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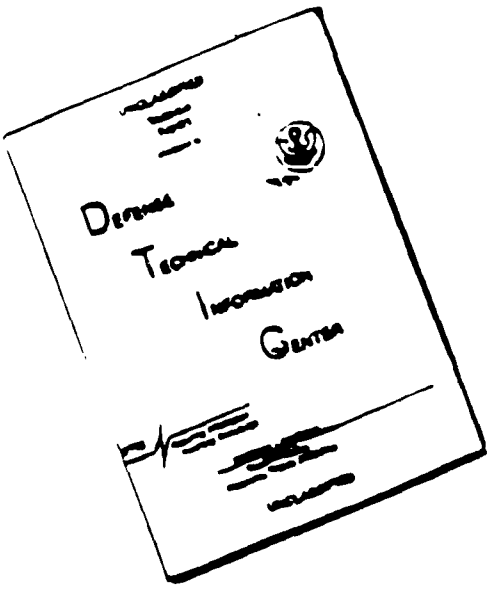


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# Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein

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ABSTRACT Immunization with irradiated sporozoites protects animals and humans against malaria, and the circumsporozoite protein is a target of this protective immunity. We now report that adjuvant-free intramuscular injection of mice with plasmid DNA encoding the *Plasmodium yoelii* circumsporozoite protein induced higher levels of antibodies and cytotoxic T lymphocytes against the *P. yoelii* circumsporozoite protein than did immunization with irradiated sporozoites. Mice immunized with this vaccine had an 86% reduction in liver-stage parasite burden after challenge with $5 \times 10^5$ sporozoites ($>10^5$ median infectious doses). Eighteen (68%) of 28 mice that received two or three doses of vaccine were protected against challenge with $10^2$ sporozoites, and the protection was dependent on $CD8^+$ T cells. These studies demonstrate the utility of plasmid DNA immunization against a nonviral infection. By obviating the requirement for peptide synthesis, expression and purification of recombinant proteins, and adjuvants, this method of immunization provides an important alternative for rapid identification of protective B- and T-cell epitopes and for construction of vaccines to prevent malaria and other infectious diseases.

The irradiated sporozoite (IrrSpz) vaccine which protects humans against malaria is not practical for the prevention of the estimated 200–500 million new infections and 1–2 million deaths caused annually by malaria. Thus, there have been considerable efforts to develop subunit vaccines that give comparable protective immunity (1). The circumsporozoite protein (CSP) is a target of this immunity. In the *Plasmodium yoelii* (Py) rodent model, passive transfer of monoclonal antibodies (mAbs) against PyCSP (2), and adoptive transfer of $CD8^+$ (3, 4) and $CD4^+$ (5) T-cell clones against PyCSP are protective. Numerous vaccines designed to protect mice against Py have induced antibodies and cytotoxic T lymphocytes (CTLs) with the specificities of the protective mAbs and T-cell clones (6–11). Only two of these PyCSP vaccines, a recombinant mastocytoma cell vaccine (10) and a combination of recombinant influenza and vaccinia virus (11) expressing PyCSP, have provided protection against blood-stage infection with this highly infectious parasite. Recombinant tumor cells can never be given to humans to prevent malaria, and immunization with two live recombinant vectors raises issues of cost, safety, and practicality. Further, neither vaccine induced levels of protective immunity comparable to that found after immunization with IrrSpz, or after transfer of mAbs or T-cell clones against PyCSP.

Immunization with plasmid DNA by intramuscular (i.m.), particle-bombardment, and intravenous (i.v.) routes induces antibodies and CTLs against foreign proteins (12–17) and protection against influenza virus in mice (15) and chickens (16, 17) and against bovine herpesvirus 1 in cattle (14). However, this potentially revolutionary approach to immunization has not been tested for induction of immunity against bacteria, fungi, or parasites. We now report that i.m. injection of BALB/c mice with PyCSP DNA induces specific antibodies and CTLs and protects against Py.

