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A Totally Synthetic Polyoxime Malaria Vaccine Containing *Plasmodium falciparum* B Cell and Universal T Cell Epitopes Elicits Immune Responses in Volunteers of Diverse HLA Types[1]

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This open-labeled phase I study provides the first demonstration of the immunogenicity of a precisely defined synthetic polyoxime malaria vaccine in volunteers of diverse HLA types. The polyoxime, designated $(T1BT^*)_4$-P3C, was constructed by chemoselective ligation, via oxime bonds, of a tetrabranched core with a peptide module containing B cell epitopes and a universal T cell epitope of the *Plasmodium falciparum* circumsporozoite protein. The triepitope polyoxime malaria vaccine was immunogenic in the absence of any exogenous adjuvant, using instead a core modified with the lipopeptide P3C as an endogenous adjuvant. This totally synthetic vaccine formulation can be characterized by mass spectroscopy, thus enabling the reproducible production of precisely defined vaccines for human use. The majority of the polyoxime-immunized volunteers (7/10) developed high levels of anti-repeat Abs that reacted with the native circumsporozoite on *P. falciparum* sporozoites. In addition, these seven volunteers all developed T cells specific for the universal epitope, termed T*, which was originally defined using $CD4^+$ T cells from protected volunteers immunized with irradiated *P. falciparum* sporozoites. The excellent correlation of T*-specific cellular responses with high anti-repeat Ab titers suggests that the T* epitope functioned as a universal Th cell epitope, as predicted by previous peptide/HLA binding assays and by immunogenicity studies in mice of diverse H-2 haplotypes. The current phase I trial suggests that polyoximes may prove useful for the development of highly immunogenic, multicomponent synthetic vaccines for malaria, as well as for other pathogens. ***The Journal of Immunology,*** **2001, 166: 481–489.**

A major focus of malaria vaccine development has been the circumsporozoite (CS)[3] protein that is present in both sporozoite and liver stages of the parasite and is the target of protective cellular and humoral immune responses (1). Earlier studies demonstrated that polyclonal and mAbs specific for the repeat region of the CS protein neutralized infectivity of sporozoites of rodent, primate, and human malaria species. These repeat sequences contain linear immunodominant B cell epitopes consisting of multiple copies of four to nine amino acid sequences, thus providing attractive candidates for synthetic peptide vaccine development.

The first clinical trial demonstrating that synthetic peptides could elicit functional anti-sporozoite Abs in humans used a peptide-protein conjugate, consisting of the *Plasmodium falciparum* CS repeat B cell epitope, $(NANP)_3$, conjugated to tetanus toxoid as a carrier (2). Second generation peptide vaccines, termed multiple Ag peptides (MAP), have been developed in which the relevant T and B cell epitopes are synthesized on a branched lysine core, eliminating the requirement for a foreign protein carrier (3). MAPs containing CS repeat epitopes induced high levels of anti-sporozoite Abs and protective immunity in the murine malarias, *Plasmodium berghei* and *Plasmodium yoelii* (4, 5), and in a Saimiri monkey/*Plasmodium vivax* primate malaria model (6).

We have recently completed phase I clinical trials to assess the safety and immunogenicity of a *P. falciparum* MAP vaccine in volunteers of defined class II genotypes (7). This vaccine, termed $(T1B)_4$ MAP, contains the $(NANP)_3$ B cell epitope synthesized in tandem with a repeat region Th cell epitope, T1, that was originally defined using $CD4^+$ T cells of a *P. falciparum* sporozoite-immunized volunteer (8). Immunization of volunteers with the $(T1B)_4$ MAP vaccine elicited high anti-sporozoite Ab titers, comparable to those obtained in volunteers immunized by multiple exposures to the bites of hundreds of irradiated *P. falciparum*-infected mosquitoes (7, 9). However, this high responder phenotype was genetically restricted to three HLA class II genotypes (DRB1*0401, *1101, and DQB1*0603), which are found in ~25–35% of the population, depending on ethnic background (7).

Effective immunoprophylaxis against malaria requires a vaccine that can elicit parasite-specific humoral and cell-mediated immunity in individuals of diverse HLA types. The ideal Th epitope would be "universal," that is capable of interacting with a large number, if not all, class II molecules. To identify a parasite-derived universal T cell epitope, we used $CD4^+$ T cell clones derived from several volunteers immunized with *P. falciparum* sporozoites who

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[3] Abbreviations used in this paper: CS, circumsporozoite; CSP, CS precipitin; SI, stimulation indices; MAP, multiple Ag peptides; PCR-SSP, PCR/sequence-specific primers; IFA, immunofluorescence assay; TCL, T cell lines; GMT, geometric mean titer.

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were protected against sporozoite challenge (9–11). These human T cell clones recognized an epitope located in the C terminus of the CS protein that was restricted by a broad range of class II molecules (11). Peptides containing this epitope sequence, designated T*, also bound with high affinity to multiple DR and DQ molecules in vitro and thus was predicted to be a potential universal T cell epitope (12). Consistent with this prediction, MAPs containing the T* peptide were found to elicit Th cells in mice of diverse genetic backgrounds (12).

The ability to construct a triepitope MAP, containing this universal T* epitope in combination with the T1B repeats, was limited by the step-wise solid-phase synthesis used to construct MAPs. This limitation was overcome by chemoselective oxime ligation, in which reciprocally modified peptide modules, consisting of the epitopic peptide and a branched core, are first synthesized and purified before linkage via oxime bonds (13). A malaria $(T1BT^*)_4$ polyoxime vaccine was constructed using a branched core modified to contain the lipopeptide palmitoyl-*S*-glyceryl cysteine (P3C), to function as an endogenous adjuvant (Refs. 14 and 15; Fig. 1). This totally synthetic vaccine formulation was of sufficient homogeneity for chemical characterization by mass spectrometry (16). Polyoxime constructs containing malaria or flu epitopes were found to be highly immunogenic in mice (16, 17), but the immunogenicity of these polyoxime synthetic peptides in humans had not yet been determined. An open-label phase 1 trial was designed to assay the safety and immunogenicity of the triepitope $(T1BT^*)_4$-P3C polyoxime vaccine in 10 volunteers of diverse HLA types.

