Irradiated Sporozoite Vaccine Induces HLA-B8-restricted Cytotoxic T Lymphocyte Responses against Two Overlapping Epitopes of the Plasmodium falciparum Sporozoite Surface Protein 2

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Summary

Vaccines designed to protect against malaria by inducing CD8⁺ cytotoxic T lymphocytes (CTL) in individuals of diverse HLA backgrounds must contain multiple conserved epitopes from various preerythrocytic-stage antigens. Plasmodium falciparum sporozoite surface protein 2 (PfSSP2) is considered an important antigen for inclusion in such vaccines, because CD8⁺ CTL against the P. yoelii SSP2 protect mice against malaria by eliminating infected hepatocytes. To develop PfSSP2 as a component of malaria vaccines, we investigated the presence of anti-PfSSP2 CTL in two HLA-B8⁺ volunteers immunized with irradiated P. falciparum sporozoites and characterized their CTL responses using PfSSP2-derived 15-amino acid peptides bearing the HLA-B8-binding motif. Peripheral blood mononuclear cells from both volunteers stimulated with recombinant vaccinia expressing PfSSP2 displayed antigen-specific, genetically restricted, CD8⁺ T cell-dependent CTL activity against autologous target cells expressing PfSSP2. Of the five HLA-B8 motif-bearing 15-mers identified in the PfSSP2 sequence, two peptides sharing a 10-amino acid overlap sensitized HLA-B8-matched target cells from both volunteers for lysis by peptide-stimulated effectors. The CTL activity was HLA-B8 restricted and dependent on CD8⁺ T cells. Analysis of the three shorter peptides representing HLA-B8 motif-bearing sequences within the two positive peptides for their ability to bind to HLA-B8 in vitro, and to sensitize target cells for lysis by effectors stimulated with the 15-mers, identified two overlapping HLA-B8-restricted CTL epitopes. Available data indicate that the sequence of one CTL epitope is conserved and the other is variant among P. falciparum isolates. Circulating activated CTL against the conserved epitope could be directly identified in one of the two volunteers. The identification of two HLA-B8-restricted CTL epitopes on PfSSP2 provides data critical to developing an epitope-based anti-liver stage malaria vaccine.

There are an estimated 300–500 million clinical cases of malaria, and 1–2 million deaths are caused by malaria each year (1). One approach to malaria vaccine development is to induce CD8⁺ CTL that recognize parasite-derived peptides complexed with class I MHC molecules on the surface of Plasmodium sp.-infected hepatocytes (2). This work is primarily based on the observations that immunization of mice (3), monkeys (4), and humans (5–10) with the respective Plasmodium sp.-irradiated sporozoites (irr spz)¹ protects against malaria, that this immunity is directed against infected hepatocytes (11), and that such protection

¹Abbreviations used in this paper: β₂m, β₂-microglobulin; B-LCL, lymphoblastoid B cell lines; irr spz, irradiated sporozoites; OM, opti-MEM I; PfSSP2, P. falciparum SSP2; SSP2, sporozoite surface protein 2, TCM, T cell medium.
is abrogated in some strains of mice by in vivo depletion of CD8+ T cells (12, 13).

Two preerythrocytic-stage *Plasmodium* sp. proteins, the circumsporozoite protein (14) and sporozoite surface protein 2 (SSP2) (15), also known as TRAP (16), have been definitively identified as targets of this CD8+ T cell–dependent protection, because CD8+ T cell clones against both proteins adoptively transfer protection against rodent malaria (17–20). It is our view that optimal development of human malaria vaccines designed to induce protective CTL will require identification of CTL epitopes on these and other preerythrocytic-stage antigens, and definition of the class I HLA molecules restricting such determinants. A peptide representing *P. falciparum* circumsporozoite protein residues 368–390 shown first to include an H-2K-restricted CD8+ CTL epitope (21) was later shown to contain CD8+ CTL epitopes recognized by humans immunized with irradiated *Anopheles stephensi* mosquitoes infected with either the NF54 strain (33) or 3D7 clone (34) of *P. falciparum*. Over a period of 23 mo, volunteers 16 and 17 were exposed to 1,415 and 1,398 infectious bites, in 11 and 10 sessions, respectively (Table 1).

**Materials and Methods**

**Human Volunteers and Immunization.** Two HLA-B8+ individuals, volunteers 16 and 17, were immunized with *P. falciparum* irradiated spz as previously described (9) (Table 1). Briefly, after giving informed consent to a protocol approved by an institutional review board, the volunteers were immunized by exposure to the bites of irradiated (1.5 x 10^4 rad) *Anopheles stephensi* mosquitoes infected with either the NF54 strain (33) or 3D7 clone (34) of *P. falciparum*. Over a period of 23 mo, volunteers 16 and 17 were exposed to 1,415 and 1,398 infectious bites, in 11 and 10 sessions, respectively (Table 1).

