

## CYTOTOXIC T CELLS RECOGNIZE A PEPTIDE FROM THE CIRCUMSPOROZOITE PROTEIN ON MALARIA-INFECTED HEPATOCYTES

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When a person is infected with malaria by the bite of a mosquito, sporozoites travel through the bloodstream and enter hepatocytes. The parasite develops within the liver cell for days to weeks, and during this period there are no symptoms. Only after the liver stage parasites mature, and merozoites are released to invade red blood cells, do the fevers, chills and lethal complications of malaria begin. Thus, a vaccine aimed at sporozoites or liver stage parasites could eliminate malaria infections before clinical disease appears.

Immunization with radiation-attenuated sporozoites can protect rodents and humans against live sporozoite challenge (1, 2). One hypothesis is that this protective immunity is mediated by cytotoxic T cells directed against malaria antigens on the infected hepatocyte. Several recently published experiments give support to this idea. Immune animals became susceptible to sporozoite infection if they were depleted of their CD8<sup>+</sup> T cells (3, 4), but the antigens recognized by these cells and their site and mode of action were not determined. Infiltrates with CD8<sup>+</sup> and CD4<sup>+</sup> T cells and other cellular elements were found in the livers of immune animals after challenge with sporozoites and these infiltrates formed after the transfer of immune CD8<sup>+</sup> cells (5). Unfractionated spleen cells from immune animals eliminated malaria-infected hepatocytes from in vitro culture (5), but the active cell types and the targeted parasite antigens were not identified. A very recent study showed that mice were protected by adoptive transfer of cloned CD8<sup>+</sup> T cells specific for an epitope on the *Plasmodium berghei* circumsporozoite (CS)<sup>1</sup> protein (6). However, it was not shown that these cells were attacking infected hepatocytes directly, or releasing

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<sup>1</sup> Abbreviations used in this paper: CS, circumsporozoite; RCAS, rat Con A culture supernatant.

systemic mediators. We now show that a small peptide fragment of the *Plasmodium yoelii* CS protein is a target of immune CD8<sup>+</sup> cytotoxic T cells, and that these T cells can kill cultured liver stage parasites in an antigen-specific and MHC-restricted manner.

### Materials and Methods

**Mice.** 6-wk-old BALB/cByJ and B10.D2 female mice were purchased from The Jackson Laboratories, Bar Harbor, ME. BALB.K mice were obtained from Biocon, Rockville, MD.

**Parasites.** Clone 1.1 of *P. yoelii* 17X (NL) was grown in *Anopheles stephensi* mosquitos and harvested as previously described (4). For some control experiments *P. berghei* ANKA clone sporozoites were similarly grown and harvested.

**Immunization Protocol.** Dissected sporozoites received 12,000 Rad of  $\gamma$  radiation from a Cesium-137 source. Mice were given sporozoite immunizations through the tail vein at 2–4-wk intervals. Either of two immunization schedules was used: two doses of 60,000 and 50,000 sporozoites, or three doses of 75,000, 25,000, and 25,000 sporozoites. BALB/c mice were protected against sporozoite challenge by either protocol.

**Cell Culture.** Splens were removed from animals 2–12 wk after their last sporozoite immunization.  $5 \times 10^6$  spleen cells were aliquoted into 2 ml of medium in wells of a 24-well tissue culture plate. Medium was RPMI 1640 supplemented with 10% FCS, L-glutamine, 50 U/ml each of penicillin and streptomycin, and 2-ME at  $5 \times 10^{-5}$  M. Peptide antigens were added at 5  $\mu$ M concentrations, and cultures were incubated for 2 d at 37°C, 5% CO<sub>2</sub>. At that time, 0.2 ml of rat Con A culture supernatant (RCAS) (Collaborative Research Inc., Bedford, MA) was added to each well, and cultures were reincubated for an additional 5 d.

**Peptides.** Using the method of Houghten (7), peptides were synthesized corresponding to amino acid sequences from the *P. yoelii* CS protein (8). Peptides were not desalted before use and were not toxic to target cells or cell cultures at concentrations below 60  $\mu$ M. They were checked for purity by HPLC analysis. The 26 peptides used for initial screening spanned the entire protein, each peptide being 20 amino acids long and overlapping its neighbors by 10 amino acids (Fig. 1). For finer analysis of the CTL epitope, a nested set of peptides was synthesized by progressively shortening the amino acid sequence 280–296 at both NH<sub>2</sub> and COOH ends.

