Synthetic Malaria Peptide Vaccine Elicits High Levels of Antibodies in Vaccinees of Defined HLA Genotypes

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A multiple antigen peptide (MAP) malaria vaccine containing minimal *Plasmodium falciparum* circumsporozoite protein repeat epitopes was assessed for safety and immunogenicity in volunteers of known class II genotypes. The MAP/alum/QS-21 vaccine formulation elicited high levels of parasite-specific antibodies in 10 of 12 volunteers expressing DQB1*0603, DRB1*0401, or DRB1*1101 class II molecules. In contrast, volunteers of other HLA genotypes were low responders or nonresponders. A second study of 7 volunteers confirmed the correlation of class II genotype and high responder phenotype. This is the first demonstration in humans that a peptide vaccine containing minimal T and B cell epitopes composed of only 5 amino acids (N, A, V, D, and P) can elicit antibody titers comparable to multiple exposures to irradiated *P. falciparum*–infected mosquitoes. Moreover, the high-responder phenotypes were predicted by analysis of peptide/HLA interactions in vitro, thus facilitating the rational design of epitope-based peptide vaccines for malaria, as well as for other pathogens.

The development of drug-resistant *Plasmodium* parasites and insecticide-resistant mosquito vectors has compromised traditional methods of malaria treatment and control, reemphasizing the critical need for an effective vaccine. Monoclonal antibodies (MAbs) directed against the repeat region of a major surface antigen, the circumsporozoite (CS) protein, can block invasion of the host cell and passively confer sterile immunity [1–3]. Clinical trials were carried out ~10 years ago with a first-generation synthetic peptide malaria vaccine based on the tetramer repeat sequence of the *Plasmodium falciparum* CS protein, $(NANP)_3$, conjugated to tetanus toxoid as a carrier protein [4]. These phase 1 and phase 2a trials demonstrated that anti–repeat antibodies reactive with *P. falciparum* sporozoites were elicited by the peptide vaccine in most of those vaccinated. Although the antibody titers were relatively low, protection against challenge by the bite of *P. falciparum*–infected mosquitoes was obtained in a small number of these volunteers, as evidenced by an absence or significant delay in the development of a patent malaria infection.

The limitations of this first-generation peptide-protein conjugate vaccine, which included carrier toxicity, low epitope density, possible epitopic suppression, and lack of parasite-derived T cell epitopes, were addressed by the development of multiple antigen peptides (MAPs) [5]. These synthetic constructs replaced the foreign protein carrier with a peptide core matrix that uses the α and ε amino groups of lysine to construct a branched scaffolding on which peptide epitopes can be synthesized. MAPs containing repeat B cell epitopes of CS proteins elicited high levels of antibodies and protection in mice challenged with rodent malaria sporozoites [5–7] and in *Saimiri* monkeys challenged with sporozoites of the human parasite *P. vivax* [8]. Moreover, recent studies that used MAPs containing other sporozoite epitopes have demonstrated $CD4^+$ T cell–dependent protective immunity [9, 10] mediated by interferon (IFN)–γ, a potent inhibitor of the intracellular hepatic stages of the parasite [11].

An effective malaria vaccine should, therefore, contain a parasite-derived epitope to elicit $CD4^+$ T cells that could function both as a source of inhibitory lymphokines and of T helper factors for antibody responses. We used a series of $CD4^+$ T

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Informed consent was obtained from all volunteers, and the phase 1 trial was approved by the institutional review board of the University of Maryland.

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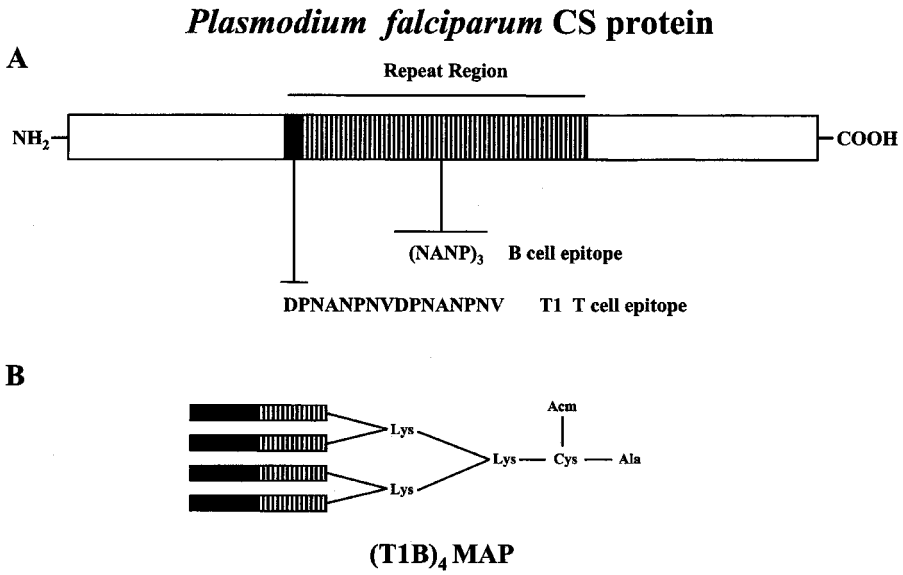

Figure 1. $(T1B)_4$ multiple antigen peptide (MAP) vaccine containing T and B cell epitopes of *Plasmodium falciparum* circumsporozoite (CS) protein. *A,* Illustration of the *P. falciparum* CS protein showing the central repeat domain containing the T cell epitope, T1, located in the 5′ repeat region and a B cell epitope, $(NANP)_3$, located in the 3′ repeat region. *B,* Diagram of the tetrabranched $(T1B)_4$ MAP that contains the T1 epitope $(DPNANPNV)_2$ synthesized in tandem with the B cell epitope $(NANP)_3$ on each branch.

cell clones, derived from a volunteer immunized by irradiated *P. falciparum* sporozoites, to identify a class II restricted T helper epitope, designated T1, in the 5′ repeat region of the CS protein [12]. This epitope consists of alternating NANP NVDP amino acid sequences that are conserved in all *P. falciparum* strains.

