0022-1767/90/1455-1557$02.00/0
THE JOURNAL OF IMMUNOLOGY
Copyright © 1990 by The American Association of Immunologists
Vol. 145, 1557-1563, No. 5, September 1, 1990
Printed in U.S.A.

SECONDARY STRUCTURE AND IMMUNOGENICITY OF HYBRID SYNTHETIC PEPTIDES DERIVED FROM TWO *Plasmodium falciparum* PRE-ERYTHROCYTIC ANTIGENS[1]

J. ARTURO LONDOÑO,[2]* HÉLÈNE GRAS-MASSE,† CHRISTINE DUBEAUX,† ANDRÉ TARTAR,† AND PIERRE DRUILHE*

*From the *Laboratoire de Parasitologie Médicale, Institut Pasteur, Paris, France; and †Laboratoire de Chimie de Bio-Molécules, URA CNRS 1309, Institut Pasteur de Lille, Lille, France*

Multicomponent synthetic vaccines containing both B and T cell epitopes belonging to two different pre-erythrocytic Ag of *Plasmodium falciparum* are presented. In a di-component hybrid, a circumsporozoite T cell epitope and a peptide representing a liver stage-specific Ag were connected to obtain a reciprocal reinforcement of helical potentials. In a tri-component hybrid, a sequence corresponding to the circumsporozoite repeat tetrapeptide (NPNA) was tandemly synthesized on the N-terminal end of the di-component hybrid. Both hybrid molecules were able to adopt a partial helical conformation in water as determined by circular dichroism studies. To analyze if the different components were immunologically functional in these vaccines, mice bearing genetic backgrounds known to respond or not to the individual components were immunized with the hybrids. The tri-hybrid peptide showed high immunogenic capacity as it elicited, in both H-2^b and H-2^k mice, high antibody responses against every separate individual sequence. Moreover, the antibodies induced by these conformationally restricted peptides were able to recognize the corresponding native proteins in the liver schizont and the sporozoite surface. H-2^d mice, in which the immune response to the individual components was genetically restricted, did respond against the di-hybrid peptide. The tri-hybrid peptide, in which NPNA repeats were present, lacked this H-2^d-priming capacity but it triggered antibody production in H-2^d mice previously primed with the di-hybrid peptide. These results indicate that multivalent vaccines can provide positive (potentiating) effects by carefully combining structurally well defined epitopes; however, negative (suppressive) effects are also possible suggesting that selection of multivalent vaccine components will require testing of combined molecules to optimize specific immune responses and avoid undesirable effects which may result from negative molecular interactions.

After inoculation of *Plasmodia* sporozoites by the mosquito, multiple parasite stages occur in the host. Expression of specific Ag has been reported in different stages of the parasite's development (1-4) and protective immunity has been shown to be stage specific (5, 6). Difficulties in the development of malaria vaccines arise from the antigenic variability among both strains and stages of the parasite and from the genetic restriction of the immune response to subunit vaccine candidates (7-11). Modern approaches aimed at overcoming some of these obstacles include the construction of multivalent vaccines by molecular biology methods (12) or chemical synthesis (13, 14) and the assessment of different ways of presenting these molecules to the immune system.

With regard to the pre-erythrocytic stages of the parasite, particular attention has been paid to the immunodominant surface Ag of the sporozoite, the CSP[3] (15). In *Plasmodium falciparum*, this protein contains a tandemly repeated tetrapeptide sequence (Asn-Ala-Asn-Pro) (16). Chemically synthesized and cloned peptide fragments representing the repeated sequence have been the object of two human vaccine trials and have shown little success (17, 18). In this context, the design of multivalent vaccines combining molecules of both sporozoite and liver stage sequences may constitute a logical complementary approach.

We previously identified and characterized a *P. falciparum* liver stage Ag, the LSA (2), which reacts specifically with most sera collected from individuals naturally exposed to malaria (2). (J. A. Londoño, manuscript in preparation). The primary structure of this protein presents a highly charged 17 amino acid repeat sequence; a synthetic peptide representing the 17 residue repeat unit is able to adopt a significant helical organization in water and is recognized by human and experimentally induced antibodies (19); smaller peptides neither do organize nor are antigenic. The possible biologic role of this molecule and the antibodies it elicits remains unknown. However, as the LSA is localized on peripheral parasite structures throughout the liver schizogony and is internally distributed in the mature liver schizont (2), it may constitute a target of specific immune mechanisms either during the hepatic phase or when liver merozoites are released to invade RBC.

Recent reports (20-25) suggest that conformationally restricted peptides used as immunogens would elicit an immune response that could be used for probing a sec-

Received for publication March 5, 1990.
Accepted for publication June 4, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by the WHO/UNDP/World Bank-TDR program, Project 840105 and 870121.
[2] Present address Department of Medical and Molecular Parasitology, New York University Medical Center, 341E 25th St. New York, NY 10010.

[3] Abbreviations used in this paper: CSP, circumsporozoite protein; LSA, liver stage Ag; TT, tetanus toxoid; IFAT, immunofluorescent antibody test; CD, circular dichroism; TFE, trifluoroethane; BOC, butyloxycarbonyl.

ondary structure on the cognate protein. Also, the study of immunogenic properties of conformationally restricted peptides may help in mimicking other biologic properties of some of the repeating structures, whose frequency in parasitic surface antigens remains poorly understood. In view of the possible utilization of the LSA sequence in multivalent vaccines, we decided to study the immunologic behavior of a hybrid molecule containing the LSA structure and the CSP repeat sequence connected together. Previous studies using mice and synthetic as well as recombinant peptides showed that the LSA structure was unable to prime or boost either specific antibody responses in vivo or T cell proliferation in vitro. Moreover, in vitro T cell proliferation assays using peripheral blood lymphocytes from individuals living in endemic areas and LSA molecules have yielded, so far, consistently negative results (J. L. Santhou, J. A. Londoño, and P. Druilhe, unpublished data). Because of this apparent lack of T cell epitope in the LSA repeat sequence, we introduced in the construct a Th epitope present in the nonrepetitive portion of the CSP sequence (sequence 326–343 of *P. falciparum* clone 7G8, referred to as Th2R) (13, 26). The Th2R was originally predicted to fold as an α-amphipathic structure and contains helper sites for H-2^k mice (13) whereas the murine immune response against the CSP repeat sequence is H-2^b restricted (7, 8).

In this study, we designed a hybrid molecule containing a peptide representing the CSP repeated region, the Th2R fragment and 27 amino acids from the LSA repeated sequence synthesized in tandem. Special attention was paid in our construct to the connection between the Th2R and LSA helical components to obtain a reciprocal reinforcement of the helical potentials (Fig. 1). The structural incidence of connecting these peptide sequences was monitored by CD measurements and the ability of the new hybrid peptides to induce specific antibodies in mice with different MHC haplotypes was tested.

