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# A Conserved Peptide Sequence of the *Plasmodium falciparum* Circumsporozoite Protein and Antipeptide Antibodies Inhibit *Plasmodium berghei* Sporozoite Invasion of Hep-G2 Cells and Protect Immunized Mice against *P. berghei* Sporozoite Challenge

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**Minutes after injection into the circulation, malaria sporozoites enter hepatocytes. The speed and specificity of the invasion process suggest that it is receptor mediated. The region II sequence of *Plasmodium falciparum* circumsporozoite (CS) protein includes a nonapeptide (WSPCSVTCG) which is highly conserved in all of the CS proteins sequenced to data, including the one from *Plasmodium berghei*. We have found that two peptides based on the *P. falciparum* region II sequence, P18 (EWSPCSVTCGNGIQVRIK) and P32 (IEQYLKKIKNS ISTEWSPCSVTCGNGIQVRIK), significantly inhibited *P. berghei* sporozoite invasion into Hep-G2 cells in vitro. This inhibition was enhanced if either peptide was preincubated with Hep-G2 cells prior to sporozoite invasion. We confirm that region II is a sporozoite ligand for the hepatocyte receptor; moreover, despite the few differences between *P. falciparum* and *P. berghei* region II sequences around the nonapeptide sequence (66% homology), the functional characteristics of the motif sequences are not affected. Since the conserved motifs represent a crucial sequence involved in *Plasmodium* sporozoite invasion of hepatocytes, antibodies to region II should inhibit sporozoite invasion into hepatocytes. Indeed, we found that polyclonal antibodies generated to the *P. falciparum*-based peptide P32 inhibited *P. berghei* sporozoite invasion of Hep-G2 cells. Furthermore, inbred mice (C57BL/6) immunized with P32 were protected against a lethal challenge of *P. berghei* sporozoites. Our results suggest that the conserved region II of the CS protein contains crucial B- and T-cell epitopes, that such peptide sequences from the human malaria parasite *P. falciparum* can be screened in the *P. berghei* rodent model, and, finally, that region II can be considered useful as one of the components of a malaria vaccine.**

One of the major problems in developing a vaccine against malaria is the different stages in the life cycle of the parasite and the variety of antigens, mostly polymorphic in nature, involved in these stages. However, several *Plasmodium falciparum* antigens from different developmental stages of the parasite have been identified and are being considered as candidates for malaria vaccine development (13, 18). Of these antigens, the circumsporozoite (CS) protein is the most well characterized, and its role in protection against malaria infection is well established (18). A striking feature of this antigen is the presence of a highly immunodominant, tandemly repeated tetrapeptide sequence which is conserved in all strains of *P. falciparum*. However, clinical trials of recombinant CS protein fragments, aimed at developing specific antibody responses to the repeats, have proved disappointing (11, 12), leading to the suggestions that there might be other antigenic sites involved in protective immunity against the sporozoite stage (10, 15).

Carboxy-terminal to the repeats, CS proteins from all species possess a stretch of conserved amino acid sequence termed region II (4). The amino acid sequence of *P. falciparum* region II contains a nonapeptide sequence (WSPCSVTCG) which has been found to be conserved in the CS proteins from *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium knowlesi*, *Plasmodium berghei*, and *Plasmodium yoelii* (Fig. 1a). This conserved sequence is also found in thrombospondin-related anonymous protein (TRAP) (22) and in an antigen from *Eimeria tenella* (3), a parasite phylogenetically related to plasmodia. These motifs are also conserved in a variety of different proteins of biological significance, such as thrombospondin (16), properdin, and components of the complement pathways (9, 16). The occurrence in the sporozoite and blood stages of *P. falciparum* of a highly conserved sequence which is also present in highly functional proteins involved in cellular interactions has led to the speculation that conserved motifs might be involved in parasite interactions with the host cell membrane (21, 22). A possible role for the major sporozoite surface protein, i.e., the CS protein, is recognition and entry into hepatocytes. Since sporozoites from different malarial parasites can infect hepatocytes from the same mammalian host (1), the ligands recognized by the putative liver receptor may not be the species-specific repeat sequences; instead, some highly conserved sequences may be used by the parasite for this purpose (2).

Indeed, this appears to be the case. Recently, the works of others have shown that synthetic peptides representing the genus-conserved region II of the CS protein, which includes the conserved motif sequences mentioned above, inhibited the interaction of CS protein with hepatocytes as well as sporozoite invasion of Hep-G2 cells (2, 6, 19, 21). Bearing in mind this information and the fact that there are some differences between *P. falciparum* and *P. berghei* region II around the non-

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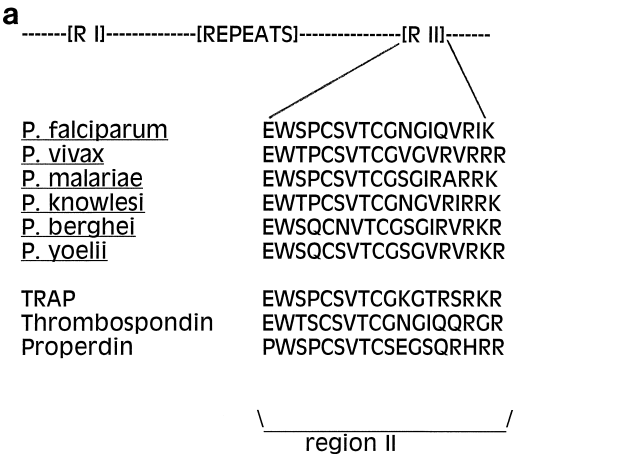