**Synthetic Peptides.** A panel of 113 synthetic 15-mers overlapping by 10 residues and spanning the entire PfSSP2 (3D7) sequence (15) was synthesized by the solid-phase “tea-bag” method (35). For these studies, five peptides were selected based on the presence of the HLA-B8-binding motif (30–32) (Table 2). HLA-B8 motif-bearing sequences within selected 15-mers contain eight to nine amino acid segments with a Lys (K) or an Arg (R) as dominant anchor residues at positions 3 (P3) and 5 (P5), and Leu (L), Ile (I), or the hydrophobic residue Val (V) as auxiliary anchor residues at positions 8 or 9 (COOH terminus). Six shorter peptides corresponding to HLA-B8 motif-bearing sequences (Table 2) were synthesized using solid-phase Fmoc chemistry. PfSSP2 peptide 99 (residues 491–505; REEHEKPDNNKKKAG), the HLA-B8–restricted influenza type A nucleoprotein peptide NP 380–388 (ELRSRYWAI) (30, 32), the HLA-A2.1–restricted hepatitis B core peptide HBcAg 18–27 (FLPSDYFPSV) (36), and the breakpoint region protein peptide BCR 1303–1311 (LT1NKEDDE) (37) were used as control peptides. Purity of peptides was assayed by reverse-phase HPLC, and the structure of shorter peptides was verified by mass spectrometric analysis. Lyophilized peptides were dissolved in DMSO, and stock solutions were stored at −20°C. Before use, peptides were diluted with RPMI 1640. Peptides were not toxic to cell cultures or target cells.

**HLA-B8–binding Assay.** rHLA-B8 H chains (residues 1–278) were prepared from *Escherichia coli* inclusion bodies as described (31, 38). HLA complexes were reconstituted with 25 µg of isolated rHLA-B8 H chains dissolved in 2.5 µl of 8.8 M urea/25 mM 4-morpholineethanesulfonic acid/150 mM NaCl, pH 6.5, 1 µl of peptide dissolved in DMSO (10 mg/ml), and 5 x 10^6 cpm of ^35^S-labeled human β₂-microglobulin (BM) (sp act 1 x 10^12–1 x 10^13 cpm/mol) in a final volume of 50 µl. Reconstituted HLA complexes were separated from unincorporated BM by HPLC gel filtration. Peptide binding was measured indirectly.

**Table 1. HLA Phenotypes of Volunteers Immunized by the Bites of Irradiated Anopheles Mosquitoes Infected with P. falciparum (NF54 or 3D7) and of Control Target Cells**

| Cells     | HLA type (class I) | Immunizing doses* | Infectious bites† |
|-----------|--------------------|-------------------|-------------------|
| Volunteers|                    |                   |                   |
| 16        | A1, A24, B8, B38, Bw4, Bw6, Cw7 | 11                | 1415              |
| 17        | A1, A3, B7, B8, Bw6 | 10                | 1398              |
| Controls  |                    |                   |                   |
| WR        | A1, A28, B44, Bw57, Cw6 |                   |                   |
| RH        | A2, B62, B65, Bw6, Cw3, Cw8 |                   |                   |
| GR        | A24, A28, B7, B14, Cw7, Cw8 |                   |                   |

*Total number of exposures to the bites of irradiated infected mosquitoes.
†Total number of infectious bites from irradiated infected mosquitoes.

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plasmid pHbAnSSP2 was used for transfection of target cells. The insert was then cloned into the expression vector pH[3APr-l-neo (40) (pHbAn), which had sequence was excised from plasmid pPfSSP2.43 (15) by digestion with SacI/Klenow and BamHI. The generation of a recombinant vaccinia virus, vP 1155, expressing full-length PfSSP2 (3D7) has been previously described (26).

The PfSSP2 Synthetic Peptides with HLA-B8 Motif

| Peptide | Sequence* | Residues |
|---------|-----------|----------|
| 15-mers |           |          |
| 7       | NIVDEIKYREEVCND | 31-45    |
| 21      | IRLHSADASKNKEKAL | 101-115 |
| 22      | DASKNKEKALIJKS  | 106-120  |
| 52      | TCGKGTTSRKREILH  | 256-270  |
| 53      | TRSRKREILHEGCTS  | 261-275  |
| 8- or 9-mers | | |
| 7.1     | EIKYREEV     | 35-42    |
| 22.1    | ASKNKEKAL    | 107-115  |
| 22.2    | KNAKEKALI    | 109-117  |
| 22.3    | KNKKEKALI    | 109-116  |
| 52.1    | GTRSRKREIL   | 260-268  |
| 52.2    | RSRKREIL     | 262-269  |

*Sequence in single-letter amino acid code. Binding motif for HLA-B8: K/R at position 3 and K/R at position 5 (primary anchor residues); L/I/hydrophobic at position 8 or 9 (auxiliary anchor residues) (30-32). Primary and auxiliary anchor residues are in bold; polymorphic residues (53) are underlined.

The stability of such complexes, the t1/2 rate of dissociation of peptides from HLA complexes was measured at 37°C as previously described (39).

Construction of Recombinant Vaccinia Virus Expressing PfSSP2.

The generation of a recombinant vaccinia virus, vP1155, expressing full-length PfSSP2 (3D7) has been previously described (26).