MATERIALS AND METHODS

Peptide synthesis. The synthesis followed the stepwise solid-phase strategy in a fully automated peptide synthesizer (Applied Biosystem model 430 A, Foster City, CA) using the recent improvements in the BOC-trifluoroacetic acid method (28). We used tBOC-Nα protected aminoacids that were coupled sequentially to a tBOC-Leu-OCH2 Pam resin or to a tBOC-OBzl-Ser-OCHZ Pam resin. At the end of the synthesis, the protecting group of histidine residue was removed by thiolysis before cleavage and deprotection by the "low-high" hydrogen fluoride procedure. The crude peptide was purified by gel filtration on TSK HW40S (Merck, Rahway, NJ), followed by HPLC ion exchange chromatography (sulfoethyl aspartimide SCX, the Nest group, CIL Clouzeau, Sainte-Foy-La-Grande, France). They were checked for homogeneity by RP-HPLC on nucleosyl C18 (Macherey Nagel, Düren, W. Germany) and TLC in different solvent systems, and for identity by amino acid analysis. Both analyses gave satisfactory results. Amino acid sequence of the peptides included in this study are represented in Table I.

TT conjugate. The three-component hybrid was conjugated to TT through an additional N-terminal cysteine using heterobifunctional reagent (N-succinimidyl 6-maleimidocaproate) (29).

CD measurements. Spectra were taken using a Roussel Jouan model II at room temperature. CD studies were performed on peptides (hydrochlorides), dissolved in NaCl 100 mM or TFE 90%, in cells of 0.01 or 0.1 cm path length, respectively. Peptide concentrations were determined by quantitative amino acid analysis after total acid hydrolysis, and were 3×10^{-3} or 1.5×10^{-2} M, respectively. CD results were reported in terms of mean residue ellipticity (θ) expressed in deg$\cdot$dmol^{-1} cm^2. Helix content was calculated from the CD spectra taking (θ)222 nm = $-35{,}700$ deg$\cdot$dmole^{-1} cm^2 for 100% helicity.

Mice and immunizing protocols. Mice with different genetic backgrounds were purchased from the Centre d'élevage du CNRS (congenic B10 and C3H/He mice) or bred in the Institut Pasteur facilities (BALB/c, C57B1/6, F1(BALB/cxB6), DBA1, and DBA2). They were 2 to 4 mo old at the beginning of the experiments. To evaluate the immunogenicity of the new hybrid peptides as well as their ability to boost antibody responses in mice primed with individual sequences, groups of four to five mice received 50 μg of one peptide $(NPNA)_4$N-Th2R-LSA_{27}, Th2R-LSA_{27}, Th2R, LSA(41aa), or $(NANP)_4$ emulsified in CFA and injected s.c. at the base of the tail. Then 60 days later they were given a booster injection (i.p.) of 25 μg of aqueous $(NPNA)_4$N-Th2R-LSA_{27} or Th2R-LSA_{27}. Bleedings were performed at the retroorbicular plexus before and 10, 30, and 60 days after the injections to measure levels of specific antibodies. Anamnestic humoral responses recorded in mice primed with individual peptides and boosted with $(NPNA)_4$N-Th2R-LSA_{27} were compared to those observed in control mice primed with saline in CFA alone and boosted with $(NPNA)_4$N-Th2R-LSA_{27}.

Complementary protocols were performed as follows: 1) B10D2 mice primed with Th2R-LSA_{27} and $(NANP)_4$ (50 μg of each peptide, mixed and emulsified in CFA) were challenged on day 60 with $(NPNA)_4$N-Th2R-LSA_{27} (aqueous, 25 μg). 2) $(NPNA)_4$N-Th2R-LSA_{27}-primed B10D2 mice were injected on day 60 with Th2R-LSA_{27}. 3) naive B10D2 mice received a single injection (i.p.) of 20 μg of $(NPNA)_4$N-Th2R-LSA_{27} conjugated to TT. Bleedings were performed as above.

Antibody specificities. Sera were tested by ELISA using each of the individual sequences as solid phase Ag and by immunofluorescent antibody test against the whole parasite Ag (sporozoite and liver schizont). For ELISA, 96-well plates (Nunc, Roskilde, Denmark) were incubated overnight with a 5 μg/ml PBS, pH 7.2, solution of each peptide and blocked afterward with a low-fat powdered milk suspension (Regilait 2.5% w/v in PBS 0.13 M, pH 7.2) during 2 h. After washing, 100 μl of twofold dilutions of sera in 1.25% milk-0.05% Tween 20-PBS 0.13 M were put in triplicate wells and incubated for 90 min at 20°C; the presence of specific antibodies was revealed after multiple washing by adding 100 μl of a 1/2000 dilution of goat anti-mouse IgG (γ specific) or IgM (μ specific) antibody (Biosys, Compiegne, France) coupled to peroxidase. After incubation (60 min) and washing, a substrate solution (citrate buffer/H_2O_2/O-phenylene diamine) was added and absorbances at 492 nm were recorded using a Titertek Multiscan (Flow Laboratories, McLean, VA) reader after 30 min of incubation. Titers are expressed as the inverse of the maximal dilution giving a signal twice as high as the negative control (day 0 samples from the same mice diluted 1/50).

For immunofluorescent antibody test, sporozoites and liver schizonts were prepared and used as described elsewhere (30, 31). Briefly, glutaraldehyde-fixed sporozoites in suspension were allowed to attach to poly-L-lysine-treated slides and were used the next day. The 5-μm tissue cuts were prepared from carnoy-fixed/paraffin-embedded hepatic tissue containing liver schizonts and used after hydration. Twofold serum dilutions in PBS were incubated with the parasite Ag for 30 min and, after washing, a 1/100 dilution of a goat anti-mouse IgG, IgA, IgM FITC-antibody (N. L. Cappel Laboratories, Cochranville, PA) was added. Slides were examined under a UV light microscope (Olympus Japan, New Hyde Park, NY). Titers are expressed as the inverse of the last dilution showing specific labeling of the parasite structures.

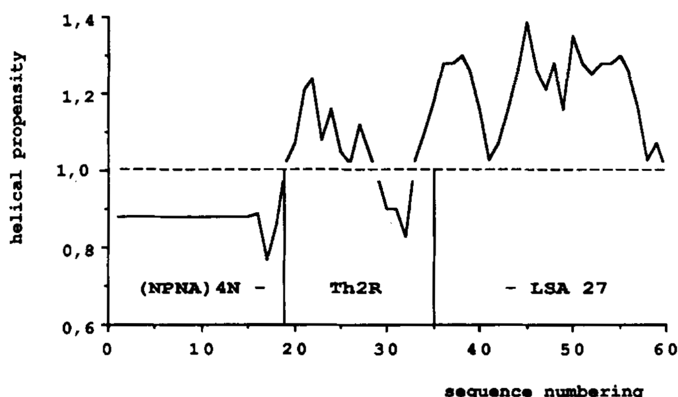

Figure 1. Helical propensity was calculated along the tri-component hybrid molecule according to the Chou and Fasman (27) method. The sequences corresponding to each component are indicated.

