in purified HLA-DRβ1* molecule binding activity by short-lived antibody-inducing peptides when compared to that of protection-inducing immunogenic peptides, suggesting a shift in their functional register reading resulting from differences in their amino acid sequence. The immunogenic, protection-inducing **13450** peptide (HABP **1585** analogue) bound to HLA-DRβ1*0301, according to characteristic motifs for HLA-DRβ1*0301 (Y4 in pocket 1, D7 in pocket 4, A9 in pocket 6, and Y12 in pocket 9) functional register reading. However, short-lived antibody-inducing analogue **15484** bound exclusively to

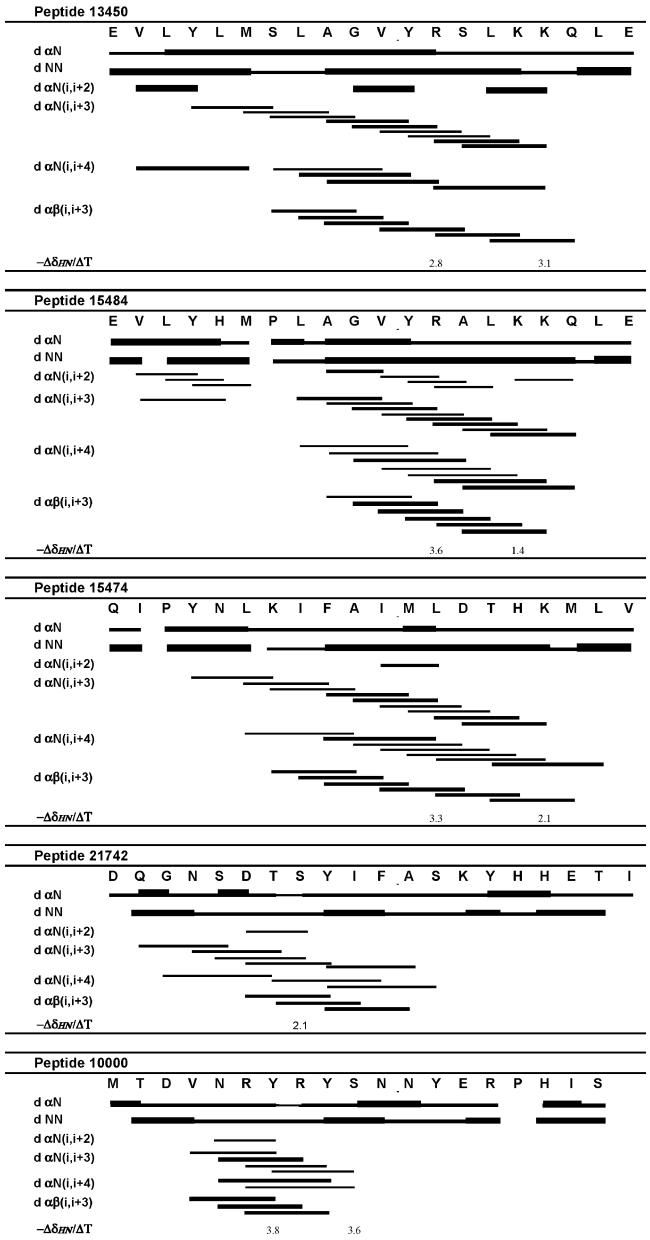

FIGURE 2: Summary of sequential medium-range NOE connectivity (NOE intensity is represented by line thickness). Low H^N amide temperature coefficients are indicated by their value.

HLA-DRβ1*1101, beginning its register reading at Y12 in pocket 1, L15 in pocket 4, K17 in pocket 6, and E20 in pocket 9, classical binding motifs for this allele. It should be mentioned that in those peptides in which modifications were made to other critical residues (such as L5H and K6M) or in noncritical residues for this HABP's binding to red blood cells (such as L3I, L19I, or E20G) (Table 1), short-lived antibodies were also induced.

The immunogenic, protection-inducing **23230** peptide bound to HLA-DRβ1*0301 in the HABP **6746** analogue with a functional register beginning at W7 in pocket 1, A10 in pocket 4, K12 in pocket 6, and L15 in pocket 9, while peptide **21742** (short-lived, antibody-inducing) bound with high affinity to HLA-DRβ1*1101 (Table 2). This peptide's functional register reading began at Y9 in pocket 1, A12 in pocket 4, K14 in pocket 6, and H17 in pocket 9. All these were residues conforming to motifs characteristic of HLA-DRβ1*1101 (*28*, *29*).