Plasmid Construction. The PfSSP2 (3D7) genomic DNA sequence was excised from plasmid pPfSSP2.43 (15) by digestion with SacI/Klenow and BamHI. The insert was then cloned into the expression vector pH[3APr-l-neo (40) (pHbAn), which had been digested with HindIII/Klenow and BamHI. The resulting plasmid pHbAnSSP2 was used for transfection of target cells.

Cell Lines. All EBV-transformed lymphoblastoid B cell lines (B-LCL), with the exception of GR cells (kindly provided by Dr. B. D. Walker, Massachusetts General Hospital/Harvard Medical School, Boston, MA), were established from freshly isolated PBMC using culture supernatant of the B95.8 marmoset cell line (41) (Table 1). Hmy2.C1R cells (42) transfected with HLA-B8 cDNA (HLA-B8-Hmy2.C1R) (31) (kindly supplied by Dr. W. E. Bidddison, National Institutes of Health, Bethesda, MD) and all B-LCL were maintained in RPMI 1640 supplemented with 10 mM Hepes, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD), and 10% heat-inactivated FCS (JILH Biosciences, Lenexa, KS) (CR). Opti-MEM I (GIBCO BRL) (OM) was used for transient transfection of target cells.

In Vitro Stimulation of PBMC. 2-4 wk after the last exposure to irradiated infected mosquitoes, PBMC were isolated from hep-arinized venous blood or from leukopheresis runs by density gradients over lymphocyte separation medium (Organon Teknika, Durham, NC). PBMC (3 × 10^6 cells/well) were cultured in 24-well plates in a final volume of 2 ml RPMI 1640 prepared as for CR, but substituting heat-inactivated pooled normal AB+ human serum for FCS (T cell medium [TCM]). For vaccinia stimulation, one-third of the PBMC in culture were infected with vP1155 (10 PFU/cell, 5 × 10^6 cells/ml in RPMI 1640, 1 h at 37°C, 5% CO2). For peptide stimulation, PBMC were cultured in the presence of individual peptides (15 μM), and, 2 d after initiation of the culture, rhIL-2 (Cetus Corp., Emeryville, CA) was added to each well (50 U/ml). Plates were incubated for 6 d at 37°C, 5% CO2. Unstimulated PBMC that did not undergo secondary in vitro stimulation were also tested as effector cells.

Target Cells. B-LCL were transiently transfected 48 h before use as targets in the 51Cr-release assay. In a final volume of 100 μl, 10 μg of pHbAnSSP2 or pHbAn DNA was mixed with 30 μg of Lipofectin reagent (1:1 [wt/vt] liposome formulation of N-[1-(2,3-dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride and dioleoyl phosphatidylethanolamine) (GIBCO BRL), and the mixture was incubated for 20 min at room temperature. DNA-Lipofectin complexes were added to a 2-ml cell suspension (1 × 10^6 cells/well, 6-well plate) in OM, incubated overnight at 37°C, and then diluted with an equal volume of OM–20% FCS. Expression of PfSSP2 by B-LCL transfected with pHbAnSSP2 was confirmed by immunofluorescence using a PfSSP2-specific polyclonal mouse serum (15, 26). Peptide-pulsed target cells were prepared by overnight incubation of B-LCL with 15 μM of individual synthetic peptides. To determine minimal CTL epitopes, HLA-B8-Hmy2.C1R cells were pulsed with each peptide at the indicated concentrations. All target cells were labeled overnight with 100 μCi (1 Ci = 37 GBq) of a sterile Na251CrO4 solution (Dupont New England Nuclear, Boston, MA).

51Cr-Release Assay. Effector cells were washed, diluted with TCN, and placed into triplicate wells of 96-well round-bottomed plates (Costar Corp., Cambridge, MA). Triplicate wells were also plated with effector cells that had been depleted of CD4+ or CD8+ T cells (>95%, by flow cytometric analysis). Immunomagnetic depletion was performed using anti-CD4- or anti-CD8–coated immunobeads (Dynal, Inc., Great Neck, NY). Target cells were washed three times and resuspended at 5 × 10^6/ml in TCM, and 5 × 10^5 cells were added to wells containing 100 μl effector cells. Effector cells were incubated (Costar Corp., Cambridge, MA), and released 51Cr was counted in a gamma counter. Percent specific lysis was calculated from the following formula: 100 (experimental release – spontaneous release)/(maximum release – spontaneous release). Maximum and spontaneous release were determined in wells containing no effectors in the presence or absence of 5% Triton X-100, respectively. Spontaneous release values varied from 19–23% for transiently transfected targets and 11–20% for peptide-pulsed targets.

Results

Antigen-specific, Genetically Restricted, and CD8+ T Cell–dependent CTL Recognition of Endogenously Synthesized PfSSP2. To determine whether the immunization with P. falciparum irr spz induced PfSSP2-specific CTL, frozen PBMC from both HLA-B8+ volunteers were in vitro stimulated with autologous cells infected with a recombinant PfSSP2 vaccinia virus (vP1155) (26), and cytotoxic activity of effector cells was tested against HLA-matched and -mis-

Table 2. PfSSP2 Synthetic Peptides with HLA-B8 Motif

| Peptide | Sequence* | Residues |
|---------|-----------|----------|
| 7       | NIVDEIKYREEVCND | 31-45    |
| 21      | IRLHSADASKNKEKAL | 101-115 |
| 22      | DASKNKEKALIJKS  | 106-120  |
| 52      | TCGKGTTSRKREILH  | 256-270  |
| 53      | TRSRKREILHEGCTS  | 261-275  |

*Sequence in single-letter amino acid code. Binding motif for HLA-B8: K/R at position 3 and K/R at position 5 (primary anchor residues); L/I/hydrophobic at position 8 or 9 (auxiliary anchor residues) (30-32). Primary and auxiliary anchor residues are in bold; polymorphic residues (53) are underlined.