RESULTS

Conformational studies. As expected, no helical organization was observed in water or in TFE for the peptide

TABLE I
Sequence of single and hybrid peptides used

Peptide	Sequence		
$(NANP)_4$	NANPNANPNANPNANP		
Th2R		PSDKHIEQYLKKIENSIS	
LSA_{41}			LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ
$(NPNA)_4N$-Th2R	NPNANPNANPNANPNAN	PSDKHIEQYLKKIENSIS	
Th2R-LSA_{27}		PSDKHIEQYLKKIENSIS	EKLQEQQSDLEQERLAKEKLQEQQSDL
$(NPNA)_4N$-Th2R-LSA_{27}	NPNANPNANPNANPNAN	PSDKHIEQYLKKIENSIS	EKLQEQQSDLEQERLAKEKLQEQQSDL

$(NANP)_4$ (data not shown). Although no clearly helical organization was detectable in water for the hybrid structure $(NPNA)_4N$-Th2R, dissolution in 90% TFE resulted in an increase of negative ellipticity at 222 nm to a value of -24×10^{-3}. The CD spectrum was characteristic of an α-helix and showed that the peptide became 65% helical in TFE (Fig. 2). This result showed that the presence of the fragment $(NPNA)_4$ did not impair the α-helical organization of the fragment Th2R. The CD spectra corresponding to the hybrid peptides Th2R-LSA_{27} (peptide 18-62) were characteristic of a helical organization, both in water (22% helical) and in 90% TFE (70% helical) (Fig. 2). As expected from the results obtained with the peptide $(NPNA)_4N$-Th2R, the partially helical organization was maintained when the CSP repeat moiety was present in the tri-component hybrid peptide $(NPNA)_4N$-Th2R-LSA_{27}, 19% helical in water and 52% in TFE (Fig. 2).

Hybrid synthetic peptides are immunogenic in mice with different H-2 haplotypes. The levels of antibody responses obtained in different strains of mice after two injections of the hybrid peptides are shown in Figure 3. High IgG antibody responses to every isolated peptide were recorded in mice with $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes after injection of $(NPNA)_4N$-Th2R-LSA_{27} (Fig. 3*A*). In addition, one single injection, even without adjuvant, was enough to induce substantial levels of antibodies in these mice (data not shown). In contrast, $H\text{-}2^d$ mice receiving two or three bimonthly injections of $(NPNA)_4N$-Th2R-

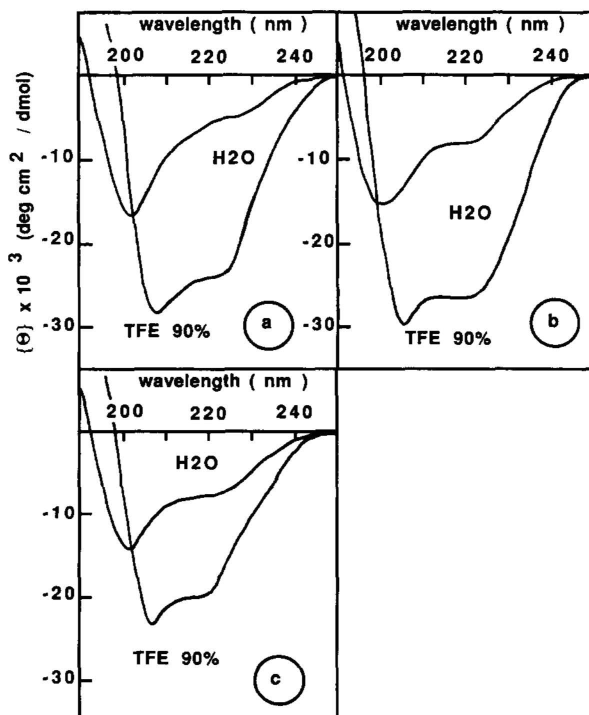

Figure 2. Circular dichroism spectra in water and in TFE 90%. *a*, $((NPNA)_4N$-Th2R; *b*, Th2R-LSA_{27}; and *c*, $(NPNA)_4N$-Th2R-LSA_{27}.

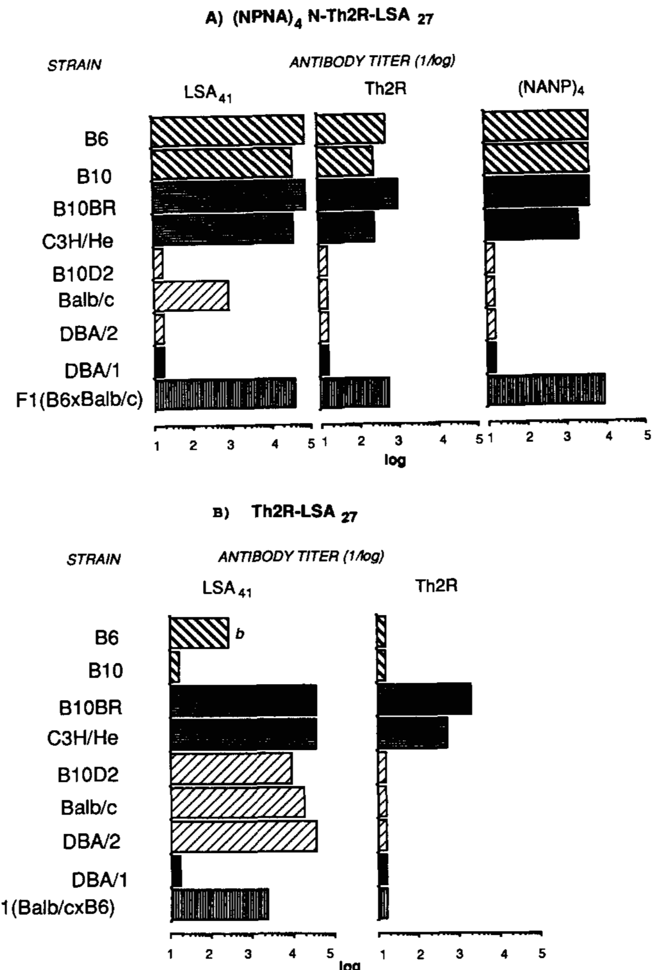

Figure 3. Specific IgG antibody levels (as measured by ELISA) against individual sequences (LSA_{41}, Th2R, $(NANP_4)$) in mice immunized twice either with (NPNA) N-Th2R-LSA (*A*) or Th2R-LSA (*B*). Sera from groups of three to five mice were taken 10 days after the last injection. Bars denote the MHC haplotype for each strain of mice as follows: ▨$H\text{-}2^b$, ▥ $H\text{-}2^k$, ▧ $H\text{-}2^d$, ■ $H\text{-}2^q$, ▤$H\text{-}2^{d/b}$. *b*, Only IgM antibodies were detected for this particular case.