Our preclinical studies demonstrated that a *P. falciparum* MAP containing the T1 epitope in combination with the $(NANP)_3$ B cell epitope, designated $(T1B)_4$ MAP (figure 1), elicited extraordinarily high levels of anti-sporozoite antibodies in mice and *Aotus* monkeys [13, 14]. Immunofluorescence assay (IFA) titers exceeding 1×10^6 were obtained in some of the murine and *Aotus* serum samples, a magnitude of antibody response not previously reported for any recombinant or synthetic CS construct. The $(T1B)_4$ MAP also elicited a significant anamnestic response when administered to *P. falciparum* sporozoite-primed mice and monkeys [14, 15], suggesting that the vaccine would significantly increase antibody levels, and concomitantly protection, in individuals living in areas where malaria is endemic.

In the murine response to $(T1B)_4$ MAP, high or low antibody responses correlate with specific murine H-2 haplotypes [13]. The genetic restriction of the human response to the T1 or B repeat peptides was defined by in vitro analysis of peptide HLA interactions. The DQB1*0603 class II molecule was shown to function as a restriction element for the presentation of the T1 peptide to $CD4^+$ T cell clones derived from a *P. falciparum* sporozoite–immunized volunteer [16]. Peptides containing the T1 epitope also blocked presentation of a cognate peptide to a DRB1*1101 restricted $CD4^+$ T cell clone in a peptide competition cellular assay [17]. In addition, low-affinity binding of the T1 peptide to soluble DRB1*0401 molecules (IC_{50} 80 μM) was detected in a peptide competition radioimmunoassay, which is consistent with the presence of a DRB1*0401 binding motif in the T1 amino acid sequence ([18]; J. Hammer and F. Sinigaglia, personal communication). The $(NANP)_3$ B cell epitope, which lacks the aliphatic or aromatic amino acid residues that function as P1 anchors in the class II peptide binding groove, did not bind to any of the class II molecules in these in vitro assays [16–18].

The current Phase I trial was designed as a prospective study, to determine whether the $(T1B)_4$ MAP vaccine could elicit immune responses in individuals expressing DQB1*0603-, DRB1*1101-, or DRB1*0401-encoded molecules. Two cohorts of volunteers of these selected class II genotypes or random HLA haplotypes were immunized with an alum adsorbed $(T1B)_4$ MAP vaccine formulation, with or without QS-21 as coadjuvant, to assess the safety and immunogenicity.

Subjects and Methods

Subjects. Cohort A consisted of 32 healthy male and female volunteers (19–40 years old) recruited at the University of Maryland, College Park. Cohort B consisted of 7 volunteers recruited from the Baltimore community. Informed consent was obtained from all volunteers before admission into the study. The clinical protocol was approved by the institutional review boards of the University of Maryland at Baltimore and at College Park.

Medical history, physical examination, and routine standard laboratory tests (complete blood count, serum chemistries, urinalysis, immunoglobulin levels, and serology) were obtained to exclude individuals with cardiovascular, hepatic, or renal functional abnormalities or a history of hepatitis or human immunodeficiency virus or malaria infection. Class II genotypes (DRB1, DRB3, DRB4, DRB5, and DQB1 genes) were determined with the standard reverse transcriptase–polymerase chain reaction sequence–specific oligonucleotide probe typing scheme of the 11th and 12th International Histocompatibility Workshops [19].

After each injection of vaccine, volunteers were observed for 60 min and examined for local and systemic side effects by a physician. Clinical examinations carried out at 24 and 48 h and at 7, 14, and 28 days after immunization included an interval history of systemic and local reactions, examination of the site of injection, and oral temperature. The detailed clinical findings, including application of immediate-type and delayed-type hypersensitivity skin tests, will be reported elsewhere (J. Kublin and R. Edelman, unpublished data).

Vaccine formulation. The $(T1B)_4$ MAP was synthesized in a stepwise fashion, as described elsewhere [5, 13], under Good Manufacturing Practices (Peninsula Laboratories, San Carlos, CA). The MAP contained equimolar amounts of the 16-mer T1 epitope, $(DPNANPNV)_2$, synthesized in tandem with the 12-mer B cell epitope, $(NANP)_3$, on each arm of a tetrabranched lysine core (figure 1). Adsorption to alum (aluminum hydroxide; Reheis, Berkeley Heights, NJ) was carried out in acetate buffer, pH 6.2, at a final concentration of 2.5 mg $(T1B)_4$ MAP and 3.1 mg aluminum per milliliter. As a coadjuvant, a purified saponin, QS-21 (Aquila Biopharmaceuticals, Framingham, MA) [20, 21], was mixed with the $(T1B)_4$ MAP/alum immediately before subcutaneous (sc) injection into the upper arm.

Study design. The cohort A study was an open-label trial to assess the dose-dependent safety and immunogenicity of the $(T1B)_4$ MAP adsorbed to alum, with or without QS-21 as coadjuvant, in volunteers of known HLA class II genotypes (table 1). Four groups of 8 individuals were immunized sc with either 500 or 1000 μg of alum-adsorbed MAP, with or without 50 or 100 μg of QS-21 as coadjuvant. Each group included 2–5 individuals of selected DR or DQ genotypes (DQB1*0603, DRB1*0401, and DRB1*1101), with the balance of volunteers of random class II haplotypes, exclusive of these 3 genotypes. Groups 1–3 were immunized on days 0, 28, and 56, whereas group 4/5 was immunized on days 0, 28, and 237. (Results for volunteers in groups 4 and 5 were pooled because the same dose of vaccine and adjuvant was administered, separated by 1 week as a safety precaution.) In the cohort B study, 7 volunteers of responder genotypes were immunized on days 0 and 28 with 500 μg of $(TIB)_4$ MAP/alum and 50 μg of QS-21.

Immunogenicity. Serological responses were measured in serum samples obtained at 2 weeks and 1 month after each immunization and at sequential times after the third and final dose. Peptide-specific antibodies were measured by ELISA by use of peroxidase-labeled antibodies specific for the Fc of IgM or IgG (Cappel, West Chester, PA). IgG subgroups IgG1–IgG4 were measured with specific MAbs (Southern Biotechnology, Birmingham, AL).

