A Linear Peptide Containing Minimal T- and B-Cell Epitopes of
Plasmodium falciparum Circumsporozoite Protein Elicits
Protection against Transgenic Sporozoite Challenge

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An effective malaria vaccine is needed to address the public health tragedy resulting from the high levels of morbidity and mortality caused by Plasmodium parasites. The first protective immune mechanism identified in the irradiated sporozoite vaccine, the “gold standard” for malaria preerythrocytic vaccines, was sporozoite-neutralizing antibody specific for the repeat region of the surface circumsporozoite (CS) protein. Previous phase I studies demonstrated that a branched peptide containing minimal T- and B-cell epitopes of Plasmodium falciparum CS protein elicited antirepeat antibody and CD4+ T-cell responses comparable to those observed in volunteers immunized with irradiated P. falciparum sporozoites. The current study compares the immunogenicity of linear versus tetrabranched peptides containing the same minimal T- and B-cell epitopes, T1BT*, comprised of a CS-derived universal Th epitope (T*) synthesized in tandem with the T1 and B repeats of P. falciparum CS protein. A simple 48-mer linear synthetic peptide was found to elicit antisporozoite antibody and gamma interferon-secreting T-cell responses comparable to the more complex tetrabranched peptides in inbred strains of mice. The linear peptide was also immunogenic in outbred nonhuman primates (Aotus nancymae), eliciting antibody titers equivalent to those induced by tetrabranched peptides. Importantly, the 48-mer linear peptide administered in adjuvants suitable for human use elicited antibody-mediated protection against challenge with rodent malaria transgenic sporozoites expressing P. falciparum CS repeats. These findings support further evaluation of linear peptides as economical, safe, and readily produced malaria vaccines for the one-third of the world’s population at risk of malaria infection.

There is a critical need for an effective malaria vaccine, as standard public health measures have been eroded by drug resistance of the parasite and insecticide resistance of the mosquito vector. Moreover, traditional control measures have failed to prevent Plasmodium infections in 300 to 600 million people worldwide, resulting in over 1 million deaths each year (55). Vaccines remain the most cost-effective means for control of infectious diseases. Over the past 30 years, an effective malaria vaccine, based on attenuated sporozoites delivered by the bites of irradiated infected mosquitoes, has been shown to elicit sterile immunity in experimental animal models and human volunteers (11, 25, 27, 45). However, large-scale production of an attenuated parasite vaccine faces significant practical limitations and logistical and regulatory hurdles that must be overcome. Sporozoites cannot be grown in culture and must be dissected from the salivary glands of infected mosquitoes that have fed on blood-stage parasites cultivated in human red blood cells. Additional challenges for attenuated sporozoite vaccines relate to cost, scale-up production, sterility, cold-chain storage, and route of immunization, which in humans has thus far been obtained only by exposure to the bites of irradiated infected mosquitoes. Nevertheless, the ability to elicit sterile protection following immunization with attenuated sporozoites provides a gold standard for development of subunit malaria vaccines that target the preerythrocytic stages of the parasite and effectively prevent initiation of the blood-stage infection responsible for clinical disease.

In contrast to whole parasites, peptide vaccines can be readily synthesized from inexpensive, well-defined amino acid components and lyophilized for storage and transport. In recent years, peptide immunotherapeutics have been developed for human autoimmune diseases and allergies (21, 31), and peptide subunit vaccines for infectious diseases and treatment of cancer have been tested in clinical trials (3, 24, 32, 33, 52). The first phase I/II trial of a malaria synthetic peptide vaccine was carried out over 20 years ago to assess the efficacy of a peptide-protein conjugate vaccine, termed (NANP)3-TT, comprised of the immunodominant B-cell repeat epitope (NANP)3 from the Plasmodium falciparum major surface circumsporozoite (CS) protein linked to tetanus toxoid (TT) as the protein carrier (26). Exposure of a small number of (NANP)3-TT-immunized volunteers to the bites of P. falciparum-infected mosquitoes demonstrated that the vaccine could elicit protective antibody responses against P. falciparum sporozoites. However, the lack of malaria T-cell epitopes in the (NANP)3-TT conjugate vaccine limited the ability of the host...
to mount anamnestic T-cell responses following sporozoite challenge (14, 23).

Subsequent studies using a CD4+ T-cell clone from a volunteer immunized with irradiated *P. falciparum* sporozoites identified a CD4+ T-cell epitope, termed T1, within the 5′ minor repeat region of *P. falciparum* CS protein (41). A branched peptide containing the T1 and the (NANP)3 B-cell epitopes from the *P. falciparum* CS repeat region, termed (T1B)4 multiple-antigen peptide, provided a macromolecular construct which did not require a foreign protein carrier and which was highly immunogenic in preclinical studies and in human volunteers (16, 37, 43). However, the T1 Th epitope contained in the vaccine was genetically restricted and recognized by inbred strains of mice and human volunteers expressing a limited number of major histocompatibility complex class II genotypes (38, 43).