LSA_{27} did not respond at all (B10D2 and DBA/2) or showed only low antibody levels (BALB/c) to the LSA sequence. When $(NPNA)_4N$-Th2R-LSA_{27} was injected in B10D2 mice as early as 45 days after homologous priming, low levels of antibodies could be detected.

Surprisingly, when the Th2R-LSA_{27} was used as immunogen (Fig. 3*B*), all mice with the $H\text{-}2^d$ haplotype showed high IgG (anti-LSA) antibody responses after two injections, as did $H\text{-}2^k$ mice in which one single injection induced antibodies able to bind LSA and Th2R sequences. An antibody response was also observed in C57B1/6 ($H\text{-}2^b$) mice; however, it was not long lasting and was entirely due to IgM (Fig. 3*B*). DBA/1 ($H\text{-}2^q$) mice showed no detectable specific antibodies after two injections with the hybrids (Fig. 3, *A* and *B*). F1(BALB/c ×

C57Bl/6) mice responded to the hybrid peptides as did the parental H-2^{d} strain to the Th2R-LSA$_{27}$ and the parental H-2^{b} strain to the (NPNA)$_4$N-Th2R-LSA$_{27}$.

We found antibody specificities directed against each of the three individual molecules in H-2^{b} and H-2^{k} mice, but higher levels were always recorded against the LSA sequence compared to the two others, i.e., Th2R and (NPNA)$_4$. H-2^{d} mice immunized with the hybrid peptides never produced antibodies against (NPNA)$_4$ nor Th2R. Whatever the level and specificity of the antibody responses and the immunogen used, most of the antibodies belonged to the IgG isotype (Fig. 3) and relatively high levels lasted up to 4 mo after the last injection in every responding strain (data not shown).

New Th function is detected in Th2R-LSA$_{27}$ hybrid peptide. To determine whether the known Th sites included in the hybrid peptides were functional, groups of mice bearing different MHC haplotypes were primed either with individual sequences or Th2R-LSA$_{27}$. Then 2 mo later all mice were injected with (NPNA)$_4$N-Th2R-LSA$_{27}$. No antibody response was detected in any group after priming injections except in H-2^{k} mice injected with the Th2R-LSA$_{27}$ as described above. Anamnestic responses were compared to those obtained after injection of NPNA-Th2R-LSA in mice primed with saline and CFA alone. The results for three groups of B10 congenic mice are shown in Figure 4. The tri-hybrid peptide only boosted antibody responses in B10 mice primed with (NPNA)$_4$ and in B10BR mice primed with Th2R or Th2R-LSA$_{27}$ indicating that the homologous sequences were functional in the hybrid molecule. In contrast, the antibody response in B10D2 mice was not primed by any of the individual peptides; only those mice primed with Th2R-LSA$_{27}$ responded to (NPNA)$_4$N-Th2R-LSA$_{27}$, strongly suggesting the presence of a new Th site in the di-hybrid peptide. The same pattern and H-2 restriction of immune responses were observed in other strains of mice (data not shown). Thus, as described by others (7, 8, 13), the immune responses elicited by NPNA repeats and Th2R were restricted to H-2^{b} and H-2^{k} haplotypes, respectively. However, when present in hybrid molecules, these sequences were able to provide help for the antibody response against other sequences that otherwise are nonimmunogenic for those strains of mice. In addition, the total absence of antibody response in H-2^{d} mice when individual peptides were used as immunogens was overcome by using the hybrid peptides. In agreement with previous observations, the LSA sequence failed to prime the antibody response in any of the strains of mice used.

Antibody priming capacity of Th2R-LSA$_{27}$ hybrid peptide when coupled to NPNA repeats is lost in H-2^{d} mice. Although, as just described, (NPNA)$_4$N-Th2R-LSA$_{27}$ was able to boost the antibody response in H-2^{d} mice primed with Th2R-LSA$_{27}$, no antibody response was detected in B10D2 or DBA/2 mice primed with (NPNA)$_4$N-Th2R-LSA$_{27}$ and challenged 2 mo later with the homologous Ag (Fig. 3*A*). Moreover, no booster effect was observed in these mice challenged with Th2R-LSA$_{27}$ after one injection of (NPNA)$_4$N-Th2R-LSA$_{27}$ (Fig. 5). When B10D2 mice were primed with a mixture of Th2R-LSA$_{27}$ and (NPNA)$_4$ (instead of the tri-hybrid peptide), and challenged with (NPNA)$_4$N-Th2R-LSA$_{27}$, they responded as the controls primed with Th2R-LSA$_{27}$ alone; this strongly suggests that a covalent link between the NPNA repeats and Th2R-LSA$_{27}$ is necessary to suppress the priming capability of the latter. A single injection in naive B10D2 mice of (NPNA)$_4$N-Th2R-LSA$_{27}$ coupled to TT induced specific antibodies against TT, but also against the LSA peptide, showing no interference by the NPNA repeats in this case (data not shown).

Hybrid synthetic peptides induce antibodies that recognized native proteins. Some of the results from the fluorescence assays are shown in Table II. Sera from

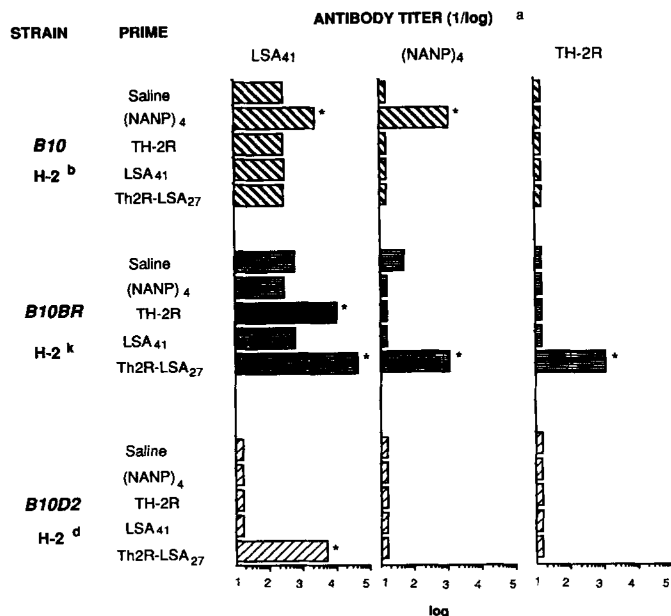

Figure 4. *a*, ELISA titers against the separate sequences LSA$_{41}$, Th2R, and NANP$_4$ in sera from groups of B10 congenic mice primed either with individual sequences, Th2R-LSA hybrid, or saline/CFA alone and challenged 2 mo later with the (NPNA)$_4$N-Th2R-LSA$_{27}$ tri-hybrid peptide. Mice were bled 10 days after challenge. * Denotes a higher level of antibodies compared to those in mice that received saline/CFA alone as priming immunization.