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## MATERIALS AND METHODS

**Plasmid Construction.** *pDIP/PyCSP.1.* The PyCSP gene (18) was obtained from plasmid B155 (19) as a 1468-bp *Dra* I–*Eco*RV fragment. This fragment was ligated into pUC18 that had been digested with *Sma*I followed by calf intestinal alkaline phosphatase, to form pUC18/PyCSP. A 1486-bp *Xba* I–*Kpn* I fragment encompassing the PyCSP gene was excised from pUC18/PyCSP and used to replace the 666-bp *Xba* I–*Kpn* I fragment of the human interleukin 2 (IL-2) expression vector pBC12/CMV/IL-2 (20) to form pDIP/PyCSP.1. DNA sequencing of the *Xba* I junction of pDIP/PyCSP.1 predicted that the entire CSP coding region was fused in frame with the sequence encoding the first 82 aa of IL-2. Plasmid DNA for injections was purified by CsCl gradient centrifugation, sterilized by ethanol precipitation, and suspended in sterile phosphate-buffered saline (PBS).

*nkCMVintPyCSP.1.* After establishment that immunization with PyCSP induces protective immune responses, all work was switched to a vector produced by Vical (San Diego), to facilitate the practical development of human malaria plasmid DNA vaccines. The *Dra* I–*Eco*RV fragment of the PyCSP gene, cloned into the *Hinc*II site of pBluescript II SK(+) (Stratagene), was transferred into the *Sal* I/Klenow-filled and *Bam*HI sites of kCMVinBL vector [modified pUC18-based plasmid pCMVintBL (ref. 21) where the ampicillin-resistance gene has been replaced with a kanamycin-resistance gene by using the pBluescript *Xho* I/Klenow-filled and *Bam*HI restriction endonuclease sites located 5′ and 3′, respectively, to the PyCSP coding sequence]. Expression of CSP was tested by *in vitro* transfection of BHK cells and immunoblot analysis of cell lysates.

**Parasites and Animals.** For immunizations, Py (17XNL) sporozoites (Spz) were separated by the discontinuous gradient technique (22) from infected mosquitoes that had been irradiated at 10 kilorads ($^{137}$Ce) (1 rad = 0.1 Gy). For challenges, Spz were obtained by hand dissection of infected mosquito glands in M199 medium containing 5% normal mouse serum. Six- to 8-week-old BALB/cByJ female mice (The Jackson Laboratory) were used in all experiments.

**Injection of Plasmid DNA.** Initially, BALB/c mice were injected i.m. in right and left tibialis anterior muscles with 100 μg of plasmid DNA dissolved in 50 μl of PBS with or without Lipofectin reagent (BRL). Lipofectin has been previously used to introduce DNA into mouse brain cells *in vivo* (23). When Lipofectin was used, each 50-μl injection consisted of 25 μl of undiluted Lipofectin vortexed for 5–10 sec with 100

Abbreviations: Spz, sporozoite(s); IrrSpz, irradiated Spz; CSP, circumsporozoite protein; Py, *Plasmodium yoelii*; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody; IL-2, interleukin 2; IFA, indirect fluorescent antibody test; ILSDA, inhibition-of-liver-stage development assay.

plasmid DNA in 25 μl of PBS and allowed to stand for 5 min. Injection with Lipofectin did not augment antibody responses measured in an indirect fluorescent antibody test (IFAT) against Spz (data not shown). Plasmid DNA was subsequently delivered i.m. in 50 μl of PBS alone. Negative control mice were injected with unmodified plasmid DNA lacking the PyCSP gene. Positive control mice were immunized i.v. with IrrSpz, $5 \times 10^4$ for the first dose and $3 \times 10^4$ for two subsequent doses (7).