FIG. 1. (a) Region II of the *P. falciparum* CS protein and the homologous regions of other plasmodia, TRAP, thrombospondin, and properdin. (b) Amino acid sequences of the *P. falciparum*-based region II peptides P18 and P32. Underlined residues in P32 show a *P. falciparum* CS protein-based T-cell epitope. (c) Comparison of the amino acid sequences of P18 and P32 with homologous sequences in *P. berghei* (Pb18 and Pb32). Amino acids shared by the two parasites are underlined.

apeptide sequence (5) (Fig. 1c), we chose to study the functional characteristics of the *P. falciparum* region II motifs in the *P. berghei* rodent model. Moreover, since the conserved motifs represent a crucial sequence involved in *Plasmodium* sporozoite invasion of hepatocytes, we thought it interesting to study whether these region II sequences could become targets of protective antibodies generated upon immunization which had the potential to inhibit sporozoite invasion into Hep-G2 cells in vitro and confer protection against challenge infection in vivo.

We have found that two peptides, an 18-mer (P18) and a 32-mer (P32), from region II of *P. falciparum* CS protein are able to inhibit *P. berghei* sporozoite invasion of Hep-G2 cells. We also show that immunization with the 32-mer produces an anti-region-II-specific antibody response and that mice immunized with this peptide were protected against a lethal challenge with *P. berghei* sporozoites. Peptide constructs like P32 may be useful as part of a malaria vaccine.

## MATERIALS AND METHODS

**Source of sporozoites.** *P. berghei* ANKA sporozoites were dissected from the salivary glands of *Anopheles stephensi* mosquitoes 21 days after an infective blood meal.

**Hep-G2 cells.** Hep-G2 cells were maintained in 25-$cm^2$ sterile culture flasks (Nunc) in 5 ml of minimal essential medium (MEM) Rega 3 (Gibco, Ghent, Belgium) containing 10% heat-inactivated fetal calf serum (Bio-Lab, Jerusalem, Israel), 2 mM L-glutamine (Gibco), and antibiotics (complete MEM Rega 3). The antibiotic mixture was composed of 10,000 U of penicillin per ml, 10 mg of streptomycin per ml, and 10 μg of amphotericin B (Fungizone) per ml. Cells were incubated in a $CO_2$ incubator at 37°C, with 5% $CO_2$ and almost 99% relative humidity. For parasite culture and peptide assays, 0.81-$cm^2$ sterile chamber slides (Lab-Tek, Nunc; eight chambers per slide) were utilized.

**Synthetic peptides.** The sequences of synthetic peptides P32 and P18 based on region II of the CS protein of *P. falciparum* are given in Fig. 1b. Corresponding sequences from the CS protein of *P. berghei* are shown in Fig. 1c. All of the peptides were synthesized by solid-phase methods with an Applied Biosystems model 430A peptide synthesizer. Peptides were cleaved by anhydrous hydrogen fluoride treatment and purified by reverse-phase high-performance liquid chromatography. Peptides were characterized by amino acid analysis.

**Animals.** Six-week-old female inbred mice of different genetic backgrounds (C57BL/6 and BALB/c) were purchased from IFFA CREDO (Brussels, Belgium).

**Antibodies.** The monoclonal antibody 3D11 (20), which recognizes the repeat-containing domain of *P. berghei* CS protein, was used in indirect immunofluorescent antibody test (IFAT) studies at concentrations of 50 μg/ml.

Anti-P32 antibodies were induced in C57BL/6 mice by intraperitoneal injection of P32 (50 μg per animal) emulsified in Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.). The priming immunization was in complete Freund's adjuvant (CFA), whereas two booster immunizations (days 14 and 28) were in incomplete Freund's adjuvant (IFA). Mice were bled from the tip of the tail, a week after the second boost, and their sera were assayed for antibodies reacting with glutaraldehyde-fixed *P. berghei* sporozoites.

Anti-sporozoite antibodies to *P. berghei* were induced by three intravenous injections in C57BL/6 mice of 30,000 irradiated sporozoites each (irradiation dose, 12 krads). Inoculations were spaced 14 days apart, and sera were obtained 1 week after the third immunization.

**Inhibition of *P. berghei* sporozoite invasion of Hep-G2 cells by peptide and antipeptide sera.** Hep-G2 cells (50,000) in 200 μl of complete MEM Rega 3 were incubated in each chamber of Nunc (eight-chamber) slides. On day 4, the medium was changed to fresh MEM Rega 3 just before starting the assay. Ten micrograms of P18 or P32 in a volume of 200 μl of complete MEM Rega 3 was then added to Hep-G2 cells in each chamber. After 4 h, 10,000 *P. berghei* sporozoites in a volume of 50 μl of a medium consisting of glucose, lactalbumin, fetal calf serum, and hemoglobin (GLSH) were added to each chamber. After incubation at 37°C for 2 h, the medium in the chambers was replaced several times. The cells were then fixed with cold methanol, and the parasites were counted by an IFAT with 3D11 as the primary antibody. These assays were read blind. Results were obtained as the arithmetic means of counts obtained in quadruplicate chambers.