ELISAs were carried out by use of 96-well plates coated with $(T1B)_4$ MAP, as described elsewhere [13, 14]. The end point was defined as the dilution of serum samples giving an optical density (OD) greater than the day 0 mean ΔOD plus 2 SD. An indirect immunofluorescence antibody assay (IFA) was carried out by use of air-dried *P. falciparum* sporozoites, incubated with 2-fold dilutions of serum samples, followed by fluorescein isothiocyanate–labeled anti–human IgG or IgM (Kierkegaard & Perry, Gaithersburg, MD) diluted in PBS/0.4% Evans blue.

Table 1. *Plasmodium falciparum* $(T1B)_4$ multiple antigen peptide (MAP) phase 1 cohort A study design.

Group	MAP/alum, μg[a]	QS-21, μg[b]	No. of volunteers	Class II genotypes: DRB1*0401, DRB1*1101, DQB1*0603	Class II genotypes: Random
1	500	—	8	4	4
2	500	50	8	2	6
3	1000	50	8	5	3
4/5	1000	100	4 + 4	5	3
Total	—	—	32	16	16

[a] All volunteers were immunized subcutaneously on days 0 and 28. The third dose of vaccine was administered on day 56 to groups 1–3 and on day 237 to group 4/5. Immunization of group 5 volunteers was initiated 1 week after that of group 4 volunteers.

[b] The 500-μg and 1000-μg doses of MAP/alum contained 625 and 1250 μg of aluminum, respectively. The QS-21 coadjuvant was mixed with the alum-adsorbed $(T1B)_4$ MAP at the time of injection.

To determine the ability of the MAP-induced antibodies to react with CS protein expressed on the surface of viable *P. falciparum* sporozoites, CS precipitin (CSP) assays were carried out [22, 23]. Dilutions of serum samples collected from volunteers 20 days after the final immunization were mixed with viable sporozoites and incubated for 45 min at 37°C. The end-point titer was the last serum dilution that could elicit a positive CSP reaction in 2 of 20 sporozoites, as determined by phase microscopy [22].

Cellular responses were measured with Ficoll-purified peripheral blood leukocytes (PBLs) in a 6-day ^{3}H-thymidine incorporation proliferation assay. Cultures were incubated with medium or with 10-fold dilutions of MAP or a recombinant *P. falciparum* CS protein that lacked only the putative signal and anchor sequences [24]. Positive controls included the recall antigen tetanus toxoid (Wyeth-Ayerst, Marietta, PA) or mitogens, phytohemagglutinin-P (Difco, Detroit) and poke weed mitogen (Life Technologies, Grand Island, NY). Negative controls included an unrelated MAP or a *P. vivax* recombinant CS protein [25]. Interleukin (IL)–2 levels in culture supernatants were measured with an IL-2–dependent T cell bioassay [12]. PBLs obtained from the volunteers before immunization (day 0) did not proliferate or produce IL-2 in response to any of the malarial antigens.

T cell lines (TCLs) were established from Cohort B PBL obtained 14 days after the second immunization. The PBL were cultured for 1 week with 4 μM $(T1B)_4$ MAP, followed by the addition of fresh medium containing IL-2 (100 U/mL) at 3–4 day intervals. After ~3 weeks' growth in vitro, the TCLs were assayed for proliferation, in response to stimulation with 4 μM $(T1B)_4$ MAP, using irradiated autologous PBL as antigen-presenting cells. The amount of IL-2 in 24-h culture supernatants was measured by bioassay. IFN-γ was measured in 48-h culture supernatants by ELISA (R&D Systems, Minneapolis).

Statistical methods. The primary analysis compared the antibody response of individuals expressing responder genotypes with