The genetic restriction of vaccine-induced anti-CS repeat antibody responses was subsequently overcome by inclusion of a universal Th epitope derived from the *P. falciparum* CS protein (6, 36). This epitope, termed T*, was originally identified using CD4+ T cells of volunteers protected against challenge following immunization with irradiated *P. falciparum* sporozoites and was considered “universal” based on its ability to bind to a broad range of murine and human class II molecules. In a small phase I trial, a tetrabranched triepitope peptide vaccine, termed (T1BT*)3-P3C, comprised of *P. falciparum* CS repeat epitopes T1 and B synthesized in tandem with the universal T* epitope, elicited antirepeat antibody and CD4+ T-cell responses in volunteers of diverse class II haplotypes (40). The fine specificity and function of CD4+ T-cell clones derived from the (T1BT*)3-P3C-immunized volunteers were comparable to those of clones derived from volunteers immunized with irradiated *P. falciparum* sporozoites (7, 36). Consistent with the broad genetic restriction of the universal T-cell epitope, the majority of CD4+ T cells in both the peptide- and sporozoite-immunized volunteers were specific for the T* epitope, with few clones recognizing the CS repeat epitopes. The T*-specific clones from both peptide- and sporozoite-immunized volunteers secreted high levels of gamma interferon (IFN-γ), a potent inhibitor of intracellular hepatic stages of the *Plasmodium* parasite (20, 34, 53). While the branched synthetic peptide containing *P. falciparum* CS T- and B-cell epitopes was safe and immunogenic in clinical trials (40, 43), scale-up synthesis of large quantities of branched peptides has presented significant technical challenges for vaccine development.

It has been shown in various experimental models of infectious diseases that linear peptides containing strong T-cell epitopes can be highly immunogenic. In rodent malaria, linear peptides containing a combination of malaria epitopes and universal T-helper epitopes, derived from either tetanus toxoid or an artificial pan-DR binding epitope, were as immunogenic as branched peptides containing the same sequences (1, 10, 15, 22, 64). However, the use of foreign protein or nonnatural universal epitopes limits the potential of linear peptides, since they would not elicit parasite-specific memory T-cell anamnestic responses in naturally infected individuals, and vaccine-induced responses would not be boosted following exposure to bites from infected mosquitoes.

We therefore determined whether the presence of the 10 μg was mixed with peptide in phosphate-

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**A.** Peptides containing a combination of malaria epitopes and universal T-helper epitopes, derived from either tetanus toxoid or an artificial pan-DR binding epitope, were as immunogenic as branched peptides containing the same sequences (1, 10, 15, 22, 64). However, the use of foreign protein or nonnatural universal epitopes limits the potential of linear peptides, since they would not elicit parasite-specific memory T-cell anamnestic responses in naturally infected individuals, and vaccine-induced responses would not be boosted following exposure to bites from infected mosquitoes.

**B.** Peptides containing a combination of malaria epitopes and universal T-helper epitopes, derived from either tetanus toxoid or an artificial pan-DR binding epitope, were as immunogenic as branched peptides containing the same sequences (1, 10, 15, 22, 64). However, the use of foreign protein or nonnatural universal epitopes limits the potential of linear peptides, since they would not elicit parasite-specific memory T-cell anamnestic responses in naturally infected individuals, and vaccine-induced responses would not be boosted following exposure to bites from infected mosquitoes.

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**MATERIALS AND METHODS**

**Peptides.** The T1 epitope (DPNANPNNV)3, located in the 5′ minor repeat and the B epitope (NANP)3, in the 3′ major repeat of the central repeat region of the *P. falciparum* CS protein (NF54 isolate) (Fig. 1). The universal T* epitope, EYLNIKNQNSLSTEWSPCSVTT, was synthesized as a branched peptide containing the triepitope (T1BT*)3, or as a 48-mer linear peptide T1BT*

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**FIG. 1.** (A) Schematic illustration of the *P. falciparum* CS protein (NF54 isolate) showing the central repeat region, containing the T1 minor repeat epitope and the major repeat B-cell epitope (NANP)3, and the universal T* epitope located in the C terminus. (B) The 48-mer triepitope T1BT* sequence, (DPNANPNNV)3 (NANP)3, EYLNIKNQNSLSTEWSPCSVTT, was synthesized on the tetrabranched lysine core of the branched peptide (T1BT*)3 or as 48-mer linear peptide T1BT*.
buffered saline (PBS). Peptide-adjuvant formulations were administered in a final volume of 50 to 100 μl. 

Mice. BALB/c (H-2\(d\)), C3H (H-2\(k\)), and C57BL/10 (H-2\(b\)) mice (Jackson Labs, Bar Harbor, ME, and Tacoma Farms, NY) were used to assay the immunogenicity of branched versus linear peptides. Mice in each group (\(n = 3\) to 4) were immunized subcutaneously with three doses of 50 μg of peptide at 20- to 30-day intervals. Peptide dosage was chosen based on previous murine and monkey studies using branched synthetic peptides as immunogens (37-39). Individual mice in each group were bled before immunization and 22 days after each immunization for analysis of antirepeat antibody responses. All rodent studies were carried out with approval of the Institutional Animal Care and Use Committee.