Materials and Methods

Subjects

Ten healthy male and female volunteers (19–29 years old) were recruited at the University of Geneva in Geneva, Switzerland. Medical history, physical examination, and routine laboratory tests (complete blood count, serum chemistries, electrocardiogram, and urinalysis) were obtained to exclude individuals with cardiovascular, hepatic, or renal functional abnormalities or past malaria infection. Serology for hepatitis B surface Ag and HIV-1 and -2 Abs was used to rule out volunteers with hepatitis or HIV infection. Women were tested for pregnancy by measuring urinary β-hCG. Informed consent was obtained from all volunteers before admission into the study, which was approved by the ethics Committee of the Geneva University Hospital.

The 10 volunteers were typed for class I and II HLA haplotypes (Table I). The class II genotypes were determined by PCR/sequence-specific oligonucleotide probes and PCR/sequence-specific primers (PCR-SSP) methodologies. DNA extracted from Ficoll-purified PBL from each volunteer was amplified by PCR using primer pairs specific for exon 2 of DRB1/B3/B4/B5 and DQB1 class II loci, respectively. The PCR products were analyzed in a microtiter-plate oligotyping assay (18) using HLA-DRB and DQB-oligodetection kits that were generously supplied by Dr. B. Mougin (BioMerieux, Lyon, France). HLA-DRB1 allelic subtypes of DR4, DR8, DR11, DR13-DR16 were determined by PCR-SSP using commercial kits (Dynal). HLA-A and -B class I Ags were first typed by serology using Ficoll-purified PBL in a standard microlymphocytotoxicity assay, and then by low resolution PCR-SSP (12th International Histocompatibility Workshop, Dynal or Biotest kits). HLA-A2 subtypes were determined by PCR/sequence-specific oligonucleotide probes as described (19).

Vaccine

The polyoxime construct $(T1BT^*)_4$-P3C contained the repeat T and B cell epitopes, T1B, in combination with a universal T cell epitope, T*, of the *P. falciparum* (NF54 strain) CS protein (Fig. 1). The T* epitope, representing the C-terminal aa 326–345 (EYLNKIQNSLSTEWSPCSVT), was originally identified using $CD4^+$ T cells derived from sporozoite-immunized volunteers (10, 11). These human T cell clones recognized the peptide in the context of DR 1,4, 7, and 9 class II molecules (11) and the T* peptide bound to the corresponding soluble DR 1, 4, and 7 class II molecules in vitro (12). In addition, the T* peptide also bound to DR 2, 3, 8, 11, and 13 and to DQ 7 and DQ 9 soluble class II molecules, as well as to cells expressing DRB1*1501 (12), suggesting the potential of T* to function as a universal T cell epitope in vivo.

The $(T1BT^*)_4$-P3C polyoxime vaccine contains a 48-mer malaria sequence $[(DPNANPNV)_2(NANP)_3$ EYLNKIQNSLSTEWSPCSVT] in each of the four branches (Fig. 1). The core was modified to contain the synthetic lipopeptide *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-cysteinyl) (P3C), to function as an endogenous adjuvant (14, 15). Each of the functional peptide components (i.e., the 48-mer malaria epitopic module and the lipopeptide-modified tetrabranched core) were purified and fully characterized by HPLC and mass spectrometry before assembly by oxime bond ligation, thus ensuring a synthetic product of high purity and defined composition (16).

Briefly, for construction of the polyoxime, the terminal lysines on each branch of the lipopeptide core module were modified with aminooxyacetyl groups (NH_2OCH_2CO). The 48-mer epitopic module was synthesized to contain Ser on a C-terminal Lys side chain, which was oxidized with periodate to create the reciprocal aldehyde group (O=CH-CO)-NH-. Spontaneous oxime bond formation (-COCH=$NOCH_2CO$) was initiated in aqueous solution at pH 4 by mixing the aldehydic peptide derivatives in a 4-fold excess over the aminooxyacetyl-modified core. After 16 h at room temperature, the tetra-oxime was isolated as a single peak by HPLC. Matrix-assisted laser desorption- time of flight mass spectrometry gave an experimental molecular mass of 23,936 (±60), in agreement with the calculated mass of 23,973, as previously found in the small-scale batches of $(T1BT^*)_4$-P3C polyoximes produced for preclinical studies (16). The polyoxime migrated as a single major band on tricine SDS-PAGE, and this band stained with a mAb specific for *P. falciparum* CS repeats (mAb 2A10) in a Western blot (data not shown). The vaccine preparation was sterilized by ultrafiltration and was negative when tested in endotoxin and bacteriological assays.

Study design

The study was an open-label trial to assess the safety and immunogenicity of $(T1BT^*)_4$-P3C in volunteers of known HLA class I and class II haplotypes. Vaccinees received 1 mg of $(T1BT^*)_4$-P3C administered s.c. in the deltoid area on days 0, 28, and 84. After each injection of vaccine, volunteers were observed for 60 min and examined for local and systemic side effects by a physician. An interval history of axillary temperature and systemic and local reactions following each vaccination were recorded by each volunteer.

CBC, serum chemistries and urinalysis were determined immediately before each immunization and at 2 wk after each vaccine dose. Blood samples for serology and T cell assays were drawn at the same time points. Additional samples were obtained ~1, 2, 5, and 10 mo after the third and

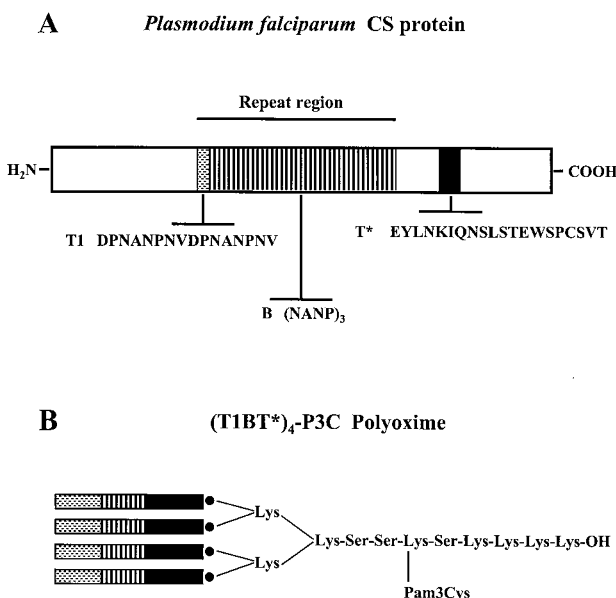

FIGURE 1. *A*, An illustration of *P. falciparum* CS protein to indicate the location of the T1 helper and the B cell epitopes within the central repeat region and the universal T* epitope in the C terminus (NF54 isolate aa 326–345). *B*, A schematic diagram of the triepitope polyoxime $(T1BT^*)_4$-P3C containing the malaria epitopic module (T1BT*) linked via oxime bonds (●) to the lipopeptide (P3C)-modified tetrabranched core.