In a separate experiment, the relevant peptide in a volume of 200 μl of complete medium and sporozoites (10,000 in 50 μl of GLSH) were added together simultaneously to Hep-G2 monolayers. After 2 h, all of the extracellular sporozoites were removed by changing the medium in the chamber slides several times. Infected cells were fixed, and IFAT was carried out as described before. An 18-residue peptide representing a sequence from the major surface antigen of a *Plasmodium* sp. (*P. yoelii* MSA-1) and containing two residues was used as a control peptide in the peptide inhibition experiments described above.

In another experiment, sporozoites were added to Hep-G2 monolayers together with 20 μl of heat-inactivated pooled serum from mice immunized with P32 at different dilutions. After incubation for 2 h, the medium was refreshed several times, and methanol fixation of Hep-G2 cells and IFAT were carried out. The percent reduction (arithmetic mean) of invasion of *P. berghei* sporozoites (ISI) into Hep-G2 cell cultures in the presence of peptide or antipeptide sera was calculated by the following formula: $ISI = [(I_c - I_t)/I_c] \times 100$, where $I_c$ is the number of sporozoites that invaded in the absence of test peptide or test sera and $I_t$ is the number of sporozoites that invaded in the presence of test peptide or test sera.

It was necessary to assess the persistence of extracellular sporozoites at the end of the sporozoite–Hep-G2 cell incubation period. An initial experiment was set up to observe whether after 2 h of incubation with Hep-G2 cells all of the extracellular sporozoites were removed by medium washes. Hep-G2 cells were fixed in situ in a first group of chambers with 1% glutaraldehyde and in a second group with methanol; the difference between the two groups was in the cell membrane permeability after fixation. In the first case, only extracellular sporozoites would be available for IFAT labeling; in the second, intracellular as well as extracellular sporozoites would be labeled. In the chambers with glutaraldehyde-fixed cells, an average of 41 ± 14 sporozoites per chamber, which were probably adhering to the Hep-G2 cell surface, were counted. In the absence of glutaraldehyde fixation, an average of 1,518 ± 86 sporozoites were counted in each chamber. The sharp difference between the two counts was due to the counting of intracellular sporozoites. It may be concluded that most of the extracellular sporozoites are removed by medium changes and that most of the parasites demonstrated by IFAT staining after methanol fixation are intracellular.

**IFAT.** Fixed Hep-G2 cells were incubated with 50 μl of monoclonal antibody 3D11 per chamber for 30 min at 37°C. Chamber slides were rinsed with phosphate-buffered saline (PBS), incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Diagnostics Pasteur) diluted 1/100 in Evans blue solution for 30 min at 37°C, and then finally rinsed again with PBS. Slides were mounted with a glass coverslip with glycerol medium, and sporozoites were observed under a fluorescence microscope.

TABLE 1. Inhibition of *P. berghei* sporozoite invasion into Hep-G2 cells in the presence of peptides P18 and P32

| Expt | Peptide or medium added | Concn[a] (μg/ml) | No. of sporozoites in Hep-G2 cells[b] | ISI (%) |
|---|---|---|---|---|
| 4-h preincubation of peptide with Hep-G2 cells, followed by sporozoite invasion | P18 | 50 | 129, 150, 146, 122 | 84 |
| | P32 | 50 | 260, 283, 285, 291 | 67 |
| | *P. yoelii* | 50 | 840, 811, 752, 821 | |
| | Medium | | 876, 910, 830, 750 | |
| | P18 | 2 | 776, 692, 689, 727 | 26 |
| | P18 | 5 | 410, 451, 435, 437 | 53 |
| | P18 | 10 | 286, 294, 292, 260 | 55 |
| | P18 | 20 | 213, 195, 180, 198 | 67 |
| | P18 | 30 | 142, 157, 159, 172 | 74 |
| | P18 | 40 | 158, 145, 156, 140 | 75 |
| | P18 | 50 | 141, 130, 157, 149 | 76 |
| | Medium | | 565, 557, 619, 651 | |
| Simultaneous addition of peptide and sporozoites | P18 | 50 | 306, 328, 300, 339 | 62 |
| | P32 | 50 | 357, 499, 392, 450 | 50 |
| | Medium | | 876, 910, 830, 750 | |

[a] A 200-μl volume of peptide dilutions was added to each chamber.
[b] Values of quadruplicate chamber counts.

Mouse sera raised against P32 were assayed for the presence of antibodies to glutaraldehyde-fixed sporozoites. Drops of sporozoite suspension were distributed on multispot slides (Wellcome) and air dried for 30 min. The slides were fixed in an 0.01% glutaraldehyde solution in PBS buffer (pH 7.2) for 30 min and then rinsed in a bath with PBS buffer. Different serum dilutions were incubated with sporozoites in wells for 30 min at 37°C. The wells were rinsed with PBS, incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G diluted 1/100 in Evans blue solution for 30 min at 37°C, and then rinsed again with PBS. Slides were mounted with a glass coverslip with glycerol medium and examined under a fluorescence microscope for sporozoites stained with antibody.

**ELISA.** Mouse sera raised against P32 were assayed in a checkerboard enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to P18 and P32. As a control, we used a synthetic peptide representing the *P. falciparum* CS protein-based T-cell epitope CST3, whose sequence, DIEKKIAK MEKASSVFNVVNS (positions 378 to 398), has been reported to be a conserved and universal T-cell epitope (25).