Aotus monkeys. Aotus nancymae monkeys were maintained at the Fundacion Instituto de Inmunologia de Colombia animal facility in Leticia, Colombia, in compliance with standards for Humane Care and Use of Laboratory Animals. Monkeys were quarantined for a 1-month conditioning period during which they were examined for concurrent infections and treated appropriately (37). Animals positive for malaria infections by Giemsa-stained blood smears and/or by serology were excluded from the trial. Monkeys were immunized with 1 mg of linear or branched peptide at 3-week intervals administered at multiple subcutaneous injections in the ventral area, and blood was collected for sera prior to and 21 days after each immunization.

Antibody assays. The antirepeat immunoglobulin G (IgG) titers were determined by enzyme-linked immunosorbent assay (ELISA) using the (T1B*) tetramer as antigen (AnaSpec, Inc.), enzyme-labeled species-specific anti-IgG antibody, and 2,2'-azinobis(3-ethylthiazolinone)sulfonic acid (ABTS) substrate. Endpoint titers, defined as the last serum dilution having an optical density greater than the optical density + 3 standard deviations of preimmune sera, were obtained for individual mice in each group and expressed as geometric mean titer (GMT). Reactivity with parasites was determined by indirect immunofluorescence assay (IFA) using P. falciparum sporozoites (39), high responders to CS repeats (14, 23, 38). Therefore, the fine specificities of the antibodies elicited by branched peptide in each group of mice were collected on day 21 days after each immunization.

IFN-γ ELISPOT. The number of IFN-γ-secreting cells in spleens of mice immunized with linear or branched peptides was measured by IFN-γ enzyme-linked immunospot assay (ELISPOT) assay (35). Briefly, 3×10^5 naive or immune spleen cells were incubated overnight with irradiated spleen cells as antigen-presenting cells, either with or without T1BT* peptide, in nitrocellulose 96-well plates coated with monoclonal antibody (Ab) anti-IFN-γ (R-6A2). Plates were washed and reacted with biotinylated anti-murine IFN-γ (BD Pharmingen) followed by washing and staining with peroxidase-labeled streptavidin and 4-chloro-3-indolylphosphate–nitroblue tetrazolium membrane phosphatase substrate. The number of spot-forming cells (SFC) was counted by an ImmunoSpot Analyzer (CTL, Cleveland, OH), and results were expressed as the number of SFC/10^6 spleen cells.

Transgenic sporozoites. Transgenic Plasmodium berghei rodent malaria parasites expressing P. falciparum CS repeats, termed PIPs, were used to assess biological activity of peptide-induced antirepeat specific immunity (49). Immunized and naive control mice were anesthetized and challenged by exposure to the bites of 10 to 15 PIP-positive mosquitoes. Livers were removed 40 h postchallenge, and total RNA was extracted using a commercial kit (Invitrogen).

The amount of parasite rRNA in each liver was determined by reverse transcription and real-time PCR of cDNA using primers specific for 18S rRNA (5). Results were expressed as the number of copies of parasite rRNA based on an 18S rRNA plasmid reference standard.

The mechanism of immune protection in peptide-immunized mice was determined by T-cell depletion prior to PfPb challenge of immunized mice. Mice were depleted of T cells by administration of MAb specific for CD4 (MAb GK1.5) or CD8 (MAb 2.43) on days −2 and −1 prior to challenge. Depletion of CD4+ and CD8+ T cells following MAb treatment was confirmed by fluorescence-activated cell sorter analysis (BD Pharmingen). Following challenge, the level of parasite 18S rRNA in livers of T-cell-depleted or intact immunized mice was determined using real-time PCR, as described above.

The function of antirepeat antibodies elicited in the peptide-immunized mice was determined using an in vitro transgenic sporozoite neutralization assay (TSNA), as previously described (29) with modifications. Preimmune or immune sera of peptide-immunized mice were incubated with 2×10^6 PfPb sporozoites for 40 min on ice prior to addition to HepG2 cells grown to confluence in 48-well culture plates. After washing to remove sporozoites that did not invade, the cell cultures were incubated in complete RPMI containing 10% fetal calf serum for 48 h at 5% CO2. Cell cultures were trypsinized, and parasite rRNA levels were determined by real-time PCR, as described above.

RESULTS

**Immunogenicity of branched (T1BT*)\(_4\) versus linear T1BT* peptide.** Peptide immunogens frequently require strong adjuvants, as they lack the pathogen-associated molecular pattern found in bacterial or viral antigens. Therefore, initial assays comparing the immunogenicity of the T1BT* linear peptide versus (T1BT*)\(_4\) branched peptide were carried out using the potent Freund’s adjuvant. Consistent with previous studies using the (T1BT*)\(_4\)P3C branched peptide (39), high anti-immunogen antibody titers were obtained in BALB/c and C57BL/10 mice immunized with the branched (T1BT*)\(_4\) peptide in Freund’s adjuvant (Table 1). Importantly, the linear T1BT* peptide elicited equally high anti-immunogen titers in both strains of mice, with peak antibody titers of 163,840 to 327,680.