**Monoclonal Antibodies.** Anti-CD8 mAb 2.43 (9) was used for in vitro complement lysis. For detection of liver stage schizonts, mAbs NYLS1 and NBS1 were developed and kindly provided by Dr. Yupin Charoenvit (Naval Medical Research Institute, Bethesda, MD).

**Target Cells.** Mouse tumor cell lines P815 (H-2d) and EL4 (H-2b) were purchased from American Type Culture Collection, Rockville, MD. 18 h before use as targets, 10<sup>6</sup> cells were placed in 2 ml of medium in single wells of a 24-well tissue culture plate. To this was added 0.1 mCi of <sup>51</sup>Cr as a sterile sodium-chromate solution (Dupont-New England Nuclear, Inc., Boston, MA) and synthetic peptide at the desired concentration. These cells were incubated overnight at 37°C, 5% CO<sub>2</sub>. In the morning they were washed twice, recultured in 1 ml of medium for 1 h at 37°C, washed twice again, and counted for use as target cells. For some experiments, peptide was not added the night before, but only during the chromium-release assay.

**Chromium-release Assay.** 5,000 target cells were placed in well of a 96-well U-bottomed plate. Effector cells and synthetic peptide were added at the desired concentrations. For lysis of CD8<sup>+</sup> cells, effector cells were incubated for 1 h at 37°C with mAb 2.43 and rabbit complement (Accurate Scientific, Westbury, NY). Normal rat serum Ig and complement were used as control. Total volume was 0.2 ml, and experimental wells were reproduced in triplicate. Plates were centrifuged at 500 g for 2 min, and incubated for 6 h at 37°C, 5% CO<sub>2</sub>. At that time supernatants were harvested using the Skatron SCS system (Skatron, Inc., Sterling, VA), and released <sup>51</sup>Cr was detected by scintillation counting. Maximum release was determined by lysing target cells with 10% SDS. Spontaneous release of <sup>51</sup>Cr was ~20% of maximum release. Calculation of percent specific lysis = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. Calculation of percent peptide

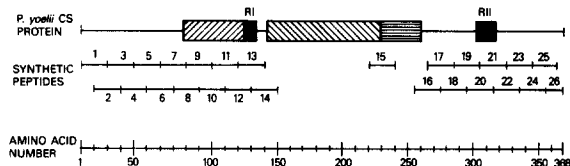


FIGURE 1. The structure of the *P. yoelii* CS protein is shown on the top line (8). It is 368 amino acids in length, with three regions of repeating sequences shown by the three types of hatching. RI and RII show the constant regions found in the CS protein of all *Plasmodia*. The middle lines show the locations of the 26 synthetic peptides.

specific lysis = (percent specific lysis of targets with peptide) – (percent specific lysis of targets without peptide).

**In Vitro Killing of Plasmodium-infected Hepatocytes.** Mouse hepatocytes were prepared using enzymatic perfusion (10, 11). The hepatocyte preparations used in screening cultures were depleted of Kupffer cells and macrophages by differential centrifugation and by cultivation in medium containing  $7 \times 10^{-5}$  M hydrocortisone hemisuccinate.  $10^5$  hepatocytes were seeded in eight-chamber plastic Lab-tek slides (Miles Research, Elkhart, IN) in MEM supplemented with 10% FCS and allowed to attach overnight at 37°C, 5% CO<sub>2</sub>. After removal of medium from the culture chambers,  $5 \times 10^4$  sporozoites were added in 50  $\mu$ l of fresh medium. 3 h after inoculation, medium containing the suspended sporozoites was removed and replaced by 300  $\mu$ l of fresh medium, and cultures were incubated for another 24 h. Medium was then replaced by medium containing the required number of cultured lymphocytes and incubated overnight. Experimental wells were repeated in triplicate. Cultures were then examined by an immunofluorescence assay to determine the numbers of sporozoites developing into schizonts (12). *P. yoelii* schizonts were detected using mAb NYLS1 specific for liver stage schizonts, and *P. berghei* schizonts were detected using mAb NBS1 specific for *P. berghei* CS protein. Inhibition was calculated by a pairwise comparison of killing by identically cultured spleen cells from sporozoite immunized and normal mice. Percent inhibition =  $[1 - (\text{number of schizonts with lymphocytes cultured from immunized mice} / \text{number of schizonts with lymphocytes cultured from normal mice})] \times 100$ .