Table 2: Conserved HABP Structural Features and Those of Their Analogues, Determined by ^{1}H NMR[a]

peptide	structural features	distance (Å)	% binding HLA-DRβ1 0101	0301	0401	1101	I	P
1585	α-helix (Y4−Y12)	14.3	5	18	7	15	−	−
15484	α-helix (A9−K17)	13.3	16	21	0	**58**	+	−
13450	α-helix (V2−Q18)	20.2	−12	**47**	−8	20	+	+
6746	α-helix (CD)	−	12	**55**	5	**76**	−	−
21742	α-helix (N4−A12)	12.7	6	13	7	**51**	+	−
23230	α-helix (N9−H16)	20.0	2	**84**	13	37	+	+
1522	α-helix (Q1−V20)	16.1	1	**88**	−3	**91**	−	−
15474	α-helix (K7−K17)	15.2	18	**73**	37	45	+	−
9782	α-helix (P3−N11)	22.1	−1	16	14	**48**	+	+
6737	α-helix (I3−N14)	14.2	0	**79**	0	38	−	−
14096	α-helix (V5−M12)	14.2	−3	**65**	14	**78**	+	−
22834	α-helix (V5−V10)	21.0	−3	4	17	**54**	+	+
6671	β-turn tendency (CD)	−	24	45	16	**61**	−	−
10000	α-helix (T2−Y9)	18.4	3	17	11	**73**	+	−
13492	α-helix (V4−R9)	24.7	−5	33	**50**	16	+	+

[a] Given are the distances between HABP residues theoretically fitting into pockets 1−9 of HLADRβ1* molecules and the percentage of these HABPs' specific binding to class II molecules. The HLADRβ1* molecule to which it binds with ≥50% affinity is shown in bold. I indicates immunogenic; P indicates protection-inducing. The three-dimensional structures of peptides 9782 (Cubillos 2003), 22834, and 14096 (Cubillos 2003) have already been described, while the structures of peptides 15484, 13450, 21742, 15474, and 10000 are described here for the first time. NMR spectra have been described for peptide 14096 but not for its three-dimensional model.

The same phenomenon of changing HLA-DRβ1* molecule binding was shown by HABP **1522** analogue, where immunogenic, protection-inducing **9782** peptide bound to HLA-DRβ1*1101 with a functional register beginning at L6 in pocket 1, R9 in pocket 4, G11 in pocket 6, and D14 in pocket 9, while peptide **15474** (short-lived antibody inducer) bound with high affinity to HLA-DRβ1*0301 (Table 2). Functional register reading for **15474** began fitting I11 in pocket 1, D14 in pocket 4, H16 in pocket 6, and L19 in pocket 9 (Table 1). All these are residues conforming to motifs characteristic of HLA-DRβ1*0301 (*28*, *29*).

HABP **6737** analogues also presented the same shifted register reading. Immunogenic, protection-inducing peptide **22834** presented high HLA-DRβ1*1101 (Table 2) binding capacity, its functional register reading starting at I11 which fits into pocket 1, N14 in pocket 4, K16 in pocket 6, and L19 in pocket 9. All of these (except L19) are characteristic peptide binding motifs for HLA-DRβ1*1101 (*28*, *29*). Meanwhile, short-lived antibody-inducing peptide **14096** bound with high capacity to HLA-DRβ1*0301 (Table 2), shifting its register reading into M12 in pocket 1, D15 in pocket 4, S17 in pocket 6, and I20 in pocket 9 with characteristic HLA-DRβ1*0301 binding motifs (*28*, *29*).

The immunogenic, protection-inducing **13492** peptide HABP **6671** analogue bound to HLA-DRβ1*0401 with high affinity, all its amino acids corresponding to allele characteristic motifs, while peptide **10000** (short-lived, antibody-inducing) bound with high affinity to HLA-DRβ1*1101 (Table 2).

DISCUSSION

Table 1 lists the amino acid sequences of modified HABPs inducing high short-lived antibody titers detected by IFA 10 or 15 days after the second immunization; these disappeared almost immediately and could not be detected 15 days after

the third immunization. These data was corroborated by Western blotting, suggesting that antibodies induced by these modified very short-lived HABPs are not associated with protection since none of the *Aotus* monkeys immunized with them became protected against experimental challenge, an outcome different from that for modified HABPs inducing long-lived antibodies which did protect some *Aotus* monkeys.