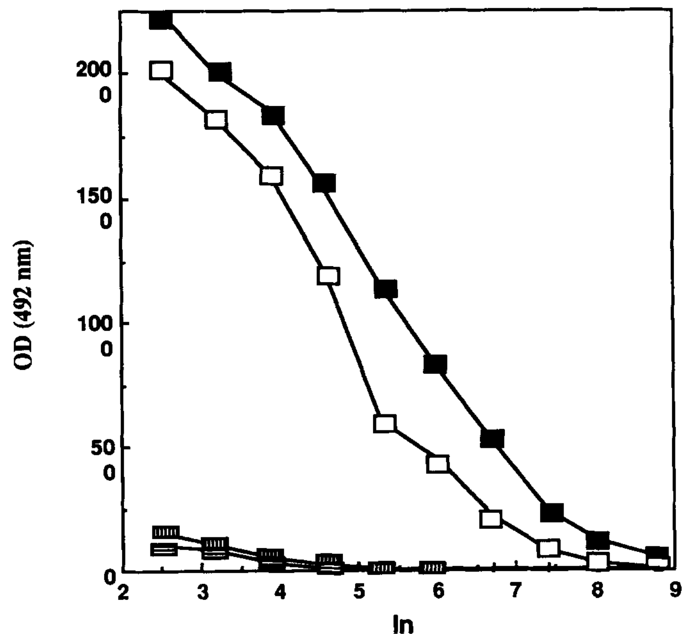

Figure 5. Antibody responses elicited by hybrid peptides in B10D2 mice. Four groups of B10D2 mice were primed 1) with 50 μg (CFA emulsified, s.c.) of peptide and boosted 2) 60 days later with 25 μg (aqueous, i.p.) as follows: □, 1) Th2R-LSA$_{27}$, 2) (NPNA)$_4$N-Th2R-LSA$_{27}$; ▥, 1) (NPNA)$_4$N-Th2R-LSA$_{27}$, 2) (NPNA)$_4$N-Th2R-LSA$_{27}$; ▤, 1) (NPNA)$_4$N-Th2R-LSA$_{27}$, 2) Th2R-LSA$_{27}$; ■, 1) (Th2R-LSA$_{27}$ + NANP$_4$), 2) (Th2R-LSA$_{27}$ + NANP$_4$). The antibody response in B10D2 mice immunized with Th2R-LSA27 (twice) is shown in Figure 3*B*.

TABLE II
Immunofluorecence titers of mice sera against native proteins

Strain	Immunogen[b]	IFAT Titer[a]	
		Liver stage	Sporozoite
B6	$(NPNA)_4N$-Th2R-LSA_{27}	512	2048
C3H/He	$(NPNA)_4N$-Th2R-LSA_{27}	1024	1024
B10BR	$(NPNA)_4N$-Th2R-LSA_{27}	2048	2048
B10D2	Th2R-LSA_{27}	1024	Neg
Balb/c	Th2R-LSA_{27}	2048	Neg

[a] Titers are the inverse of the last dilution still showing specific labeling of parasite structures either using whole sporozoites or infected liver tissue cuts.
[b] Sera were taken 10 days after the last of two injections; the corresponding antibody titers against specific sequences are those shown in Figure 3.

mice immunized with the hybrid peptides specifically bound the sporozoite surface and the most external structures of the 5-day-old liver schizont. These results indicate that the antibodies induced by these synthetic peptides were able to recognize the corresponding sequences in the native proteins.

DISCUSSION

We synthesized hybrid peptides that contained either two or three different sequences from two proteins of the pre-erythrocytic phase of *P. falciparum*: the CSP (1) and the LSA (2). Theoretically, the synthesis of a hybrid molecule containing those sequences had three major advantages. 1) The Th2R would provide help for the antibody production against the LSA α-helix repeated structure which apparently lacks T cell epitopes. 2) The α-helix structure initiated by the LSA component (19) would bring more stability to the Th2R organization and therefore favor the overall immunogenicity of the hybrid (32, 33). 3) The size of the construct allows its use as immunogen without carrier protein. To achieve a maximum of restricted conformation in the hybrid construct, the amino acid composition of the individual sequences at the connecting points was carefully chosen.

Choice of CSP repeat component. Recently, ^{1}H-NMR studies carried out in water-methanol mixtures on synthetic peptide models of the CSP repeated sequence (34) suggested the occurrence of an extended structure, set up of a mixed sequence of βI-turns and half turns around the positions N_{i-1}-P_i-N_{i+1}, evolving toward a left-handed spiral. This conclusion is in agreement with previous predictions based on conformational energy minimization (35). In this context, we can consider that the conformation subunit is the tetrapeptide NPNA rather than NANP. The selected CSP repeat component was represented by a sequence $(Asn\text{-}Pro\text{-}Asn\text{-}Ala)_4$.

Th2R component. In its native context, the helical propagation in Th2R is stopped on the N-terminal end with an asparagine-rich sequence and Pro 326. An additional Asn was added to the end of the CSP repeat component to connect the Th2R N-terminal Pro according to the periodicity of the CSP repeat sequence. On the C-terminal end, the helical propagation is probably impaired in the isolated fragment by the presence of consecutive helix breakers (Asn 340, Pro 348), or indifferents (Ser 341, 343, 347, Thr 344, Cys 349). However, the Trp 346 may be included in a hydrophobic cluster, thus contributing to the stabilization of this amphiphilic structure.

Choice of LSA repeat component. The helical nucleation patterns contained in the primary structure of the LSA repeat may initiate an α-helical folding that can propagate alone a lengthened helical structure synthesized in tandem. The connection between the Th2R sequence and the LSA repeat component was devised to place a hydrophobic residue in place of the Trp (a Leu residue), and to maintain a maximum helical propensity immediately adjacent to the Th2R sequence—the repeated structure was thus started on Glu-Lys-Leu-Gln-Glu sequence. The complete 17 amino acid sequence, previously showed as partially helical in water (19), was then introduced and lengthened with the hexapeptide sequence of a second repeat unit, to introduce another strong helix pattern formed by Gln-Glu-Gln-Gln and to avoid the destabilizing effect of the negatively charged Asp toward the negative end of the helix dipole.