The fine specificities of the antibodies elicited by branched (T1BT*), or linear T1BT* peptides were also similar in both strains and were predominantly directed against the CS repeat (Table 1). The peptide-induced antirepeat titers elicited by both branched and linear peptide correlated with titers to P. falciparum sporozoites as measured by IFA. BALB/c mice (H-2\(^b\)), which are nonresponders to CS repeats, developed peak ELISA and IFA titers similar to C57BL/10 (H-2\(^b\)), which are high responders to CS repeats (14, 23, 38). Therefore, the presence of multiple Th epitopes, i.e., within CS repeats and...
T* epitope, did not affect the magnitude of the antirepeat or antispore antibody responses elicited by linear peptide.

While the antirepeat antibodies predominated, antibodies to the T* epitope were also detected in the peptide-immunized mice, depending on the immunogen and murine strain. All of the mice immunized with the branched peptide developed anti-T* antibodies, reaching peak titers of 5,120 in BALB/c and 20,480 in C57BL/10 mice. In contrast, the response to linear peptide was mainly to CS repeats, and significantly lower anti-T* antibody in BALB/c (1,280 titer) and no antibodies to T* in C57BL/10 (<80) were detected.

Immunogenicity of linear peptides requires the universal epitope. The immunogenicity of the linear peptide was dependent on Th provided by the universal T* epitope. BALB/c mice, which are nonresponders to both the T1 and B repeats but high responders to the T* epitope (6, 38), developed high antirepeat antibodies when immunized with the linear peptide (Table 1).

To determine if other CS-derived Th epitopes could substitute for the universal T* epitope and overcome the requirement for a branched peptide configuration, C57BL/10 and C3H mice were immunized with linear or branched peptides containing only the T1 and B repeat sequences. Both C57BL/10 (H-2b) and C3H (H-2k) mice can recognize Th epitopes within the T1 minor repeats and branched peptides differed, depending on the murine strain. In C57BL/10 mice, both branched and linear peptides were immunogenic in the absence of adjuvant, eliciting antirepeat titers of $10^3$ to $10^4$ copies when administered in PBS (Fig. 3). In contrast, in BALB/c mice only the branched (T1BT*)$_4$ peptide was immunogenic in the absence of adjuvant.

Addition of adjuvant increased antibody titers 1 to 2 logs higher than those elicited by peptide in PBS. Branched peptide formulated in Montanide ISA 720, ISA 51, or CpG adjuvants elicited antirepeat ELISA and IFA titers of $10^3$ to $10^4$ in both C57BL/10 and BALB/c mice (Fig. 3A). Similar results were obtained with MF59 adjuvant in C57BL/10 mice. In contrast, the antibody response to linear peptide was more adjuvant dependent (Fig. 3B). In both strains of mice, optimal levels of antibodies were obtained with linear peptides formulated in ISA 51 or ISA 720 adjuvant, reaching peak titers equal to or greater than those obtained with Freund's adjuvant formulations (Table 1). The magnitude of antibody induced by linear T1BT* in ISA 720 or ISA 51 was equivalent to that obtained with the branched peptide administered in the same adjuvants. However, in contrast to the oil emulsion adjuvants, CpG-formulated linear peptide was less immunogenic than branched peptides in C57BL/10 mice and poorly immunogenic in BALB/c mice.

For all adjuvant formulations, antirepeat antibody titers elicited by either linear or tetrabranched peptide correlated with reactivity with P. falciparum sporozoites as measured by IFA (Fig. 3, dark versus hatched bars). These results indicate that neither the peptide configuration nor the adjuvant formulation altered the peptide-induced antirepeat antibody cross-reactivity with native CS protein on sporozoites.

Antibody IgG subtypes. The linear and branched peptides, either with or without adjuvant, elicited a predominantly Th2 type IgG1 antirepeat antibody response (Fig. 4). Predominance of IgG1 antibody subtype was also observed in sera of mice immunized with peptides in oil-in-water adjuvant MF59 and oligonucleotide dinucleotide CpG (data not shown).

The ability to elicit a mixed Th1/Th2 response varied between the strains of mice and was independent of peptide configuration. BALB/c mice immunized with either branched or linear peptide, administered in water-in-oil adjuvants (Freund's, ISA 720, or ISA 51), developed Th1 type IgG2a antibodies. In contrast, only low levels of IgG2a antibodies were detected in sera of peptide-immunized C57BL/10 mice. Adjuvant was critical for induction of Th1 type responses to linear peptide, as mice immunized with linear peptides in the absence of adjuvant did not develop IgG2a antirepeat antibodies.

Linear versus branched peptides formulated in adjuvants suitable for humans. The immunogenicity of linear peptides was comparable to that of branched peptides when administered in Freund's, a potent adjuvant which is not acceptable for human use. The peptides were therefore tested in various adjuvants suitable for human vaccines, including the water-in-oil adjuvants Montanide ISA 51 and ISA 720, the oil-in-water adjuvant MF59, and a CpG oligonucleotide, and results were compared to peptide without adjuvant (PBS).