**Measurement of Antibodies to Spz.** To evaluate antibody response after immunization with the plasmid construct in mice, an IFAT and an ELISA were used (2, 7). In the IFAT, diluted sera were allowed to react with air-dried Spz, and anti-Spz antibodies were detected with fluorescein-labeled rabbit anti-mouse immunoglobulin. A synthetic peptide, $(QGPGAP)_2$, and a recombinant fusion protein, PyCS.1 (2), produced in *Escherichia coli* that includes aa 64–321 of PyCSP were the antigens used in the ELISA. The synthetic peptide includes only the major central repeat of PyCSP, which is the only known target of protective antibodies on PyCSP (2). PyCS.1 contains the authentic major repeat and two minor Py repeat domains and the conserved region 1 sequence fused to 81 aa from the nonstructural protein of influenza A (2, 7). To determine whether antibodies were induced to the nonrepeat portion of the recombinant protein, a competition ELISA was carried out (2). Various concentrations of $(QGPGAP)_2$ or of PyCS.1 were incubated with the immune sera. The sera were then tested for reactivity with PyCS.1 by ELISA as above.

To assess the biological activity of the antibodies, the inhibition-of-liver-stage-development assay (ILSDA) (24) was used. Hepatocytes isolated from mice were seeded in eight-chamber Lab-Tek plastic slides at $10^5$ cells per chamber. After 24 hr at 37°C in an atmosphere of 5% $CO_2$ in air, medium was removed, and $5 \times 10^4$ salivary-gland-dissected Spz in 25 μl of medium were added, along with 25 μl of various dilutions of sera from immunized or control mice. After 3 hr, the cultures were washed to remove Spz that did not invade hepatocytes, and fresh medium was added. At 24 hr the medium was changed, and at 48 hr the cultures were fixed and incubated with a mAb directed against liver-stage parasites of Py (NYLS3) (gift from Y. Charoenvit, Naval Medical Research Institute) before incubation with fluorescein-labeled goat anti-mouse immunoglobulin. Liver-stage schizonts in each culture were counted with an Olympus UV microscope. The average number of liver schizonts in triplicate cultures was recorded, and percent inhibition was calculated as [(1 − mean no. of parasites in cultures with immune serum)/mean no. of parasites in cultures with control)] × 100.

**CTL Assay.** CTL assays were performed as described (9). In brief, spleen cells were obtained 2 weeks after the last immunization. Cells ($5 \times 10^6$) in 24-well plates were stimulated *in vitro* for 5 days with the CTL peptide PyCSP-(281–296) (SYVPSAEQILEFVKQI) at 2.5 μM in RPMI 1640 medium with 10% inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 units/ml), and 50 μM 2-mercaptoethanol. Two days after cultures were set up, all cultures received 10% rat concanavalin A supernatant (RCAS) (Collaborative Research) as a source of IL-2. On the night before the assay, $10^6$ P815 mastocytoma ($H\text{-}2^d$) or EL-4 thymoma ($H\text{-}2^b$) cells (American Type Culture Collection) were placed in 2 ml of medium in a well of 24-well plate. The CTL peptide was added at 2.5 μM, with control wells receiving a control peptide from the *P. falciparum* CSP [PfCSP-(368–390) (25)] or no peptide. Targets were labeled with 0.1 mCi (3.7 MBq) of $^{51}Cr$ (NEN) and incubated at 37°C. On the day of assay, targets were washed three times, and various ratios of effector cells were added to 5000 targets in 96-well U-bottom plates. Peptide was added at 2 μM during the assay. After 6 hr the supernatants were harvested (SCS system, Skatron, Sterling, VA) and the $^{51}Cr$ released was measured in a γ counter. Percent specific lysis was defined as [(experimental cpm − spontaneous cpm)/(maximum cpm, obtained by lysis with 10% SDS) − spontaneous cpm)] × 100%. All assays were carried out in triplicate. In experiments where $CD8^+$ cells were depleted, 0.9 ml of plain medium, 0.01 ml of mAb 2.43 (a mAb to $CD8^+$ cells), and 0.1 ml of rabbit complement were added to pelleted effector cells. After a 20-min incubation, cells were washed and used as effectors in the assay.

**Protection Against Challenge.** *Protection against liver-stage infection.* Mice that had received three doses of pDIP/PyCSP.1 were challenged i.v. with $5 \times 10^5$ Py Spz. Since the median infectious dose ($ID_{50}$) for Py Spz is often <2 Spz (10), this is an enormous challenge, $>10^5$ the $ID_{50}$. Forty-two hours later livers were removed, single-cell suspensions were prepared, and the liver schizonts were counted (26).