A*Numbering system refers to the PfSSP2 (3D7) sequence.
Effector cells from volunteers 16 and 17 expressed 62 and B8-restricted CTL epitopes, five PfSSP2-derived 15-mers largely dependent on CD8+ T cells. Lysis against autologous target cells transfected with infected autologous cells at a 2:1 responder/stimulator ratio. After depletion, respectively, were thawed and cocultured for 6 d with vP1155- and after 583 and 652 cumulative bites from irradiated infected mosquitoes from volunteers 16 (A) and 17 (B) obtained 2 and 3 wk after the last dose from frozen PBMC that were isolated from the same volunteer. Of CTL activity remained unchanged when the CD4+ T cell subset was depleted in both effector cell populations (E/T ratio 80:1) (Fig. 1). Using the same recombinant vaccinia/transient transfection system, effector cells obtained from frozen PBMC that were isolated from the same volunteers before the initiation of the immunization were unable to kill similarly prepared target cells (data not shown). Thus, naturally processed PfSSP2 presented by stimulator and target cells provided an efficient system to demonstrate that exposure to the irr spz vaccine induced a CTL response that was PfSSP2 specific, genetically restricted, and largely dependent on CD8+ T cells.

**CTL Responses to PfSSP2-derived Peptides Bearing HLA-B8-binding Motif.** The CD8+ T cell–dependent CTL response observed against endogenously generated PfSSP2 peptides could have been restricted by any of the HLA class I alleles expressed on the target cells of each volunteer. Therefore, to identify regions of PfSSP2 containing HLA-B8–restricted CTL epitopes, five PfSSP2-derived 15-mers (Table 2) bearing sequences conforming to the HLA-B8 allele–specific binding motif (30–32) were used to stimulate PBMC in in vitro bulk cultures. After 6 d, each peptide-stimulated effector cell population was assayed for its ability to lyse autologous target cells sensitized with the homologous peptide.

PfSSP2 peptide–specific cytotoxic activity could be demonstrated in both HLA-B8+ volunteers. The CTL activity was detected against two of the five peptides. Effector cells obtained after stimulation with overlapping peptides 21 (IRL-HSDASKNEKAL) and 22 (DASKNEKALIIIKS) displayed significant CTL activity (range of 23–28%, E/T ratio 50:1) against autologous target cells pulsed with the homologous peptide but not against autologous target cells pulsed with PfSSP2 peptide 99 (Fig. 2). Similar results were obtained in two and three additional assays from a total of six and eight experiments conducted for volunteers 16 and 17, respectively. In each experiment and for each volunteer, stimulation with peptides 52 and 53 failed to generate effector cells with cytotoxic activity. Significant peptide–specific CTL activity (volunteer 16, 27.6%; volunteer 17, 23.9%; E/T ratio 50:1), however, was detected in one experiment for PBMC stimulated with peptide 7 (NIVD-EIKYREEVCND). Additional experiments to reproduce such results proved unsuccessful. These data indicate that immunization with irr spz induces a PfSSP2 peptide–specific CTL response in both HLA-B8+ individuals. In addition, these results suggest the presence of a CTL epitope within the 10–amino acid sequence that is shared between peptides 21 and 22.

**HLA Restriction Analysis.** To determine the HLA class I restriction element responsible for the presentation of peptides 21 and 22, CTL were generated from the two HLA-B8+ volunteers by culture of PBMC with each peptide and were tested for their ability to kill peptide-pulsed allogeneic B-LCL that partially shared the HLA class I alleles present on effector cells. In the first part of the analysis, we determined whether peptide 21- and 22–stimulated PBMC from each volunteer were able to recognize the respective peptide-pulsed B-LCL target cells from both volunteers. Target cells from volunteers 16 and 17 sensitized with peptides 21 and 22 were lysed by the respective peptide-stimulated effector cells from volunteer 16 (Fig. 3A). Similarly, peptide-pulsed target cells from volunteers 17 and 16 were recognized equally well by the respective peptide-stimulated effector cells from volunteer 17 (Fig. 3B). Because, in addition to the HLA-B8 allele, volunteers 16 and 17 share the HLA-A1, HLA-Bw6 alleles (Table 1), it is possible that one, two, or all three class I alleles could have served as the restriction elements for the epitopes within peptides 21 and 22. However, vP1155-stimulated PBMC were unable to kill mismatched HLA-A1+, HLA-B8+, HLA-Bw6+ RH cells (Table 1) transfected with the PfSSP2 gene (Fig. 1). The failure of peptide 21– and 22–specific CTL from both volunteers to recognize peptide-pulsed WR or GR cells indicates that the class I alleles HLA-A1 (shared by volunteers 16 and 17 and WR cells) (Fig. 3, A and B), HLA-A24 and HLA-Cw7 (shared by volunteer 16 and GR cells) (Fig. 3, A), and HLA-B7 (shared by volunteer 17 and GR cells)
(Fig. 3 B) do not participate in the restricted presentation of these epitope-bearing peptides. These results, and the fact that the stimulating peptides contain sequences conforming to the HLA-B8-binding motif (30-32), whose features differ markedly from the motifs described for alleles HLA-A1 (31), HLA-A24 (43), HLA-B7 (44), and HLA-Cw7 (45), demonstrate that the CTL response from both volunteers to PfSSP2 peptides 21 and 22 is HLA-B8 restricted.