In the absence of adjuvant, the immunogenicity of the linear and branched peptides differed, depending on the murine strain. In C57BL/10 mice, both branched and linear peptides were immunogenic in the absence of adjuvant, eliciting antirepeat titers of $10^3$ to $10^4$ copies when administered in PBS (Fig. 3). In contrast, in BALB/c mice only the branched (T1BT*)$_4$ peptide was immunogenic in the absence of adjuvant.
Cellular responses elicited by branched versus linear peptides. The cellular responses elicited by the T1BT* branched or linear peptide formulated in the optimal Montanide ISA 720 adjuvant were evaluated by IFN-\(\gamma\)/H9253 ELISPOT assay. Immunization of C57BL/10 mice with branched (T1BT*) 4 peptide induced high levels of IFN-\(\gamma\)/H9253-secreting cells (Fig. 5). Importantly, similar levels of IFN-\(\gamma\)/H9253-secreting cells were also observed in spleen cells of mice immunized with T1BT* linear peptide. Responses were specific for malaria peptides, as minimal IFN-\(\gamma\) SFC were found in spleen cells of naïve mice or mice immunized with ISA 720 adjuvant only.

Immunogenicity of linear peptides in Aotus monkeys. While comparable levels of immune responses were observed in the inbred mice immunized with branched or linear peptide, no information was available on the immunogenicity of small linear peptides in larger animals of diverse genetic background. Outbred Aotus nancymae monkeys were therefore immunized with the T1BT* linear or branched peptides in different adjuvant formulations.

Aotus monkeys immunized with (T1BT*) 4-P3C branched peptide developed positive antibody responses detected by ELISA and IFA (Fig. 6). These antibody titers were of the same magnitude as those found in human volunteers immunized with this vaccine formulation (40), which suggests that this Aotus model may be relevant for preclinical analysis of potential vaccine constructs. Similar levels of antirepeat antibody titers (Fig. 6, hatched bars) and IFA titers (Fig. 6, solid bars) were obtained in Aotus monkeys immunized with the T1BT* 4 branched peptide administered in Freund’s.

Importantly, three monkeys immunized with the linear peptide T1BT* administered in Montanide ISA 51 developed high antirepeat and antisporozoite antibody titers, with GMT of 32,510 and 51,606, respectively. These titers were comparable to those observed in the monkeys immunized with the tetra-branched peptide (T1BT*) 4-P3C, which reached peak GMT of 81,920 in ELISA and 28,963 by IFA.

Protective immunity induced by linear peptides. Aotus monkeys, as well as other nonhuman primates, are poorly susceptible to P. falciparum sporozoites, which limits the ability to determine the protective efficacy of immune responses elicited by the peptide vaccines. We therefore utilized a transgenic P. berghei rodent parasite, termed PfPb, in which the homologous P. berghei CS repeat region was replaced with the P. falciparum CS repeat region (29, 49). These transgenic sporozoites are functionally rodent parasites, infecting mice in vivo and hepatoma cells in vitro, but are antigenically P. falciparum, since they express the immunodominant P. falciparum repeat region, thus providing a means to assay the inhibitory activity of vaccine-induced anti-P. falciparum repeat responses.

Mice immunized with branched or linear peptide in
Freund's or ISA 720 adjuvant were challenged by exposure to the bites of mosquitoes infected with the transgenic PfPb parasites. In four control mice receiving only Freund's adjuvant, parasite rRNA was detectable in livers at 40 h postchallenge (Fig. 7A). In contrast, the level of parasite rRNA was reduced 61% in livers of four mice immunized with branched peptides (T1BT*)₄-Freund's adjuvant \( P/H_{11005} 0.01 \) and 95% in mice immunized with (T1BT*)₄-P3C \( P/H_{11005} 0.002 \) compared to control mice. Importantly, the level of hepatic parasite rRNA was reduced 96% in mice immunized with the T1BT* linear peptide administered in Freund's adjuvant \( P/H_{11005} 0.004 \).

When peptides were tested in an adjuvant acceptable for human use, Montanide ISA 720, protection against viable sporozoite challenge was also obtained (Fig. 7B). At 40 h after PfPb sporozoite challenge, parasite rRNA levels were reduced 90% in the mice immunized with branched peptide (T1BT*)₄-ISA 720 compared to adjuvant control \( P = 0.04 \). The linear peptide was as effective as branched peptide in protecting against a natural sporozoite challenge delivered by the bite of infected mosquitoes. Mice immunized with T1BT* linear peptide in ISA 720 demonstrated a reduction of 97% of hepatic-stage development \( P = 0.01 \). Of particular importance, in three of the four mice immunized with T1BT*-ISA 720, there was no parasite rRNA detectable in the liver as measured by

**FIG. 4.** IgG subtype of antirepeat antibody responses elicited by immunization with (A) branched (T1BT*)₄ peptide or (B) linear T1BT* peptide administered without adjuvant (PBS), in Freund's adjuvant, or in adjuvants ISA 720, ISA 51, or CpG, which are suitable for human use. Results are shown as the optical density obtained with BALB/c or C57BL/10 serum (1:100 dilution) incubated with (T1B)₄ peptide-coated ELISA plates and reacted with IgG subtype-specific antibodies.

**FIG. 5.** The number of ELISPOT IFN-γ-positive cells in 10⁶ spleen cells of C57BL/10 mice immunized with three doses of tetrabranched (T1BT*)₄ or linear T1BT* peptide administered in Montanide ISA 720. Negative controls included mice that received ISA 720 adjuvant only and naïve mice.
the highly sensitive real-time PCR assay, indicating that peptide immunization elicited sterile immunity in these mice.