Briefly, wells of a flat-bottom microtiter plate (Greiner, Nuringen, Germany) were coated with different concentrations (2, 4, 6, 8, and 10 μg) of the relevant peptide, in a 50-μl volume of $Na_2CO_3$-$NaHCO_3$ coating buffer (0.06 M; pH 9.6). The plates were incubated overnight at 37°C until dry and then washed thrice with PBS-Tween (pH 7.2); uncoated reactive sites in the wells were then blocked by incubation with PBS containing 5% albumin. After incubation at 37°C for 1 h, the plates were washed with PBS-Tween, and 50 μl of suitably diluted (1/100 to 1/12,800) mouse anti-P32 serum was added to each well. The plates were incubated for 90 min in a humid chamber. After further washing, 50 μl of a goat anti-mouse antibody conjugated to horseradish peroxidase was added to each well at a 1/1,000 dilution. The plates were incubated for an additional 90 min in a humid chamber and washed with PBS-Tween, and 100 μl of a substrate solution containing $H_2O_2$ and *O*-phenylenediamine dihydrochloride was added to each well. The optical density at 450 nm ($OD_{450}$) of the reaction product was measured with an ELISA reader. Control values were obtained by incubating dilutions of normal mouse serum in peptide-coated wells.

The presence in the sera of mice immunized with *P. berghei* sporozoites of antibodies cross-reactive with the synthetic peptides was also assayed by checkerboard ELISA, with different concentrations (2 to 10 μg per well) of the relevant peptide used as the capture antigen. Fifty microliters of suitably diluted (1/100 to 1/12,800) mouse antisporozoite serum was added to each well of a flat-bottom well plate that was coated with the relevant antigen, and the ELISA was carried out as described above.

**T-cell proliferation assay.** Mice were immunized in the footpads with 35 μg of P32 emulsified in CFA on day 0. On day 8, the popliteal lymph nodes were excised from these mice and placed in a glass homogenizer with 1 ml of RPMI 1640 (Gibco, Grand Island, N.Y.). Popliteal lymph node cells were extracted by homogenization, washed twice in RPMI 1640, and adjusted to a concentration of $2 \times 10^6$ lymphocytes per ml in complete RPMI 1640 containing 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) 5% heat-inactivated fetal calf serum, and $5 \times 10^{-5}$ M mercaptoethanol. As controls, popliteal lymph node cells were isolated from popliteal lymph nodes of naive adjuvant-primed mice.

Two hundred microliters of cell suspension ($2 \times 10^6$ lymphocytes per ml) was deposited in each well of a 96-well microtiter plate (Falcon). Different peptide concentrations of P32 ranging from 0.2 μg to 4 μg per well were added to the cells. Each peptide dilution was assayed in triplicate. Lymph node cells were incubated with peptide for 2 days at 37°C in a 5% $CO_2$ incubator. Each well was then pulsed with 0.5 μCi of [$^3$H]thymidine (Amersham, Sydney, Australia). The contents of each well were harvested 18 h later onto nitrocellulose filters, and the extent of proliferation was determined by measuring the uptake of $^3$H by liquid scintillation counting with an LKB/βPlate system (Pharmacia, Espoo, Finland).

**Challenge of immunized mice with *P. berghei* sporozoites.** Groups of four to six mice (C57BL/6 and BALB/c) were immunized by intraperitoneal injections of P32 (50 μg per animal) emulsified in Freund's adjuvant. The priming immunization was in CFA, whereas booster immunizations were in IFA. Mice were bled from the tip of the tail a week after the second boost. Control mice received only the adjuvant. All immunized and control mice were challenged by intravenous inoculations of 100 (for C57BL/6 mice) and 1,000 (for BALB/c mice) live *P. berghei* sporozoites. From day 4 after challenge, blood smears were obtained from tails of infected mice, and the percentage of blood-stage parasitemia was determined by Giemsa staining and counting parasites in several fields under a microscope. Observations were repeated on days 7, 10, 25, and 30.

## RESULTS

**Peptides P18 and P32 inhibit *P. berghei* sporozoite invasion of Hep-G2 cells.** To determine whether peptides P18 and P32 were able to interfere with sporozoite invasion of Hep-G2 cells, the cells were preincubated with different concentrations of the peptide and the sporozoites were then added. At a peptide concentration of 50 μg/ml, ISI values of 84 and 67% were obtained for P18 and P32, respectively. As shown in Table 1, the inhibition of sporozoite invasion is dependent on the peptide concentration. In another experiment, when the peptide and sporozoites were added together to Hep-G2 cells, the inhibition was somewhat lower; at 50 μg of peptide per ml, the ISI values of 62 and 50% were obtained for P18 and P32, respectively. To check for any possible toxic effects of peptides on sporozoite invasion into Hep-G2 cells, we carried out the inhibition experiment with a synthetic peptide corresponding to a sequence from the C-terminal region of *P. yoelii* MSA-1. This 18-mer peptide sequence (RCLLGYKKGEGNTCVENN) spanning amino acids 1686 to 1703 of *P. yoelii* MSA-1 also contained two cysteine residues, like the sequences of P18 and P32. In the presence of this peptide at a concentration of 50 μg/ml, no inhibition of sporozoite invasion of Hep-G2 cells was observed. These results indicate that the inhibition observed with the CS region II-based peptides is specific to their structures.