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Table I. *HLA genotypes of volunteers in phase I study of (T1BT*)$_4$-P3C malaria vaccine*

Volunteer	A*	B*	DRB1*	B3*	B4*	B5*	DQB1*
03	26/30	35/53	0701/1104	02	01	–	02/0301
04	01/x	57/15	1103/1501	02	–	0101	0301/0602
05	0201/6901	44/55	0404/1301	0101	01	–	0302/0603
06	32/8001	35/55	0801/1501	–	–	0101	0402/0602
07	01/0201	08/57	0301/0701	0101	01	–	02/03032
08	24/29	35/44	1401/1602	02	–	02	0301/05031
09	0201/11	35/44	0401/1501	–	01	0101	0302/0602
10	23/24	44/15	0403/0701	–	01	–	02/0302
14	0201/03	07/15	0301/0408	0101	01	–	02/0304
15	0201/03	08/35	0301/0404	0101	01	–	02/0302

final dose of vaccine to measure persistence of the Ab and cellular immune responses.

Serological assays

Peptide-specific Ab titers were measured by ELISA using peroxidase-labeled Abs specific for IgM or IgG (Cappel, West Chester, PA) and IgE (Hybridoma Research Lab, Baltimore, MD). Qualitative determination of IgG subgroups, IgG1 through IgG4, was obtained by ELISA using sera diluted 1:100 and enzyme-labeled murine mAb specific for each human IgG subgroup (Southern Biotechnology Associates, Birmingham, AL).

ELISA were conducted using 96-well plates coated with the (T1BT*)$_4$ polyoxime or the corresponding triepitope MAP construct. The fine specificity of the Ab response was determined using the T1B repeats or universal T* peptide as Ag in the ELISA. Following incubation for 1 h with 2-fold dilutions of sera in PBS/0.05% Tween/2.5% BSA, and washing, the presence of bound Ig was detected by incubation with peroxidase-labeled anti-IgG or IgM Abs followed by the addition of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The end point titer was defined as the dilution of sera giving a Δ OD (OD peptide-coated wells $-$ BSA-coated wells) that was greater than the mean + 2 SD obtained with day 0 sera. IgE titers were determined using a murine mAb specific for human IgE, followed by sequential incubations with biotinylated anti-murine IgG (Vector Laboratories, Burlingame, CA), HRP-labeled strepavidin (Calbiochem, La Jolla, CA), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate.

An indirect immunofluorescence assay (IFA) was conducted using *P. falciparum* (NF54 isolate) sporozoites, dissected from the salivary glands of infected *Anopheles* mosquitoes. The slides containing air-dried sporozoites were incubated with 2-fold dilutions of sera, followed by FITC-labeled anti-human IgG or IgM (Kierkegaard & Perry Laboratories) diluted in PBS/0.4% Evans blue.

Reactivity with viable *P. falciparum* sporozoites was assayed using the CS precipitin (CSP) assay (20, 21). Twofold dilutions of sera, obtained 20 days after the final immunization, were mixed with viable *P. falciparum* sporozoites and incubated for 45 min at 37°C. The endpoint titer was the last serum dilution that could elicit a positive terminal precipitate visible by phase microscopy, the CSP reaction, in 2/20 sporozoites.

Cellular assays

Peptide-specific T cell responses were measured by proliferation and cytokine assays using PBL obtained at various time points during immunization. Ficoll-purified PBL were incubated in triplicate wells with medium, or with 10-fold dilutions of polyoximes in RPMI 1640/10% human serum starting at 1 μM concentration. The triepitope (T1BT*)$_4$ immunogen, as well as polyoximes representing the individual epitopes, (T1)$_4$, (B)$_4$, or (T*)$_4$, were tested in each assay. Positive controls included T cell mitogens PHA (Difco, Detroit, MI) or PWM (Life Technologies, Grand Island, NY) and a control recall Ag, tetanus toxoid (kindly provided by Wyeth-Ayerst Laboratories, Marietta, PA). For the proliferation assays, cultures received 10 U/ml IL-2 on day 7 and were pulsed on day 9 with 1 μCi [^{3}H]Tdr for 18 h before harvesting and scintillation counting. Significant responses were taken as >1100 δ cpm (cpm in cultures stimulated with peptide $-$ cpm in cultures without peptide), representing the mean + 2 SD of responses obtained with preimmune PBL from the 10 volunteers.

T cell lines (TCL) were established using PBL depleted of CD8$^+$ T cells by magnetic cell sorting (Miltenyi Biotec, Auburn, CA). The purified CD4$^+$ T cells were expanded in vitro by stimulation with 4 μM T1BT* linear peptide for 7 days followed by the addition of fresh medium containing 100 U/ml IL-2 at 3- to 4-day intervals. After ~3 wk in vitro, the TCL were assayed for proliferation and lymphokine production in response to stimulation with 4 μM T1BT*, or equimolar concentrations of the monoepitope peptides containing T1, B, or T*, using irradiated autologous EBV-immortalized B cells as APCs.

IL-2 assays were conducted using PBL culture supernatants collected at 72 h, or TCL culture supernatants obtained 24 h after peptide stimulation. The amount of IL-2 was measured by bioassay using an IL-2-dependent T cell line (10, 11). Stimulation indices (SI) were calculated as follows: cpm induced by supernatants from peptide stimulated cultures/cpm induced by supernatants from medium only cultures. SI > 3.2 were considered positive for the triepitope peptide and SI > 2.5 positive for the mono-epitope peptides (corresponding to 35 mU/ml rIL-2 standard). The IFN-γ levels were measured in TCL supernatants collected at 48 h using a commercial ELISA (R&D Systems, Minneapolis, MN).