## Results

To look for CTL epitopes on the *P. yoelii* CS protein, we synthesized 26 peptides that spanned the molecule (Fig. 1). We pooled these peptides into five groups, (1-6, 7-11, 12-16, 17-21, 22-26) and cultured spleen cells from sporozoite-immunized mice with these pools and RCAS as a source of IL-2. After 7 d viable cells from these cultures were used as effector cells in an in vitro cytotoxicity assay. The target cells were <sup>51</sup>Cr-labeled syngeneic tumor cells pulsed with a single 1 of the 26 peptides. Spleen cells that had been cultured with each peptide pool were then added to targets pulsed with individual peptides from that pool. Peptide 19 (281–300), and to a lesser degree peptide 18 (271–290), could sensitize target cells for lysis (Fig. 2).

When immune spleen cells from BALB/c mice were cultured with peptide 18 alone, no CTL activity could be seen (data not shown). However cultures with peptide 19 as stimulating antigen produced CTL activity (Fig. 3). Both peptide 19 and RCAS were necessary to generate this activity, which was MHC restricted, as peptide-pulsed EL-4 cells (H-2b) were not lysed (Fig. 3, Exp. 1). Another strain of mice also carrying the H-2d alleles, B10.D2, also made CTL against peptide 19 when immunized with *P. yoelii* sporozoites (data not shown). Spleen cells from unimmunized BALB/c mice would not produce CTL when cultured with peptide 19 (Fig. 3, Exp. 2). Neither would peptide 19 stimulate CTL from mice immunized with *P. berghei* sporozoites

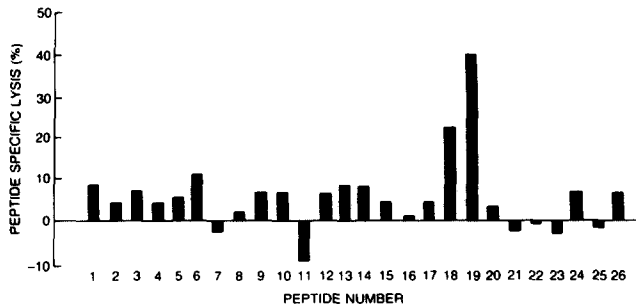


FIGURE 2. Screening peptides for CTL activity. Spleen cells from sporozoite-immunized BALB/c mice were cultured with groups of synthetic peptides (1-6, 7-11, 12-16, 17-21, or 22-26) and used as effector cells in a 6-h  $^{51}\text{Cr}$ -release assay. Targets were P815 cells pulsed overnight with a single one of the synthetic peptides at a  $5\ \mu\text{M}$  concentration. E/T ratios were 40:1.

(Fig. 3, Exp. 2). Therefore, the CTL do not represent an *in vitro* primary response to peptide. Interestingly, the homologous region to peptide 19 in *P. berghei* (13) contains two amino acid changes (Fig. 4), and a syngeneic peptide with these two substitutions could not label target cells for lysis by CTL reactive with peptide 19 (data not shown). Lysis of target cells was mediated by  $\text{CD8}^+$  T cells as pretreatment of cell cultures with anti- $\text{CD8}$  mAb and complement eliminated CTL activity (Fig. 3, Exp. 3). Thus, the CTL activity was from  $\text{CD8}^+$  cells that were MHC restricted, and were highly specific to *P. yoelii*.