It is clear that certain modifications to native nonimmunogenic, non-protection-inducing HABPs make them able to induce high very short-lived antibody titers which are not associated with protection. It can be seen in Table 1, Western blotting, and Table 2 that modified HABPs inducing very early appearing antibodies (II_{10}) (being a minority, which then definitively disappear) are associated with the HLA-DR53 haplotype's genetic characteristics (including HLA-DRβ1*0401 and HLA-DRβ1*0701 alleles). It can also be seen that those modified HABPs inducing medium-lived, later-appearing antibodies lasting 10 or 15 days (II_{10} and II_{15}) after the second immunization and then definitively disappearing are almost totally associated with HLA-DR52 (including HLA-DRβ1*0301 and HLA-DRβ1*1101 alleles) and HLA-DR8 (HLA-DRβ1*0801) genetic characteristics. It can be suggested that there is an association between HLA-DR's genetic characteristics and appearing times for non-protection-inducing short-lived antibodies.

^{1}H NMR structural analysis has shown clear differences between immunogenic and protection-inducing peptides **13450**, **23230**, **9782**, **22834**, and **13492** and short-lived antibody-inducing peptides **15484**, **21742**, **15474**, **14096**, and **10000**.

Modified long-lived, antibody- and protection-inducing HABPs (shown in Table 2) presented α-helices having different extension and localization when compared to short-lived antibody-inducing peptides. It can be seen that peptide **15484** inducing short-lived antibodies had an α-helix between A9 and K17, while immunogenic, protection-inducing **13450** had an α-helix between V2 and Q18. Peptide **15474** (short-lived, antibody inducing) had an α-helix between K7 and K17, while its analogue (protection-inducing peptide **9782**) had a shorter α-helix between amino acids P3 and N11. Another example of short-lived antibody-inducing peptide helix displacement can be observed in peptide **22834** having a shorter α-helix between residues V5 and V10, while its analogue (short-lived antibody-inducing **14096**) had a helical region between V5 and M12. Peptide **21742** (short-lived, antibody-inducing) presented displacement in its α-helical region between N4 and A12, when compared to its protection-inducing analogue **23230** which has a helical region between residues N9 and H16. A similar phenomenon occurred with other peptides involved in this study.

Analyzing the binding of different modified short-lived, antibody-inducing HABPs to HLA-DRβ1* molecules showed that there was a change in the binding of these peptides to other HLA-DRβ1* molecules. This variation was associated with a change in binding register and a shortening of the distance (in short-lived, antibody-inducing peptides) between residues theoretically binding to pockets 1−9 of these molecules, when compared to modified immunogenic, protection-inducing HABP distances.

Peptide **15474** (short-lived, antibody-inducing), for example, bound to HLA-DRβ1*0301 with a 15.2 Å distance between pockets 1 and 9 (obtained from the structural model), while its analogue (protection-inducing peptide **9782**) bound to HLA-DRβ1*1101 with a 22.1 Å distance between amino acids binding to the same pockets (6.9 Å difference when comparing these distances between the two peptides).

Another example of this phenomenon is modified short-lived antibody-inducing HABP **21742** which bound to HLA-DRβ1*1101 with a 12.7 Å distance between pockets 1 and 9 and its **23230** protection-inducing peptide analogue which bound to HLA-DRβ1*0301, presenting a 20.0 Å distance between the same pockets (7.3 Å difference).

A similar phenomenon was observed for other peptides where protection-inducing, short-lived antibody-inducing peptides bound to different HLA-DRβ1* molecules and where the difference in distance was 6.8 ± 0.5 Å between the residues fitting into pockets 1 and 9, when comparing each pair of peptides.

It is worth stressing the longer distances between those residues which could putatively be fitting into pockets 1−9 of immunogenic, protection-inducing peptides, cf., those inducing short-lived antibodies (Figure 3) identified according to previously defined binding motifs for these HLA-DRβ1* molecules (*28*, *29*). This difference was 6.8 ± 0.5 Å, making short-lived antibody-inducing peptides shorter in this region and more similar to native HABPs than immunogenic, protection-inducing peptides (Table 2).