*Protection against blood-stage infection.* Immunized mice were challenged 2–3 weeks after the last immunization by i.v. injection of $10^2$ Py Spz. Protection was defined as absence of Py parasites on blood smears obtained on days 4, 7, 8, 9, 11, and 14 after challenge.

**Dependence of Protection on $CD8^+$ T Cells.** In additional studies (M.S., unpublished work), the protective efficacy of various doses of nkCMVintPyCSP.1 plasmid DNA administered at various intervals has been evaluated. Five of 6 mice administered three doses of 40 μg or 200 μg nkCMVintPyCSP.1 at 6-week intervals were protected against sporozoite challenge. Sixteen days after challenge (30 days after last immunization), the 10 protected mice were randomized into two groups. On each of the next 3 days, mice in one group received a single intraperitoneal dose of 0.5 mg of the rat IgG2b anti-$CD8^+$ (mAb 2.43; ref. 27), and mice in the other group received a control rat IgG2b (mAb J1.2; gift of Fred Finkelman (Uniformed Services University of the Health Sciences) who produced the mAb from a cell line supplied by John Abrams, DNAX). On day 4 the mice were challenged with $10^2$ Spz.

## RESULTS AND DISCUSSION

**Antibodies to Spz.** Initially, antibody responses were inconsistent. After three doses, 9 of 13 mice had antibodies to Spz, but 7 of these 9 had low levels of antibodies. However,

Table 1. Antibodies against Spz in mice after immunization with PyCSP plasmid DNA

| Mouse | Time of immunization, weeks | IFAT titer | | |
|---|---|---|---|---|
| | | 5 weeks | 8 weeks | 10 weeks |
| 1A | 0, 8 | 40 | 40 | 20,480 |
| 2A | 0, 8 | 320 | 160 | 20,480 |
| 3A | 0, 8 | 320 | 160 | 20,480 |
| 7A | 0, 8 | 160 | 320 | 20,480 |
| 5A | 0, 8 | <10 | <10 | 10,240 |
| 6A | 0, 8 | 320 | 320 | 2,560 |
| 4A | 0, 8 | 80 | 80 | 2,560 |
| 3B | 0, 5, 8 | 640 | 20,480 | 20,480 |
| 4B | 0, 5, 8 | 640 | 10,240 | 20,480 |
| 5B | 0, 5, 8 | 160 | 2,560 | 20,480 |
| 1B | 0, 5, 8 | 320 | 5,120 | 5,120 |
| 2B | 0, 5, 8 | 160 | 2,560 | 2,560 |
| 6B | 0, 5, 8 | 160 | 2,560 | 2,560 |
| Controls ($n$ = 6) | 0, 8 | <10 | <10 | <10 |
| Controls ($n$ = 6) | 0, 5, 8 | <10 | <10 | <10 |

Mice were immunized with pDIP/PyCSP.1 at 0 and 8 weeks or at 0, 5, and 8 weeks. Sera were tested for antibodies to air-dried Spz by IFAT (7) 5, 8, and 10 weeks after the first immunization. Control mice received the pBC12/CMV/IL-2 plasmid without the PyCSP insert. Pooled sera taken 2 weeks after the third immunization with Py IrrSpz and tested at the same time had an IFAT titer of 1280.