CD8$^+$ T cells were assayed by IFN-γ ELISPOT using target cells pulsed with an HLA-A*0201-restricted CTL epitope of the CS protein (22, 23). Purified CD8$^+$ T cells were isolated by magnetic cell sorting (Miltenyi Biotec) of cryopreserved PBL from five HLA-A*0201 volunteers, obtained 2 wk after the second or third dose of vaccine. The cells were expanded by in vitro culture with a 9-mer HLA-A*0201-restricted CD8$^+$ CTL epitope, which is present within the universal T* peptide sequence (23). As a positive control, parallel cultures were stimulated with a HLA-A*0201-restricted 9-mer peptide derived from influenza hemagglutinin (24). After 7–10 days of in vitro expansion, varying concentrations of CD8$^+$ effector cells were cocultured with 10^4 HLA-A*0201-positive T2 target cells, pulsed with or without the CS or flu peptides. After overnight incubation of the cells in anti-IFN-γ mAb-coated nitrocellulose microtiter wells, lymphokine secretion was detected by staining the washed wells with peroxidase-labeled anti-IFN-γ, followed by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazoluim substrate (Kirkegaard & Perry). The number of spots in triplicate wells was counted and the results were expressed as mean number of CD8$^+$ IFN-γ-secreting cells/10^5 cells. Responses were considered significant if this number was at least 2-fold greater than that obtained with the negative control at the cell concentration used.

Results

Safety and reactogenicity

The (T1BT*)$_4$-P3C vaccine was well tolerated and elicited minimal local and systemic reactions in the majority of volunteers (Table II). None of the vaccinees developed fever (defined as axillary temperature ≥ 37.5°C) and laboratory safety parameters remained

Table II. *Reactogenicity of (T1BT*)$_4$-P3C polyoxime vaccine*

	Vaccine Dose		
Symptom[a]	1st	2nd	3rd
Local pain	5	5	3
Redness	1	1	4
Induration	1	1	1
Local itch	0	1	2
Fatigue	3	1	2
Headache	1	2	2
Nausea	1	0	0
Arthralgia	1	2	1

[a] Summary of the number of vaccinees reporting local or systemic symptoms after each vaccine dose.

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within normal ranges. Systemic side effects included mild to moderate fatigue, mild arthralgia, headache, and nausea, which resolved spontaneously without treatment. One individual (volunteer no. 7) had an increase of C-reactive protein with intercurrent streptococcal angina on day 7, unrelated to the vaccine.

Mild local reactions, including pain at the injection site, induration, and redness, were common. Two participants reported localized itching 2–3 days after the second and third immunization, suggesting a delayed-type hypersensitivity reaction. Local pain following injection decreased with increasing immunizations, while redness and local itch increased with additional booster inoculations. All local reactions resolved without treatment and all participants were willing to receive further inoculations if requested.

Humoral Immune Response

Polyoxime elicits Ab responses in individuals of diverse HLA. Following three immunizations with $(T1BT^*)_4$-P3C, all of the volunteers developed detectable levels of IgG Abs reactive with the triepitope immunogen (Fig. 2). The Ab titers ranged from 160 to 20,240, with peak anti-peptide titers exceeding 10^3–10^4 in the majority (8/10) of the vaccinees.

High Ab responses were not limited to volunteers expressing HLA class II genotypes known to function as restriction elements in vivo. Five of the volunteers were DRB1*0401, DRB1*0701, or DQB1*0603 genotypes (Fig. 2, ■), which had been shown to function in T1- or T*- specific $CD4^+$ T cell responses in *P. falciparum* sporozoite-immunized volunteers (11, 12). The IgG geometric mean titer (GMT) in the five volunteers who expressed these known class II responder alleles was not significantly different from the titers of five vaccinees expressing HLA molecules of unknown in vivo function ($p = 0.8$; Fig. 2, ▨). Consistent with the broad spectrum of T* binding to soluble HLA class II molecules, all of the volunteers in this latter group expressed at least one class II molecule that was predicted to function as a restriction element based on peptide binding studies in vitro (12).

Isotypes and kinetics of anti-polyoxime response. The majority of the volunteers seroconverted following a single dose of the $(T1BT^*)_4$-P3C vaccine. Volunteers (8/10) had peptide-specific IgM Abs (GMT 320) and 9/10 volunteers had IgG (GMT 296) 1 mo after the first immunization. Following a booster inoculation, all of the volunteers were seropositive with IgM GMT 529 and IgG GMT 1670 (Fig. 3). Ab titers declined 2- to 4-fold over a 2-mo interval. Administration of a third dose of vaccine at this time point (day 84), lead to a rapid increase in Ab titers, which reached IgM GMT of 802 and IgG GMT 2605. The peptide-specific Ab levels decreased over the 10-mo period following the final immunization, falling to an IgG GMT 137 on day 373 when the study was terminated.

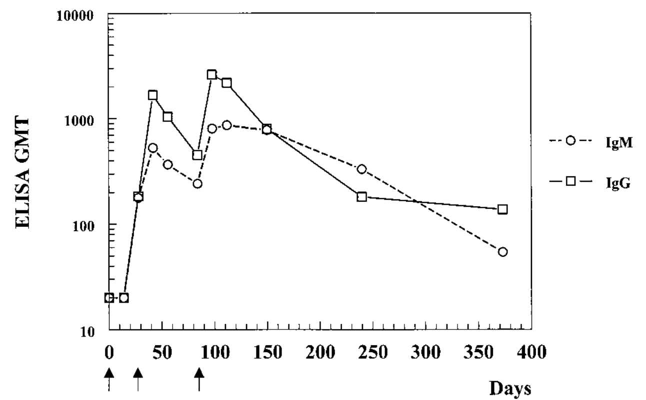

FIGURE 3. Kinetics of the IgM (○) and IgG (□) Ab response to $(T1BT^*)_4$ measured in sera of the polyoxime-immunized volunteers. ELISA results are expressed as GMT for the 10 volunteers. The arrows indicate days of immunization.

The anti-peptide Abs elicited by $(T1BT^*)_4$-P3C immunization were predominately of IgG1 and IgG3 subtypes in the majority of the volunteers (Fig. 4). Volunteers with the highest IgG titers also had increased levels of IgG2 and IgG4 peptide-specific Abs. The two volunteers with the lowest Ab titer, volunteers no. 03 and no. 06, had only IgG1 subtype Abs detectable.