General Considerations. The presence of short-lived antibodies induced by these modified HABPs could be due to the skewed induction of short-lived subclass antibodies, such as human IgG3 whose $t_{1/2}$ or half-time lasts 6.8 days, as happens in response to *P. falciparum* glycosylphosphatidylinositol (GPI) (30) or merozoite surface protein 2 (MSP2) (*31*), suggesting that continuous stimulation is necessary for achieving a certain degree of protection (*32*). However, the disappearance of these *Aotus* antibodies obeys more rapid kinetics than IgG3 half-time, and they do not reappear with further immunizations.

Another alternative could be the presence of anti-idiotypic antibodies in late sera (obtained III_{15}) which could be neutralizing the reactivity of these antibodies. Nevertheless, adding these late sera (even in excessive amounts) to the sera showing short-lived antibodies did not block or remove their reactivity (determined by IFA or Western blotting) (data not shown).

These short-lived antibodies are not *Aotus* IgM since antisera were specifically produced against purified *Aotus* IgG with a specific methodology that removes IgM as shown by immunoelectrophoretic analysis (data no shown).

These peptides could also be inducing antigen-presenting cell (APC) (monocytes and macrophages) apoptosis depending on the degree of APC maturity (*32*).

The fact that *Plasmodium chavaudii* can also induce selective deletion and apoptosis of antigen-specific CD4+ T-cells (*33*), leading to selective suppression of immune responses against this malarial parasite (preventing later protective immune responses), favors this hypothesis. Total parasite immunosuppression has also been shown in *Plasmodium yoelii* malaria infection; such suppression depends on the presence of regulatory CD25 CD4+ T-cells (*34*). In essence, specific global immunosuppression occurs during malarial infection, and such suppression could be mediated by selective apoptosis of APC or CD4+ T-lymphocytes or induction of CD25+ CD4+ T-cells.

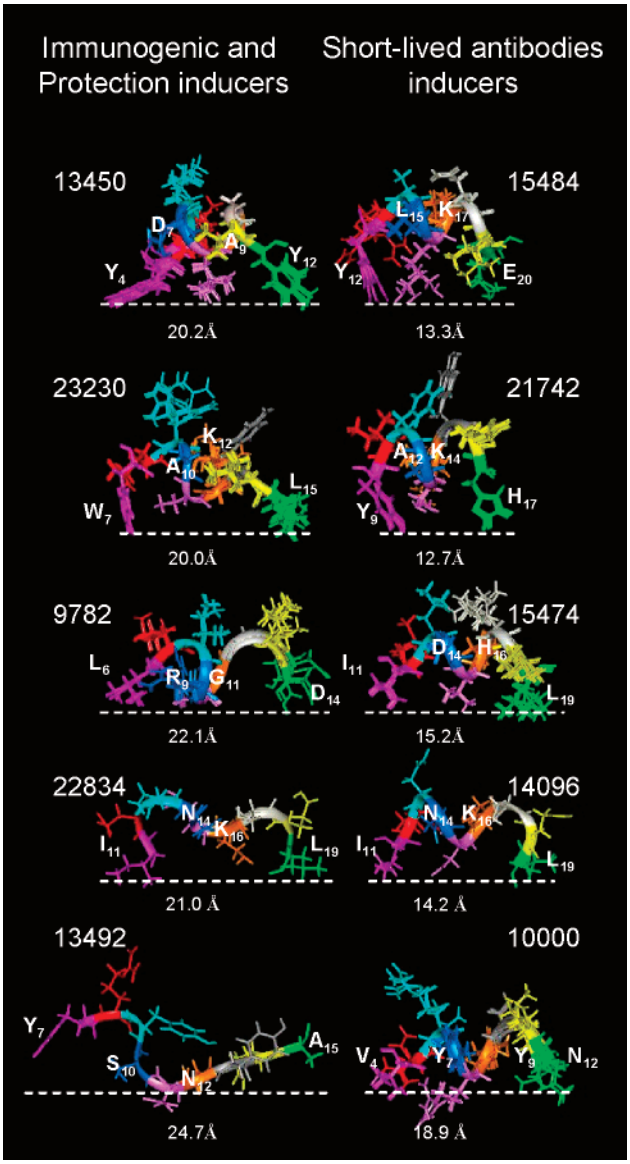

FIGURE 3: Immunogenic, protection-inducing high long-lasting antibody titer-inducing modified HABPs (left) and modified HABPs which only induced high short-lived antibody titers (right). The amino acid color code followed the HLA-DRB1* binding activities of immunogenic and protection-inducing modified HABPs: fuchsia for pocket 1, red for pocket 2, cyan for pocket 3, dark blue for pocket 4, rose for pocket 5, brown for pocket 6, gray for pocket 7, yellow for pocket 8, and green for pocket 9. Also shown are those amino acids which fit into pockets 1, 4, 6, and 9 in each of these peptides.

