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# Emerging Rules for Subunit-Based, Multiantigenic, Multistage Chemically Synthesized Vaccines

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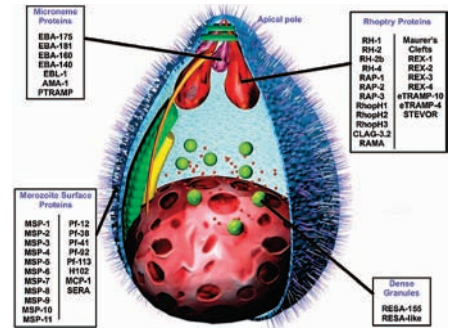
## CONSPECTUS

Seventeen million people die of transmittable diseases and 2/3 of the world's population suffer them annually. Malaria, tuberculosis, AIDS, hepatitis, and reemerging and new diseases are a great threat to humankind. A logical and rational approach for vaccine development is thus desperately needed.




Protein chemistry provides the best tools for tackling these problems. The tremendous complexity of microbes, the different pathways they use for invading host cells, and the immune responses they induce can only be resolved by using the minimum subunit-based (chemically produced ~20-mer peptides), multiantigenic (most proteins involved in invasion), multistage (different invasion mechanisms) vaccine development approach.

The most lethal form of malaria caused by *Plasmodium falciparum* (killing 3 million and affecting 500 million people worldwide annually) was used as target disease since many of its proteins, its invasion pathways, and its genome have been described recently. A New World primate (the *Aotus* monkey) is highly susceptibly to human malaria; its immune system molecules are 80–100% identical to those of its human counterpart, making it an excellent model for vaccine development.

Chemically synthesized ~20-mer peptides, covering all the *P. falciparum* malaria proteins involved in red blood cell (RBC) invasion were synthesized by the classical t-Boc technology (based on synthetic SPf66 antimalarial vaccine information for identifying targets) and assayed in a highly sensitive, specific, and robust test for detecting receptor–ligand interactions between high-activity binding peptides (HABPs) and RBCs. HABPs were identified, some in which the molecule displays genetic variability (to be discarded due to their tremendous complexity) and elicits a strain-specific immune response and others that are conserved (no amino acid sequence variation).

Conserved HABPs were synthesized in a polymeric form by adding cysteines at their N- and C-terminal ends to be used for monkey immunization. They became nonimmunogenic (no antibodies were induced) nonprotection inducers (monkeys were not protected against *P. falciparum* malaria challenge with a highly infective strain) suggesting a code of immunological silence or nonresponsiveness for these conserved HABPs.

A large number of monkey trials involving a considerable number of *Aotus* monkeys were performed to break this code of immunological silence by replacing critical residues (determined by glycine peptide analogue scanning) to find that the following amino acid changes had to be made to render them antibody and protection inducing: F↔R; W↔Y; L↔H; I↔N; M↔K; P↔D; Q↔E; C↔T.

The three-dimensional (3D) structure of >100 of these native modified HABPs (determined by $^1$H NMR) revealed that the following structural changes had all to be achieved to allow a better fit into the major histocompatibility complex class II (MHC II)−peptide−TCR complex to properly activate the immune system: $\alpha$-helix shortening, modifying their $\beta$-turn, adopting segmental $\alpha$-helix configuration, changing residue orientation, and increasing the distance of those residues fitting into the MHC II molecules from antigen-presenting cells. More than 100 such highly immunogenic, protection-inducing (against *P. falciparum* malaria) modified HABPs have been identified to date with this methodology, showing that it could lead to developing a highly effective subunit-based, multiantigenic, multistage synthetic vaccine against diseases scourging humankind, malaria being one of them.

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## Rationale for Multiantigenic Multistage Vaccine Development

The tremendous complexity of microbes and the way they interact with host cells combined with the immune responses elicited by them have led to the search for a logical and rational methodology for vaccine development including the multiple proteins (multiantigenic) or their fragments involved in the different stages of microbial invasion of susceptible cells (multistage). Rather than administering the whole microorganism as a vaccine, which could induce undesired effects, chemical synthesis allows the delivery of multiple functionally relevant (~20 amino acid) peptides, establishing the basis for minimal subunit-based synthetic vaccine development.

This task has proved to be extremely difficult in the case of malaria due to this parasite's complex life cycle within both mammal and invertebrate hosts (humans and *Anopheles* mosquitoes, respectively; Figure 1A), requiring the interaction of at least 58−90 proteins in just one of it stages, the red blood cell (RBC) invasion process.[1]

A brief description of the *Plasmodium falciparum* (the most lethal malarial parasite) life cycle is required to better understand its molecular interactions in invasion. Following the bite of a malarial parasite-infected mosquito, the inoculated **sporozoites** (larva-like parasites present in the mosquito's salivary glands) migrate to the liver and invade hepatocytes through the interaction of a yet unknown large number of proteins[2−4] [step a in Figure 1A].

After entering a hepatocyte, each parasite differentiates and proliferates within a week, ending up with the release of 30 000 parasites having a pear-shaped morphology, named **merozoites** [step b in Figure 1A and Figure 1B], and a new set of molecules,[5,6] which will target RBCs or erythrocytes for their reproduction.