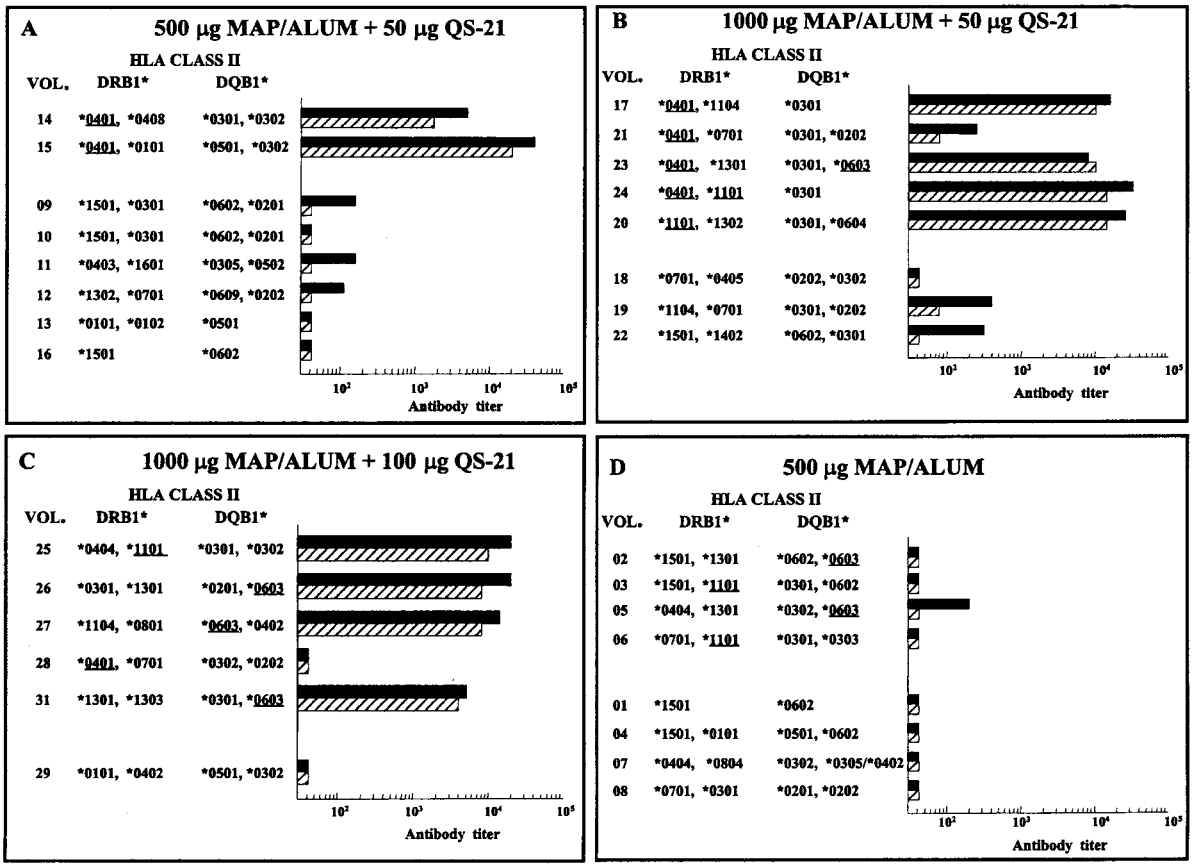

Figure 2. Correlation of high antibody responder phenotype with class II genotypes. IgG geometric mean titers were measured by multiple antigen peptide (MAP) ELISA *(solid bars)* or indirect immunofluorescence assay with *Plasmodium falciparum* sporozoites *(diagonal bars)*. Serum samples were obtained after 3 immunizations with 500 μg MAP/alum and 50 μg QS-21 *(A);* 1000 μg MAP/alum and 50 μg QS-21 *(B);* 1000 μg MAP/alum and 100 μg QS-21 *(C);* or 500 μg MAP/alum without QS-21 *(D)*. Underlined genotypes—DRB1*0401, DRB1*1101, and DQB1*0603—encode HLA class II molecules that bind the malaria T1 epitope in vitro.

individuals of random genotypes. Additional comparisons were made of the antibody response to different concentrations of MAP/alum with or without QS-21 as coadjuvant. Between-group differences were assessed by 2-sided *t* tests after log transformation of antibody titers. The correlation coefficient was calculated for ELISA and IFA titers.

Results

Safety and reactogenicity of the $(T1B)_4$ MAP/alum with or without QS-21. The vaccine and adjuvant formulations were well tolerated at all doses and elicited minimal local and systemic reactions in most volunteers. Local inflammation at the vaccination site was more frequent in response to higher concentrations of the vaccine and adjuvants.

Two of 8 volunteers in group 3 developed local and systemic immediate hypersensitivity reactions (urticaria) after the third dose of vaccine. Neither volunteer developed signs of anaphylaxis, and the pruritic hives resolved after treatment with oral antihistamines. Because of the development of urticaria in 2 volunteers in group 3, administration of the final dose of vaccine to the group 4/5 volunteers was delayed. At 7 months after the second dose, the volunteers tested negative by skin test, and MAP-specific IgE antibodies were not detectable by ELISA (R. Edelman, unpublished data). Subsequent administration of the third dose of 1000 μg MAP/alum and 100 μg QS-21 at this time did not elicit any adverse local or systemic reactions in any of the volunteers.

Eosinophilia (>500 eosinophils per microliter of blood) was not induced by any of the vaccinations. No clinically significant laboratory changes were noted in any of the immunized volunteers.

Serologic responses of volunteers immunized with $(T1B)_4$ MAP/alum with or without QS-21: correlation of high antibody responses with specific class II genotypes. After 3 sc immunizations, antibody levels in the serum samples of the vaccinees were measured by ELISA, using MAP as antigen, or by IFA, using *P. falciparum* sporozoites. High IgG titers were detected in a number of the volunteers immunized with either 500 or

Table 2. Serological and cellular responses in cohort B volunteers.

Volunteer	Responder genotype	ELISA GMT[a]	CSP titer[b]	$(T1B)_4$ MAP[c]		rPfCS	
				Proliferation	IL-2	Proliferation	IL-2
1	DRB1*0401	8127	10	3.7	38.6	24.3	39.9
2	DRB1*0401	10,240	10	3.9	36.9	16.9	27.8
3	DRB1*1101	8127	20	3.8	23.8	7.4	5.8
4	DQB1*0603	<80	Negative	0.7	1.0	3.3	3.2
5	DRB1*1101	20,480	20	19.9	90.5	9.7	10.3
6	DRB1*1101	12,902	20	11.2	237.9	10.2	63.9
7	DQB1*0603	10,240	5	21.0	6.1	13.8	0.7

NOTE. CSP, circumsporozoite precipitin; GMT, geometric mean titer; IL-2, interleukin-2; MAP, multiple antigen peptide; rPfCS, recombinant *Plasmodium falciparum* CS protein.

[a] IgG ELISA GMT was determined in serum obtained 14 days after the second dose of vaccine (day 42), using $(T1B)_4$ MAP as antigen. The corresponding immunofluorescence assay titers against air-dried *P. falciparum* sporozoites ranged from 1280 to 20,480, with a GMT of 4580 (data not shown).

[b] The CSP titer represents the last dilution of day 42 serum samples that elicited positive CSP reactions on 2 of 20 viable *P. falciparum* sporozoites, as detected by phase microscopy.

[c] Proliferation shown as stimulation index (SI) obtained after incubation of peripheral blood leukocytes with 4 μM $(T1B)_4$ MAP or 25 μg/mL rPfCS. Control wells incubated with unrelated MAP, recombinant *P. vivax* CS protein, or medium gave means of 951 ± 297, 2234 ± 1154, and 942 ± 365 cpm, respectively. IL-2 levels in 72-h culture supernatants are shown as SI of an IL-2–dependent T cell line.

1000μg of $(T1B)_4$ MAP/alum coadjuvanted with either 50 or 100 μg of QS-21 (figure 2*A*–2*C*). These high antibody responses were dependent on the presence of QS-21 as coadjuvant. Only 1 of 8 vaccinees seroconverted after immunization with 3 doses of 500 μg $(T1B)_4$ MAP/alum without QS-21 (figure 2*D*).

The pattern of response in the volunteers immunized with the $(T1B)_4$ MAP/alum/QS-21 vaccine formulation was bimodal, consisting of high responders and low or nonresponders. In all groups, the high-responder phenotype correlated with specific class II genotypes predicted by in vitro analyses of peptide/HLA interactions (figure 2*A*–2*C, underlined*). Only the volunteers who expressed class II molecules encoded by DQB1*0603, DRB1*0401, or DRB1*1101 developed high anti-peptide and anti-sporozoite antibody titers. No correlation of antibody responses with class II DRB3, DRB4, or DRB5 genotypes was observed in these volunteers.