Transient peptide-specific IgE Abs were detected in two volunteers, no. 05 and no. 09, following the third dose of vaccine (day 98; data not shown). Volunteer no. 09 also had a transient elevated

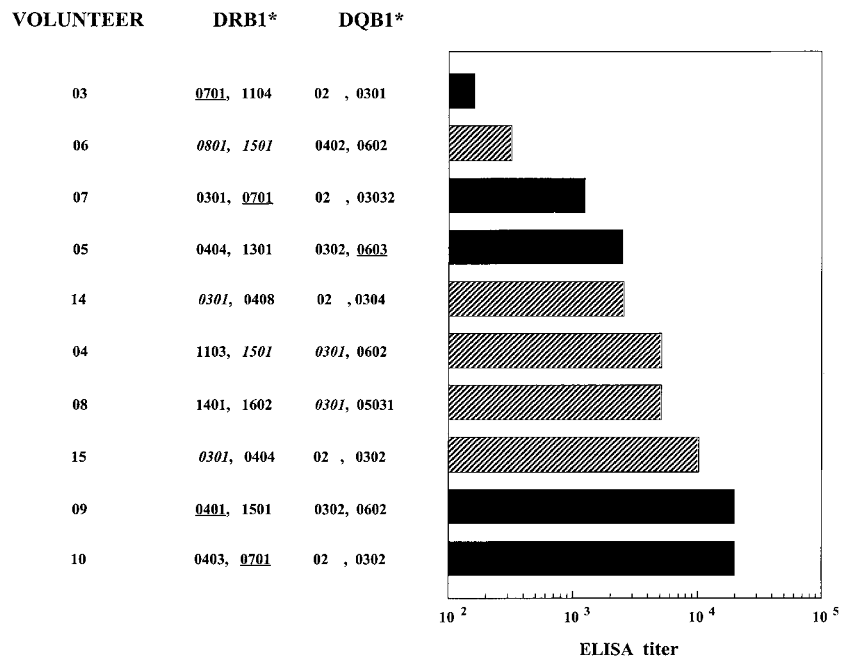

FIGURE 2. HLA class II haplotypes and corresponding Ab titers of volunteers immunized with $(T1BT^*)_4$-P3C. Sera obtained +14 days following the third and final dose of vaccine was assayed by ELISA using $(T1BT^*)_4$ as Ag. The solid bars (■) represent volunteers expressing class II genotypes known to function as restriction elements in vivo (underlined), based on the genetic restriction of human T cell clones derived from volunteers immunized with *P. falciparum* sporozoites. The hatched bars (▨) represent volunteers who lack these known responder genotypes, but who express DR or DQ alleles predicted to function as restriction elements based on binding of T* epitope to class II molecules in vitro (italics) (12).

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IgE response after the second dose of vaccine (day 42), which decreased to background level by day 84, when the third dose of vaccine was administered. No immediate-type hypersensitiviy responses were noted in any of the volunteers at any time point during immunization.

Fine specificity of Ab response. Peptides representing the individual B or T cell epitopes contained in the vaccine (i.e., the T1B repeats or the T* universal T cell epitope) were used to determine the fine specificity of the anti-peptide Ab response (Table III). For all sera, the titers to the (T1B) repeats were within a 4-fold dilution of the Ab titer against the entire triepitope $(T1BT^*)_4$ immunogen, indicating the repeats contained the immunodominant B cell epitope. In contrast to the anti-repeat response, little or no reactivity was detected against the T* epitope, with low titers measured in only five volunteers (GMT 320). The polyoxime core sequence also was not immunogenic as comparable ELISA titers were obtained using a $(T1BT^*)_4$ MAP, which differed in the structure and amino acid composition of the branched core (data not shown). Moreover, no positive reactions were observed when the lipidated Pam3Cys-modified core was used as Ag in the ELISA (data not shown).

A positive correlation of anti-repeat Abs with reactivity with native CS on *P. falciparum* sporozoites was demonstrated using both IFA and CSP reactions (Table III). 8/10 volunteers seropositive for anti-repeat Abs (GMT 1114) were also positive by IFA conducted using air-dried *P. falciparum* sporozoites (GMT 830). However, volunteer no. 07, despite good titers to the peptide immunogen, reacted less well with the repeat peptide and poorly with *P. falciparum* sporozoites. Volunteers no. 03 and no. 06, the two individuals with the lowest anti-repeat ELISA titers (160–320), did not have detectable IFA reactions (<80).

The biological relevance of the peptide-induced Abs was also demonstrated using viable *P. falciparum* sporozoites. When tested in the CSP assay, 7/10 volunteers with highest anti-repeat ELISA titers gave positive CSP reactions, at sera dilutions of 1:5–1:10 (Table III). Consistent with the requirement for high levels of Ab to induce the terminal precipitin reaction on the viable parasite, the sera of volunteers with low or negative IFA titers were CSP negative (volunteers no. 03, no. 06, and no. 07).

Cellular immunity

PBL proliferation and IL-2 production. Proliferative responses to the $(T1BT^*)_4$ peptide were detectable after multiple inoculations of the vaccine. None of the PBL obtained after the first immunization (day 14), and 1/10 after the second dose of vaccine (day 42), proliferated in response to peptide stimulation (Fig. 5). At 2–8 wk after the third and final dose of vaccine (days 98, 112, and 150), the PBL of the majority of volunteers (7/10) proliferated in response to $(T1BT^*)_4$ peptide. When retested 10 mo after the third dose (day 373), PBL of 57% (4/7) of these volunteers still had peptide-specific T cell responses detectable in the proliferation assay.

Volunteers whose PBL proliferated in response to $(T1BT^*)_4$ peptide, also had detectable levels of IL-2 in culture supernatants, indicating that peptide-specific T cells were responding (Fig. 6). The fine specificity of the cellular response was determined using supernatants from cultures stimulated with monoepitope peptides, $(T)_4$, $(B)_4$, or $(T^*)_4$. PBL of the majority of volunteers produced lymphokine only in response to the T* peptide (SI range, 4.5–21.5). IL-2 was not detected in supernatants from cultures stimulated with the T1 or B cell peptides (mean SI for T1 peptide 1.3 ± 0.4; for B peptide 1.0 ± 0.2). Volunteers no. 03 and no. 07 did not produce significant IL-2 in response to any peptide, consistent with the lack of proliferative response. The PBL of these volunteers gave normal responses to PHA and PWM in all of the assays.

The positive peptide-specific cellular responses correlated with the presence of high Ab titers in the serum of these vaccinees. The seven volunteers whose PBL proliferated and produced IL-2 following stimulation with the (T1BT*) or T* epitope had ELISA GMT of 6241. In contrast, the three volunteers (no. 03, no. 06, and no. 07) whose cells failed to proliferate or produce significant IL-2 following peptide stimulation had a GMT of 403.