Invasion of the RBC begins with the merozoite rolling over the RBC surface, a process mediated by weak receptor−ligand interactions carried out by the merozoite surface protein (MSP) family, of which 10 members (MSP1 to MSP10) have been described in *P. falciparum* to date,[7−10] as well as some other proteins named Pf12, Pf38, Pf41, Pf92 Pf113, and SERA [Figure 1B].

The parasite then, in a process mainly mediated by apical merozoite antigen-1 (AMA-1) protein,[11] orientates its apical pole to bring it into direct contact with the RBC. Parasite entry and invasion follows proteins being released from intracytoplasmic organelles (named micronemes and rhoptrias) [Figure 1B], a process taking place in less than 40 s.

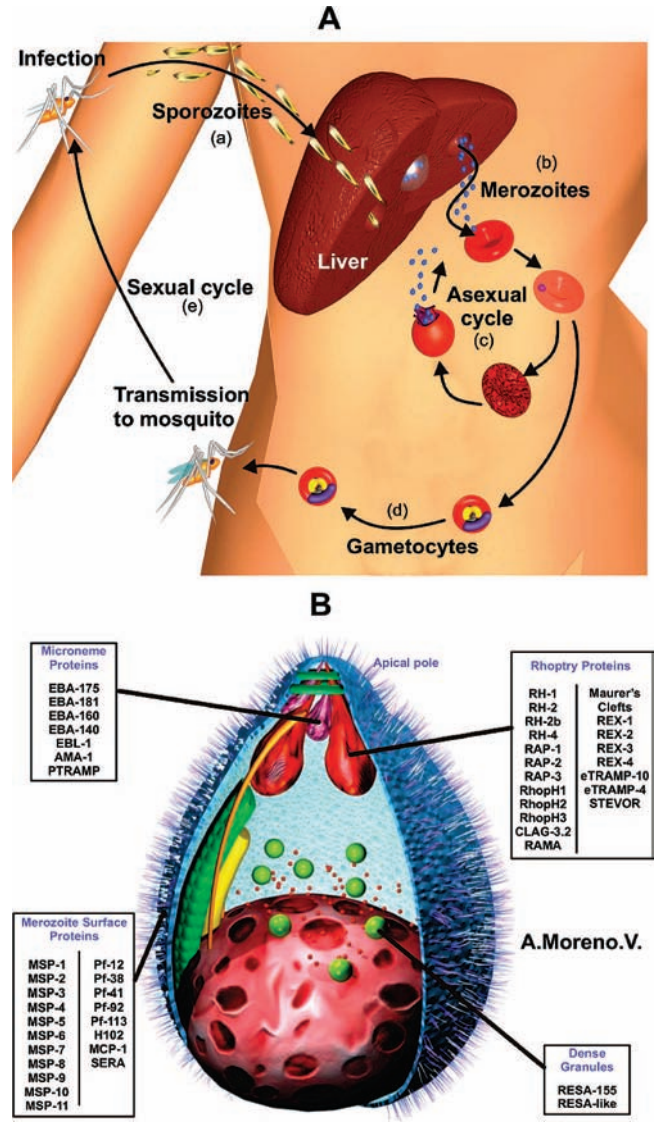


**FIGURE 1.** (A) The *P. falciparum* malaria-parasite life cycle in its human and mosquito hosts and (B) the merozoite structure with its most important cellular compartments and organelles, as well as the most important molecules involved in invasion of RBCs.

The erythrocyte binding antigen (EBA) family (including EBA-175, -180, -140 and -160 kDa proteins)[12−14] is of particular interest among microneme molecules [Figure 1B], since this group of proteins has been shown to be essential in invasion.

Some of these membrane and microneme merozoite proteins are anchored to the surface by a glycosyl phosphatidyl inositol (GPI) tail or a transmembrane domain, establishing a strong interaction with RBC and aiding formation of the parasitophorous vacuole (PV) in which the merozoite multiplies.

Other equally important rhoptry-localized proteins (found to be invasion mediators) are the rhoptry proteins (Rhop-1−3),[15] rhoptry-associated proteins (RAP1−3),[16] rhoptry H1−148/

CLAG9, and the CLAG family (CLAG-1−9)[17] [Figure 1B]. However, their role has still to be fully established.

Multiple molecules are thus involved in different parasite invasion stages, even more so when the parasite is able to switch-on or turn-off the expression of different proteins when sensing an immune response to an already expressed molecule or whenever the target receptor is not present on the host cell. This occurs when receptors for merozoite EBA-175, -140, and -181 proteins are not present on RBCs and the PfRh2b molecule is thus synthesized for invasion instead.[18] This also occurs in field isolates where some use high expression levels of Rh1 versus Rh2b proteins, or EBA-175 versus Rh4 proteins to mediate RBC invasion.[19] *P. falciparum* parasites are thus able to use multiple pathways to invade their target cells.

Following such adhesion and invasion processes, the parasite's intraerythrocyte development cycle continues through different stages (ring, trophozoite, schizont) [step c in Figure 1A], all of them being inside the RBC and difficult for the immune system to reach because they are hidden within the host cell. A vaccine against the whole array of proteins expressed during these intraerythrocyte maturation stages would thus have little or very limited efficacy.