Immunogenicity of constructs. As expected, the $(NPNA)_4N$-Th2R-LSA_{27} hybrid peptide was immunogenic in H-2^b and H-2^k mice, as they responded to the component sequences (NANP)x and Th2R, respectively (7, 8, 13). The tri-hybrid peptide induced high and long lasting levels of antibodies after a single injection without adjuvant or carrier protein, indicating a potent Th function for these haplotypes. There were antibodies specifically directed against each of the individual components of the hybrid. The assumption that known MHC-restricted Th sites (included in the hybrid molecule) provided help for antibody production was supported by the ability of the tri-hybrid peptide to boost secondary antibody responses in mice primed with individual sequences. The experiments also indicated that each sequence containing an active Th site induced antibodies against itself and helped antibody production against neighboring sequences, overcoming the genetic restriction of the immune response to the latter. Although no antibody production was expected in other H-2 haplotypes (14), BALB/c (and in some cases B10D2) mice produced weak but detectable antibody levels after two injections of the tri-hybrid molecule.

When the di-hybrid peptide Th2R-LSA_{27} was used as immunogen, all H-2^d mice, as well as H-2^k mice, gave good antibody responses. The data indicated that a fully active helper site was created in the hybrid peptides as individual sequences failed to prime the antibody production in H-2^d mice. As a H-2^d-restricted T proliferative site (without helper activity) is present in the Th2R sequence (26, 36), we do not know whether a H-2^d helper function was reinforced in the Th2R epitope by the LSA sequence or if an artificial Th site was created in the overlapping portion of the hybrid peptide. In any case, the genetic restriction of the immune response observed with individual peptides in H-2^d mice was again overcome using hybrid peptides. The better Th activity of the hybrid compared to Th2R was also present in H-2^k mice that made antibodies to the NPNA and Th2R components when they were primed with Th2R-LSA_{27} and challenged with $(NPNA)_4N$-Th2R-LSA_{27} but not when they were primed with Th2R alone and then challenged with the tri-hybrid construct (Fig. 4).

An unexpected finding was that the capacity of Th2R-LSA_{27} to effectively prime the antibody response in H-2^d mice was abrogated (or nearly) when it was covalently linked to the NPNA repeat sequence in the tri-hybrid molecule. The reasons for this phenomenon were not explored but some hypothesis may be proposed. First, the NPNA repeats could induce in H-2^d mice a specific T cell-

mediated suppression of the antibody response against the rest of the components in the hybrid. A specific effect such as this has never been reported despite numerous experimental studies concerning the NPNA repeated sequence (7, 8, 37–39). Field studies have recently shown that human T cell proliferative responses in vitro to different T cell epitopes of the CSP (including Th2R) do not correlate with levels of anti-NPNA antibodies in the same naturally exposed individuals (40, 41). Although it is not known whether these T epitopes actually contain helper sites for humans, it was suggested that suppressor cells modulating the antibody response against the repeats could be activated by exposure to the native protein (41). There is at the moment no evidence that the NPNA repeats can mediate that "immuno-modulating" effect but our results show that, at least in our murine model, a $(NPNA)_4$ sequence may have a negative control in antibody production when coupled to a Th2R-related Th epitope.

Whether the effect that we described would be observed only when $(NPNA)_x$ is coupled to the homologous T cell epitopes (i.e., T cell epitopes of the CSP) or also detectable when it is coupled to other immunogenic molecules needs to be further explored. The fact that good levels of anti-TT as well as anti-LSA antibodies were induced in $H\text{-}2^d$ mice after one single injection of $(NPNA)_4N\text{-}Th2R\text{-}LSA_{27}$ conjugated to TT showed that antibody suppression did not occur in this case. Similarly, in previous studies reported by others (7, 8), high anti-$(NPNA)_x$ antibodies have been obtained in the same mice when the repeats were injected coupled to molecules such as keyhole limpet or tet32. All these results suggest that "strong" Th sites for $H\text{-}2^d$ mice would overcome the supposed (MHC restricted) suppressive effect of NPNA repeats. Similar phenomena of Th and Ts epitope dominance have been described using murine models and protein antigens like *Escherichia coli* β-galactosidase and hen egg white lysozyme (42). Alternatively, the modulating effect of the repeats could be overcome by a previous exposure to the fully immunogenic hybrid molecule (i.e., without NPNA repeats), because $(NPNA)_4N\text{-}Th2R\text{-}LSA_{27}$ triggered specific antibody responses in $Th2R\text{-}LSA_{27}$-primed $H\text{-}2^d$ mice. This may indicate that when $Th2R\text{-}LSA_{27}$-specific T and/or B cells have undergone previous stimulation, they can specifically react and produce antibodies when challenged with the NPNA-containing hybrid, the antibody specificity remaining apparently unchanged. That, together with the need of a covalent link between $Th2R\text{-}LSA_{27}$ and NPNA, is reminiscent of some models of hapten-specific B cell suppression (43) where previously specifically stimulated B cells become refractory to negative control.

Other possible explanations for the differences in the priming capacity between the two hybrids, specifically related or not to the NPNA repeats, include: 1) differences at the level of the cognate interaction between T and B cells (44) or in the profile of IL secreted by T cells (45); 2) differences in the Ag processing and presentation by APC (46) that may be closely related to Th and Ts dominance expression (42); 3) major alterations in the tri-hybrid peptide of the predicted α-helix secondary structure of $Th2R\text{-}LSA_{27}$; as we showed here, this possibility can be ruled out because a similar degree of organization measured by CD is present in both di- and tri-hybrid peptides.

Antibody specificities induced by hybrid peptides. In view of the potential use of these peptides as synthetic vaccines, another important aspect of these experiments is the specificity of the antibodies induced. As noted before, hybrid peptides elicited antibodies in $H\text{-}2^b$ and in $H\text{-}2^k$ mice against every separate individual sequence. In many cases, most of the antibody specificities were directed against the LSA sequence that in natural conditions of exposure is also highly antigenic (2) (J. A. Londoño, manuscript in preparation). More important, the hybrid peptides induced anti-LSA antibodies that were able to recognize the native protein in liver schizonts suggesting an adequate presentation of the LSA α-helix-related B epitope (19) in the synthetic molecule. However, the lower levels detected against the native protein suggest a more rigid configuration present in the structure of the original molecule and/or a still high flexibility of the peptides when presented to the immune system. Alternatively, antibodies induced by hybrid peptides containing CS-related sequences reacted with the native CS protein at relatively (compared to the ELISA titers) higher levels suggesting a better presentation of the corresponding epitopes in the hybrid peptides or a more flexible structure in the original molecule (22–24).