**Anti-P32 antibodies inhibit *P. berghei* sporozoite invasion of Hep-G2 cells.** To determine whether anti-P32 antibodies could

also inhibit the sporozoite invasion, mice (C57BL/6) were immunized with P32. Sera from the immunized mice were pooled and assayed for the presence of antibodies to glutaraldehyde-fixed sporozoites by IFAT. Serum antibodies showed weak reactivity; immunofluorescence was observed only with serum dilutions up to 1/20. In an ELISA, the same anti-P32 serum showed strong reactivity to P32 and P18, as compared with the $OD_{450}$ values of wells coated with a peptide representing a T-cell epitope (Fig. 2a). In our experiment, no differences in reactivity were observed within the range of concentrations (2, 4, 6, 8, and 10 μg per well) used for each peptide. We therefore present data obtained with 10 μg of relevant peptide per well. As a negative control, P32-coated wells were also incubated with dilutions of normal mouse serum, and the $OD_{450}$ values in these wells did not exceed 0.02.

Anti-P32 serum, at a 1/100 dilution, also showed reactivity to a *P. berghei* sporozoite lysate. The lysate was prepared by repeated freeze-thaw of a sporozoite suspension in GLSH. After further dilution in coating buffer, a final volume of 50 μl of lysate, corresponding to about 700 sporozoites, was dispensed in each microtiter well. This result suggested that anti-P32 serum antibodies recognized epitopes on P32 as well as on the native protein in an ELISA.

Incubation of anti-P32 serum dilutions with sporozoites in chambers with Hep-G2 monolayers significantly inhibited sporozoite invasion of Hep-G2 cells (Table 2). As a positive control, 3D11, a monoclonal antibody to *P. berghei* CS repeat sequences, was also incubated with sporozoites and Hep-G2 cells in separate chambers. A level of inhibition of sporozoite invasion, similar to that in the case of the anti-P32 serum was also observed with 3D11. No such inhibition was observed when Hep-G2 cells were incubated with a control adjuvant-primed mouse serum.

***P. berghei* antisporozoite antibodies cross-react with *P. falciparum* peptides P18 and P32.** Since anti-P32 serum antibodies reacted with *P. berghei* sporozoites, we wondered if antisporozoite (*P. berghei*) antibodies would also recognize peptides P18 and P32 (Fig. 1b). Mice (C57BL/6) were immunized with irradiated *P. berghei* sporozoites, and serum antibodies showed reactivity to *P. berghei* sporozoite lysate in an ELISA. The reactivity of these antisporozoite serum antibodies to the peptides was checked in a checkerboard ELISA. Significant reactivity of antibodies to P18 and P32 was observed. These results (Fig. 2b) show the cross-reactivity of anti-*P. berghei* antibodies with *P. falciparum*-based peptide sequences. Similar levels of reactivity were observed at all concentrations tested for each peptide; thus, only values obtained with 10 μg of relevant peptide per well are presented. Negative control values obtained from OD readings of peptide-coated wells incubated with normal mouse serum did not exceed 0.02.

**Cellular proliferative responses to P32.** Peptide P32 contains a well-known T-helper epitope sequence at its N-terminal end (7). To determine whether this T epitope was functional during the peptide immunizations, groups of mice were primed with P32. Popliteal lymph node cells were separated and restimulated with different dilutions of the same peptide in vitro. [$^3$H]thymidine uptake experiments revealed strong T-cell-proliferative responses at all of the P32 dilutions used in vitro (Fig. 3). Lymph node cells from adjuvant-primed mice also did not trigger any proliferative response when stimulated with P32.

**Protection in mice immunized with P32.** To determine if immunization with P32 could provide protection in mice, groups of mice (C57BL/6 and BALB/c; six per group) were immunized with the peptide and challenged with *P. berghei* sporozoites. Blood smears were obtained periodically from the tails of the immunized mice to monitor the course of infection.