New merozoites are released following RBC rupture [step c in Figure 1A] to invade new RBCs, such process repeating itself every 48 h (in the case of *P. falciparum*), sometimes leading to death due to the host's excessive physiological and immunological response against such proliferation.

Some merozoites (pear-shaped parasite forms) differentiate into **male and female gametes** displaying a banana-like form [step d in Figure 1A] and then are transported to a mosquito's midgut during its blood meal. They mate and undergo a series of transformations there in a sexual cycle [step e in Figure 1A] culminating in the development of new sporozoites, which migrate to the mosquito's salivary glands to be inoculated into another human host [Figure 1A], starting the cycle over again.

A completely effective vaccine must therefore contain not one but most of the molecules involved in the different stages of parasite invasion of host cells (i.e., it must be multiantigenic and multistage).

## Rationale for Subunit-Based Synthetic Peptide Vaccine Development

The parasite's tremendous genetic variability represents another formidable obstacle to overcome when developing a completely effective vaccine (there are only small protein fragments with no genetic amino acid sequence variations or **conserved** regions in MSP-1, -2, -3, and -6 proteins or AMA-1).[7,8,20−22]

Including all the parasite's genetic variants (thousands) involved in invasion, thereby trying to saturate the immunologic system for preventing a single parasite's escape from the immune response would be an impossible task, even more so when these genetic variants are found very close to functionally relevant amino acid sequences (such as those binding to host cells), thus biasing the immune system toward responding against functionally irrelevant regions.

These genetic variants induce clearly established biased parasite escape mechanisms (such as immune system nonresponsiveness), suppressing the immune response to other parasite genetic variations of the protein,[23] inducing or programming the death (or apoptosis) of specific immune response cells, etc.[24] This data clearly suggests the need for designing minimal subunit-based vaccines (preferentially synthetic ones) so as not to fall into the genetic variant-induced immunological traps described above.

## The Basis of Our Approach

Our institute thus began developing synthetic-peptide-based (15−25-mer), chemically synthesized vaccines containing several peptides derived from different parasite proteins and stages. The first such multiantigenic, multistage subunit-based chemically synthesized vaccine was produced 20 years ago after testing dozens of synthetic peptides in intravenously challenged *Aotus* monkeys, after vaccination, with a highly infective *P. falciparum* strain.[25] Those partially protective peptides were later synthesized in a single 45-mer synthetic peptide polymerized via N- and C-terminal cysteine addition, named SPf66.[26] After all monkey trials and safety and immunogenicity trials involving thousands of humans, this first synthetic vaccine (produced 20 years ago) was safe and immunogenic and provided ~35% protection in people older than 1 year in large field trials carried out in different parts of the world.[27−29] One lot produced elsewhere proved nonprotective.[30]

It was also found that two out of the three merozoite-derived amino acid sequences included in this vaccine[31] were derived from peptides that bound with high affinity to RBC. The fourth one was a sporozoite-derived peptide that bound with equally high affinity to hepatic cells, suggesting that what had been included in this vaccine were amino acid sequences having high host cell binding ability.

Strong genetic control of the immune response was also found when using SPf66; this was associated with major histocompatibility complex class II (MHC II) molecules, particularly HLA-DR, since individuals immunized with SPf66 who did not produce antibodies against the vaccine nor the parasite (nonresponders) and developed malaria in natural conditions were typed as being individuals carrying the HLA-DR$\beta$1* 0401 genetic characteristic.[32] It was also observed that nonresponders were preferentially using T-lymphocyte receptor (TCR) V$\beta$3, -10, and -11 families.[33] This suggested an imperfect SPf66 vaccine fit into the complex formed by MHC II−peptide−TCR molecules in such nonresponding individuals.

## Our Approach

Based on the fact that SPf66 synthetic vaccine included peptides having high RBC binding ability, a highly robust, specific, and sensitive methodology was designed for recognizing those relevant peptides in all proteins involved in RBC invasion. Sequences of 20 amino acids having high host cell binding ability[34] were thus assayed, leading to identification of peptides having high specific binding activity (HABPs).[35] Analyzing the polymorphism of HABPs led to us to find that some of these HABPs had conserved amino acid sequences[34−40] and others had variable ones, in addition to regions neighboring conserved HABPs having tremendous genetic polymorphism, perhaps used by the parasite as a distraction mechanism for evading the immune response. Faced by such tremendous genetic polymorphism and related immunological biasing effects, the rational and logical development of vaccines focused on producing a conserved HABP-induced protective immune response.

However, another insurmountable immunological problem hindered finding a rapid solution; conserved HABPs are immunologically silent (i.e., they are not seen by the immune system), and therefore, in natural infection conditions, they do not induce antibody, cellular, or protective immune responses directed against them. On the other hand, variable HABPs are highly antigenic (visible by the immune system during natural infection) inducing very high but strain-specific antibody levels.

Very strong antibody-mediated (humoral) or cellular immune responses have been found by our institute against variable sequences (irrespective of their binding property) and humoral or cellular immune response against conserved sequences that did not bind to the host cell; however, no immune response has so far been found against conserved HABPs. The immunological code of silence (or immunological nonresponsiveness) is thus mainly directed toward conserved HABPs.