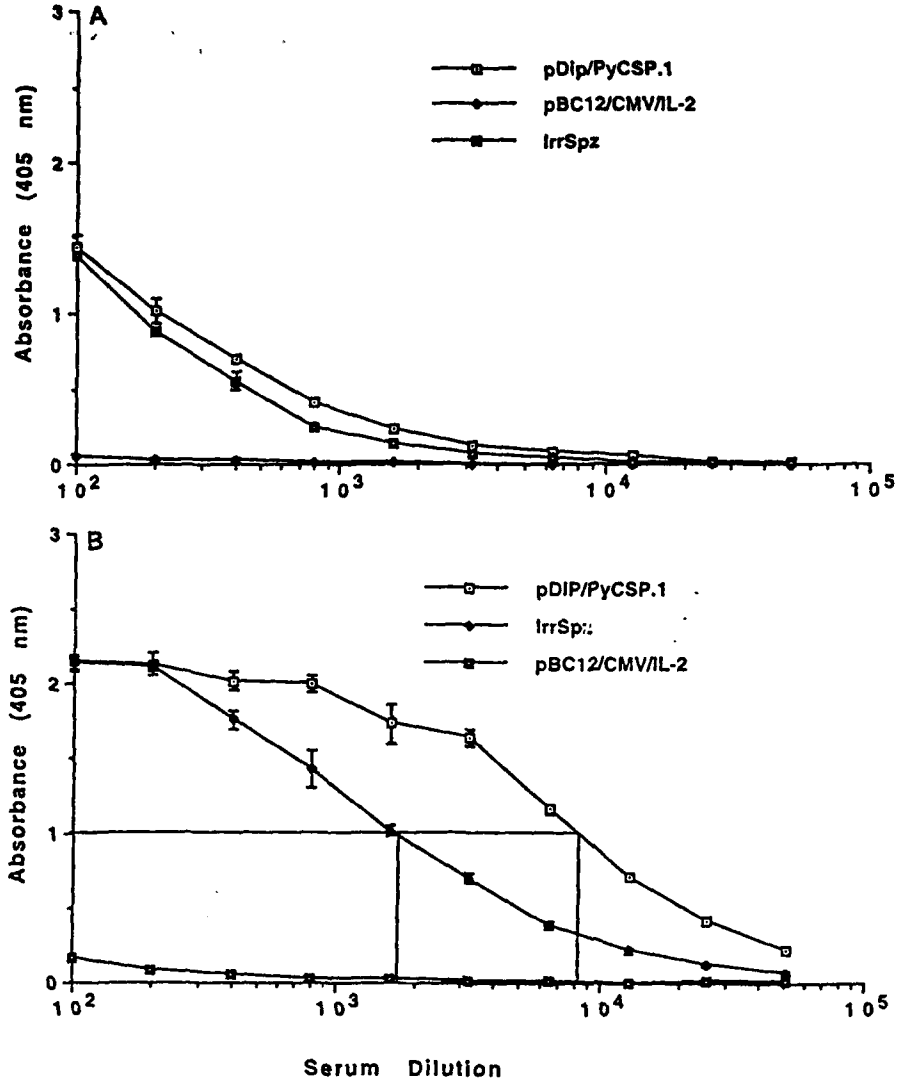


FIG. 1. Induction of antibodies to PyCSP by immunization with pDIP/PyCSP.1. Pooled sera taken 2 weeks after the third dose from three mice immunized with the pDIP/PyCSP.1 vaccine, six mice immunized with Py IrrSpz, and six mice immunized with pBC12/CMV/IL-2 plasmid control were assessed by ELISA (2) for antibodies to the synthetic peptide $(QGPGAP)_2$ (*A*) and PyCS.1, a recombinant protein, including aa 64–321 of PyCSP (*B*).

after four doses, 12 of the 13 mice had moderate to high antibody titers (data not shown). To minimize leakage from the injection site, the caliber of the injection needle was reduced from 26 to 30 gauge, and the frequency of antibody response increased subsequently (Table 1).

