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# Complete Protection against *Plasmodium yoelii* by Adoptive Transfer of a CD8$^+$ Cytotoxic T-Cell Clone Recognizing Sporozoite Surface Protein 2

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**BALB/c mice immunized with irradiated *Plasmodium yoelii* sporozoites produce antibodies and cytotoxic T lymphocytes against the circumsporozoite protein and against a 140-kDa protein, sporozoite surface protein 2 (PySSP2). Approximately 50% of mice immunized with P815 cells transfected with the gene encoding PySSP2 are protected against malaria, and this protection is reversed by in vivo depletion of CD8$^+$ T cells. To determine if CD8$^+$ T cells against PySSP2 are adequate to protect against malaria in the absence of other malaria-specific immune responses, we produced three CD8$^+$ T-cell clones by stimulating spleen cells from mice immunized with irradiated *P. yoelii* sporozoites with a mitomycin-treated P815 cell clone transfected with the PySSP2 gene. Adoptive transfer of clone TSLB7 protected 100% of mice against *P. yoelii*. The second clone protected 58% of mice, and the third clone provided no protection. Clone TSLB7 protected even when administered 3 h after sporozoite inoculation at a time when sporozoites had entered hepatocytes, suggesting that it is recognizing and eliminating infected hepatocytes. These studies demonstrate that cytotoxic T lymphocytes against PySSP2 can protect against *P. yoelii* sporozoite challenge in the absence of other parasite-specific immune responses.**

Vaccination with radiation-attenuated sporozoites protects mice, monkeys, and humans against malaria (reviewed in reference 7). In some mice, this immunity is eliminated by depletion of CD8$^+$ T cells (24, 29), indicating that cytotoxic T lymphocytes (CTL), probably directed against malaria antigens on the surface of infected hepatocytes (8, 28), are required for this immunity. Mice (10, 23, 28) and humans (12) immunized with irradiated sporozoites produce CD8$^+$ CTL against the major sporozoite surface protein, the circumsporozoite protein (CSP). Such CTL against the *Plasmodium yoelii* CSP (PyCSP) eliminate malaria-infected hepatocytes in culture (28), and CTL clones against defined epitopes on the *Plasmodium berghei* (23) and *P. yoelii* (20, 27) CSPs adoptively transfer protection. Accordingly, the CSP is a target for vaccine development (7). However, vaccines based on the CSP alone have not provided protection comparable to that found after immunization with irradiated sporozoites, and there has been a search for additional targets of protective immunity (reviewed in reference 7).

Mice immunized with irradiated *P. yoelii* sporozoites produce antibodies (2) and CTL (9) against a 140-kDa protein called sporozoite surface protein 2 (PySSP2). We have reported the nucleotide sequence of the gene encoding the 826-amino-acid PySSP2 (6, 22) and that 33 to 67% of mice immunized with P815 cells (mastocytoma cells) transfected with a 1.5-kb gene fragment of PySSP2 were protected against sporozoite challenge (9). As with the irradiated sporozoite vaccine, the immunity induced by immunization with this PySSP2 vaccine was eliminated by in vivo depletion of CD8$^+$ T cells. The current studies were undertaken to determine if CD8$^+$ CTL against PySSP2 can protect against malaria in the absence of other parasite-specific immune responses.

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## MATERIALS AND METHODS

**Mice.** Six-week-old female BALB/cByJ mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*.

**Parasite.** *P. yoelii* 17X, nonlethal strain (clone 1.1), was used for all experiments. For in vitro and in vivo infections, salivary glands of *Anopheles stephensi* were dissected by hand. For immunizations, sporozoites were isolated by discontinuous gradient centrifugation (16) from infected mosquitoes that had been exposed to 10,000 rads from a $^{137}$Cs source. The dose of this parasite that infects 50% of mice is usually between two and eight sporozoites (9).

**PySSP2 transfectant.** PySSP2-transfected P815 cell clone 3.9 (PySSP2-P815 3.9) (9) was used in all experiments. This clone was obtained by cotransfecting the mouse tumor cell line P815 (H-2$^d$ mastocytoma) by calcium phosphate precipitation with *P. yoelii* DNA including a 1.5-kb fragment of the gene encoding PySSP2 cloned into expression vector pcEXV-3 and pSV2neo DNA as described previously (9). The clones were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum, 8 mM L-glutamine, 4 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 50 U of penicillin and streptomycin per ml (complete DMEM medium) plus 200 μg of G418 (Geniticin; Gibco) per ml.