$CD4^+$ TCL. To further analyze the T cell responses, $CD4^+$ TCL were established from five volunteers by expanding PBL using a single cycle of (T1BT*) peptide stimulation in vitro. TCL that specifically recognized the (T1BT*) peptide were obtained from 4/5 volunteers, including individuals with low PBL responses, such as volunteer no. 15 (Table IV). Consistent with the fine specificity noted with PBL, the $CD4^+$ TCL proliferated and/or produced IL-2 when stimulated with the T* peptide, but not the repeat, T1, or B peptides (data not shown). TCL from volunteer no. 07 did not proliferate or produce lymphokine in response to any of the peptides, consistent with the lack of response noted with bulk PBL cultures.

Supernatants from the peptide-stimulated TCL cultures were also examined for IFN-γ, a potent inhibitor of intracellular hepatic

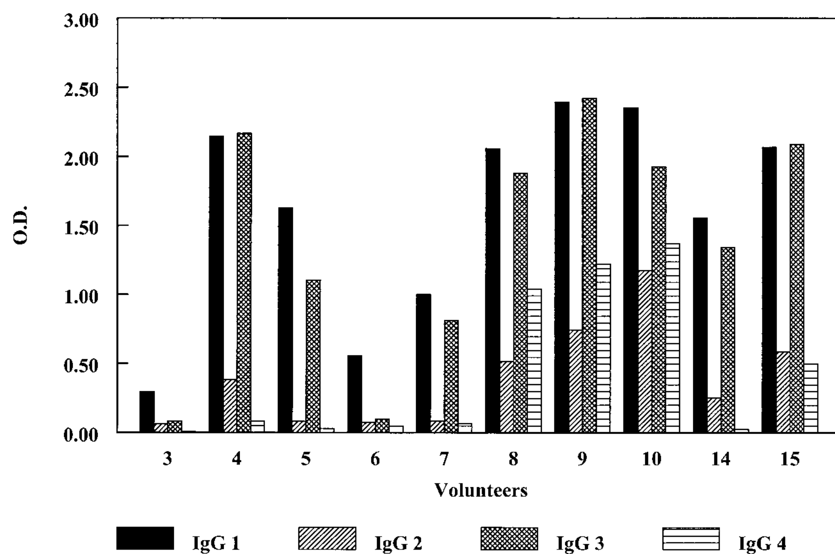

FIGURE 4. IgG subtypes of peptide-specific Ab responses elicited in volunteers immunized with three doses of vaccine. Results are expressed as Δ OD (OD in wells coated with $(T1BT^*)_4$ − OD in wells coated with BSA) obtained with sera at 1:100 dilution.

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Table III. *Fine specificity of antibody responses in (T1BT*)$_4$-P3C vaccinees*[a]

Volunteer	HLA DRB1*	Peptide ELISA			IFA	CSP
		(T1BT*)$_4$	(T1B)$_4$	(T*)$_4$		
03	0701/1104	160	160	<80	<80	Negative
04	1103/1501	5,120	2,560	160	1,280	5
05	0404/1301	2,560	1,280	<80	320	5
06	0801/1501	320	320	<80	<80	Negative
07	0301/0701	1,280	320	<80	80	Negative
08	1401/1602	5,120	1,280	160	640	5
09	0401/1501	20,480	5,120	640	2,560	10
10	0403/0701	20,480	5,120	640	5,120	10
14	0301/0408	2,560	1,280	<80	640	5
15	0301/0404	5,120	1,280	320	1,280	5

[a] Results are shown as IgG Ab titers measured in sera collected +14 days after the third and final s.c. injection of (T1BT*)$_4$-P3C polyoxime vaccine. IFA and CSP were carried out using air-dried or viable *P. falciparum* (NF54) sporozoites, respectively.

stages of the parasite (25). All of the CD4$^+$ TCL, with the exception of volunteer no. 07, produced IFN-γ in response to T1BT* and/or the T* peptide, suggesting a Th1- or Th0-type cellular response. Further analysis of the cytokines and fine specificity of cellular responses of the polyoxime-immunized volunteers is in progress using peptide-specific T cell clones (J.M.C.-C., manuscript in preparation).

CD8$^+$ T cells. The T* epitope contains, in addition to a Th cell epitope, an HLA-A*0201-restricted CD8$^+$ CTL epitope that is recognized by cells of peptide-immunized mice and naturally infected individuals (23). The P3C lipopeptide adjuvant has been shown to enhance CD8$^+$ T cell responses in peptide-immunized murine and primate hosts (26, 27).

To determine whether malaria-specific CD8$^+$ T cells were elicited by immunization with (T1BT*)$_4$-P3C, CD8$^+$ T cells were isolated from PBL of five HLA-A*0201-positive vaccinees. The purified CD8$^+$ T cells were expanded in vitro by coculture with a 9-mer peptide containing the CD8$^+$ CTL epitope, or a control peptide from influenza hemmagglutinin, and tested in a IFN-γ ELISPOT assay. In the control cultures expanded with the flu peptide, CD8$^+$ T cells of all five HLA-A*0201 volunteers produced IFN-γ when assayed using HLA-A*0201 target cells pulsed with flu peptide (mean 2472 spots/10^5 cells). An additional cycle of in vitro peptide expansion further increased the levels of flu-specific CD8$^+$ T cells (> 4000 spots/10^5 cells). In contrast, despite multiple cycles of in vitro expansion with the malaria CS peptide, CD8$^+$ T cells of the five HLA-A*0201 volunteers failed to produce positive ELISPOT when tested against malaria peptide-pulsed target cells (data not shown).

Discussion

The current phase I trial is the first demonstration in man of the immunogenicity of a (T1BT*)$_4$-P3C polyoxime malaria vaccine. This peptide represents a precisely defined synthetic construct containing a universal T cell epitope and repeat B cell epitopes of the *P. falciparum* CS protein ligated to a branched core modified with the lipopeptide P3C as an endogenous adjuvant. The composition of this totally synthetic vaccine formulation could be confirmed by mass spectroscopy, facilitating the reproducible production of vaccine batches for experimental studies and clinical testing.