**PfSSP2 Peptide-specific, Genetically Restricted CTL Activity Is Dependent on CD8+ T Cells.** Peptide 22 not only contains the same HLA-B8-motif-bearing 9-amino acid sequence present in peptide 21, but also contains two additional sequences with such a motif (Table 2). To determine if the cytolytic activity was dependent on CD8+ CTL, PBMC from volunteers 16 and 17 were stimulated in vitro with peptide 22, and the activity of the effector cells was studied in depletion experiments. Only undepleted and CD4+ T cell-depleted effector cell populations from both volunteers recognized autologous target cells pulsed with peptide 22 (Fig. 4). The cytotoxic activity was dependent on CD8+ T cells because the killing was eliminated by the depletion of this T cell subset (Fig. 4). Autologous and WR target cells pulsed respectively with PfSSP2 peptides 99 and 22 were not lysed by both undepleted effector cell populations, confirming the genetically restricted and specific nature of the CTL response (Fig. 4).

**Binding to HLA-B8 and CTL Recognition of Predicted Minimal Epitopes.** Based on the foregoing results, six shorter peptide analogues representing the HLA-B8 motif-bearing sequences within the five 15-mers (Table 2) were synthesized to determine the ability of predicted epitopes to bind to HLA-B8 class I molecules in an in vitro assay that measures the peptide-dependent incorporation of 125I-β2m into HLA complexes (39). Peptides 22.1 (ASKNKEKAL), 22.2 (KNKELALII), and 22.3 (KNKEKALI), representing the putative CTL epitopes within peptides 21 and 22, formed stable HLA H chain-β2m-peptide heterotrimeric complexes. The t1/2 for dissociation of 125I-β2m from complexes made with these shorter analogues was 0.5, 0.5 and 4 h, respectively (Table 3). Although CTL responses were not reproducibly detected for peptide 7, peptide 7.1 (EIKYREEV) bound detectably to HLA-B8 with a t1/2 of 2 h (Table 3). Similarly, the octamer 52.2 (RSK.KREIL) sequence, which maps within the 10-amino acid overlap of negative peptides 52 and 53, was shown to bind to HLA-B8 with a dissociation rate of t1/2 = 1 h (Table 3). Only peptide 52.1
Table 3. Peptide Binding to HLA-B8

| Peptide | Percentage of incorporation of $^{125}$I-$\beta$m $^4$ (h at $37^\circ$C)$^8$ | $t_{1/2}$ |
|---------|-------------------------------------------------|--------|
| 7.1     | EIKYREEV                                        | 60     | 2     |
| 22.1    | ASKNKEKAL                                       | 50     | 0.5   |
| 22.2    | KNKEKALI                                        | 40     | 0.5   |
| 22.3    | KNKEKAL                                         | 50     | 4     |
| 52.1    | GTRSRKRKEI                                      | 10     | —     |
| 52.2    | RSRRKREI                                       | 50     | 1     |
| NP 380–388$^f$ | ELRSRYWAI                                       | 50     | 3     |
| BCR 1303–1311$^f$ | LTINKEDDE                                      | 7     | —     |
| No peptide |                                                  | 10     | —     |

*Sequence in single-letter amino acid code.

$^1$Percent of human $\beta$m incorporated into HLA-B8 complexes using HLA-B8 heavy chains, peptide, and $^{125}$I-$\beta$m. HLA complexes were detected by gel filtration (39). Incorporation of $^{125}$I-$\beta$m at levels $>$20% indicates specific binding.

$^2$Dissociation rate ($t_{1/2}$) of $^{125}$I-$\beta$m from reconstituted complexes measured at $37^\circ$C (38, 39). Values are accurate within a factor of two.

$^3$Positive control peptide (30, 32) (with HLA-B8-binding motif).

$^4$Negative control peptide (37) (without HLA-B8-binding motif).

Figure 4. Cytotoxic response to PfSSP2 peptide 22 is dependent on CD8$^+$ T cells. After 1,127 and 1,163 bites of irradiated infected mosquitoes during 18 mo and 2 wk after the last exposure, PBMC from volunteers 16 (A) and 17 (B) were stimulated in vitro with peptide 22 (15 $\mu$M). After 6 d of culture, effector cells were depleted of CD4$^+$ or CD8$^+$ T cells. Cytotoxic activity of depleted and undepleted effector cells was tested at an E/T ratio of 60:1 in a 6-h $^{51}$Cr-release assay against matched (autologous) and mismatched WR target cells pulsed with the same peptide (15 $\mu$M). Autologous B-LCL pulsed with peptide 99 (15 $\mu$M) were used as a negative control for peptide-specific lysis.