## How To Resolve the Problem

Many studies have been carried out on a significant number of *Aotus* monkeys to resolve the problem of the immunological code of silence toward conserved HABPs. These primates are highly susceptible to developing human malaria, besides having an almost identical immune system to that of humans, as assessed by cloning and sequencing the most relevant immune system molecules, such as immunoglobulins, T cell receptors $\alpha\beta$ and $\gamma\delta$, MHC class I and II proteins, cytokines, etc. They were immunized with polymerized modified conserved HABPs (homogenized with Freund's adjuvant) in which some critical residues in RBC binding (previously identified by glycine analogue peptide scanning) were replaced in the search for some immune response and thereby a break in the HABP immunological code of silence.[41]

Most of these modifications provided totally negative results (group C in Tables 1 and 2); however, some changes made to these modified HABPs during the last 10 years have led to induction of high short-lived antibody titer production (determined by immunofluorescence (IFA) and Western blot)[42] and the absence of protection against experimental challenge performed 20 days after the third immunization, when they were experimentally challenged using a 100%-infective dose of an *Aotus*-adapted *P. falciparum* strain (group B in Tables 1 and 2).

Some changes in these modified HABPs induced high long-lived antibody titers without inducing protection against experimental infection of *Aotus* monkeys (group B in Tables 1 and 2), phenomena related to the residues' orientation and peptide configuration in modified HABPs.[43]

However, certain specific amino acid replacements did induce high long-lived antibody titers associated with protection against experimental challenge (group A in Tables 1 and 2).

Physicochemical and biological principles began to emerge from these studies performed in the *Aotus* monkey; one of them stressed that the critical amino acids in RBC binding (as assessed by glycine analogue scanning)of these conserved HABPs must be replaced by others having similar mass, volume, and surface, but opposite polarity to make these conserved and immunologically silent HABPs become immunogenic and protection-inducing. Therefore, the following amino acid pairs can be mutually substituted: F↔R; W↔Y; L↔H; I↔N; M↔K; P↔D; Q↔E; and

**TABLE 1.** Antibody-Mediated Immune Response and Protective Efficacy Induced by 5501 (MSP-1 derived HABP) and Its Analogues[58] [a]

| polymerized peptide no. | peptide sequence | IFA 2nd[b] | IFA 3rd[c] | nr. of protected monkeys | group |
|---|---|---|---|---|---|
| 5501 | MLNISQHQCVKKQCPQNS | 0/5 | 0/5 | 0/5 | |
| 12926 | --------S----IS--- | 0/5 | 1 (640) | 1/5 | |
| 12924 | --------S----AD--- | 0/5 | 1 (640) | 1/5 | |
| 12920 | --------S----SD--- | 1 (160) | 0/5 | 1/5 | A |
| 15522 | --------T-MMMT---- | 2 (640) | 0/3 | 1/3 | |
| 24148a | -----ML-T-MMMT--K- | 2 (2560) | *d* | 2/8 | |
| 24148b | -----ML-T-MMMT--K- | 1 (640) | *d* | 1/8 | |
| 15968 | -H----L-T----TD--- | 1 (320) | 0/6 | 0/5 | |
| 13466 | ------M-S----SD--- | 3 (1280) | 0/6 | 0/6 | |
| 13978 | --------S-M-MSD--- | 3 (640) | 0/5 | 0/5 | |
| 15524 | -H------T-MMMT---- | 1 (320) | 0/3 | 0/3 | |
| 22788 | ------M-V----SD-K- | 1 (640) | *d* | 0/10 | B |
| 22810 | ------M-V----VD-K- | 1 (640) | *d* | 0/10 | |
| 23754 | -H----L-V---MV--K- | 1 (640) | *d* | 0/8 | |
| 13984 | -H------S-MMMSD--- | 0/6 | 2 (320) | 0/5 | |
| 13980 | -H------S-M-MSD--- | 0/6 | 1 (320) | 0/4 | |
| 13982 | --------S-MMMSD--- | 0/6 | 1 (640) | 0/6 | |
| 7556 | -G------S-G------- | 0/5 | 0/5 | 0/4 | |
| 7560 | -G------S---G----- | 0/5 | 0/5 | 0/5 | |
| 7562 | -G------S----G---- | 0/5 | 0/5 | 0/4 | |
| 7370 | --------S---G-G--- | 0/5 | 0/5 | *d* | |
| 9582 | --------S----GG--- | 0/5 | 0/5 | 0/5 | |
| 9576 | --------S---GG---- | 0/5 | 0/5 | 0/5 | |
| 9608 | --------S-G-GG---- | 0/5 | 0/5 | 0/5 | |
| 12922 | --------S----VD--- | 0/5 | 0/5 | 0/5 | |
| 13468 | ------L-S----SD--- | 0/5 | 0/4 | 0/4 | |
| 13470 | -H----M-S----SD--- | 0/6 | 0/6 | 0/6 | |
| 13472 | -H----L-S----SD--- | 0/6 | 0/6 | 0/6 | |
| 13750 | ------M-S-M-MSD--- | 0/5 | 0/5 | 0/5 | |
| 13746 | ------M-S---MSD--- | 0/5 | 0/5 | 0/5 | |
| 13754 | ------M-S----VD--- | 0/5 | 0/5 | 0/5 | |
| 13756 | -H----M-S----VD--- | 0/5 | 0/5 | 0/5 | |
| 13760 | -H----M-S---MVD--- | 0/5 | 0/5 | 0/5 | |
| 13762 | ------M-S-M-MVD--- | 0/5 | 0/5 | 0/5 | C |
| 13764 | -H----M-S-M-MVD--- | 0/5 | 0/5 | 0/5 | |
| 15520 | -H------T-M-MT---- | 0/5 | 0/4 | 0/4 | |
| 15528 | -H------T-M-MTD--- | 0/5 | 0/5 | 0/5 | |
| 24326 | -----ML-T-M-MT--K* | 0/7 | *d* | 0/7 | |
| 15530 | --------T-MMMTD--- | 0/5 | 0/5 | 0/5 | |
| 15532 | -H------T-MMMTD--- | 0/4 | 0/4 | 0/4 | |
| 13758 | ------M-S---MVD--- | 0/5 | 0/5 | 0/5 | |
| 13478 | -H----L-S----PV--- | 0/6 | 0/6 | 0/6 | |
| 15970 | -H------T----TD--- | 0/6 | 0/4 | 0/4 | |
| 13474 | -H----M-S----PV--- | 0/6 | 0/6 | 0/6 | |
| 17946 | ------M-S---ISD*-- | 0/6 | 0/6 | 0/6 | |
| 15966 | -H------T----T---- | 0/6 | 0/6 | 0/6 | |
| 15972 | --------T----TD--- | 0/6 | 0/4 | 0/4 | |
| 22446 | -H----L-S----ADK* | 0/6 | *d* | 0/6 | |
| 2808 | -H----M-V----VD-K* | 0/10 | *d* | 0/10 | |
| 3282 | ------M-S---MAD-K* | 0/10 | *d* | 0/10 | |
| 24074 | -H----M-V---MVD-K* | 0/9 | *d* | 0/9 | |
| controls | | 0/100 | 0/100 | 0/100 | |