All 10 volunteers, of diverse HLA haplotypes, seroconverted following two doses of the (T1BT*)$_4$-P3C polyoxime vaccine (Fig. 2). The majority of Abs elicited in the vaccinees specifically recognized the CS repeats (Table III). The immunodominance of the CS repeat B cell epitope, and absence of significant responses to the nonrepeat T* helper epitope, is consistent with the fine specificity of the Ab responses elicited by immunization with *P. falciparum* sporozoites in human volunteers and murine hosts (1). Of particular importance was the ability of the peptide-induced Abs to react with *P. falciparum* sporozoites. Sera of the seven volunteers with high anti-repeat Ab titers all had positive IFA titers and CSP reactions. The CSP reaction effectively immobilizes the sporozoite

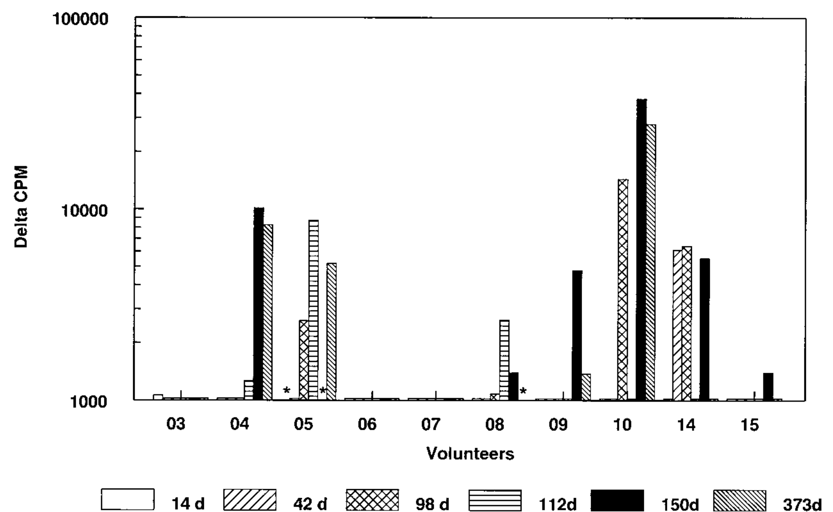

FIGURE 5. PBL obtained after the first (day 14), second (day 42), or third dose of vaccine (days 98–373) were stimulated with 1 μM (T1BT*)$_4$ polyoxime in a 10-day proliferation assay. Results are expressed as Δ cpm (cpm in triplicate wells with Ag − cpm in wells without Ag). Δ cpm > 1100 (mean + 2 SD of PBL obtained from the 10 volunteers before immunization) were considered positive. The asterisks indicate dates when samples were not available.

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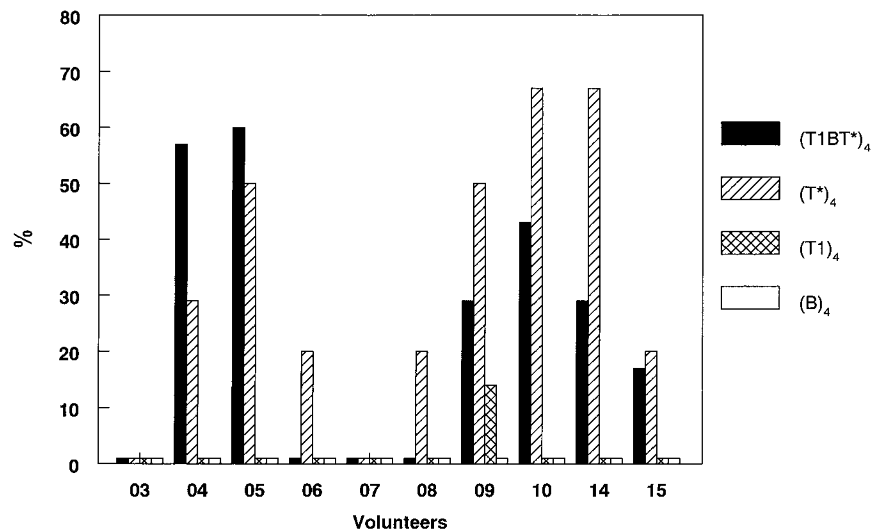

FIGURE 6. IL-2 was measured in supernatants collected from PBL cultures at the same time points described in Fig. 5. The PBL were stimulated without or with 1 μM $(T1BT^*)_4$ or equimolar concentrations of the monoepitope $(T1)_4$, $(B)_4$, or $(T^*)_4$ peptides. The percent of samples that induced significant proliferation (triepitope SI > 3.2, monoepitope SI > 2.5) of an IL-2-dependent T cell line is shown for each volunteer. Mean cpm induced by supernatants from PBL cultures without peptide was 353 ± 107.

(28), thereby inhibiting invasion of the hepatocytes, which is dependent on parasite motility (29). High levels of Abs reactive with the sporozoite surface may also function by sterically blocking host cell receptor interactions mediated by the CS ligand (30).

The 100% seroconversion of the polyoxime vaccinees is most likely due to inclusion of the parasite-derived T* epitope. The T* peptide functions as a Th cell epitope in 8/8 inbred strains of mice and can bind to a broad range of HLA class II molecules in vitro (12). These murine and in vitro studies suggested that the T* epitope could function as a universal T cell epitope in humans. These predictions were confirmed in the present study, as high Ab titers were detectable in the majority of volunteers (7/10) of diverse HLA types following immunization with the $(T1BT^*)_4$-P3C vaccine. In contrast, a $(T1B)_4$ MAP, which contains the same CS repeat epitopes but lacks the universal T* epitope, could elicit high Ab titers in only limited class II genotypes (DRB1*0401, *1101, and DQB1*0603; Ref. 7).

The suggestion that the T * epitope was functioning as a universal Th epitope in the polyoxime-immunized volunteers is also supported by the correlation of Ab and cellular responses. T*-specific proliferation, IL-2 and IFN-γ production were observed in PBL and/or TCL derived from all volunteers who developed high levels of anti-parasite Abs. The inability to detect cells of this specificity before immunization indicate that these T*-specific cellular responses were induced by the polyoxime vaccine.

In contrast to the T* epitope, significant cellular responses to T1, a second malaria Th epitope contained in the vaccine, were not detected using either bulk PBL or TCL. This was despite the fact that two volunteers, no. 05 and no. 09, were of the high responder genotype for the T1 epitope (DQB1*0603 and DR *0401, respectively). In contrast, $CD4^+$ T cells specific for the T1 helper epitope were detected in volunteers of these genotypes following immunization with $(T1B)_4$ MAP (Ref. 7; G.A.O., manuscript in preparation).

These findings indicate that T* was the immunodominant T cell epitope within the $(T1BT^*)_4$ polyoxime vaccine. Although the mechanisms controlling immunodominance remain to be defined (31), it is noteworthy that the HLA binding affinity of the T* epitope was significantly higher than that of the T1 peptide, as measured by peptide binding to soluble class II molecules (12). The absence of detectable T1-specific T cells in the polyoxime-immunized volunteers expressing T1 responder genotypes (DRB1*0401 and DQ603) suggest that peptide competition may occur in vivo, as has been found in vitro (J.M.C.-C., unpublished observations). The correlation of broad spectrum T* peptide HLA binding in vitro and high levels of immunogenicity of the $(T1BT^*)_4$ polyoxime in vivo support the use of computer-driven algorithms that predict T cell epitopes with high affinity for multiple class II molecules to identify potential T cell epitopes for inclusion in peptide vaccines (32).