(GTRSRKREI), a nonamer within peptide 52, failed to form complexes in the in vitro reconstitution assay (Table 3). From this analysis, it can be concluded that the octamer and two nonamers representing the putative HLA-B8-restricted CTL epitopes within peptides 21 and 22 bind effectively to HLA-B8.

To determine whether the HLA-B8-binding peptides within overlapping peptides 21 and 22 represent the minimal CTL-defined epitopes, PBMC from volunteer 17 were stimulated in vitro with peptides 21 and 22 and were assayed for their ability to kill HLA-B8-Hmy2.C1R cells sensitized with the stimulating peptide (15-mer) and with the shorter peptides 22.1 (9-mer), 22.2 (9-mer), and 22.3 (8-mer) over a range of concentrations. Peptide 21–stimulated PBMC recognized HLA-B8-Hmy2.C1R cells pulsed with 10 $\mu$M of the homologous peptide (19%, E/T ratio 60:1) or with as little as 0.1 $\mu$M of peptide 22.1 (23%, E/T ratio 60:1), and they failed to recognize target cells pulsed with peptides 22.2 or 22.3 at any of the concentrations tested (Fig. 5 A). Effector cells generated by stimulation with peptide 22 recognized equally well HLA-B8-Hmy2.C1R cells individually pulsed with 10 $\mu$M of peptides 22, 22.1, 22.2, and 22.3 (range of 19–25%, E/T ratio 60:1). While the CTL activity against target cells pulsed with each shorter peptide was not diminished by decreasing the pulsing concentration to 1 $\mu$M, the activity against target cells pulsed with peptide 22 was sharply reduced to the cut-off value considered as positive (from 22 to 10.4%, E/T ratio 60:1). At 0.1 $\mu$M, only target cells pulsed with each of the shorter peptides remained susceptible to lysis (Fig. 5 B). Control target cells pulsed with no peptide or with peptides representing the HLA-B8–restricted epitope of influenza NP 380–388 and the HLA-A2.1–restricted epitope of HBVc 18–27 were not lysed (Fig. 5, A and B). These findings indicate that PfSSP2–derived peptides 21 and 22 contain at least two minimal HLA-B8–restricted CTL epitopes: the nonamer 22.1 (ASKNKEKAL) and the octamer 22.3 (KNKEKAL), a truncated form of the nonamer 22.2 (KNKEKAL). Because Hmy2.C1R cells express only low levels of HLA-B35 and normal levels of HLA-Cw4 (46), and none of the volunteers expressed HLA-B35 or HLA-Cw4 (Table 1), the lysis of HLA-B8–Hmy2.C1R transfectants (31) substantiates the conclusion that the observed CTL responses are HLA-B8 restricted.

Cytotoxic Activity of Circulating Activated CTL. PBMC from volunteer 16 were isolated after 1,127 bites of irradiated infected mosquitoes (2 wk after the last exposure to 145 bites), and an aliquot of the cells was directly tested at an E/T ratio of 100:1 for their ability to kill $^{51}$Cr-labeled autologous B-LCL coated with the 15-mers 21 and 22. Significant CTL activity was detected against target cells sensitized with peptide 21 (25.4%) or peptide 22 (27.9%) but not against target cells alone (4.9%) or pulsed with peptide 99 (7.2%). To determine the fine specificity of circulating activated CTL, a similar experiment was conducted using the same volunteer’s PBMC obtained after 1,415 infectious bites (2 wk after the last exposure to 122 bites) and directly cultured with $^{51}$Cr-labeled target cells coated with peptides 21 and 22 as well as with the shorter peptide analogues, 22.1, 22.2, and 22.3. At an E/T ratio of 100:1, freshly isolated PBMC lysed autologous B-LCL pulsed with peptides 21 (18.4%), 22 (19.6%), and 22.1 (24.3%), but did not lyse target cells pulsed with peptides 22.2 (1.9%) and 22.3 (5.7%). The lack of killing of target cells pulsed with HLA-B8–restricted NP 380–388 (2.6%) and HLA-A2–restricted HBVc 18–27 (1.5%) peptide epitopes indicated that the CTL activity is PfSSP2 specific.
Discussion

These studies demonstrate that immunization of HLA-B8+ volunteers with radiation-attenuated *P. falciparum* sporozoites induces antigen-specific, CD8+ T cell-dependent, HLA-B8-restricted cytotoxic activity against a nine-amino acid (ASKNKEKAL) and an eight-amino acid (KNKEKALI) PfSSP2 peptide. Furthermore, circulating activated CTL against the ASKNKEKAL peptide were detected in the peripheral blood of one of the two volunteers.