[a] Antibody titers were assayed by immunofluorescence antibody test (IFA) 15 days after the second ($II_{15}$) and 20 days after the third ($III_{20}$) immunization. Protection was defined as being the complete absence of parasites in immunized monkey blood for 15 days following challenge. Control as well as nonprotected monkeys developed patent parasitemia by days 5 or 6 that required treatment by days 8 or 9. [b] Number of monkeys with antibody titer by IFA >1:160 after the second immunization. [c] Number of monkeys with antibody titer by IFA >1:160 after the third immunization. [d] Not determined.

C↔T. S, A, and G, being very small amino acids lacking counterparts having opposite polarity, need special consideration. Modifications to critical binding residues would thus seem to have broken the immunological code of silence of the conserved binding sequences (Tables 1 and 2).

## Native and Modified HABP 3D Structure

It has been argued for a long time that short synthetic peptides do not display the same structure that they show in native proteins, casting doubts on a minimal subunit-based

**TABLE 2.** Antibody Response and Protection Induced by 4325 (AMA-1 Derived HABP) and Its Analogues in *Aotus* Monkeys[54] [a]

| polymerized peptide no. | peptide sequence | IFA 2nd[b] | IFA 3rd[c] | nr. of protected monkeys | group |
|---|---|---|---|---|---|
| 4325 | MIKSAFLPTGAFKADRYKSH | 0 | 0 | 0/6 | |
| 13486 | ---AS-D------SP----- | 3 (2560) | 2 (1280) | 2/5 | |
| 15516 | ---A--H-----MS--W--- | 1 (1280) | 1 (160) | 1/4 | |
| 20034 | ---A--------M------- | 2 (320) | 1 (160) | 2/8 | A |
| 20032 | ---A---------------- | 1 (160) | 1 (160) | 1/8 | |
| 22784 | ---VG-D------SP----- | 1 (2560) | *d* | 1/10 | |
| 23404 | ---VS-D-----MS------ | 2 (320) | *d* | 1/10 | |
| 23406 | ---VS-DD----MS------ | 1 (320) | *d* | 1/10 | |
| 14518 | ---VG-H------SP-W--- | 1 (320) | 0 | 0/3 | |
| 14520 | ---VG-H-----MSP----- | 2 (640) | 0 | 0/3 | B |
| 17934 | ---AS-HV----MS------ | 2 (320) | 0 | 0/7 | |
| 17936 | ---AS-HV----MSP-W--- | 2 (640) | 0 | 0/5 | |
| 9192 | ----G--------------- | 0 | 0 | 0/5 | |
| 9196 | -------------G------ | 0 | 0 | 0/5 | |
| 10100 | ---RG--------------- | 0 | 0 | 0/5 | |
| 10102 | ---FG--------------- | 0 | 0 | 0/5 | |
| 10104 | ---GT--------------- | 0 | 0 | 0/5 | |
| 12708 | ----------------W--- | 0 | 0 | *d* | |
| 15512 | ---A---------S--W--- | 0 | 0 | 0/4 | |
| 15510 | ---A---------S------ | 0 | 0 | 0/4 | |
| 16000 | ----S--------------- | 0 | 0 | 0/5 | |
| 16008 | ------H-------P----- | 0 | 0 | 0/4 | C |
| 16002 | ----S-H------------- | 0 | 0 | 0/5 | |
| 16004 | ----S-H-------P----- | 0 | 0 | 0/5 | |
| 16006 | ----S---------P----- | 0 | 0 | 0/5 | |
| 17932 | ---AS-D------SP-W--- | 0 | 0 | 0/7 | |
| 23178 | ---VS-D----------G- | 0 | *d* | 0/9 | |
| 23774 | ---VS-HD---MS------ | 0 | *d* | 0/9 | |
| 22424 | ---SS-HV------P----- | 0 | *d* | 0/8 | |
| 23000 | ---V--D------SP----- | 0 | *d* | 0/7 | |
| controls | | 0/60 | 0/60 | 0/60 | |