To determine whether the antibodies produced by immunization with PyCSP plasmid DNA inhibited invasion of Spz into hepatocytes, serum (IFAT titer, 20,480) from a mouse that had received three doses of pDIP/PyCSP.1 was tested in the ILSDA. This serum inhibited Spz invasion and development by 80% compared with serum from a mouse immunized with plasmid control (9.3 ± 2.5 vs. 46.0 ± 3.6 schizonts per well; $P = 0.001$, Student's $t$ test, two-tailed). The inhibitory activity dropped to 46% when the serum was diluted 1:20. Since sera from mice immunized with Py IrrSpz do not inhibit Spz invasion and development in this assay (24), the inhibitory activity of these sera, although low, was encouraging. However, this relatively poor inhibitory activity was inconsistent with the extremely high level of antibodies to Spz observed by IFAT with these same sera. The only known target of protective antibodies on the PyCSP is the major central repeat region sequence, $(QGPGAP)_n$ (2). We therefore measured antibodies to $(QGPGAP)_2$ by ELISA. Sera from mice immunized with the PyCSP plasmid DNA had >10 times the level of antibodies to sporozoites as did mice immunized with IrrSpz (Table 1), but had similar levels of antibodies against $(QGPGAP)_2$ by ELISA (Fig. 1*A*). This suggested that the DNA vaccine was inducing antibodies to the central repeat as well as to other epitopes on PyCSP. To determine whether the plasmid DNA immunization was inducing antibodies against regions of PyCSP flanking the repeats, we performed an ELISA using the recombinant protein PyCS.1. The serum dilution at which absorbance was 1.0 by ELISA was 7.4 times higher in mice immunized with pDIP/PyCSP.1 than in mice immunized with IrrSpz (Fig. 1*B*), indicating that the plasmid DNA had eliminated the immunodominance of the central repeats and induced high levels of antibodies to the flanking regions. To confirm that immunization with pDIP/PyCSP.1 induced antibodies to the flanking regions, sera from immunized mice were incubated with peptide $(QGPGAP)_2$ or with recombinant protein PyCS.1 and then studied in an ELISA for reactivity to PyCS.1. The synthetic peptide $(QGPGAP)_2$ at 500 μg/ml reduced absorbance by only 50%, while PyCS.1 at 15 μg/ml reduced absorbance to baseline (data not shown). These data suggest that the poor *in vitro* biological activity of the high-titer anti-Spz sera from the plasmid DNA-immunized mice is due to relatively low levels of antibodies against the important B-cell epitopes within the sequence $(QGPGAP)_n$. Such data also suggest that the plasmid DNA vaccine-

duced antibodies play little, if any, role in the protective immunity provided by the vaccine, and that if such vaccines are designed to produce protective antibodies, they may have to be constructed to only include DNA sequences coding for defined B-cell epitopes.

**Genetically Restricted, CD8⁺ T-Cell-Dependent Cytolytic Activity After Immunization with PyCSP Plasmid DNA.** Immunization with pDIP/PyCSP.1 induced classical cytolytic activity. The cytotoxicity was genetically restricted, antigen specific, and dependent on CD8⁺ T lymphocytes. The *H-2*$^d$ effectors did not lyse mismatched EL-4 cells (*H-2*$^b$) pulsed with PyCSP-(281–298), P815 cells pulsed with control peptide PfCSP-(368–390), or P815 cells pulsed with PyCSP-(281–298) after the effectors had been treated with anti-CD8 antibody and complement (Fig. 2). The cytolytic activity was significantly greater in mice immunized with pDIP/PyCSP.1 (Fig. 2*B*) than in those immunized with IrrSpz (Fig. 2*C*).

Before achieving consistency of antibody induction by modifying injection techniques, we tested two immunized mice without antibodies to Spz and four immunized mice with antibodies to Spz for CTLs. The mice with antibodies had demonstrable CTLs (45–72% specific lysis at 80:1 effector/target ratio) whereas the mice without antibodies did not. These results suggest that when this vaccine induces immune responses, it induces both antibodies and CTLs.

**Protection After *in Vivo* Challenge.** *Protection against liver-stage infection.* To determine whether immunization protected against liver-stage infection *in vivo*, mice that had received three doses of pDIP/PyCSP.1 and had high IFAT titers (mice 3B, 4B, and 5B in Table 1) and three mice that had received control plasmid were challenged with $5 \times 10^5$ Py Spz, and liver-stage infection was assessed 43 hr later. There was an 85.6 ± 4.0% (mean ± SD) reduction in numbers of schizonts in the group that received pDIP/PyCSP.1 as compared with the mice that received the plasmid without the PyCSP insert (12.7 ± 3.5 vs. 88.0 ± 17.8 schizonts per $1.4 \times 10^6$ hepatocytes; $P = 0.002$, Student's $t$ test, two-tailed).