Concluding remarks. We generated by chemical synthesis hybrid peptides containing structurally well defined T and B cell epitopes. As predicted, they behaved as organized structures in solution and exhibited high immunogenicity (without carrier) in mice, although a direct relationship between these two properties was not established. It was shown, however, that although favorable (potentiating) effects are observed with these constructs in some H-2 haplotypes (overcoming genetic restriction, inducing significant antibody response after one single injection), the addition of NPNA repeats to the $Th2R\text{-}LSA_{27}$ hybrid had a suppressive effect in one of them. A similar negative interaction has already been reported for a recombinant malaria vaccine (47) suggesting again that selection of multivalent vaccine components derived from parasite targets will require testing of combined molecules to optimize specific immune responses and to avoid detrimental consequences. Epitopes that induce specific suppression should be clearly identified and eventually excluded from multivalent vaccines. Finally, other potential problems these constructs may pose as human vaccines include their ability to prime or boost immune responses elicited by the parasite itself in naturally exposed individuals, not only because the probability of carrying artificial epitopes (i.e., Th site at the junction of the $Th2R\text{-}LSA_{27}$ sequence) but also because immune responses evoked by purified (synthetic or recombinant) molecules seem to be qualitatively different, at least in mice, from those induced by the live microorganism (48).

Acknowledgments. The authors are grateful to Dr. Genevieve Milon and Dr. Richard L. Beaudoin for helpful discussions and constant encouragement and to Dr. Ruth Nussenzweig for critically reading the manuscript.

REFERENCES

1. **Nussenzweig, V., and R. S. Nussenzweig.** 1985. Circumsporozoite proteins of malaria parasites. *Cell 42:401.*
2. **Guerin, C., P. Druilhe, B. Galey, J. A. Londono, J. Patarapotikul, R. L. Beaudoin, C. Dubeaux, A. Tartar, O. Mercereau-Puijalon, and G. Langsley.** 1987. A liver-stage-specific antigen of Plasmodium falciparum characterized by gene cloning. *Nature 329:164.*