[a] IFA titers are the maximum reciprocal dilution in this group of monkeys, where 3 (2560) means that 3/10 monkeys had a 1:2560 titer. [b] Number of monkeys with antibody titer by IFA >1:160 after the second immunization. [c] Number of monkeys with antibody titer by IFA >1:160 after the third immunization. [d] ND= not determined, since they received only two doses instead of three.

synthetic vaccine approach. This could be true for very short 5−10-mer peptides; however, we have observed that our 18−25 amino acid conserved native HABP three-dimensional (3D) structures determined by $^1$H NMR studies could be practically superimposed on the corresponding amino acid sequences in the few recombinant malarial molecules whose 3D structures had been determined by X-ray crystallography. An example of this can be seen when superimposing conserved HABP 1783 structure [shown in blue in Figure 2] determined by $^1$H NMR[44] on top of its corresponding residues 580−599 (red) from EBA-175 region II recombinant protein fragment (yellow) determined by X-ray crystallography[45] [Figure 2]. Root mean square deviation (rmsd) was 0.89. Furthermore, the same crystallographic studies have shown that glycan residues from RBC glycophorin A specifically bind to this conserved HABP.[45] The same occurs with other conserved HABPs such as AMA-1-derived 4325 and MSP-derived 4044.

The biological functions that have been determined for these native conserved HABPs (i.e., host cell binding ability[34−40]) and immunological characteristics such as an immunological code of silence could thus be equal to or very similar to those presented by native proteins, strongly supporting our approach aimed at working with 15−25-mer, chemically synthesized peptides as a logical and rational methodology for minimal subunit-based synthetic vaccine development.

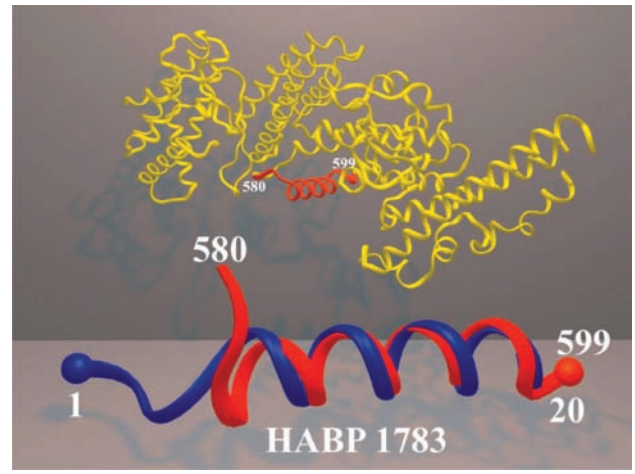


**FIGURE 2.** X-ray crystallography determined 3D structure of the recombinant EBA-175 region II (yellow),[45] and localization of HABP 1783 (red) in residues 580−599 (top panel). At the bottom, the almost complete superimposition of EBA-175 recombinant region II amino acid sequence (red) on conserved HABP 1783 3D structure (blue) as assessed by $^1$H NMR,[44] displaying 0.89 rmsd.

## Structural and Binding Characteristics of HLA-DR Molecules

A peptide must be appropriately adjusted in the binding groove of antigen-presenting cell MHC class II molecules, consisting of two membrane-anchored chains [α and β], to become immunogenic, thereby allowing residues interacting with amino acids from the $P_1$, $P_4$, $P_6$, and $P_9$ pockets of these molecules[46] to fit appropriately into such pockets[47] [Figures 3A,B] and thereby leading to H-bond formation between the backbone atoms of the peptide and class II amino acid residues.[46–49] These 11 H-bonds stabilize peptide binding to this molecule, and if the other residues ($-P_2$, $-P_1$, $P_2$, $P_3$, $P_5$, $P_7$, $P_8$, and $P_{10}$) are appropriately orientated in the opposite direction to the class II molecule groove, then these residues interact with the different variable regions of the TCR chains (α and β) called $CDR_1$, $CDR_2$, and $CDR_3$ to form the correct MHC II−peptide−TCR complex, thereby inducing appropriate immune system activation[47–49] and antibody production.

Independently of native conserved HABP three-dimensional structure (α-helix, β-turn, random structure), these peptides' fundamental characteristic is that they do not bind to class II molecule groove or do so imperfectly, thus hampering the appropriate and stable MHC−peptide−TCR complex formation for inducing an appropriate immune response.