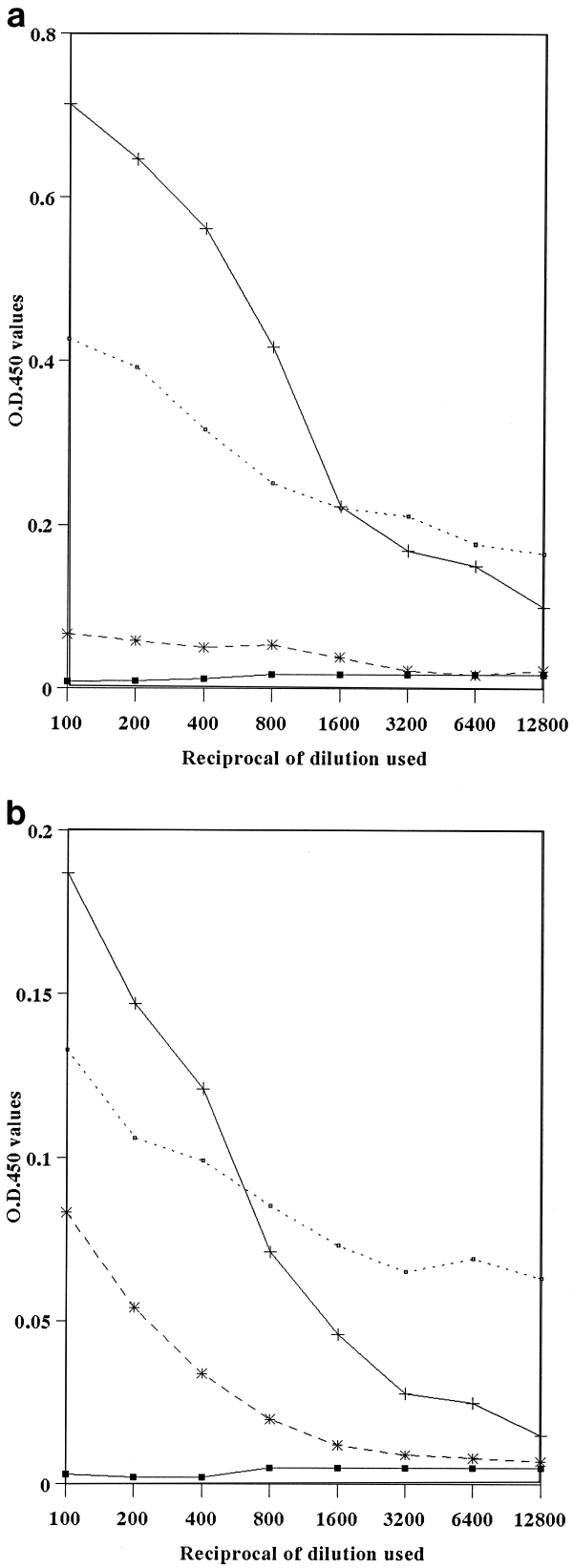


FIG. 2. Checkerboard ELISA to show serum reactivity to different peptide fragments, i.e., P18 (- - □ - -), P32 (+), and T epitope (*). Negative control (—■—) values were obtained by incubating peptides with normal mouse serum dilutions and did not exceed 0.02. (a) Sera from C57BL/6 mice immunized with P32; (b) sera from C57BL/6 mice immunized with 12-kilorad-irradiated *P. berghei* sporozoites.

TABLE 2. Inhibition of *P. berghei* sporozoite invasion into Hep-G2 cells in the presence of anti-P32 sera[a]

| Addition to sporozoites | Dilution or concn[b] | No. of sporozoites in Hep-G2 cells[c] | ISI (%) |
|---|---|---|---|
| Anti-P32 serum | 1/10 | 121, 133, 116, 142 | 89 |
| | 1/100 | 112, 159, 155, 107 | 88 |
| 3D11 | 50 μg/ml | 68, 75 | 93 |
| Control mouse serum | 1/10 | 1,021, 1,132, 1,228, 1,011 | 2 |
| Medium | | 1,326, 1,049, 1,077, 1,007 | |

[a] This experiment involved incubation of sera with sporozoites and Hep-G2 cells.
[b] A 20-μl volume of serum was added to each chamber.
[c] Values of duplicate or quadruplicate chamber counts.

Five of the six C57BL/6 mice did not show any parasitemia and survived a challenge of 100 *P. berghei* sporozoites. In one immunized mouse, blood-stage parasites were seen on day 4; this mouse died on day 25. All of the six control mice showed a steady increase in parasitemia, and all died by day 30 (Fig. 4a). On the other hand, none of the immunized BALB/c mice survived upon challenge with 1,000 *P. berghei* sporozoites, although there was a marked delay in the onset of blood-stage parasitemia in all of the immunized mice until day 7 (Fig. 4b). All of the immunized and control BALB/c mice died by day 14.

## DISCUSSION

Our results from the present study show that synthetic peptides P18 and P32, representing region II of the CS protein of *P. falciparum* and containing the highly conserved nonapeptide motif WSPCSVTCG, significantly inhibit *P. berghei* sporozoite invasion into Hep-G2 cells in vitro. This inhibition is somewhat enhanced if either peptide is preincubated with Hep-G2 cells prior to sporozoite invasion. The malaria parasite invasion of hepatocytes is highly efficient and specific and in all probability is a receptor-mediated process. Two *P. falciparum* sporozoite surface proteins, the CS and the TRAP, have been identified as candidate ligands for the putative hepatic receptor, and the conserved motif sequence, common to both of the proteins, has been implicated to be crucial for this function (2, 17). Our finding that *P. falciparum*-based peptides P18 and P32 inhibit *P. berghei* sporozoite invasion of Hep-G2 cells further supports the view that the conserved motif represents a crucial peptide sequence involved in all *Plasmodium* sporozoite invasion of the hepatocytes (2). Furthermore, since there are a few differences between *P. falciparum* and *P. berghei* region II sequences, including the conserved nonapeptide, it is clear that these changes (Fig. 1a) do not greatly affect the functional characteristics of the motif sequences. The observation that P32 is also effective in sporozoite invasion inhibition reflects that the conformational integrity of the conserved motif sequence is maintained even when the peptide sequence is elongated at its N terminus by 14 residues, although our experiments do not provide any direct evidence for it.

Serum antibodies produced by immunization with 12-kilorad-irradiated *P. berghei* sporozoites cross-reacted with P18 and P32 in a peptide ELISA, indicating that in the context of natural infection, there is a B-cell epitope sequence within the structure of these peptides. Given the extent of the homology between *P. berghei* and *P. falciparum* sequences in P18 and P32, the cross-reactivity observed in the peptide ELISA may not be surprising. We also found that at least 30% of the serum samples collected from individuals living in *P. falciparum*-infected areas in India reactived with P18 and P32 in an ELISA, although the reactivity was lower than that with other immunodominant peptides representing the repeat sequence of CS protein or ring-infected erythrocyte surface antigen (RESA) of *P. falciparum* (2a). This also suggests that the conserved motifs may represent a B-cell epitope sequence, which is not as immunodominant as some other sequences from malarial antigens, during the course of *P. falciparum* infection.