**Mechanism of immune resistance elicited by peptide immunization.** The C57BL/10 mice immunized with T1BT* linear or branched peptides developed high antirepeat antibody titers (Table 1 and Fig. 3) as well as peptide-specific cells that produced IFN-γ (Fig. 5), a potent inhibitor of exoerythrocytic form development (20, 53). Therefore, CS-specific cells and/or antibodies could potentially function in protection against PfPb challenge in mice immunized with T1BT* linear peptide.

To explore the role of T-cell-mediated immunity, in a second experiment, mice immunized with T1BT*-ISA 720 were depleted of CD4+ or CD8+ T cells prior to challenge. In the nondepleted mice immunized with T1BT*-ISA 720, mean parasite rRNA levels were reduced 85% (5,060 ± 9,809 copies) compared to control mice (35,102 ± 14,380 copies) (P = 0.007) (Fig. 8A). Three of four of the immunized mice were totally protected, with a mean rRNA copy number of 156 ± 47, similar to the no-parasite control (189 ± 95 copies).

Depletion of CD4+ or CD8+ T cells prior to challenge did not abrogate immune resistance. Parasite levels were reduced 98% (mean number of rRNA copies, 826 ± 1,369) and 99% (mean number of rRNA copies, 200 ± 110) in the livers of CD4+- and CD8+-T-cell-depleted immune mice, respectively, which was not significantly different from the level of protection observed in the nondepleted immunized mice (P = 0.2). Three of four mice in the anti-CD4 MAb-treated group and four of four mice in the anti-CD8 MAb-treated group had <350 rRNA copies detectable by PCR, similar to the no-parasite control, indicating that the mice were totally protected. Therefore, resistance to PfPb sporozoite challenge in the T1BT* linear peptide-immunized mice did not require the presence of repeat-specific T cells.

To determine the role of antibodies, the serum from the peptide-immunized mice was tested for biological activity using an in vitro TSNA (29). Compared to day 0 sera, the sera of eight mice immunized with T1BT*-ISA 720 significantly blocked sporozoite invasion of hepatoma cells (Fig. 8B). For all eight sera, the mean number of rRNA copies in cultures receiving sporozoites incubated in day 0 serum was 34 × 10⁶ ± 17 × 10⁶, compared to 0.4 × 10⁶ ± 0.9 × 10⁶ rRNA copies in cultures containing sporozoites incubated with immune serum (P = 0.001). The sporozoite-neutralizing activity in the immune sera correlated with the high levels of antirepeat antibodies that developed in all of the C57BL mice immunized with T1BT*-ISA 720 (mean titer, 54,613; range, 20,480 to 163,840). The presence of strong sporozoite-neutralizing activity in sera of all of the peptide-immunized mice, combined with the failure of T-cell depletion to abrogate immune resistance, indicates that protection elicited by linear peptide was mediated by high levels of antirepeat antibodies.

![FIG. 7. Challenge by exposure to the bites of PfPb-infected mosquitoes of C57BL/10 mice immunized with tetrabranched (T1BT*)₄ peptide or linear T1BT* peptide administered in (A) Freund’s adjuvant or (B) ISA 720 adjuvant. Liver cells were obtained at 40 h postchallenge, and the number of parasite 18S rRNA copies was determined by real-time PCR.](http://iai.asm.org/)

![FIG. 6. Antirepeat ELISA (hatched bars) and anti-*P. falciparum* sporozoite IFA (solid bars) titers in sera of *Aotus nancymaeae* monkeys immunized with three doses of tetrabranched or linear peptides administered in Freund’s or ISA 51 adjuvant. Each set of bars represents endpoint titers for sera of individual monkeys.](http://iai.asm.org/)
cell interactions in the liver and inhibiting sporozoite motility required for hepatocyte invasion (8, 57, 59). Recent intravital microscopy studies have shown that antirepeat antibodies also immobilize extravascular sporozoites at the site of the mosquito bite and prevent their egress into the circulation and transit to the target cells in the liver (62). Antibodies can also enhance phagocytosis of sporozoites, and the presence of opsonizing antibodies specific for CS repeats has been correlated with protection in volunteers immunized with a CS subunit vaccine (13, 47, 54).

In a phase I trial, a triepitope branched synthetic peptide, \((T1BT^+)\)\(_a\)P3C, comprised of the \(P. falciparum\) CS protein universal T* epitope synthesized in tandem with CS repeats, was shown to be safe and immunogenic (40). This branched peptide elicited antispore antibody and CD4+ T cells with fine specificity and function comparable to those found in volunteers immunized by irradiated \(P. falciparum\) sporozoites (7, 36). In the current studies, a 48-mer linear peptide containing these minimal \(P. falciparum\) CS T- and B-cell epitopes was as immunogenic as a branched peptide when tested in inbred strains of mice and outbred nonhuman primates. This simple T1BT+ linear peptide elicited sporozoite-neutralizing antibodies and protection against viable sporozoite challenge comparable to levels obtained by immunization with the more complex \((T1BT^+)\) tetrameric branched peptide.