Any vaccine designed to induce CD8+ CTL for the prevention or treatment of infectious diseases and malignancies must include protective or therapeutic epitopes recognized by CTL from individuals of diverse HLA backgrounds. We consider PfSSP2 (15), also known as TRAP (16), as an important target protein for malaria vaccine development, because work in BALB/c (H-2b) mice demonstrates that adoptive transfer of CD8+ CTL against PySSP2 provides sterile immunity against highly infectious *P. yoelli* sporozoites in the absence of other parasite-derived immune responses (20). Furthermore, it has been shown that immunization of mice with recombinant mastocytoma cells expressing PySSP2 induces protective immunity that is eliminated by in vivo depletion of CD8+ T lymphocytes (47). We consider identification of HLA-B8-restricted epitopes as an important component of our overall strategy, because the HLA-B8 allele is expressed in Caucasian (18.1%), Black (6.3%), Hispanic (9.0%), and Chinese (3.6%) populations (48).

Our strategy for identifying class I HLA-restricted CTL epitopes recognized by PBMC of volunteers with specific HLA types begins with the assessment of CTL activity against endogenously produced epitopes in target cells transiently transfected with the full-length PfSSP2 gene (Fig. 1). Once a volunteer is determined to be a responder, we then proceed to identify specific epitopes. Because of the cost and time required for synthesis of peptides, the requirement for identifying multiple epitopes restricted by different class I HLA alleles, and our interest in identifying B cell and class II-restricted epitopes, we synthesized 113 overlapping 15–amino acid peptides representing the entire sequence of PfSSP2 to produce a set of reagents useful for numerous studies. PBMC from volunteers immunized with *P. falciparum* irr spp are then studied to determine if they have evidence of CTL against peptides that include sequences conforming to class I HLA–binding motifs corresponding to the alleles expressed by each individual. We next determine the ability of shorter peptides to bind to the specific HLA molecule and assess their capacity to sensitize target cells in cytotoxicity assays.

Five PfSSP2-derived 15-mers containing sequences conforming to the HLA-B8 motif were selected for these studies. CTL responses were detected in both volunteers against two peptides, 21 and 22 (Fig. 2). In each assay where peptide-specific CTL responses were detected, activity was associated with both peptides. This finding suggested that the HLA-B8 motif-bearing sequence ASKNKEKAL found within the 10–amino acid overlap of peptides 21 and 22 could represent a CTL epitope. Additional evidence for the presence of an epitope within the overlapping sequence of both peptides was provided by an experiment demonstrating the ability of effectors stimulated with peptide 21 to recognize and kill target cells pulsed with peptide 22 and vice versa (Fig. 3).

We next demonstrated that PBMC from each of the volunteers that were stimulated in vitro with peptide 21 and 22 generated CTL activity that was restricted by HLA-B8 class I molecules (Fig. 3) and mediated by CD8+ T cells (Fig. 4). HLA-B8 restriction was demonstrated using peptide-pulsed allogeneic B-LCL sharing class I HLA alleles with the effector cells of both volunteers (Fig. 3). However, in the case of volunteer 17, restriction analysis did not include B-LCL target cells sharing the HLA-A3 allele. Since PfSSP2 peptide 21 contains a 10–amino acid segment that conforms to the HLA-A3–binding motif (49) (RLHS-DASKNK) (Table 2), it is therefore theoretically possible that, in addition to the presence of an HLA-B8–restricted...
CTL epitope, peptide 21 could contain a determinant restricted by the A3 allele.

It is possible that, by studying 15-mers instead of a direct analysis of motif-bearing 8–10-amino acid peptides, we may have missed the presence of CTL responses against sequences within peptides 7, 52, and 53. The longer peptides may have inherent resistance or increased susceptibility to extracellular serum proteases (50, 51), which could lead to minimal or extensive processing of the longer peptides and, therefore, to a failure to adequately stimulate effectors or sensitize targets. We therefore synthesized the six eight to nine-amino acid peptides within peptides 7, 21, 22, 52, and 53 that conform to the HLA-B8-binding motif. Five of the six bind to HLA-B8 (Table 3). However, peptides 7.1 and 52.2 (Table 2), both of which were shown to bind to HLA-B8 (Table 3), were unable to generate effectors or sensitize targets for cytolytic activity (data not shown). Thus, there is no evidence that use of the 15-mers was associated with decreased sensitivity in detecting CTL responses.

The short peptides were then used to map the minimal CTL epitope within peptides 21 and 22. These studies clearly demonstrate the presence of at least two PfSSP2-derived peptides that bind to HLA-B8 and are recognized by CD8+ human CTL: a nonamer, ASKNKEKAL (peptide 22.1), and an octamer, KNKEKAL1 (peptide 22.3). Inasmuch as the nonamer KNKEKAL1 (peptide 22.2) also sensitized HLA-B8-Hmy2.C1R cells for lysis by peptide 22-stimulated effector cells at levels comparable to those obtained for target cells pulsed with peptide 22.3, it is possible that this peptide represents a distinct epitope. However, it is likely that the ability of both peptides to be similarly recognized by CTL is a result of the extracellular processing of peptide 22.2 by serum peptidases (50, 51) that remove the COOH-terminal Ile, thereby generating the octamer represented in peptide 22.3. Moreover, although high-affinity peptide ligands can have values of t1/2 for dissociation at 37°C as short as 0.5 h (52), peptide 22.3 bound to HLA-B8 molecules with an eightfold higher affinity than peptide 22.2 (Table 3). We thus favor peptides 22.1 and 22.3 as the minimal HLA-B8-restricted epitopes.