**T-lymphocyte clones.** BALB/c mice were immunized intravenously three times at 2- to 4-week intervals with live irradiated (10,000 rads; $^{137}$Cs) sporozoites ($5 \times 10^4$, $3 \times 10^4$, and $3 \times 10^4$ sporozoites). Two weeks after the last immunization, T-cell lines were initiated by stimulating $5 \times 10^6$ spleen cells

from these mice with $2 \times 10^6$ SSP2-P815 3.9 cells previously treated with 50 μg of mitomycin per ml for 30 min in 2 ml of DMEM complete medium containing $5 \times 10^{-5}$ M 2-mercaptoethanol for 7 days. Viable cells were separated from dead cells by density gradient centrifugation with lymphocyte separation medium (Organon Teknika, Durham, N.C.) and then restimulated with mitomycin-treated SSP2-P815 3.9 cells for 7 days in complete medium containing $5 \times 10^{-5}$ M 2-mercaptoethanol and 50 U of human recombinant interleukin-2 (rIL-2; Cetus Corp., Emeryville, Calif.) per ml. At 3- to 4-day intervals, the medium was replaced with fresh medium containing 50 U of human rIL-2 per ml. After the third in vitro stimulation, T cells were cloned by limiting dilution at 10, 3, and 1 cells per well in 96-well plates (Costar, Cambridge, Mass.) in the presence of irradiated, syngeneic, unprimed spleen cells as feeders and mitomycin-treated SSP2-P815 3.9 cells as stimulators. Cells from wells with signs of growth were expanded in the DMEM complete medium containing 20 U of human rIL-2 per ml and then tested for CTL activity against $^{51}$Cr-labeled SSP2-P815 3.9 cells. Recloning was done once by limiting dilution. Once established, the CTL clones were maintained and stimulated on the schedule described above. Before each assay, viable cloned T cells were isolated by density gradient centrifugation. Clones were tested for phenotype by microfluorometry.

**CTL assay.** After in vitro stimulation for 6 to 7 days, cytolytic activity of the effector cells was measured as described previously (9) by using a 6-h assay with $^{51}$Cr-labeled targets. The CD8$^+$ T-cell clones were mixed at effector-to-target (E/T) cell ratios of 10:1, 3.3:1, 1.1:1, and 0.3:1 with 5,000 targets, namely, $^{51}$Cr-labeled PySSP2-P815 3.9 cells, $^{51}$Cr-labeled pSV2neo-transfected P815 cells, and $^{51}$Cr-labeled P815 cells. Cultures were incubated for 6 h at 37°C in an atmosphere of 5% $CO_2$. Supernatants were harvested by using the SCS system (Skatron, Inc., Sterling, Va.), and released $^{51}$Cr was detected by a gamma counter (Clinigamma-1272; LKB, Gaithersburg, Md.). Experimental wells were reproduced in triplicate. The percent specific $^{51}$Cr release was calculated as 100(experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated without effector cells.

**Adoptive transfer of T-cell clones.** PySSP2-specific CTL clones, harvested 7 days after restimulation with PySSP2-P815 3.9 and feeder cells, were adoptively transferred to irradiated 8- to 10-week-old BALB/c mice (500 rads; $^{137}$Cs) by intravenous (i.v.) injection. Immediately before transfer, the CTL clones were washed and resuspended in 0.5 ml of DMEM medium without serum and containing $10^3$ U of human rIL-2. A second dose ($10^3$ U) of rIL-2 was administered intraperitoneally 20 h after cell transfer. The mice were challenged 24 h after cell transfer by i.v. injection of *P. yoelii* sporozoites. A final dose of $10^3$ U of rIL-2 was administered i.v. 24 h after the sporozoite challenge. As controls, mice were injected i.v. with either the human immunodeficiency virus (HIV)-specific CTL clone RT-1 (26) or with spleen cells from normal, uninfected BALB/c mice. Prior to administration, the RT-1 cells ($2 \times 10^5$ cells) were stimulated in vitro every 2 weeks with BALB/c 3T3 cells ($5 \times 10^5$ cells) expressing HIV gp160 (transfected cell line 15-12) (26) pretreated with 100 μg of mitomycin per ml for 40 min at 37°C in medium containing 10 U of rIL-2 per ml. Thin blood films were prepared on days 7, 10, and 14 after challenge, stained with Giemsa, and examined by light microscopy to detect parasitized erythrocytes. Protection was defined as absence of parasitemia.