Importantly, there was an excellent correlation between anti–repeat antibodies measured by ELISA and reactivity with *P. falciparum* sporozoites, as detected by IFA ($r = 0.9$). A geometric mean titer (GMT) of 10,568 by ELISA and a corresponding IFA GMT of 5511 were obtained in the 11 of 12 volunteers of high-responder genotypes. In contrast, for the 5 of 10 volunteers of random genotypes who seroconverted, the corresponding GMTs were 206 when measured by ELISA and 80 by IFA. The difference in the IFA and ELISA antibody titers in volunteers of responder versus random genotypes was statistically significant ($P < .0001$). No significant difference was noted in the titers of volunteers of responder genotypes immunized with different concentrations of MAP/alum (500 vs. 1000 μg; $P = .1$) with QS-21 coadjuvant (50 vs. 100 μg; $P = .5$).

It is of particular importance that the MAP-induced antibodies also reacted at high levels with viable *P. falciparum* sporozoites, producing CSP reactions at serum dilutions of 1:5 to 1:20 (table 2). CSP responses were similar in cohort A volunteers of responder genotypes (data not shown). CSP titers of this magnitude have not been reported for any previous CS subunit vaccine. The IFA, ELISA, and CSP titers measured in serum samples of the MAP vaccinees were comparable to those obtained in volunteers who were protected after immunization by exposure to the bites of large numbers of irradiated *P. falciparum*–infected mosquitoes [23, 26]. These titers in the MAP-immunized volunteers were also orders of magnitude higher than those obtained in a previous phase 1 trial of $(NANP)_3$–tetanus toxoid, the first generation malaria peptide-protein conjugate vaccine [4].

The antibody response to the malaria CS repeats appears to be controlled by a single autosomal dominant gene. Individuals expressing any 1 of the responder genes, DQB1*0603, DRB1*0401, or DRB1*1101, were high responders (figure 2*A*–2*C*). The presence of 2 responder alleles, as in volunteer 23 (DRB1*0401 and DQB1*0603) or volunteer 24 (DRB1*0401 and DRB1*1101), did not increase the antibody response above those observed in the individuals expressing a single responder gene. It is noteworthy that of the 2 volunteers who were DRB1*0401 and DRB1*0701 heterozygous, 1 developed the lowest antibody titer of all the responders (volunteer 21) and the other (volunteer 28) did not seroconvert.

Kinetics and IgG subgroups of anti-MAP antibody response. All the volunteers of responder genotypes developed a similar pattern of IgG subclasses in their anti-MAP antibody responses. IgG1 and IgG3 predominated after the second dose of $(T1B)_4$ MAP/alum/QS-21 (figure 3*A*), which is consistent with the known adjuvant effect of QS-21 [20, 21]. After the third dose of vaccine, increased levels of MAP-specific antibodies of IgG2 subtype were also observed, as well as low levels of IgG4 in some volunteers (figure 3*B*). This pattern, along with the development of IgE in the 2 volunteers (volunteers 17

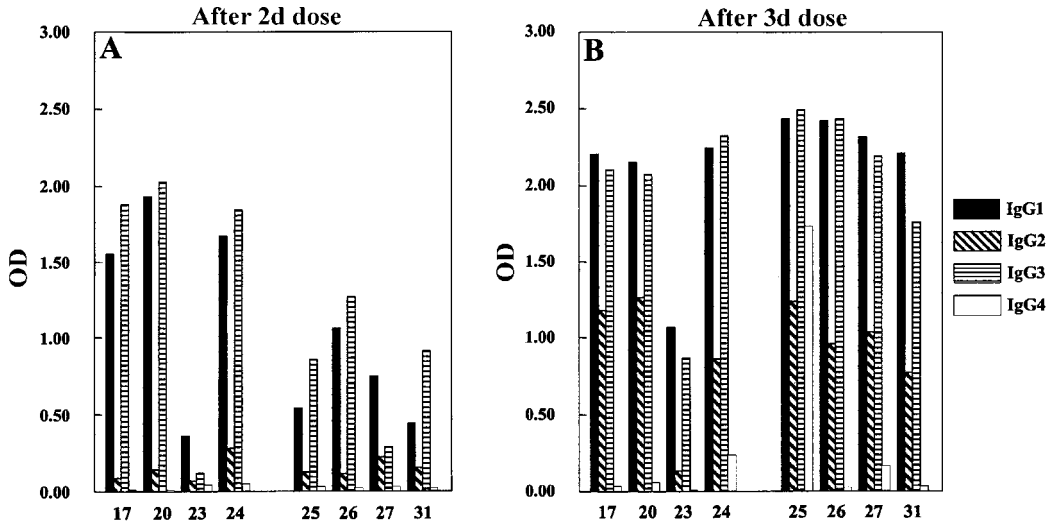

Figure 3. IgG subclasses of anti–repeat antibodies in volunteers of responder genotypes. Serum samples were obtained after immunization with 2 doses *(A)* or 3 doses *(B)* of MAP vaccine. Group 3 volunteers (volunteers 17, 20, 23, and 24) received 1000 μg $(T1B)_4$ MAP/alum with 50 μg QS-21, and group 4/5 volunteers (volunteers 25–27 and 31) received the same amount of MAP/alum with 100 μg QS-21. Seropositive volunteers of random genotypes developed low titer IgG1 and IgG3 antibodies (data not shown). Serum samples of seronegative volunteers did not give detectable responses with any of the IgG subgroup–specific reagents.

and 24) who developed urticaria (data not shown), suggests that a shift to Th2 type responses occurred with continued peptide immunization.

The kinetics and persistence of the IgG anti-MAP antibody response were also similar in all the volunteers, regardless of the dose of MAP/alum or QS-21. As illustrated by the volunteers in groups 2 and 3, peak IgG antibody responses were observed 14 days after the third and final dose of vaccine in responder, as well as random, HLA genotypes (figure 4*A,* 4*B*). By ~7 months after the final dose of vaccine, the high ELISA titers gradually decreased to GMT 905 in group 2 and GMT 368 in group 3 volunteers (figure 4*A,* 4*B*), with a corresponding IFA of 160 GMT in each group (data not shown).