In early vaccine studies, peptides containing \(P. falciparum\) CS repeat epitopes were poorly immunogenic, and optimal immune responses required conjugation to carrier proteins or synthesis as macromolecular branched peptides (9, 61, 66). Phase I trials have demonstrated the safety and immunogenicity of the first-generation \(P. falciparum\) CS repeat peptide-protein conjugate, (NANP)3-TT (26), and branched peptides containing CS repeats either alone [(T1B)\(_a\) multiple-antigen peptide] or combined with the universal T* epitope [(T1BT*)\(_a\)P3C] (40, 43). While the highest levels of antibody were elicited with the branched peptides, the technical challenges of synthesizing large quantities of homogeneous, physiochemically characterized branched peptides prevented scale-up for production of vaccines.

In the current studies, we show that incorporation of the \(P. falciparum\) CS universal T* epitope overcomes the requirement for a branched configuration, providing a highly immunogenic linear peptide vaccine. Consistent with previous studies using foreign protein or artificial universal Th cell epitopes (10, 22, 63), the inclusion of the strong \(P. falciparum\) CS universal T* epitope was essential for immunogenicity of the linear CS peptide. Other CS-derived Th epitopes could not substitute for the universal T* epitope, as shown by the failure of linear peptides containing the CS repeat T1 Th epitope to elicit immune responses in C57BL/10 or C3H mice (Fig. 2). The T1 Th epitope, in contrast to the T* universal epitope, is recognized by only a limited number of HLA class II genotypes in mice and humans (6, 38, 43). While the mechanism by which universal epitopes overcome peptide configuration requirements remains to be defined, the presence of multiple overlapping T-cell epitopes within the universal T* epitope (6, 7, 36) may elicit a broad range of Th cells to enhance the immunogenicity of linear peptides.

While comparable levels of antirepeat antibodies were induced by branched or linear peptides, the fine specificity of the

**DISCUSSION**

Recent studies based on CS transgenic mice have shown that the CS protein is a primary target of immune responses elicited by irradiated sporozoites (48a), the “gold standard” for preerythrocytic-stage vaccine development. Although CD8+ T cells play a critical role in sporozoite-induced immunity in some murine strains (19, 65), we have shown that sporozoite-immunized mice lacking CD8+ T cells can develop high levels of sterile immunity mediated by antibody and CD4+ T cells (G. A. Oliveira and E. H. Nardin, submitted for publication).

Antibodies specific for the immunodominant repeat region of the CS protein were the first immune mechanism identified in the sporozoite-immunized host (48). In early studies, incubation of sporozoites of rodent, simian, or human species of \(P. falciparum\) with immune sera containing antirepeat antibodies led to loss of infectivity, as determined by the failure of the incubated sporozoites to induce a patent infection when injected into a susceptible host (12, 42, 46). Antirepeat antibodies neutralize sporozoite infectivity by blocking parasite-host interactions in the liver and inhibiting sporozoite motility required for hepatocyte invasion (8, 57, 59). Recent intravital microscopy studies have shown that antirepeat antibodies also immobilize extravascular sporozoites at the site of the mosquito bite and prevent their egress into the circulation and transit to the target cells in the liver (62). Antibodies can also enhance phagocytosis of sporozoites, and the presence of opsonizing antibodies specific for CS repeats has been correlated with protection in volunteers immunized with a CS subunit vaccine (13, 47, 54).

In a phase I trial, a triepitope branched synthetic peptide, \((T1BT^+)\)\(_a\)P3C, comprised of the \(P. falciparum\) CS protein universal T* epitope synthesized in tandem with CS repeats, was shown to be safe and immunogenic (40). This branched peptide elicited antispore antibody and CD4+ T cells with fine specificity and function comparable to those found in volunteers immunized by irradiated \(P. falciparum\) sporozoites (7, 36). In the current studies, a 48-mer linear peptide containing these minimal \(P. falciparum\) CS T- and B-cell epitopes was as immunogenic as a branched peptide when tested in inbred strains of mice and outbred nonhuman primates. This simple T1BT+ linear peptide elicited sporozoite-neutralizing antibodies and protection against viable sporozoite challenge comparable to levels obtained by immunization with the more complex \((T1BT^+)\) tetrameric branched peptide.

In early vaccine studies, peptides containing \(P. falciparum\) CS repeat epitopes were poorly immunogenic, and optimal immune responses required conjugation to carrier proteins or synthesis as macromolecular branched peptides (9, 61, 66). Phase I trials have demonstrated the safety and immunogenicity of the first-generation \(P. falciparum\) CS repeat peptide-protein conjugate, (NANP)3-TT (26), and branched peptides containing CS repeats either alone [(T1B)\(_a\) multiple-antigen peptide] or combined with the universal T* epitope [(T1BT*)\(_a\)P3C] (40, 43). While the highest levels of antibody were elicited with the branched peptides, the technical challenges of synthesizing large quantities of homogeneous, physiochemically characterized branched peptides prevented scale-up for production of vaccines.