On the other hand, Muller et al. (17) have reported that antisera raised against recombinant TRAP constructs did not react in an ELISA with a synthetic peptide spanning the WSP CSVTCG sequence attached to carrier proteins. Other workers have also found it difficult to elicit anti-region-II antibodies capable of reacting with native proteins by immunization in animals with relevant peptides coupled to carrier proteins (2). The reasons for these seemingly contradictory observations are not clear. It may be possible that the conserved motif sequences form a highly ordered structure in the intact proteins; the complete conservation of the two cysteine residues in all of the region II sequences (Fig. 1a) would support such a possibility, and such a structure may be lost during the preparation of peptide-carrier protein conjugates. In our ELISA experiments, the peptides P18 and P32 were not conjugated to any carrier protein and used as such as the capture antigens. However, in any case, it is clear that the conserved region II sequence does not represent a highly immunodominant region in the corresponding proteins.

The sequence of P32 contains a T-cell epitope (7) which partly overlaps with the conserved nonapeptide sequence, and in this sense, this peptide represents a single, linear, T-cell and

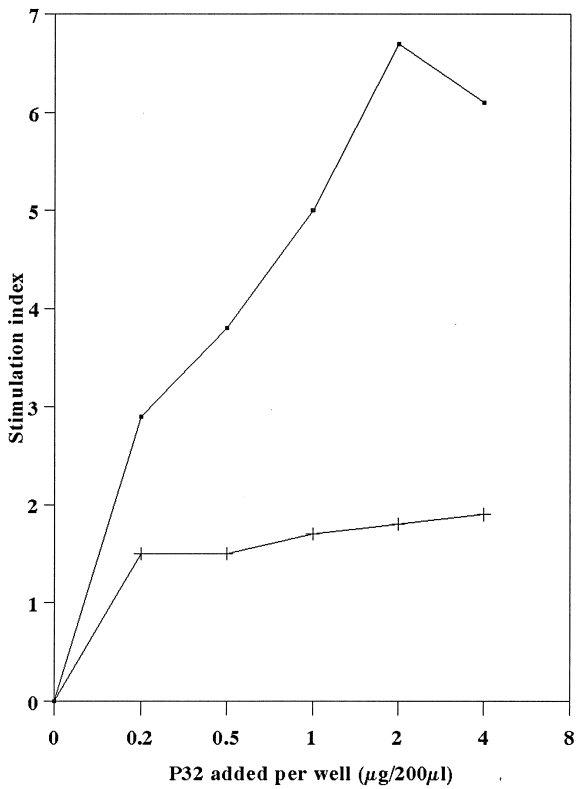


FIG. 3. T-cell-proliferative responses to P32. C57BL/6 mice were immunized with P32, and the proliferation was assessed by [$^3$H] thymidine uptake (counts per minute [cpm]) after 3 days of culture with different concentrations of the peptide. Results are presented as stimulation indices (cpm in stimulated cultures/cpm in unstimulated culture). Values are given as means of triplicate cultures. Counts in unstimulated P32-primed (■) and control adjuvant-primed cells (+) alone were 7,029 and 7,011, respectively.