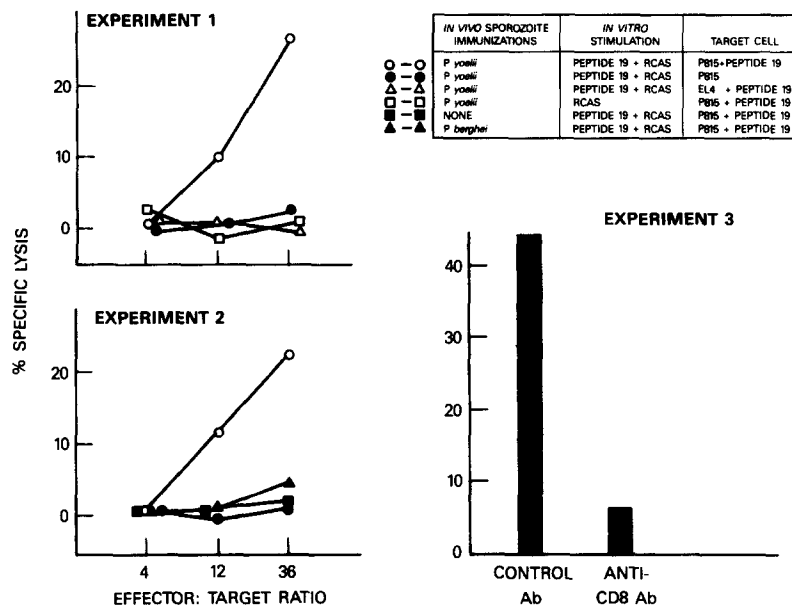


FIGURE 3. The specificity of reaction with peptide 19. In Exp. 1, spleen cells from immunized BALB/c mice were cultured with or without the addition of peptide 19 at  $5\ \mu\text{M}$  concentration. Target cells were either P815 cells without peptide, P815 cells pulsed with  $5\ \mu\text{M}$  peptide 19, or EL-4 (H-2b) mouse tumor cells pulsed with  $5\ \mu\text{M}$  peptide 19. In Exp. 2, spleen cells from normal mice, and mice immunized with *P. yoelii* or *P. berghei* sporozoites, were cultured with peptide 19 at  $5\ \mu\text{M}$  concentration. Target cells were P815 cells with or without pulsing with peptide 19. In Exp. 3, spleen cells from immunized mice were cultured with peptide 19 and treated with anti- $\text{CD8}$  mAb and complement before addition to target cells at an E/T ratio of 40:1. Controls were treated with normal rat serum Ig and complement.

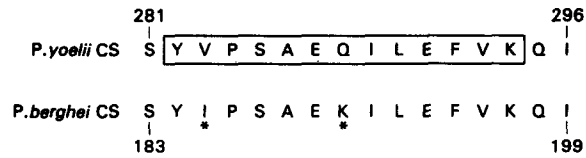


FIGURE 4. The upper line shows the sequence from amino acid 281-296 of the *P. yoelii* CS gene (8), which we have called PYCTL1. The second line shows the *P. berghei* CS amino acid sequence (13) with the two differences indicated by asterisks. The box contains the core sequence of PYCTL1 defined by experiments in Fig. 5.

To further define this CTL epitope, we synthesized a nested set of peptides by deleting single amino acids from the NH<sub>2</sub> and COOH ends of the *P. yoelii* CS 280-300 sequence. Immune spleen cells cultured with peptide 19 (281-300) were then tested against tumor targets pulsed with the nested peptides at various concentrations (Fig. 5). Shortening or lengthening by a single NH<sub>2</sub>-terminal amino acid reduced target cell labeling, and shortening by two amino acids abolished all lysis. At the COOH terminus, truncated peptides had full activity until the isoleucine at position 296 was removed. Complete loss of target sensitization occurred with