On the other hand, modified HABPs acquire structural characteristics slightly different from their native parental ones, such as shortening their α-helices, adopting short α-helical structures in those peptides presenting a random structure, modifying their β-turns, elongating those in which molecules are adjusted to certain HLA-DR alleles by 21.5 ± 1.5 Å or elongating to 25.5 Å those that bind to others, changing residue orientation, or changing affinity for certain alleles.[41,44,50–58] This distance was 6.5 ± 0.5 Å and 4.5 ± 1.5 Å shorter in short-lived and long-lasting antibody-inducing but non-protection-inducing modified HABPs, respectively, than in immunogenic, protection-inducing ones; residue orientation was also different.[42,43] In essence, immunogenic protection-inducing modified conserved HABPs have been modified so that they can fit perfectly into the MHC II−peptide−TCR complex for triggering an appropriate immune response, providing tremendous support for using chemically synthesized, specifically modified conserved HABPs in vaccine development.

Because innumerable physicochemical and biological factors are involved in developing a totally protective vaccine, it must be based on small conserved subunits to avoid falling into the trap of microbial genetic variability. It must be multiantigenic to cover all microbial host cell ligands (and their switch on and turn off strategy to evade the immune response or host cell genetic polymorphism)[18,19] and must include all modified HABPs derived from the microbe's different invasive stages (multistage). Chemical synthesis represents the most stable, rapid, economic, easily reproducible way of doing this.

Modifications made to conserved HABPs have shown an appropriate fit in the groove of HLA-DRβ1* 0403 molecules [Figure 3C] suggesting that such modifications led to the appropriate formation of the MHC II−peptide−TCR complex for inducing an immune response, which was not just immunogenic but also induced protection against experimental challenge. This occurred when *Aotus* monkeys were immunized with peptide 24112 analogue modified from MSP-2 protein HABP 4044.[52] This modified HABP induced very high antibody titers and complete protection in 66% of the immunized *Aotus* carrying the HLA-DR β1* 0403 genetic characteristic, as assessed by DNA sequencing. Molecular modeling and docking of this modified 24112 HABP structure (as determined by $^1$H NMR)[52] into the HLA-DRβ1* 0401 (as reported by X-ray crystallography studies)[47] allowed the spontaneous formation of seven out of the eleven H-bonds between the backbone atoms of this modified HABP and the corresponding atoms of the interacting amino acids of the HLA-DR molecule. This supported the suggestion that modifications performed on this modified HABP allowed a better fit into the MHC II−peptide−TCR complex despite the different methodologies used for determining their 3D structure. It is worth stating that native parental HABP 4044 did bind to anyone of the HLA-DR molecules studied, did not induce antibodies against the *P. falciparum* parasite or protection against experimental challenge (manuscript in preparation), and displayed a completely different 3D structure as assessed by $^1$H NMR.[52]

These results essentially show that conserved HABPs must be modified to achieve the appropriate formation of the MHC−peptide−TCR complex to induce an appropriate immune response and thereby break the immunological code of silence of these highly relevant amino acid sequences required for two very important microbial survival mechanisms: adhesion and invasion.

Elegant proteomic and transcriptome studies have shown how a minimum of 58 proteins mediate merozoite invasion of RBC;[1] a similar number of molecules must thus be blocked.[34–40] Our group has identified numerous conserved HABPs from these proteins; many of these modified HABPs have been proved to be able to induce a long-lived antibody response as well as their protection-inducing ability when faced by experimental challenge.[41,44,50–58] The 3D structures for more than a hundred of them (native and modified) have