*Protection against blood-stage infection.* In our initial challenge experiments, three mice (1A, 2A, and 5A in Table 1) were challenged with $10^2$ Spz and monitored for 14 days. Two of the three were completely protected (Table 2). In an attempt to increase protection, mice were immunized with three doses of vaccine by two regimens (Table 2) and with four doses at 0, 8, 10, and 12 weeks. Mice in the three groups were challenged at 14 weeks. Seven of 13 mice (54%) that received three doses of vaccine were protected (Table 2). Antibody levels decreased after the fourth dose in the group that received four doses, and none of the six mice that received four doses were protected (data not shown). Further studies are necessary to determine why the fourth dose caused immunosuppression.

**CD8⁺ T-Cell Dependence of Protective Immunity.** Mice immunized with nkCMVintPyCSP.1 and shown to be protected were depleted of their CD8⁺ T cells (97% depletion) or treated with a control mAb. Thirty-four days after the last immunization, the mice were challenged with $10^2$ Spz. Depletion of CD8⁺ T cells eliminated protection in five of five mice studied, whereas four of the five mice that received the control mAb were still protected.

These studies demonstrate that immunization with PyCSP plasmid DNA induces high levels of specific antibodies and CTLs and protects against malaria in an extremely rigorous challenge model system. As after immunization with radiation-attenuated Py Spz (27) and other PyCSP vaccines (10, 11), the protective immunity is completely dependent on CD8⁺ T cells, indicating that vaccine-induced CTLs are eliminating infected hepatocytes (28, 29). The protective immunity induced by immunizing with PyCSP plasmid DNA is not comparable to the sterile immunity against challenge with thousands of Spz induced by immunization with the