**IFN-γ, IL-2, IL-4, and IL-5 production by the CTL clones.** Cloned T cells ($10^6$) prestimulated in vitro with PySSP2-P815 3.9 cells were washed twice with DMEM medium and restimulated in vitro with irradiated SSP2-P815 3.9 cells as described previously in complete DMEM medium without human rIL-2. After 48 h of incubation, the supernatant was harvested and assayed for gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-5 by standard methods (4, 15, 25).

## RESULTS

**In vitro characterization of CTL clones.** To isolate T-cell clones, mice were immunized with irradiated sporozoites, and their spleen cells were stimulated in vitro with PySSP2-P815 3.9 cells. Ten clones were derived by limiting dilution and tested for CTL activity against PySSP2-P815 3.9. All 10 CTL clones lysed PySSP2-P815 3.9 in vitro (data not shown). The three most active CTL clones, TSLB7 (Fig. 1a), TSL14.18 (Fig. 1b), and TSL10.21 (Fig. 1c) were selected for further study. All were CD8$^+$ and CD4$^-$. All had greater than 50% cytolytic activity at an E/T cell ratio of 3.3:1 (Fig. 1). All three clones produced IFN-γ (TSLB7, 2.7 ng/ml; TSL14.18, 1.2 ng/ml; TSL10.21, 0.7 ng/ml), but none produced detectable IL-2, IL-4, or IL-5.

**In vivo activity of CD8$^+$ CTL clones.** When mice are immunized with PySSP2-P815 3.9, about 50% are consistently protected against malaria, and the protection is completely eliminated by in vivo depletion of CD8$^+$ T cells (9). Our previous data demonstrated a requirement for SSP2-specific CTL in SSP2-P815 3.9-induced protective immunity but did not prove that CTL against SSP2 were adequate to protect on their own. SSP2-specific CTL clones were therefore produced to determine if CTL against SSP2 could protect against malaria independent of any other antigen-specific acquired immune responses. The data presented in Table 1 indicate that this is the case. Adoptive transfer of CTL clone TSLB7 consistently protected 100% of recipients, while transfer of CTL clone TSL14.18 protected 50 to 67% of recipients. Transfer of clone TSL10.21, which has the same apparent cytolytic activity as the other two clones (Fig. 1) but produces much less IFN-γ, did not protect (Table 1).

Having established that two of the CTL clones provide protection after adoptive transfer to naive, irradiated mice, we wondered where in the life cycle this activity was occurring. Infected hepatocytes are the logical target for such activity (7, 8, 19, 28). Within several minutes after i.v. inoculation, most sporozoites enter hepatocytes. To determine if the CTL were acting after the sporozoites had entered the hepatocytes, we adoptively transferred CTL clone TSLB7 3 h after sporozoite inoculation; TSLB7 provided 83% protection (Table 2). In contrast, passive transfer of NYS1, a highly protective monoclonal antibody against the CS protein (3) that must interact with sporozoites prior to the invasion of hepatocytes to protect, does not have any protective activity when transferred 5 min after sporozoite inoculation (1).

## DISCUSSION

These studies establish that CD8$^+$ CTL against PySSP2 can protect against malaria in the absence of other parasite-specific immune responses. Since CTL against PySSP2 provide protection against malaria, even when administered as much as 3 h after sporozoites have invaded hepatocytes, these studies suggest that these CTL recognize PySSP2 epitopes on the surface of infected hepatocytes. Thus, like the CSP (28), PySSP2 is also a liver stage antigen and CD8$^+$ CTL against both the PyCSP