Because we did not synthesize and test truncated peptides from the sequences of 22.1 and 22.3, it could be argued that they do not represent the minimal CTL epitope. Sequence information from endogenous HLA-B8–associated peptides have revealed that, although the majority of peptides were eight or nine amino acids in length, a small proportion of naturally occurring peptides yielded sequences of seven amino acids (31). Thus, the sequence KNKEKAL, which is present within peptides 21, 22, 22.1, 22.2, and 22.3 (Table 2), could theoretically represent the minimal epitope. However, this is unlikely because peptide 21–stimulated effectors cells lysed HLA-B8-Hmy2.C1R cells pulsed with peptide 22.1, but not the same cells pulsed with peptide 22.2 or 22.3 (Fig. 5 A).

Besides MHC polymorphism, antigen variation represents an additional obstacle to development of peptide-based vaccines. Indeed, analysis of the natural sequence variation of the PfSSP2/TRAP gene revealed that this antigen is highly polymorphic (53). However, it is of considerable importance that, from the sequence data on 14 P. falciparum isolates, all the residues within the nonamer peptide ASKNKEKAL are conserved. In contrast, the COOH-terminal Ile in the octamer peptide KNKEKAL1 has been shown to vary. This variation is limited, since the replacement of Ile for Ser at such position is present in only 1 of the 14 published sequences (53). Whether the substitution of this auxiliary anchor residue will interfere with the binding of such peptide to HLA-B8 and thereby abrogate the CTL response remains to be determined. Because the nonamer peptide ASKNKEKAL1 is a target of CTL in HLA-B8 individuals and is conserved among various P. falciparum isolates, it may become an important component of malaria vaccines designed to attack infected hepatocytes. Further work must be carried out to confirm that this sequence is conserved and to establish that CTL against this peptide recognize and eliminate parasitized hepatocytes in vivo.

Thus far, HLA-B8–restricted CTL epitopes have been identified in the influenza A virus nucleoprotein (30, 52), EBV nuclear antigen 3 (54), HIV-1 gp41 (55), HIV-1 pol protein (56), and the HIV-1 gag protein (30, 55, 57, 58). All these epitopes are eight or nine amino acids long. This is the first report identifying HLA-B8–restricted epitopes for an antigen of nonviral origin.

Although the selection of PfSSP2-derived peptides with sequences conforming to the HLA-B8–binding motif proved useful as a strategy for the eventual identification of CTL epitopes (ASKNEKAL and KNKEKAL), it is clear from our analysis that the presence of primary and auxiliary anchor residues is not sufficient for binding to HLA-B8 (GTRSRKREI), and that the ability of motif-bearing peptides to bind HLA-B8 molecules in the reconstitution assay (EIKYREEV and RSRKREI) does not guarantee that the peptides will be biologically relevant. Therefore, substitution analysis using a large panel of synthetic analogues may be required to refine the HLA-B8–binding motif by defining the contribution of residues other than those at positions 3 and 5 and the COOH terminus in the binding capacity of peptide ligands to HLA-B8 molecules. Such an analysis has been successfully conducted for the HLA-A2.1 allele, for which the binding motif has been adjusted by identifying the nonanchor residues that favor or interfere with binding to A2.1 molecules (59).

During the course of this study, the two HLA-B8+ volunteers were found to be protected against malaria after exposure to the bites of five nonirradiated mosquitoes carrying P. falciparum sporozoites in their salivary glands. The challenge was conducted after 1,127 (volunteer 16) and 1,163 (volunteer 17) bites of irradiated infected mosquitoes over a period of 18 mo and 2 wk after the last exposure. We believe that the protection is primarily mediated by CTL-recognizing peptides from PfSSP2 and other preerythrocytic-stage antigens in the context of HLA class I molecules on the surface of infected hepatocytes. The HLA-B8–restricted and PfSSP2-specific CTL we identified may have contributed to this sterile protective immunity. Nonethe-
less, it is difficult to conclude that the CTL activity demonstrated in PBMC after in vitro restimulation is indicative of CTL activity in the liver. However, the demonstration of circulating activated CTL against peptide 22.1 in volunteer 16 suggests that the CTL we are detecting may be able to traffic to the liver and eliminate infected hepatocytes. We have demonstrated similar activity against HLA-A*0201-restricted PfSSP2 CTL epitopes (60). This appears to be the first published evidence identifying circulating activated CTL against a nonviral antigen.

Genetic restriction of T cell responses and polymorphism of target epitopes pose enormous problems for development of malaria vaccines designed to induce protective CTL responses. The identification of a conserved HLA-B8-restricted CTL epitope on PfSSP2 constitutes a component of our comprehensive strategy to develop peptide-based vaccines that will include multiple, conserved P. falciparum preerythrocytic-stage determinants restricted by class I HLA alleles that are expressed at moderate to high frequency among different ethnic groups. Work is now in progress to map CTL epitopes on PfSSP2 that are restricted by a range of HLA alleles and to carry out the same studies for other P. falciparum proteins (25, 61–64) expressed in infected hepatocytes.

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