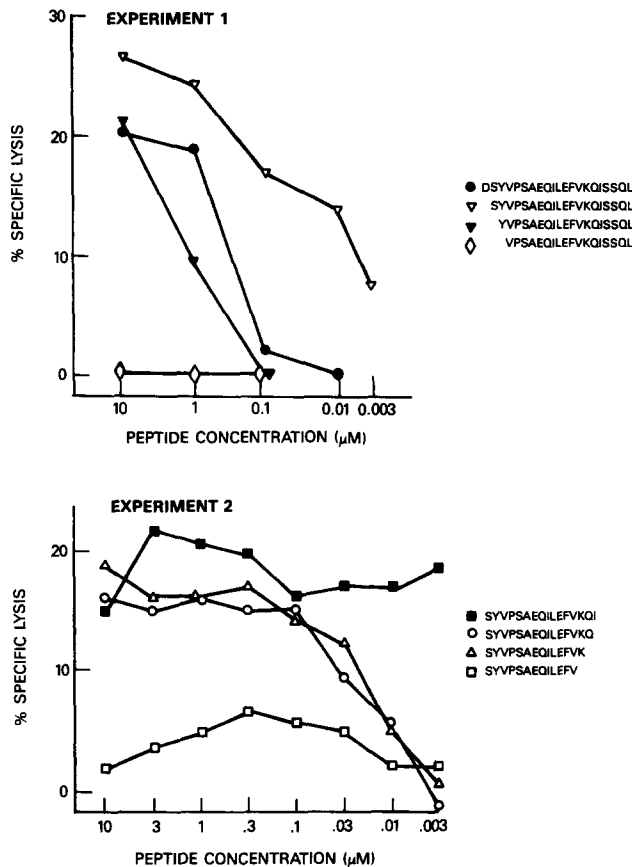


FIGURE 5. Fine mapping of the CTL epitope of peptide 19. Spleen cells from sporozoite immunized BALB/c mice were cultured with peptide 19 (amino acids 281-300) and then tested in a CTL assay against targets in the presence of each of the nested peptides at several concentrations.

deletion of the lysine at position 294. Thus a 13-amino acid core seems to be essential for sensitization but optimal activity requires a peptide containing one additional NH<sub>2</sub>-terminal, and two additional COOH-terminal residues (Fig. 4). We have named this 16-amino acid sequence PYCTL1.

Although infected hepatocytes can be killed by immune T cells (5), no malaria antigens, including the CS protein, had been previously identified on the surface of infected liver cells. We wished to know if PYCTL1 was a target antigen on malaria-infected hepatocytes. Mouse hepatocytes were cultured in monolayers and infected with *P. yoelii* sporozoites (5, 10–12). 24 h later spleen cell cultures from sporozoite-immunized or normal mice stimulated with PYCTL1 or a control (peptide 15) were added and allowed to remain overnight. The hepatocyte cultures were then fixed and stained, and the number of liver stage malaria parasites was counted. Spleen cell cultures stimulated with PYCTL1 substantially decreased the number of liver stage parasites present in the cultures (Fig. 6). This effect was not seen when control peptide was used to stimulate cultures of immune spleen cells. Antiparasitic activity was MHC restricted, as parasites grown in BALB.K liver cells were not affected (Fig. 6). The effector cells were also antigen specific, as *P. berghei* liver stage parasites were not killed.

### Discussion

These data demonstrate that PYCTL1, a 16-amino acid fragment from the *P. yoelii* CS protein, is a biologically significant epitope recognized by CTL on infected liver cells. Immunization with irradiated sporozoites induces CD8<sup>+</sup>, MHC-restricted CTL to PYCTL1, and specific, MHC-restricted CTL to this peptide eliminate parasites from hepatocyte culture. PYCTL1 is thus the first known malaria antigen presented on the surface of infected hepatocytes. It may derive from CS protein sloughed into the liver cell cytoplasm during sporozoite invasion (14–16), or represent material passing through the parasitophorous vacuole. In either case, our data show that PYCTL1 is presented with class I molecules on the hepatocyte surface.

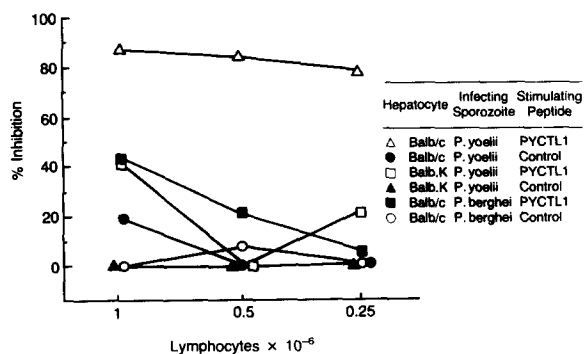


FIGURE 6. Inhibition of parasite growth in hepatocytes. Hepatocytes from BALB/c (H-2d) or BALB.K (H-2k) mice were cultured in monolayers, infected with *P. yoelii* or *P. berghei* ANKA sporozoites, and incubated for 24 h. At that time, spleen cells from normal BALB/c or *P. yoelii* sporozoite immunized BALB/c mice cultured with either PYCTL1 or peptide 15 (control) were added to the infected hepatocytes at several concentrations. 24 h later the hepatocyte monolayers were fixed and stained, and the numbers of liver stage parasites was counted. Numbers of parasites in cultures with immune and normal spleen cells were compared, and percent inhibition was calculated. Negative inhibition is marked as zero.