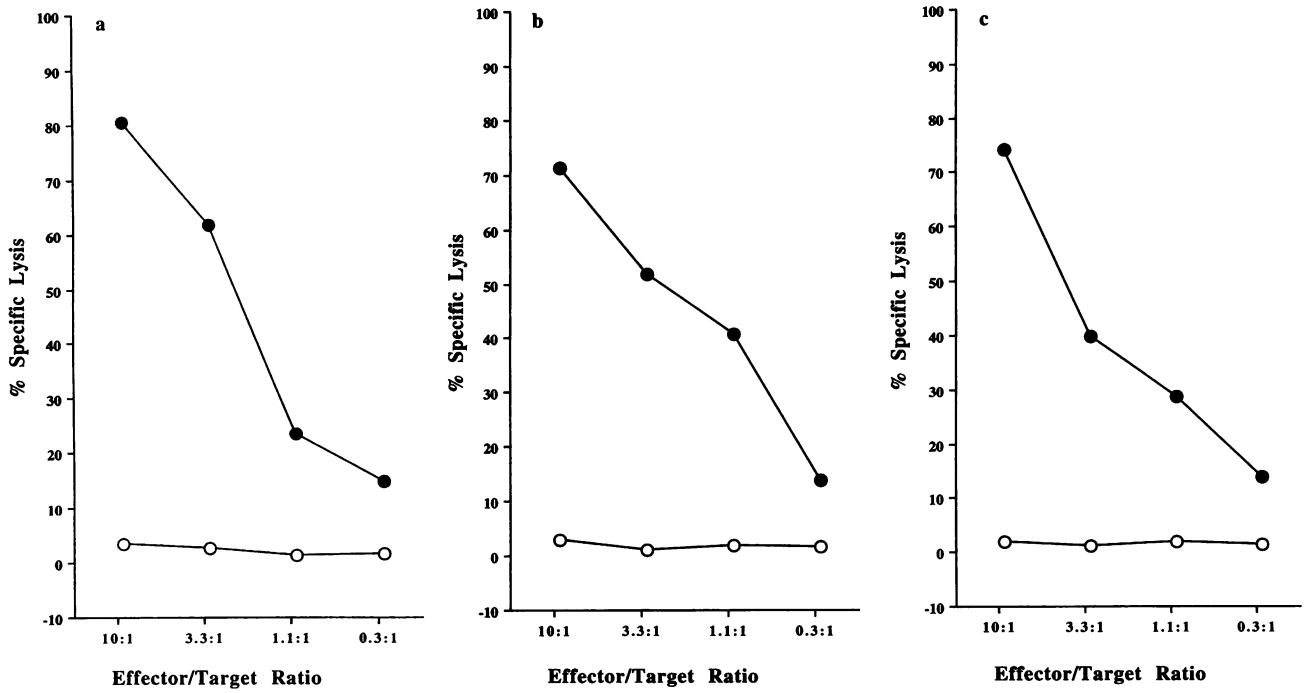


FIG. 1. In vitro lytic activity of CD8$^+$ SSP2-specific CTL clones generated by immunization with irradiated *P. yoelii* sporozoites. The CTL clones (TSLB7 [a], TSL14.18 [b], and TSL10.21 [c]) stimulated in vitro by SSP2-P815 3.9 cells were incubated with $^{51}$Cr-labeled SSP2-P815 3.9 (●) at different E/T cell ratios, and cytolytic activity was measured in a 6-h chromium release assay. The control targets were pSV2neo-transfected P815 cells (○).

and PySSP2 almost certainly protect against malaria by eliminating malaria-infected hepatocytes.

The mechanism of this killing, the epitopes recognized by the CTL clones, and the major histocompatibility complex restriction elements of these clones remain to be established. These CTL-like protective anti-PyCSP CTL (27) clones produce IFN-γ. IFN-γ eliminates malaria-infected hepatocytes from culture and, when administered i.v., provides some protection against sporozoite-induced malaria (5, 11, 13, 14). The anti-PySSP2 and anti-PyCSP CTL may directly lyse infected hepatocytes. Alternatively, the T cells could be activated by recognition of parasite antigen on the surface of the infected hepatocyte and then release IFN-γ or other cytokines that induce the hepatocyte to produce nitric oxides that destroy the parasite (13). It is interesting to note that in a single experiment with the three clones, there was a direct correlation between the quantity of IFN-γ released in vitro and the protective activities of the clones (TSLB7, 100% protection and 2.7 ng of IFN-γ released per ml; TSL14.18, 50 to 67% protection and 1.2 ng/ml; TSL10.21, no protection and 0.7 ng/ml). Further work is required to determine the meaning of this finding. Protective and nonprotective CTL clones against the CSP have been described in the *P. berghei* (23) and *P. yoelii* (19) models. In addition to production of IFN-γ, specificity of