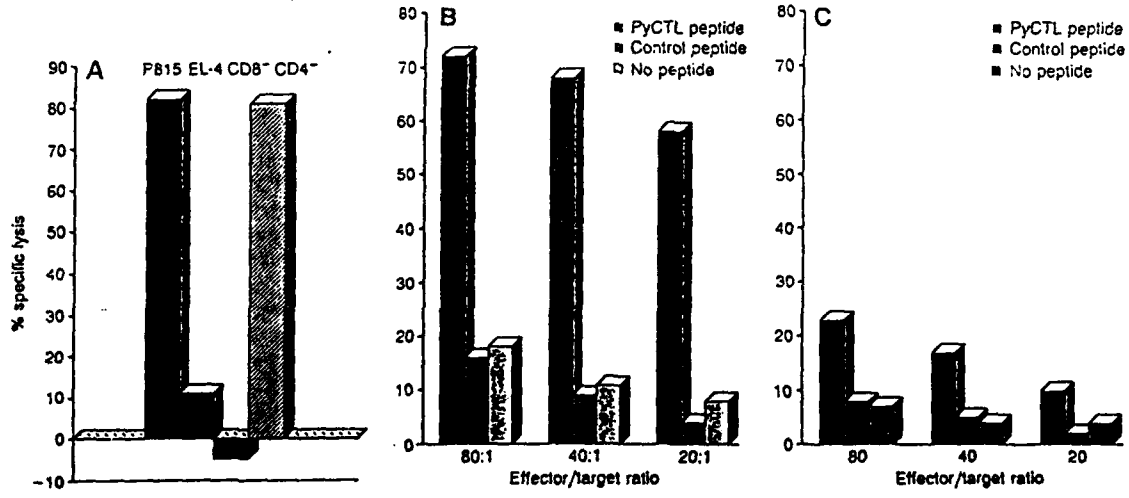


FIG. 2. Induction of genetically restricted, CD8⁺ CTLs against PyCSP by immunization with pDIP/PyCSP.1 and IrrSpz. (*A*) Two weeks after a third dose of pDIP/PyCSP.1, two mice were euthanized and spleen cells were isolated, stimulated *in vitro* for 5 days with peptide PyCSP-(281–296), (SYVPSAEQILEFVKQI), and assessed for cytolytic activity (4, 9, 27). At an effector/target ratio of 60:1, T cells lysed major histocompatibility complex-matched P815 cells (*H-2*$^d$) pulsed with PyCSP-(281–296) but did not lyse peptide-pulsed EL-4 cells (*H-2*$^b$). Cytolytic activity was eliminated by depletion of CD8⁺ (CD8⁻) T cells but was unaffected by depletion of CD4⁺ T cells (CD4⁻) (9). PyCSP-(281–296) was used to label targets instead of the H-2K$^d$-restricted decapeptide SYVPSAEQIL, because we have shown that bulk spleen cultures, in contrast with T-cell clones, more efficiently lyse targets pulsed with the longer peptide (4). (*B* and *C*) Mice were immunized with four doses of pDIP/PyCSP.1 (*B*) or 3 doses of Py IrrSpz (7) (*C*), and a CTL assay was performed 18 days after the last immunization. Significantly more cytolytic activity against P815 cells pulsed with the PyCSP-(281–296) peptide (PyCTL) was demonstrated with cells from mice immunized with pDIP/PyCSP.1. These effectors did not lyse targets pulsed with a control peptide from the *P. falciparum* CSP, PfCSP (368–390) (KPKDELDY-ENDIEKKICKMEKCS), that includes a CTL epitope (25) and did not lyse P815 cells that had not been exposed to peptide. Cytolysis was dependent on immunization, since spleen cells from naive mice and from mice immunized with the plasmid control and stimulated *in vitro* with PyCSP-(281–296) had no greater activity against P815 cells pulsed with PyCSP-(281–296) than against targets pulsed with the PfCSP-(368–390) control peptide or targets not exposed to peptide (data not shown).

Table 2. Protection against Spz challenge

| Mouse | Experiment/ regimen | IFAT titer at challenge | Protection |
|---|---|---|---|
| 1A | 1/A | 20,480 | Yes |
| 2A | 1/A | 20,480 | Yes |
| 5A | 1/A | 10,240 | No |
| Plasmid controls ($n$ = 3) | 1/A | <10 | No |
| Naive controls ($n$ = 7) | 1 | <10 | No |
| 1 | 2/B | 10,240 | Yes |
| 2 | 2/B | 5120 | Yes |
| 3 | 2/B | 5120 | Yes |
| 4 | 2/B | 1280 | Yes |
| 5 | 2/B | 10,240 | No |
| 6 | 2/B | 5120 | No |
| 7 | 2/B | 5120 | No |
| 8 | 2/B | 640 | No |
| 9 | 2/C | 5120 | Yes |
| 10 | 2/C | 5120 | Yes |
| 11 | 2/C | 5120 | Yes |
| 12 | 2/C | 640 | No |
| 13 | 2/C | <10 | No |
| Plasmid controls ($n$ = 6) | 2/B | <10 | No |

Mice were immunized with pDIP/PyCSP.1 at 0 and 8 weeks (experiment 1, regimen A); at 0, 4, and 12 weeks (experiment 2, regimen B); and at 0, 8, and 11 weeks (experiment 2, regimen C). Mice were challenged by i.v. injection of $10^2$ Py Spz 2 weeks (experiments 1 and 2) or 3 weeks (experiment 2, regimen C) after the last immunization. Plasmid controls received the pBC12/CMV/IL-2 plasmid without the PyCSP insert, and naive controls were not immunized.

radiation-attenuated Spz. Further work is required to determine whether protection can be improved by altering the immunization regimens or by immunizing with several genes or short portions of genes encoding protective B- and T-cell epitopes from PyCSP and/or other proteins such as PySSP2 (30, 31). In addition, it remains to be established at a molecular level how DNA immunization induces protective immune responses, to determine what risks, if any, this method of immunization poses, and to demonstrate comparable immunogenicity in nonhuman primates.

Previous work has established the protective efficacy of this method of immunization in viral systems (14–17). The findings in this protozoan parasite system indicate that immunization of mice with DNA also provides a method for analyzing immune responses against complex microorganisms. DNA immunization circumvents the often difficult and time-consuming requirement to produce peptides, recombinant proteins, and recombinant live vectors as immunogens and the need for adjuvants to enhance immune responses. More importantly, DNA vaccines provide an approach to developing multicomponent vaccines against the microorganisms that cause malaria and other poorly controlled infectious diseases.

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