PYCTL1 appears to be the only class I-restricted CTL epitope on the *P. yoelii* CS protein recognized by sporozoite-immunized H-2d mice. We have mapped a 13-amino acid minimal core required for sensitization of targets for lysis, and 3 adjacent amino acids that enhance activity to maximal levels. In our initial screening experiments, a 20-amino acid peptide (281–300) containing this epitope was the most effective of all peptides at sensitizing targets for lysis. This peptide containing PYCTL1 was able to stimulate immune cells to become CTL in vitro, as well as to sensitize targets for lysis by these cells. The neighboring peptide (271–290), containing 10 of the 13 core amino acids of PYCTL1, had some reduced ability to label targets in some experiments, but was unable to stimulate immune cells to have cytotoxic activity. Therefore, we do not believe that this activity in peptide 271–290 of the *P. yoelii* CS represents a separate epitope in BALB/c mice.

Synthetic peptides provided an easy means of defining this CTL epitope. There are two complementary strategies for using peptides to find T cell epitopes. The first is to synthesize peptides chosen by predictive algorithms (17, 18). PYCTL1 was predicted to fold as an amphipathic  $\alpha$ -helix, and was a T epitope candidate on that basis. The second strategy is to empirically produce peptides that are screened for reactivity with T cells. Typically, it is not practical to synthesize all possible peptides, and overlapping peptides are used. This works well if T cell epitopes are as short as eight or nine amino acids, but screening is difficult for longer T cell epitopes. Our series of overlapping peptides, 20 amino acids long overlapping by 10, reproduces all possible sequences of 11 amino acids or shorter. However, we are missing 10% of all possible 12-amino acid sequences, and 20% of all 13-amino acid sequences. Our core sequence of PYCTL1 has 13 amino acids, and 16 amino acids are necessary for optimal activity. By these calculations there is a small chance that another long H-2d-restricted CTL epitope might exist on the *P. yoelii* CS protein, but this seems unlikely as most T cell epitopes are shorter than 13 amino acids.

PYCTL1 is distinct from, but overlaps by nine amino acids, the recently described CTL epitope 249–260 on the *P. berghei* CS protein (NDDSYIPSAEKI) (6). Four of our experiments illustrate their differences. First, the *P. berghei* peptide corresponding to PYCTL1 (Fig. 4) cannot sensitize targets for killing by CTL to PYCTL1. Second, epitope mapping experiments using nested peptides (Fig. 5) show that the four COOH-terminal amino acids of PYCTL1 are required for activity. These four are absent from the *P. berghei* epitope. Also, addition of NH<sub>2</sub>-terminal amino acids to PYCTL1 decreases its ability to sensitize targets, although lengthening increases its overlap with the *P. berghei* epitope. Third, spleen cells from BALB/c mice immunized with *P. berghei* sporozoites could not be stimulated to make CTL to PYCTL1. Lastly, *P. berghei* infected hepatocytes were not killed by PYCTL1 reactive cultures that did kill *P. yoelii* liver stage parasites. We conclude that *P. yoelii* and *P. berghei* contain different epitopes mapping to a small region of the CS molecule.

This region, adjacent to the NH<sub>2</sub> terminal of constant region II, may be a center for T cell reactivity on all CS proteins. It is the site of TH2R, a dominant CD4 T cell epitope on the *P. falciparum* CS protein (19). In *P. yoelii*, *P. berghei*, and *P. falciparum* this region has the amino acid sequence consistent with folding as an amphipathic  $\alpha$ -helix (17). This may reflect a structural requirement for the normal function of the CS protein on sporozoites, which incidently makes it a conspicuous target antigen for T cells. In *P. falciparum*, naturally occurring variation at the Th2R site

shows the importance of selective pressure directed at this region (20). Interestingly, these variant sequences of TH2R can still be recognized by T cells of different specificity (21). The parasite appears unable to modify this part of the protein sufficiently to destroy the structure recognized by T cells.