Although peptide vaccines can be engineered to include universal epitopes from bacterial or viral proteins or artificial universal epitopes (5, 6), these foreign epitopes are not functionally equivalent to a parasite-derived universal T cell epitope. The inclusion

Table IV. *Specificity of $CD4^+$TCL derived from $(T1BT^*)_4$-P3C vaccinees*

Volunteer TCL[a]	T1BT* Peptide			T* Peptide		
	Proliferation[b] (SI)	IL-2[c] (SI)	IFN (pg/ml)	Proliferation (SI)	IL-2 (SI)	IFN (pg/ml)
05	21	22	0	21	71	13
07	1	1	0	1	1	0
09	20	17	1956	17	23	1102
14	5	4	14	11	22	48
15	18	13	76	16	20	128
Control TCL	1	1	0	1	1	0

[a] $CD4^+$ T cell lines (TCL) were derived, following a single in vitro expansion with (T1BT*) peptide, using PBL obtained 1 mo after the third dose of vaccine. Control TCL were established in parallel using PBL from a naive donor.

[b] Proliferation is shown as stimulation (SI) in response to 4 μM T1BT*, T*, T1, or B peptides. SI ≥ 2.5 was considered positive, with mean cpm for wells without peptide, 1332 ± 653. Stimulation of TCL with T1 or B repeat peptides did not elicit detectable proliferation or cytokines (data not shown).

[c] IL-2 in 24-h culture supernatants was measured using an IL-2-dependent T cell line. SI > 2.5 was considered significant. Mean cpm induced by supernatants from TCL cultures without peptide was 717 ± 198. IFN was measured in 48-h culture supernatants.

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of T* in a malaria vaccine provides the potential to elicit anamnestic responses in individuals living in endemic areas, and to maintain vaccine induced responses following exposure to the bites of infected mosquitoes. The $(T1BT^*)_4$ polyoxime induced strong anamnestic responses (250-fold increase in titer) when injected in mice primed with *P. falciparum* sporozoites (J.M.C.-C., unpublished observations).

In addition to the requirement for universal Th epitopes, other factors may play a role in determining the magnitude of the Ab response to CS peptide vaccines. It is noteworthy that volunteers no. 03 and no. 07, who had low anti-sporozoite Ab titers and no detectable peptide-specific PBL or TCL responses, were heterozygous for the DR*0701 allele. Similarly, in our recent phase I MAP trial, individuals of high responder genotypes who were heterozygous for DR7 also failed to develop the expected high levels of Ab and T cell responses (7). Poor humoral responses following immunization with hepatitis and measles vaccines have also been noted in DR7 heterozygotes (33, 34). However, one of the three DR7-positive polyoxime vaccinees, volunteer no. 10, developed high levels of Ab (Fig. 2), as well as strong cellular responses to the immunogen and the T* epitope (Figs. 5 and 6). The comparative analysis of DR7-linked genes expressed in low vs high responder haplotypes may help identify additional genetic mechanisms that function in immunoregulation.

The identification of regulatory cell populations would facilitate the rationale design of vaccines by ensuring that only functional T and B cell epitopes are included in peptide vaccines. However, such minimal peptide epitopes lack the nonspecific immunostimulatory properties of more complex recombinant protein or viral subunit vaccines and thus peptide vaccines require potent adjuvants to elicit optimal immune responses. In the current trial, the lipophilic adjuvant P3C, which can enhance peptide-induced Ab as well as $CD4^+$ and $CD8^+$ T cell responses (14, 17), was tested as a synthetic endogenous adjuvant.

The inclusion of the P3C adjuvant may have helped to broaden the range of IgG subgroups elicited by the vaccine. The majority of the volunteers developed IgG1 and IgG3 anti-peptide Abs, and IgG4 Abs were obtained in 50% of these vaccinees (Fig. 4), suggesting T cell help was provided by both Th1 and Th2 subsets. In contrast, the $(T1B)_4$ MAP formulated with alum and QS21 as co-adjuvants, elicited IgG1 and IgG3 anti-repeat Abs with little or no IgG4, suggesting a predominantly Th1 response (7). To directly determine the T cell subset(s) induced by the lipidated polyoxime, the cytokine profiles of peptide-specific T cell clones are being analyzed.

The inclusion of the P3C adjuvant also provided, in principle, the potential to elicit class I-restricted T cells. However, malaria-specific $CD8^+$ T cells were not detectable in the five HLA-A*0201-positive vaccinees when assayed by IFN-γ ELISPOT. Whether $CD8^+$ T cells are required for protection of man against *P. falciparum* sporozoite-induced infection is currently unknown. Although there is ample evidence that $CD8^+$ T cells are protective in the experimental murine model, additional studies have shown that sterile immunity against sporozoites can also be obtained in the absence of $CD8^+$ T cells (35). High levels of anti-repeat Abs alone can protect against sporozoite challenge in MAP-immunized mice and monkeys (5, 6). In the murine model, peptide-induced protective immunity against sporozoite challenge can also be mediated by IFN-γ produced by $CD4^+$ T cells (36).

Additional support for the role of class II-restricted responses in protection has also been provided by recent phase I/II studies of a recombinant CS/hepatitis B surface Ag protein, termed RTS,S (37). High levels of CS-specific Abs and IFN-γ producing $CD4^+$ T cells could be detected in volunteers protected against challenge with *P. falciparum* sporozoites, in the absence of malaria-specific $CD8^+$ T cells (37, 38). Of particular relevance to vaccine design, protection in that study was dependent on a combination of strong adjuvants, consisting of a mixture of MPL and QS21 in an oil-in-water emulsion (37). Although encouraging results were obtained with the P3C adjuvant in the current trial, it is expected that peptide vaccines will also require more potent adjuvants to elicit optimal immune responses.

The use of multiepitope polyoximes as immunogens can overcome the limitations of chemical synthesis and genetic restrictions that have hindered the development of synthetic peptide malaria vaccines. The results of this phase I trial demonstrate the immunogenic potential of polyoximes in humans and support further studies to develop precisely defined, broadly applicable synthetic peptide vaccines for malaria immunoprophylaxis.

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