Because the final immunization of the group 4/5 volunteers was delayed due to the development of urticaria in 2 volunteers in group 3, the effect of prolonging the interval between the second and third doses of vaccine could be assayed. Of the 5 volunteers of responder genotype in group 4/5, 4 individuals had IFA antibody titers >1000 after receiving the second dose of vaccine (figure 5, *diagonal bars*) and similar levels of anti-peptide antibodies by ELISA (data not shown). The volunteers of random haplotypes did not have IgG antibodies detectable by ELISA or IFA after the second dose of vaccine.

By ~7 months after the second dose of vaccine, the IgG IFA titers had declined to <1000 in all volunteers (figure 5, *cross bars*). At this time, the volunteers were found to be negative by skin test, and MAP-specific IgE antibodies were not detectable by ELISA (R. Edelman, unpublished data). The third dose of vaccine was administered to these volunteers without any adverse reactions. A strong anamnestic antibody response was obtained in 4 of 5 volunteers of responder genotypes, with reactivity to *P. falciparum* sporozoites increasing from low or negative (figure 5, *cross bars*) to 5120–10,240 IFA titer after this third dose of vaccine (figure 5, *solid bars*). The peak anamnestic responses in volunteers 25 and 27 were 5–8-fold higher than titers obtained after the second dose of vaccine, whereas in the remaining 2 volunteers (volunteers 26 and 31), titers returned to the peak levels obtained after the second dose. The ELISA titers directly correlated with IFA titers at all time points studied (data not shown).

The exception to these strong anamnestic responses was volunteer 28 (DRB1*0401 and DRB1*0701). No significant IFA (figure 5) or ELISA (data not shown) responses could be detected after the third dose of vaccine. Volunteer 29, of random haplotype, also remained seronegative after the final immunization.

Cellular responses in volunteers immunized with MAP/alum/QS-21. Immunization of a second group of 7 volunteers (cohort B), all of whom had responder genotypes, confirmed the correlation of class II genotypes and high-responder phenotypes. Six of 7 volunteers developed high levels of anti–repeat and anti–sporozoite antibodies after 2 injections of the vaccine (table 2). PBLs of these seropositive volunteers proliferated and secreted IL-2 in response to in vitro challenge with the $(T1B)_4$ MAP immunogen. Moreover, these immune cells recognized the repeats in the context of a recombinant *P. falciparum* CS protein, raising the expectation that MAP-sensitized T cells would proliferate and release inhibitory lymphokines, as well as helper factors, to maintain high levels of antibodies, after exposure to *P. falciparum* sporozoites. No proliferation or lym-

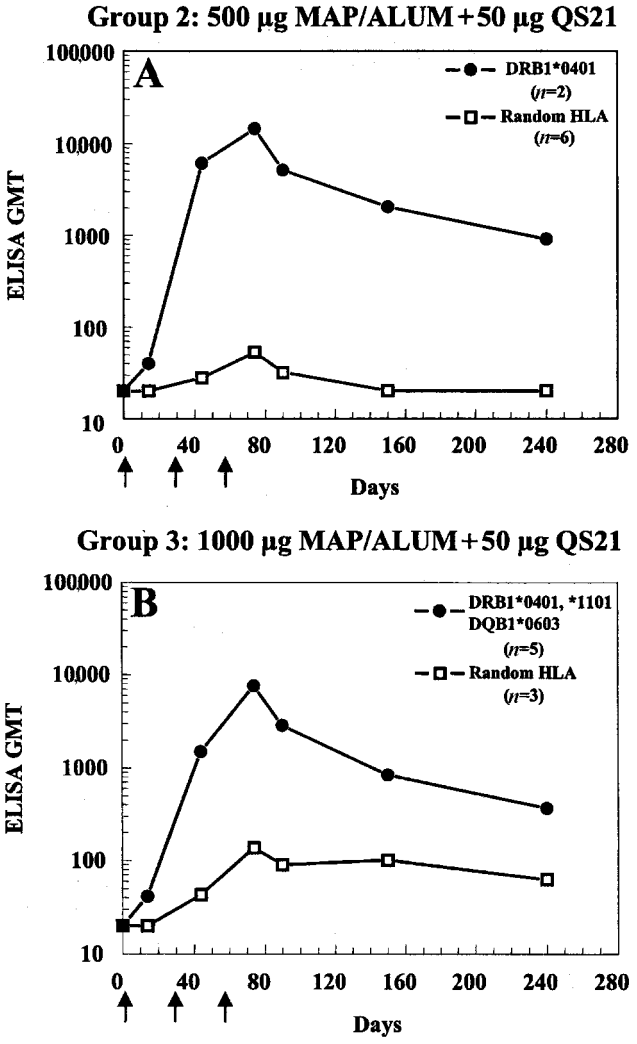

Figure 4. Kinetics and persistence of IgG anti–multiple antigen peptide (MAP) antibody response. Antibody titers were measured in volunteers of responder genotypes *(closed symbols)* or random haplotypes *(open symbols)* in group 2 *(A)* and group 3 *(B)*. Days on which volunteers were immunized are indicated *(arrows)*.

phokine production could be detected with PBLs obtained from the seronegative volunteer 4.

The kinetics of the cellular responses paralleled the humoral responses and increased with each dose of vaccine (figure 6). Low but positive proliferative responses were detected in 2 of 7 volunteers after the first immunization, and all the seropositive volunteers had detectable cellular responses after a second dose of vaccine. Moreover, $CD4^+$ TCLs could be derived by short-term peptide expansion of the volunteers' PBLs, with the exception of seronegative volunteer 4 (table 3). These MAP-specific TCLs secreted both IL-2 and IFN-γ after peptide stimulation, suggesting a Th1/Th0 response. A Th1-type T helper cell response in the cohort B volunteers was also suggested by the predominance of IgG1 and IgG3 anti–repeat antibodies, with little or no IgG2 or IgG4, which is consistent with the pattern of IgG subtypes observed in cohort A volunteers (figure 3).

Discussion

It has not been possible, for the most part, to define the basis of high and low antibody responders in humans, although these phenotypes have been noted in the response to other vaccines [27–29]. Similarly, in individuals living in areas in which malaria is endemic, antibody responses to *P. falciparum* CS repeats do not correlate with class II haplotypes [30, 31]. In these naturally infected individuals, HLA associations may be obscured by the presence of multiple T cell epitopes in the CS protein and by concomitant immune responses to the numerous sporozoite, liver, and blood stage antigens produced during malaria infections.