A vaccine that induced CTL to the CS protein might be expected to protect mice against malaria by killing infected liver cells. However, there may be many obstacles to this approach. Although CTL to PYCTL1 are induced by irradiated sporozoites and are active against infected hepatocytes, it is quite possible that other non-CS proteins in *P. yoelii* have CTL epitopes that are more prominent in the protective immune response. We cannot be sure from these studies whether PYCTL1 is a major player in immunity to sporozoite challenge. We have found that although both BALB/c (H-2d) and B10.D2 (H-2d) mice make CTL against PYCTL1 when immunized with *P. yoelii* sporozoites, only the BALB/c mice are solidly protected against sporozoite challenge (22). Also, both recombinant *Salmonella* and pseudorabies virus containing the *P. yoelii* CS gene, successfully induced CTL to PYCTL1 in BALB/c mice but the animals were not protected against infection by sporozoites (Flynn, J., W. R. Weiss, K. A. Norris, H. S. Seifert, S. Kumar, and M. So, manuscript in preparation). The CS protein may be a more important antigen in *P. berghei*. Recombinant *Salmonella* containing the *P. berghei* CS can partially protect mice against *P. berghei* sporozoites (23). As mentioned previously, CTL clones to the *P. berghei* CS can also protect mice (6). We conclude that the CS protein is a more significant antigen quantitatively or qualitatively in some malarias than in others. What determines this remains unknown, as does its role in immunity to the human malarias.

Other difficulties cloud the path toward CTL-based vaccines against malaria. We have found only a single CTL epitope recognized by H-2d mice on the *P. yoelii* CS protein. Similarly, H-2d mice recognize only one CTL epitope on the *P. berghei* CS protein (6). The *P. falciparum* CS protein contains a single CTL epitope seen by H-2k mice and no CTL epitopes seen by H-2d mice (24). This paucity of CTL epitopes has also been seen when other proteins have been analyzed (25–27), but it is not understood why they are so much less frequent than T helper epitopes. An ideal CTL vaccine would contain multiple parasite CTL epitopes, which will probably need to be drawn from many parasite antigens which are at this point undefined. This multiplicity would circumvent the lack of presentation by certain MHC alleles, and parasite variation of T cell epitopes (20). Immunization with *P. berghei*-irradiated sporozoites protects all strains of mice, indicating that many such antigens may exist (28). However, in the case of *P. yoelii*, even immunization with the whole irradiated sporozoite only protects a few strains of congenic mice (22). If the human malarias behave as *P. yoelii* instead of *P. berghei*, any vaccine may have to do better than the whole radiation-attenuated parasite to overcome genetic low responsiveness to malaria antigens.

Some answers to these problems are on the horizon. T helper epitopes have been found that are recognized by a large number of MHC alleles (29), and it is possible that widely reactive CTL epitopes may exist as well. In addition, there is much work currently aimed at defining other target antigens on infected liver cells besides the CS protein (30, 31). It is our hope that the addition of these factors to T epitopes from the CS protein will result in a vaccine that will overcome the limitations of genetic control and parasite variation.



### Summary

Irradiated malaria sporozoites can induce CD8<sup>+</sup> T cells that are required for protection against infection. However, the parasite antigens targeted by this immune response are unknown. We have discovered a 16-amino acid epitope from the *Plasmodium yoelii* circumsporozoite (CS) protein that is recognized by cytotoxic T cells from immune mice. Lymphocytes stimulated with this peptide can kill *P. yoelii* liver stage parasites in vitro in an MHC-restricted, antigen-specific manner. Thus, epitopes from the CS protein are presented on the surface of infected hepatocytes and can be targets for T cells, even though intact CS protein has not been detected on the surface of the infected hepatocyte. A vaccine that induced CTL to parasite antigens might protect humans against malaria by eliminating liver stage parasites.

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