TABLE 1. Protection against sporozoite-induced malaria by adoptive transfer of CD8$^+$ T-cell clones against SSP2

| Expt | T-cell clone | No. of cells transferred | No. of sporozoites injected | No. of mice infected/ no. of mice challenged | % Protection |
|---|---|---|---|---|---|
| 1 | TSLB7 (SSP2) | $27.5 \times 10^6$ | 200 | 0/6 | 100 |
| | TSL14.18 (SSP2) | $25.8 \times 10^6$ | 200 | 2/6 | 66.7 |
| | TSL10.21 (SSP2) | $30.0 \times 10^6$ | 200 | 6/6 | 0 |
| | RT-1 (HIV) | $17.5 \times 10^6$ | 200 | 6/6 | 0 |
| | NSC[a] | $30.0 \times 10^6$ | 200 | 6/6 | 0 |
| | None | 0 | 200 | 6/6 | |
| | None | 0 | 40 | 6/6 | |
| | None | 0 | 8 | 4/6 | |
| | None | 0 | 1.6 | 4/6 | |
| 2 | TSLB7 (SSP2) | $29.2 \times 10^6$ | 200 | 0/6 | 100 |
| | TSL14.18 (SSP2) | $30.0 \times 10^6$ | 200 | 3/6 | 50 |
| | RT-1 (HIV) | $30.0 \times 10^6$ | 200 | 6/6 | 0 |
| | None | 0 | 200 | 6/6 | |
| | None | 0 | 40 | 6/6 | |
| | None | 0 | 8 | 6/6 | |
| | None | 0 | 1.6 | 4/6 | |

[a] NSC, normal spleen cells.

TABLE 2. Protection against malaria by adoptive transfer of $CD8^+$ T-cell clones 3 h after sporozoite inoculation[a]

| T-cell clone | No. of cells transferred | No. of sporozoites injected | No. of mice infected/no. of mice challenged[b] | % Protection |
|---|---|---|---|---|
| TSLB7 (SSP2) | $30 \times 10^6$ | 200 | 1/6 | 83% |
| RT-1 (HIV) | $30 \times 10^6$ | 200 | 6/6 | 0 |
| None | 0 | 200 | 6/6 | 0 |

[a] Cells were transferred to irradiated, naive mice 3 h after challenge with sporozoites. Experimental and control mice received $10^3$ U of rIL-2 by i.v. injection at the time of adoptive transfer of the CTL clones and 24 and 48 h after transfer.

[b] Six of six naive control mice injected with eight sporozoites became infected.

epitopes, the pattern of cytokine production, homing patterns, and cell surface expression of adhesion proteins could all play a role in modulating protective activity. It will be of interest to determine whether the cell surface expression of CD44 correlates with protection as it has with other anti-PyCSP clones (19).

One of the major obstacles faced by malaria vaccine developers is that the malaria parasite changes its character as it passes through the different stages of its life cycle. If a single parasite escapes from a specific protective immune response directed against a single target, it is possible that infection will proceed unencumbered, since that target may not be present in the next stage of the parasite life cycle. Antibodies against the CSP (1a, 3, 17), $CD8^+$ CTL against SSP2 and CSP (19, 20, 23, 27), and $CD4^+$ T cells against the CSP (18) are each independently protective against malaria, and immunization with a combination of PyCSP and PySSP2 provides additive protection (9). The *Plasmodium falciparum* homolog of PySSP2 has been identified (23), and B epitopes (unpublished data) and CTL epitopes (30) on this protein have been defined. Work is now in progress to develop human vaccines that protect by inducing antibodies and T cells directed against multiple, short, defined B- and T-cell epitopes from CSP and SSP2 expressed on the surface of sporozoites and the surface of infected hepatocytes.

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