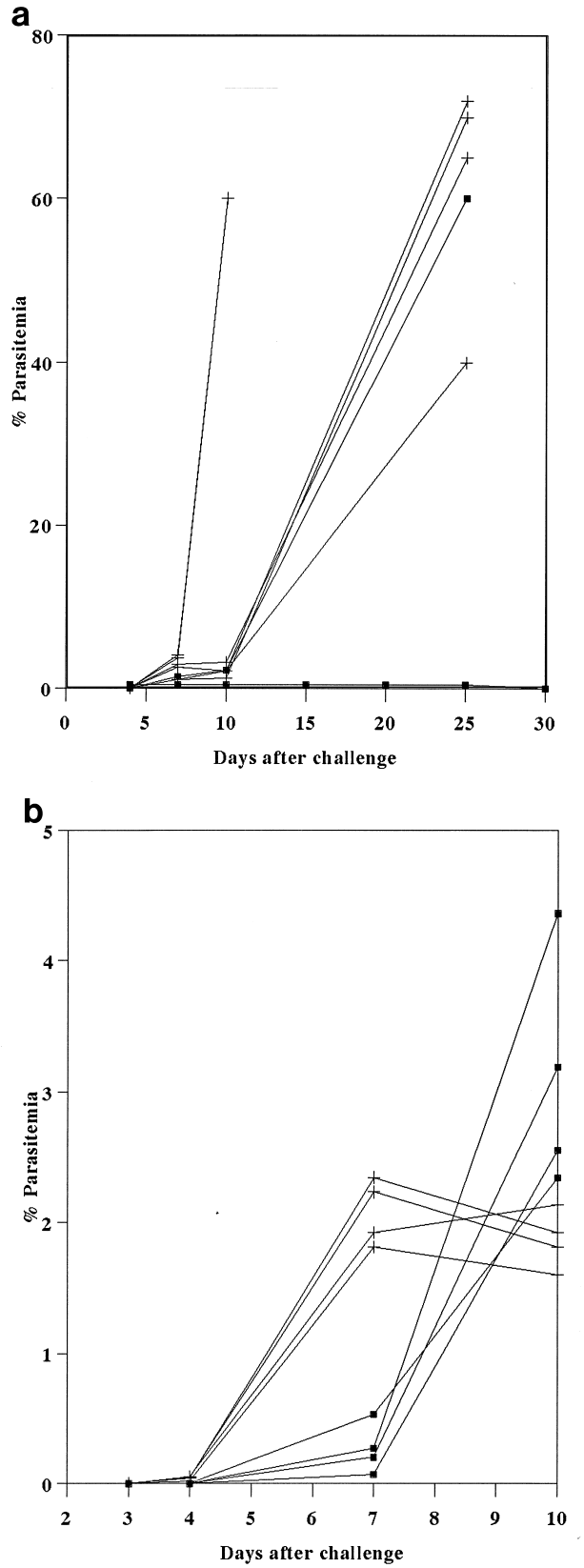


FIG. 4. Protective immunity in mice immunized with P32. Immunized mice were challenged with *P. berghei* sporozoites and monitored for blood-stage parasites (see Materials and Methods). Parasitemia is expressed as the percentage of infected erythrocytes. (a) C57BL/6 mice challenged with 100 *P. berghei* sporozoites. Symbols: +, control mice ($n = 6$); ■, immunized mice ($n = 6$). One immunized mouse developed parasitemia, while the other five did not pick up any parasitemia. (b) BALB/c mice challenged with 1,000 *P. berghei* sporozoites. Symbols: +, control mice ($n = 4$); ■, immunized mice ($n = 4$). Immunized mice showed a delayed onset of parasitemia but did not survive the challenge.

B-cell epitopic sequence. Our results show that in C57BL/6 mice, P32 is capable of stimulating memory T cells on priming in vivo and that the memory T cells can be expanded upon in vitro stimulation. The presence of a functional T-cell epitope in P32 was further supported by the fact that immunization with P32 in C57BL/6 mice produced an anamnestic antibody response without the use of a carrier protein. Moreover, as observed in the case of 3D11, a monoclonal antibody to a *P. berghei* repeat sequence, the anti-P32 serum was effective in inhibiting the *P. berghei* sporozoite invasion into Hep-G2 cells. These findings support the suggestion that a conserved motif sequence in region II is crucial for sporozoite invasion of hepatocytes (2, 17). Cerami et al. have shown that the immunoglobulin G fraction of antiserum raised against a region II peptide, at concentrations of 700 μg/ml, inhibited sporozoite invasion of Hep-G2 cells, although only one antiserum from several hyperimmunized rabbits showed weak reactivity with sporozoites (2). Our studies have provided somewhat similar results since anti-P32 serum antibodies show weak reactivity to sporozoites, yet the polyclonal antibodies even at dilutions of 1/100 significantly inhibit sporozoite invasion of Hep-G2 cells.

Perhaps the most significant finding of the present study was that C57BL/6 mice immunized with a *P. falciparum*-based peptide, P32, were protected against a lethal challenge with 100 *P. berghei* sporozoites. Considering the high susceptibility of C57BL/6 mice to *P. berghei* sporozoites, we find the protective ability of P32 to be significant. Our results are in agreement with the finding of Sina et al., who observed that immunization with *P. falciparum* sporozoites induced protective immune responses against *P. berghei* sporozoite infection (24). These workers showed that the observed cross-protection was mediated, at least in part, by antisporozoite antibody. However, since immunization with the recombinant *P. falciparum* CS protein repeat vaccine candidate $R_{32}$ $tet_{32}$ did not provide protection against *P. berghei* sporozoite infection, it was suggested that antibody-mediated protection may not be due to cross-reaction between *P. falciparum* and *P. berghei* CS proteins (24). Our results suggest that antibody responses against conserved, nonrepeat regions of the CS protein such as region II not only are cross-reactive but also may be more relevant for protection against sporozoite infection. This would be in agreement with the recent findings that it is the conserved region II sequence rather than the repeats in the CS protein which is crucially involved in the invasion of hepatocytes (2, 17).

A different pattern of protection against sporozoite challenge was observed in the BALB/c strain of mice. Because of the innate resistance of BALB/c mice to infection with *P. berghei* sporozoites (14), the challenge dose to immunized mice was raised to 1,000 *P. berghei* sporozoites. We found that in this strain, while significant delay in the onset of blood-stage parasitemia was observed in all of the immunized mice, none survived the challenge. Since immunization with P32 also produced a high antipeptide antibody response in BALB/c mice (data not shown), we are not clear about the reasons for the altered nature of protection in this strain of mice. It may be that the peptide-specific antibodies are not able to effectively inhibit the invasion of the relatively large number of sporozoites (1,000 compared with 100 in the case of C57BL/6 mice) into BALB/c hepatocytes. It is also possible that besides anti-

bodies, which do play a role in protection against sporozoite challenge, there may be other parasite- and host-specific factors which contribute to protection. For example, it is known that in the *P. yoelii* murine malaria model, the ability to mount a protective immune response against sporozoites is limited to a relatively small number of strains. Moreover, within the protected strains, the effector mechanisms of protection differ (26). Similarly, in *P. berghei* infection, the parasite-host responses are known to be different in different strains of mice. In this regard, the course of *P. berghei* infection in BALB/c mice is not well understood (23). According to a recent study, this strain, although commonly used, may not be a suitable model to study the immunology of *P. berghei* liver-stage parasites. Thus, we did not pursue any studies with this strain.

Although vaccination with irradiated sporozoites protects animals against malaria infection, immunizations in animals with recombinant malaria antigens have not always provided the expected protection. A high degree of polymorphism among these antigens and immunodominance of sequences over the ones which may provide protective immunity are considered to be some of the reasons for this observation (8, 18). Synthetic peptides may allow immune responses to be focused on the regions which may not be easily accessible to the immune system during immunization with recombinant antigens. Peptides like P32, which represent highly conserved regions of vaccine target antigens and are immunogenic without the use of a carrier protein, may be useful as one of the components of a malaria vaccine.

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