3. **Myler, P., A. Saul, and C. Kidson.** 1983. The synthesis and fate of stage-specific proteins in *Plasmodium falciparum* cultures. *Mol. Biochem. Parasitol. 9:37.*
4. **Vermeulen, A. N., J. van Deursen, R. H. Brakenhoff, T. H. W. Lensen, T. Ponnudurai, and J. H. Meuwissen.** 1986. Characterization of sexual stage antigens and their biosynthesis in synchronized gametocytes cultures. *Mol. Biochem. Parasitol. 20:155.*
5. **Nussenzweig, R. S., J. Vanderberg, H. Most, and M. Orton.** 1969. Specificity of protective immunity produced by x-irradiated *Plasmodium berghei* sporozoites. *Nature 222:488.*
6. **Mendis, K. N., and G. A. T. Targett.** 1981. Immunization to produce a transmission blocking immunity in *Plasmodium yoelii* malaria infection. *Trans. R. Soc. Trop. Med. Hyg. 75:158.*
7. **Del Giudice, G., J. A. Cooper, J. Merino, A. S. Verdini, A. Pessi, R. Togna, H. D. Engers, G. Corradin, and P-H. Lambert.** 1986. The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is I-A^b restricted: possible implications for malaria vaccine. *J. Immunol. 137:2952.*
8. **Good, M. F., J. A. Berzofsky, W. L. Maloy, Y. Hayashi, N. Fujii, W. T. Hockmeyer, and L. H. Miller.** 1986. Genetic control of the immune response in mice to *Plasmodium falciparum* sporozoite vaccine. Widespread non-responsiveness to single malaria T epitope in highly repetitive vaccine. *J. Exp. Med. 164:655.*
9. **Lew, A. M., C. Langford, D. Pye, S. Edwards, L. Corcoran, and R. Anders.** 1989. Class II restriction in mice to the malaria candidate vaccine ring infected erythrocyte surface antigen (RESA) as synthetic peptides or as expressed in recombinant vaccinia. *J. Immunol. 142:4012.*
10. **Nardin, E., P. Barr, E. P. Heimer, and R. Etlinger.** 1988. Genetic restriction of the humoral response to a recombinant *Plasmodium vivax* circumsporozoite protein. *Eur. J. Immunol. 18:1119.*
11. **Good, M. F., L. H. Miller, S. L. Kumar, I. A. Quakyi, D. B. Keister, J. Adams, B. Moss, J. A. Berzofsky, and R. Carter.** 1988. Limited immunological recognition of critical malaria vaccine candidates antigens. *Science 242:574.*
12. **Langford, C., D. Smith, L. Keam, L. Corcoran, G. Peterson, P. McIntyre, D. Kemp, R. Anders, S. Edwards, and D. Pye.** 1988. "Cocktail" vaccines against *falciparum* malaria. In *Vaccines 88.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 89.
13. **Good, M. F., W. L. Maloy, M. N. Lunde, H. Margalit, J. L. Cornette, G. L. Smith, B. Moss, L. H. Miller, and J. A. Berzofsky.** 1987. Construction of synthetic immunogen: use of new T-helper epitope on malaria circumsporozoite protein. *Science 235:1059.*
14. **Patarroyo, M. E., R. Amador, P. Clavijo, A. Moreno, F. Guzman, P. Romero, R. Tascon, A. Franco, L. A. Murillo, G. Ponton, and G. Trujillo.** 1988. A synthetic vaccine protects humans against challenge with asexual blood stages of Plasmodium *falciparum* malaria. *Nature 332:158.*
15. **Nussenzweig, R., and V. Nussenzweig.** 1986. Development of a sporozoite malaria vaccine. *Am. J. Trop. Med. Hyg. 35:678.*
16. **Enea, V., J. Ellis, F. Zavala, D. E. Arnot, A. Asavanich, A. Masuda, I. Quakyi, and R. Nussenzweig.** 1984. DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino-acid sequence of repetitive epitope. *Science 225;628.*
17. **Ballou, W. R., S. L. Hoffman, J. Sherwood, M. R. Hollingdale, F. Neva, W. T. Hockmeyer, D. M. Gordon, R. A. Wirtz, J. F. Young, P. Reeve, C. L. Diggs, and J. D. Chulay.** 1987. Safety and efficacy of recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet 1:1277.*
18. **Herrington, D. A., D. F. Clyde, G. Losonsky, M. Cortesia, F. R. Murphy, J. Davis, A. Baqar, A. M. Felix, E. P. Heimer, D. Gillessen, E. Nardin, R. S. Nussezweig, V. Nussenzweig, M. R. Hollingdale, and M. M. Levine.** 1987. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature 328:257.*
19. **Dubeaux, C., J. A. Londono, H. Gras-Masse, A. Brack, A. Tartar, and P. Druilhe.** 1989. A liver stage-specific repeat of *Plasmodium falciparum*: correlations between secondary structure and antigenicity of synthetic peptides. In *Peptides 1988.* Jung-Bayer, ed. Walter de Gruyter & Co, Germany.
20. **Dyson, H. J., K. J. Cross, R. A. Houghten, I. A. Wilson, P. E. Wright, and R. A. Lerner.** 1988. The immunodominant site of a synthetic immunogen has a conformational preference in water for a type II reverse turn. *Nature 318:480.*
21. **Dyson, H. J., M. Rance, R. A. Houghten, P. E. Wright, and R. A. Lerner.** 1988. Folding of immunogenic peptide fragments of proteins in water solution. II: The nascent helix. *J. Mol. Biol. 201:201.*
22. **Satterthwait, A. C., T. Arrhenius, R. A. Houghten, R. A. Hagopian, F. Zavala, V. Nussenzweig, and R. A. Lerner.** 1988. Conformational restriction of peptidyl immunogens for the hydrogen bond. *Vaccine 6:99.*
23. **Shulze-Gahmen, U., H. Prinz, A. Glatter, and K. Beyreuther.** 1985. Towards assignment of secondary structures by antipeptide antibodies. Specificity of the immune response to a b-turn. *EMBO J. 4:1731.*
24. **Gras-Masse, H., M. Jolivet, H. Drobecq, J. P. Aubert, E. H. Beachey, F. Audibert, L. Chedid, and A. Tartar.** 1988. Influence of helical organization on immunogenicity and antigenicity of synthetic peptides. *Mol. Immunol. 25:673.*
25. **Waltho, J. P., V. A. Feber, R. A. Lerner, and P. E. Wright.** 1989. Conformation of a T-cell stimulating peptide in acqueous solution. *FEBS Lett. 250:400.*
26. **De la Cruz, V., W. L. Maloy, L. H. Miller, M. F. Good, and T. F. McCutchan.** 1989. The immunologic significance of variation within malaria circumsporozoite protein sequences. *J. Immunol. 142:3568.*
27. **Chou, P. Y., and G. D. Fasman.** 1974. Prediction of protein conformation. *Biochemistry 13:2.*
28. **Kent, S. B.** 1988. Chemical synthesis of peptides and proteins. *Annu. Rev. Biochem. 57:957.*
29. **Lee, A. C., J. E. Powell, G. W. Tregear, H. D. Niall, and V. C. Stevens.** 1980. A method for preparing b-hcg COOH peptide carrier conjugates of predictable composition. *Mol. Immunol. 17:749.*
30. **Druilhe, P., O. Pradier, J. P. Marc, F. Miltgen, D. Mazier, and G. Parent.** 1986. Levels of antibodies to *Plasmodium falciparum* sporozoite surface antigens reflect malaria transmission rates and are persistent in the absence of reinfection. *Infect. Immun. 53:2.*
31. **Druilhe, P., R. M. Puebla, F. Miltgen, L. Perrin, and M. Gentilini.** 1984. Species and stage specific-antigens in *Plasmodium falciparum* exoerythrocytic stages. *Am. J. Trop. Med. Hyg. 33:3.*
32. **Margalit, H., J. L. Spouge, J. L. Cornette, K. B. Cease, C. Delisi, and J. A. Berzofsky.** 1987. Prediction of immunodominant helper T-cell antigenic sites from the primary sequence. *J. Immunol. 138:2213.*
33. **Spouge J. L., H. R. Guy, J. L. Cornette, H. Margalit, K. B. Cease, J. A. Berzofsky, and C. DeLisi.** 1987. Strong conformational propensities enhance T cell antigenicity. *J. Immunol. 138:204.*
34. **Esposito, G., A. Pessi, and A. S. Verdini.** 1989. 1H-NMR studies of synthetic polypeptide models of *Plasmodium falciparum* circumsporozoite protein tandemly repeated sequence. *Biopolymers 28:225.*
35. **Gibson, K. D., and H. A. Sheraga.** 1986. Predicted conformations for the immunodominant region of the circumsporozoite protein of the human malaria parasite *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA 83:5649.*
36. **Good, M. F., D. Pombo, W. L. Maloy, V. F. DeLa Cruz, L. H. Miller, and J. A. Berzofsky.** 1988. Parasite polymorphism present within a minimal T-cell epitope of *Plasmodium falciparum* circumsporozoite protein. *J. Immunol. 140:1645.*
37. **Young, J. F., W. T. Hockmeyer, M. Gross, W. R. Ballou, R. A. Wirtz, J. Trosper, R. L. Beaudoin, M. R. Hollingdale, L. H. Miller, C. L. Diggs, and M. Rosenberg.** 1985. Expression of *Plasmodium falciparum* circumsporozoite protein in *Escherichia coli* for potential use in human malaria vaccine. *Science 228:958.*
38. **Ballou, W. R., J. Rothbard, R. A. Wirtz, D. M. Gordon, J. S. Williams, R. W. Gore, I. Schneider, M. R. Hollingdale, R. L. Beaudoin, W. L. Maloy, L. H. Miller and W. T. Hockmeyer.** 1985. Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum. Science 228:996.*
39. **Zavala, F., J. P. Tam, M. R. Hollingdale, A. H. Cochrane, I. Quakyi, R. S. Nussenzweig, and V. Nussenzweig.** 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science 228:1436.*
40. **Good, M. F., D. Pombo, I. Quakyi, E. M. Riley, R. A. Houghten, A. Menon, D. W. Alling, J. A. Berzofsky, and L. H. Miller.** 1988. Human T-cell recognition of the circumsporozoite protein of *Plasmodium falciparum*: immunodominant T-cell domains map to the polymorphic regions of the molecules. *Proc. Natl. Acad. Sci. USA 85:1199.*
41. **De Groot, A. S., A. H. Johnson, W. L. Maloy, I. A. Quakyi, E. M. Riley, A. Menon, S. M. Banks, J. A. Berzofski, and M. F. Good.** 1989. Human T cell recognition of polymorphic epitopes from malaria circumsporozoite protein. *J. Immunol. 142:4000.*
42. **Krzych, U., N. Nanda, and E. Sercarz.** 1989. Specificity and interactions of CD8+ T suppressor cells. *Res. Immunol. 140:302.*
43. **Pritchard-Briscoe, H., and R. H. Loblay.** 1989. Suppression in an adoptive hapten-carrier system. *Res. Immunol. 140:313.*
44. **Mitchinson, N. A.** 1971. The carrier effect in the secondary response to hapten-protein conjugates. *Eur. J. Immunol. 1:18.*
45. **Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. Coffman, T. R. Mosmann, and E. S. Vitetta.** 1988. Regulation of antibody isotype secretion by subsets of antigens-specific helper T-cells. *Nature 334:225.*
46. **Unanue, E. R., and P. M. Allen.** 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. *Science 236:551.*
47. **Russo, D. M., J. S. Sundy, J. F. Young, H. C. Maguire, and W. P. Weidanz.** 1989. Cell-mediated immune responses to vaccine peptides derived from the circumsporozoite protein of *Plasmodium falciparum. J. Immunol. 143:655.*
48. **Schofield, L., and P. Uadia.** 1990. Lack of Ir control in the immune response to malaria. I. A thymus-independent antibody response to the repetitive surface protein of sporozoites. *J. Immunol. 144:2781.*