In the current studies, we show that incorporation of the \(P. falciparum\) CS universal T* epitope overcomes the requirement for a branched configuration, providing a highly immunogenic linear peptide vaccine. Consistent with previous studies using foreign protein or artificial universal Th cell epitopes (10, 22, 63), the inclusion of the strong \(P. falciparum\) CS universal T* epitope was essential for immunogenicity of the linear CS peptide. Other CS-derived Th epitopes could not substitute for the universal T* epitope, as shown by the failure of linear peptides containing the CS repeat T1 Th epitope to elicit immune responses in C57BL/10 or C3H mice (Fig. 2). The T1 Th epitope, in contrast to the T* universal epitope, is recognized by only a limited number of HLA class II genotypes in mice and humans (6, 38, 43). While the mechanism by which universal epitopes overcome peptide configuration requirements remains to be defined, the presence of multiple overlapping T-cell epitopes within the universal T* epitope (6, 7, 36) may elicit a broad range of Th cells to enhance the immunogenicity of linear peptides.

While comparable levels of antirepeat antibodies were induced by branched or linear peptides, the fine specificity of the

**FIG. 8.** Mechanism of protective immunity in C57BL/10 mice immunized with linear T1BT+ISA 720. (A) Effector cell function was determined by depletion of CD4+ or CD8+ T cells \((n = 4\) mice/group) prior to challenge by PiPb-infected mosquitoes compared to nondepleted immunized mice or naive mice \((n = 2\) to 4 mice/group). (B) Sporozoite-neutralizing antibody was measured by TSNA (29). PiPb sporozoites were preincubated with individual serum from eight mice collected on day 0 (preimmune) or after the third immunization with linear peptide. The ability of serum-incubated sporozoites to invade and develop in HepG2 cells was measured in 48-h cultures by real-time PCR, and the results are expressed as the number of copies of 18S rRNA.

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anti-peptide antibody responses differed. Branched peptide elicited antibody to the T* epitope as well as to the repeat epitopes, while the linear peptide elicited significantly lower or no anti-T* antibody (Table 1). Thus far, antibodies to non-repeat epitopes of CS have not been shown to play a significant role in sporozoite immunity. The linear peptide may therefore be more effective than branched peptides in focusing immune responses on the functional CS repeats contained within the vaccine.

Adjuvants are critical for peptide immunogenicity, as the absence of appropriate stimulus for maturation of dendritic cells may result in tolerogenic, rather than immunogenic, peptides (17, 56). The 48-mer linear T1BT* peptide was immunogenic in all adjuvant formulations tested, including Freund’s adjuvant and adjuvants suitable for human use. Formulation of the linear as well as the branched peptide in the water-in-oil adjuvants Montanide ISA 51 and ISA 720 elicited high levels of antirepeat antibodies in both strains of inbred mice. Consistent with murine responses, Aotus monkeys immunized with T1BT* linear peptide in ISA 51 developed antisporozone antibody titers similar to those induced by branched peptide (T1BT*)4 containing either the P3C lipopeptide or Freund’s adjuvant (Fig. 6). The finding that outbred Aotus monkeys immunized with (T1BT*)4-P3C developed antibody titers similar to those observed in phase I trials (40) and the similarity in peptide binding motifs of Aotus class II and human DR and DQ molecules (4, 18, 44) support the use of this nonhuman primate model for preclinical assessment of peptide vaccine candidates. The correlation of the Aotus and human immune responses to branched peptides and the immunogenicity of the linear peptide in the Aotus monkeys suggest the potential for immunogenicity of small linear peptides in humans.

However, studies in Aotus monkeys, as well as human phase I trials, provide limited information on the biological function of the immune responses elicited by P. falciparum peptide vaccines. In the current studies, we demonstrate that mice immunized with branched (T1BT*)4, using either the P3C lipopeptide adjuvant or Freund’s adjuvant, were protected against sporozoite challenge with epitopes from other preerythrocytic-stage and/or blood-stage antigens to elicit antibodies to target merozoites released from exoerythrocytic forms. While CD8+$+T$ cells play critical roles in murine malaria models, an essential role for human CD8+$+T$-cell responses has not been demonstrated in subunit vaccine trials and naturally infected individuals (30, 51, 58). Peptide vaccines eliciting strong class II-restricted CD4+$+T$-cell and antibody responses may therefore provide effective vaccines that can be readily optimized, in contrast to vaccines requiring both class I and class II antigen-presenting pathways.

The finding that branched peptide malaria vaccines were safe and well tolerated in phase I trials (40, 43) encourages the expectation that a linear peptide will demonstrate a similar safety profile. Synthetic peptides provide safety advantages over attenuated parasite or recombinant viral vector vaccines for vaccination of immunocompromised individuals, in addition to eliminating potential adventitious agents acquired during cell culture and the need for cold-chain storage, which significantly increase costs of attenuated vaccines. In recent years, immunotherapy with linear peptides has been used in treatment of autoimmune diseases and allergy (21, 31), and the industrial standards for synthesis, characterization, and large-scale production of peptides for clinical applications are well established. These factors encourage further development of linear peptides containing P. falciparum minimal T- and B-cell epitopes as cost-effective, safe, and easily produced malaria vaccines.

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