Since antibody production and protection do not recover over time or with later immunizations (such as the third), or new challenges, the apoptosis or selective deletion hypothesis (whether dealing with *Aotus* APC, CD4+ T-lymphocytes, or CD25+ CD4+ regulatory cell equivalents) is favored.

Since immunity against malaria is associated with MHC class II molecules (*9*), we thus suggest that inducing short-lived antibodies could be associated with imperfect or inappropriate fitting into the MHC II−peptide−TCR complex, due to a shift in register reading.

It is a very well-known phenomenon that peptides binding to class II molecules can be read in different functional registers in different scenarios. Some peptides are read within the context of different class II molecule isotypes from the same individual, for example, in the murine system with peptide Hb (residues 64−76 from hemoglobin) which is read from I68 to K76 by mouse I-E^k molecules (more similar to HLA-DRβ), but it is also read from V67 to I75 by mouse I-A^k molecules (more similar to HLA-DQβ) (*35*).

It has also been shown the same peptide can be presented within the context of different alleles from the same HLA-DRβ1* molecules, as happens with the myelin basic protein peptide (MBP 84−102). This peptide has been seen to bind to HLA-DRβ5*0101 and DRβ1*1501 molecules in two totally different functional registers (*36*).

The same class II molecule can also read the same peptide in two totally different functional registers as happens with peptide OVA 323−339 and the I-A^d molecule, where this peptide can be read by the same molecule from functional register 323−335 or alternatively from functional register 325−336. The first is unable to activate a T-cell hybridoma which can be activated by the latter. All these data show that a single peptide can bind to class II molecules in different functional registers, inducing different immune responses (*37*).

Selective use of TCR β genes has been found when T-cells are activated by alloreactive cells presented by certain HLA-DR (*38*) molecules, suggesting that peptides presented in a different functional register can activate T-lymphocytes with a different TCR receptor. As a result of this functional register reading shift, amino acids interact differently with the TCR, changing the hierarchy of TCR-contacting residues and producing different immunological responses.

This work shows that certain modifications made to HABPs can induce high levels of short-lived antibodies which are not associated with protection when faced with experimental challenge; production of such antibodies is not re-induced by new immunizations. On the basis of these data, we suggest that these particular peptide analogues could be malaria immunosuppression-inducing peptides, since antibodies did not reappear with further immunizations and monkeys had not become protected when rechallenged with the same strain 3 months later.

In this work, we show that these short-lived, peptide-inducing antibodies have a more compact α-helical structure, having a 6.8 ± 0.5 Å shorter distance than immunogenic and protection-inducing peptides between those residues theoretically fitting into pockets 1−9 of the HLA-DRβ1* molecule to which they bind (these distances were measured using the structural model obtained from the peptides) (Figure 3). This makes them structurally more similar to native peptides than to immunogenic, protection-inducing modified HABPs, suggesting a very interesting way for microbes to subvert the immune system. These peptides may theoretically allow *P. falciparum* malaria parasites to induce immunological suicide (apoptosis) of the host, CD4+ T-cells, immunosuppression, and therefore survival of the parasite. This shift in functional register reading and switching of allele may induce different MHC II peptide complex formation that could eventually lead to apoptosis. Special care thus has to be taken when designing and developing vaccines in the presence of these peptides.

On the basis of the fact that these antibodies did not begin to become produced again over time or with later immunizations, we suggest that the shifts in functional register reading of different alleles from the same haplotype, as shown here,

induced the formation of an inappropriate or unstable MHC II−peptide−TCR complex capable of inducing selective apoptosis of MHC II molecule-bearing cells (APC or CD4+ lymphocytes), partly explaining the total loss of the immune response to *P. falciparum*.

We therefore stress to those working on vaccine development (whether using biological or chemical approaches) that close attention must be paid to these peptides when developing new vaccines, since their presence in natural conditions or inappropriate design can lead to very short-lived immunogenic and/or protective response (or none at all) and long-term or permanent immunosuppression.

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