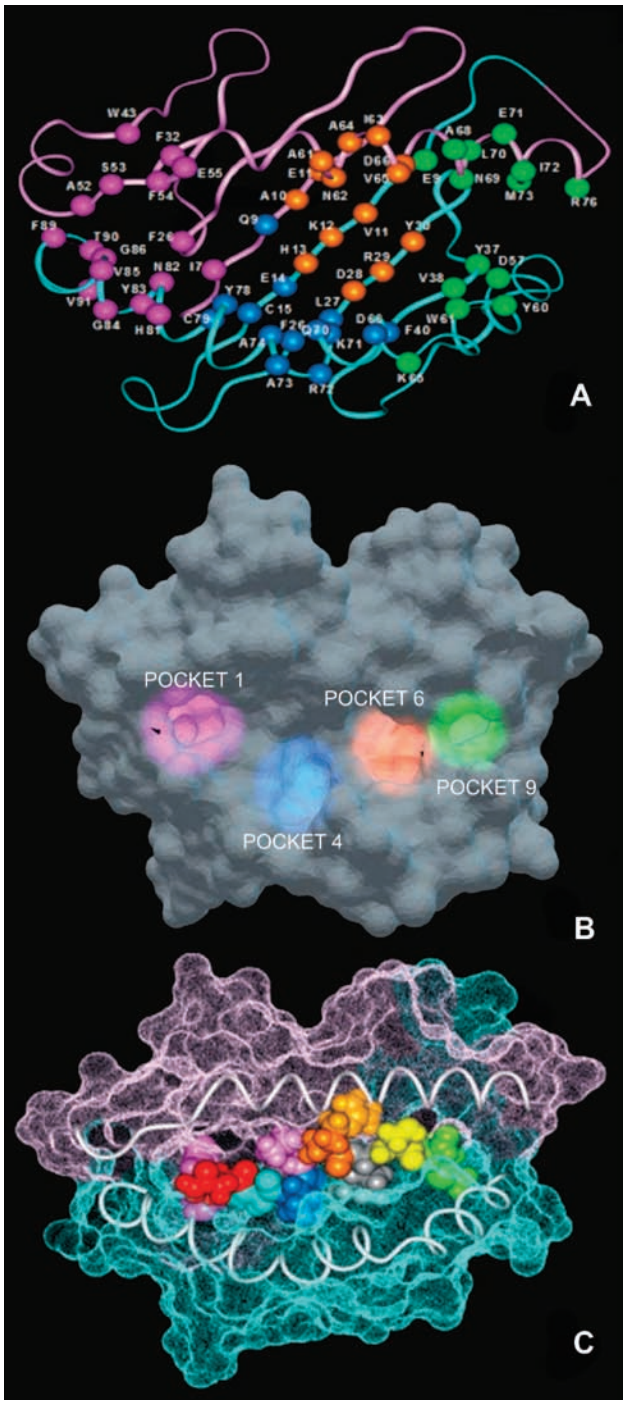


**FIGURE 3.** (A) Ribbon diagram of the HLA-DRB1*0401 molecule, showing the α-chain in pink and the β-chain in light blue. Amino acids and their surfaces forming pocket 1 are displayed in fuchsia, those of pocket 4 in blue, those of pocket 6 in light brown, and those of pocket 9 in green.[64] (B) HLA-DRβ1*0401 molecule surface highlighting the pockets. (C) Docking immunogenic *P. falciparum* malaria protection-inducing peptide 24112 into the HLA-DRB1*0401 molecule. Van der Waals surface color code for 24112 peptide amino acids and those conforming the MHC II–peptide–TCR complex are the same as those fitting into pockets 1, 4, 6 and 9, with the other residues in red (P2), turquoise (P3), rose (P5), gray (P7), and yellow (P8).

been determined by our $^1$H NMR studies,[41,44,50–58] leading to emphasis of the universality of the principles defined here. It has not escaped us that this methodology can be used for any other type of microbial agent, as ongoing work in our institute is being directed toward this end.[59–62]

Basically, developing particular vaccines against malaria, which causes more than 500 million cases annually, killing 3 million people, mainly children aged less than 5 in sub-Saharan Africa,[63] is a very complex event involving an extremely elaborate chemical, physical, biological, and immunological approach in an attempt to resolve it.

This Account provides logical and rational reasoning for developing multiantigenic, multistage, subunit-based vaccines by chemically synthesizing peptides and establishing principles for this, opening up a promising field for controlling humanity's main transmissible diseases, which take more than 17 million lives annually, malaria being one of them.

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## BIOGRAPHICAL INFORMATION

**Manuel Elkin Patarroyo** (born in 1946) received his M.D. degree from the Universidad Nacional de Colombia (National University of Colombia, School of Medicine) in 1971, where he is Full Professor for Molecular Pathology. He did postdoctoral studies at Rockefeller University in the United States with Professor Henry Kunkel and on Tumor Immunology at Karolinska Institute, Sweden, with Professor George Klein. In 1976, he founded the Instituto de Inmunología at the San Juan de Dios Hospital, fully devoted to the development of chemically synthesized vaccines, malaria being one of them, with the advice of Professors Bruce Merrifield and David Andreu (Rockefeller University), and Professor Richard Lerner (Scripps Research Institute). The first chemically synthesized vaccine against this scourging disease was published in *Nature* in 1987 followed by a large series of clinical and field trials in different parts of the world that allowed the conclusion of the feasibility of chemically synthesized vaccines. He has received numerous awards including the Award of the Third World Academy of Science (TWAS) in 1988, the Prince of Asturias Award in Science and Technology (Spain) in 1994, the Robert Koch Award (Germany) in 1994, the Medicin de l'année Award (France) in 1995, the Edinburgh Medal (England) in 1995, the National Science Award (Colombia) in 1986, 1984, 1982, and 1978, and 26 Honoris Causa Doctor degrees from different universities throughout the world. He is founder and current director of the Fundación Instituto de Inmunología de Colombia since 2001.

**Manuel Alfonso Patarroyo** (born in 1972) obtained both his M.D. and Dr.Sc. degrees from the National University of Colom-

bia. He is currently Full Professor at the Universidad del Rosario School of Medicine in Bogotá, Colombia, and Adjunct Professor at the National University of Colombia School of Medicine. His research interests have been mainly focused on developing vaccines against *Plasmodium vivax* malaria and tuberculosis. He and his group have successfully tested a recombinant vaccine candidate against *P. vivax* in *Aotus* monkeys, becoming one of the leading groups in vaccine development against this scourging disease. Besides his work on infectious diseases, Professor Patarroyo has also been working on the molecular characterization of the immune system components of the *Aotus* monkey, an ideal experimental model for testing vaccines for humans. He is currently the head of the Molecular Biology Department at the Fundacion Instituto de Inmunologia de Colombia.

## FOOTNOTES

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