In the present phase 1 studies, we demonstrate that a synthetic malaria MAP vaccine, containing only minimal epitopes of the *P. falciparum* CS protein, elicits high antibody responses in individuals of specific HLA genotypes. Our ability to demonstrate the correlation of responder phenotype with class II genotype was most likely due to the use of an immunogen of limited antigenic complexity. The $(T1B)_4$ MAP is composed of only 5 different amino acids, N, A, P, V, and D, exclusive of the nonimmunogenic lysine core. The original demonstration of class II *Ir* gene control of murine antibody responses was also dependent on the use of branched peptide polymers containing only 3 or 4 amino acids, such as (T,G)AL and (H,G)AL [32].

As found in the early murine studies of *Ir* genes, a single autosomal dominant gene appears to control the high-responder phenotype in humans immunized with $(T1B)_4$ MAP. Volunteers expressing only 1 responder allele developed high levels of antibody after immunization with 2 or 3 doses of $(T1B)_4$ MAP/alum/QS-21. Although individuals homozygous for responder alleles were not available, the presence of 2 responder alleles in volunteers 23 (DRB1*0401 and DQB1*0603) and 24 (DRB1*0401 and DRB1*1101) did not result in higher levels of antibody, compared with individuals with 1 responder allele (figure 2*B*).

Although the numbers of vaccinees are small, the results of our study also suggest that additional genetic factors may play a role in determining the magnitude of the anti–repeat antibody response. In cohort A, 2 DRB1*0401-positive volunteers who were heterozygous for DRB1* 0701, volunteers 21 and 28 (figure 2*B*, 2*C*), developed significantly lower antibody responses than the other responder haplotypes. Similarly, in cohort B, a DRB1*0701/DQB1*0603 heterozygote (volunteer 4) did not develop detectable levels of antibody after immunization with 2 doses of vaccine (table 2). HLA haplotypes containing the DRB1*0701 allele have been associated with poor antibody responses to hepatitis and measles vaccines [28, 29]. Analysis of suppressor or regulatory T cell responses in the MAP-immunized

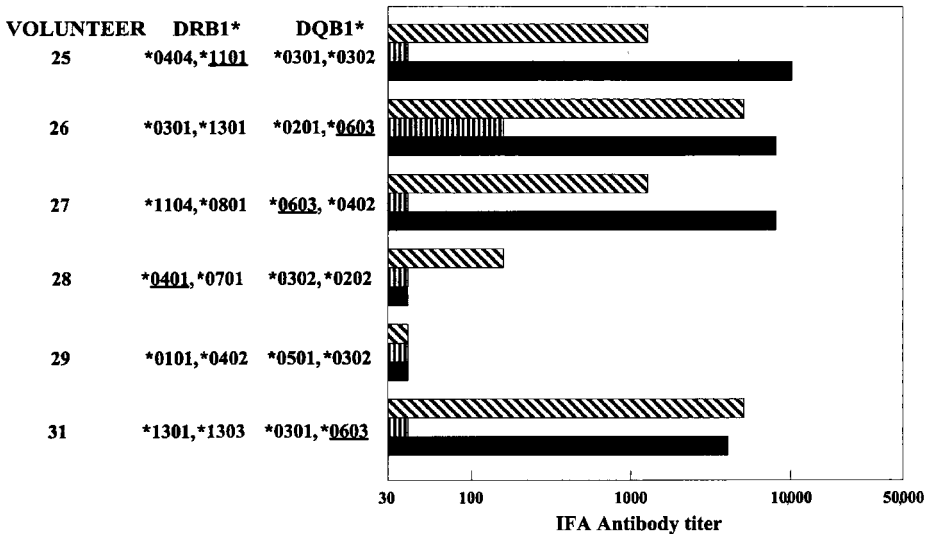

Figure 5. Anamnestic responses in multiple antigen peptide–immunized volunteers. Immunofluorescence assay (IFA) titers of 6 volunteers in group 4/5 were measured 2 weeks after the second immunization *(diagonal bars),* ~7 months later on the day of the third immunization *(cross bars),* and 2 weeks after the third immunization *(solid bars).* Two seronegative individuals of random HLA haplotypes, volunteers 30 and 32, did not receive the third dose of vaccine.

vaccinees may help define other HLA-associated factors that regulate antibody responses to *P. falciparum* CS repeats.

The cohort A study also emphasizes the importance of adjuvant formulation, especially for peptide immunogens that lack the nonspecific costimulatory properties of vaccines based on intact parasites, recombinant vectors, or complex carrier proteins. In the absence of the coadjuvant QS-21, minimal or no ELISA or IFA responses were detected in group 1 volunteers, even though 4 of these vaccinees were of responder genotype (figure 2*D*). Moreover, peptide-specific T cell responses could not be detected in PBLs of these 4 individuals (data not shown). In contrast, peptide-specific proliferation and lymphokine production was detected in PBLs of all volunteers who developed high antibody titers after immunization with the MAP/alum/QS-21 vaccine formulation (table 2). These findings suggest that one role of the QS-21 coadjuvant is to stimulate the production of costimulatory molecules, such as IL-12 [33], to expand the peptide-specific T helper cells to levels sufficient for optimal antibody production, thus facilitating detection by in vitro assays. These findings illustrate that even a potent antigen tested in a high -responder genotype may be mistakenly identified as nonimmunogenic in the absence of the correct adjuvant formulation.

Sporozoites circulate in the blood for only a brief period of time before invading the host hepatocyte, and this limited window for immunological attack necessitates the presence of high levels of antibody. The current phase 1 studies demonstrate for the first time that a synthetic peptide vaccine containing minimal CS epitopes, when presented in the correct adjuvant formulation to individuals of appropriate genotype, can elicit high levels of antibodies reactive with *P. falciparum* sporozoites. The magnitude of this response was comparable with that obtained in human volunteers immunized with large numbers of *P. falciparum* sporozoites, administered by multiple exposures to the bites of irradiated infected mosquitoes [23, 26], which represents the current "gold standard" for pre-erythrocytic malaria vaccine development.

Although it is currently unknown what immune mechanisms are required for sterile immunity in humans, high antibody titers alone may be sufficient for protection. The passive transfer of polyclonal anti–repeat antibodies derived from MAP-immunized rodents can protect naive recipients against sporozoite challenge [6, 7]. Anti-sporozoite immune serum samples can elicit a precipitin reaction on the surface of the parasite, termed the CSP reaction, which immobilizes the sporozoite [34], thereby inhibiting invasion of the hepatocytes that is dependent on parasite motility [35]. In the current study, positive CSP

Table 3. T cell lines derived from multiple antigen peptide (MAP)–immunized volunteers.

Volunteer	Responder genotype	Proliferation	IL-2	IFN-γ
1	DRB1*0401	18.7	29.0	25.2
2	DRB1*0401	52.0	79.7	57.2
3	DRB1*1101	14.7	28.2	32.6
4	DQB1*0603	1.0	0.8	0
5	DRB1*1101	23.4	41.2	642.4
6	DRB1*1101	55.9	35.5	77.2

NOTE. Proliferation of T cell lines stimulated with 4 μM $(T1B)_4$ MAP is shown as a stimulation index (SI). Interleukin (IL)–2 in culture supernatants was assayed by bioassay, and results were expressed as SI. The levels of interferon (IFN)–γ (pg/mL) in culture supernatants were measured by ELISA.

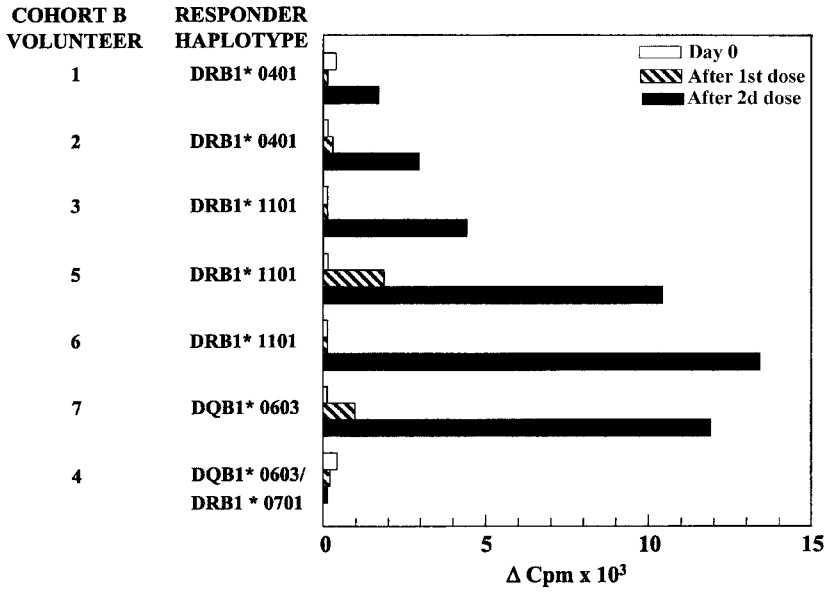

Figure 6. Kinetics of multiple antigen peptide (MAP)–specific proliferative responses. Proliferation after stimulation with $(T1B)_4$ MAP (4 μM) measured in peripheral blood leukocytes of cohort B volunteers on day 0 *(open bars),* 2 weeks after the first immunization *(diagonal bars),* or 2 weeks after the second immunization *(solid bars).* Results are shown as Δ counts per minute (cpm; mean cpm in quadruplicate wells with antigen minus mean cpm in quadruplicate wells with medium).

reactions correlated with high anti-peptide and anti-sporozoite titers measured by ELISA and IFA, respectively (table 2).

Alternatively, parasite-specific $CD4^+$ T cells could mediate protection through the release of IFN-γ, a potent inhibitor of the intracellular liver stages of the parasite [11]. In the murine model, $CD4^+$ T cell– and IFN-γ–dependent protective immunity against sporozoite-induced malaria can be elicited by MAP immunization [9, 10]. In the current studies, $CD4^+$ T cell lines derived from PBLs of the MAP-immunized volunteers were shown to produce IFN-γ after MAP stimulation in vitro (table 3). Whether repeat-specific antibodies and/or $CD4^+$ T cells are sufficient to protect against *P. falciparum* sporozoite challenge can be determined only by phase 2 studies. However, recent clinical trials of a hybrid CS recombinant protein, termed RTS,S, have clearly demonstrated that CS subunit vaccines can elicit protective immunity that correlates with high levels of anti-CS antibody and $CD4^+$ T cells in the absence of detectable $CD8^+$ T cells [36, 37].

The population frequency of the DQB1*0603, DRB1*0401, and DRB1*1101 responder genotypes suggests that the $(T1B)_4$ MAP/alum/QS-21vaccine formulation would be highly immunogenic in ~20%–35% of individuals, depending on ethnic background [19]. However, the design of effective vaccines for malaria, as well as other pathogens, requires the ability to elicit immune responses in individuals expressing a broad range of HLA molecules. To address this limitation of peptide vaccines, we have used peptide/HLA analyses to identify a CS-derived T cell epitope that can bind to multiple DR and DQ molecules in vitro [16]. The incorporation of this epitope into a CS repeat–based MAP provided T cell help for anti–repeat antibody responses in 8 of 8 strains of mice [16]. A synthetic peptide containing this malaria universal T cell epitope in combination with the *P. falciparum* CS repeats [38] recently has been shown to elicit cellular and humoral immune responses in volunteers expressing a broad range of HLA class II genotypes [39].

In contrast to more complex delivery systems such as viral particles or bacterial vectors, synthetic peptides containing minimal epitopes provide a means to define precisely the parasite-specific immune mechanisms regulating humoral responses, thus facilitating the rational design of highly immunogenic malaria vaccines. The empirical approach used in this study to define peptide class II restrictions—that is, human T cell clones or peptide competition assays—has been advanced by the recent development of more sophisticated, computer-driven algorithms that can predict T cell epitopes capable of interacting with a broad range of HLA class II molecules [40]. The in vitro and in vivo correlates defined in the current study raise the hope that these new methods of predicting highly immunogenic T cell epitopes in known protective antigens, as well as in the large number of new proteins that will be identified by genome sequencing projects, will facilitate the rational design of epitope-based vaccines for malaria, as well as other pathogens.

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