Immunogenicity and Protective Efficacy of a \textit{Plasmodium yoelii} Hsp60 DNA Vaccine in BALB/c Mice

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The gene encoding the 60-kDa heat shock protein of \textit{Plasmodium yoelii} (PyHsp60) was cloned into the VR1012 and VR1020 mammalian expression vectors. Groups of 10 BALB/c mice were immunized intramuscularly at 0, 3, and 9 weeks with 100 μg of PyHsp60 DNA vaccine alone or in combination with 30 μg of pmurGM-CSF. Sera from immunized mice but not from vector control groups recognized \textit{P. yoelii} sporozoites, liver stages, and infected hepatocytes in an indirect fluorescent antibody test. Two weeks after the last immunization, mice were challenged with 50 \textit{P. yoelii} sporozoites. In one experiment the vaccine pPyHsp60-VR1012 used in combination with pmurGMCSF gave 40% protection (Fisher's exact test; \( P = 0.03 \), vaccinated versus control groups). In a second experiment this vaccine did not protect any of the immunized mice but induced a delay in the onset of parasitemia. In neither experiment was there any evidence of a protective effect against the asexual erythrocytic stage of the life cycle. In a third experiment mice were primed with PyHsp60 DNA, were boosted 2 weeks later with \( 2 \times 10^5 \) irradiated \textit{P. yoelii} sporozoites, and were challenged several weeks later. The presence of PyHsp60 in the immunization regimen did not lead to reduced blood-stage infection or development of parasites in hepatocytes. PyHsp60 DNA vaccines were immunogenic in BALB/c mice but did not consistently, completely protect against sporozoite challenge. The observation that in some of the PyHsp60 DNA vaccine-immunized mice there was protection against infection or a delay in the onset of parasitemia after sporozoite challenge deserves further evaluation.

The World Health Organization estimates that each year there are approximately 300 to 500 million new cases of malaria and 1.5 to 2.7 million deaths due to this disease. Most of the deaths occur in African children, but malaria remains a major health and economic problem in the tropics and sub-tropics of almost all underdeveloped countries. In view of the spread of drug resistance and the resistance of the mosquito vectors to insecticides, many countries of the world in which malaria is endemic have been experiencing a deterioration of the malaria situation in the last two decades (31). The availability of a cost-effective vaccine would be a valuable asset to any malaria control initiative (36). To develop a vaccine against malaria, several problems have to be taken into consideration. The identification of protective immune mechanisms capable of eliminating the parasite, identification of antigens and epitopes able to stimulate those immune mechanisms, and the selection of a vaccine delivery system able to present the antigen to the immune system in order to generate these protective immune responses are some of the key issues (14).

Immunization of mice with radiation-attenuated sporozoites induces complete protection against sporozoite challenge, and the protective role of CD8+ T cells and cytokines in this model has been clearly demonstrated (10, 20). DNA vaccines have provided an antigen delivery system able to generate the mechanisms of immunity that confer protection against the challenge of mice with sporozoites. They also provide a method for developing multivalent, multi-immune response vaccines (8). A DNA vaccine expressing the \textit{Plasmodium yoelii} circumsporozoite protein (PyCSP) protects BALB/c mice against sporozoite challenge (25). The genetic restriction of protection elicited by this vaccine can be overcome by combining it with other antigens (9). The protective efficacy of the PyCSP DNA vaccine was further improved by coadministering it with a plasmid encoding the murine granulocyte-macrophage colony-stimulating factor (pmurGM-CSF) (35) or by boosting the PyCSP DNA vaccine-generated immune responses with a recombinant vaccinia virus expressing PyCSP (26). Combining GM-CSF and a recombinant poxvirus boost increases immunogenicity and protection more than either intervention alone (27). Incorporation of more target antigens/epitopes to protect a genetically heterogeneous population in areas where malaria is endemic requires the identification and evaluation of the protective capacity of new antigens.

Heat shock proteins (Hsp) are a highly immunogenic and conserved family of proteins that have been identified as prominent antigens in the immune response to a wide variety of infections (38). They are able to transfer exogenous peptides to major histocompatibility complex class I molecules and prime cytotoxic T lymphocytes (29), induce the expression of cyto-
kines and adhesion molecules, induce antigen-specific CD8+ and/or CD4+ T cells, and mediate antigen-specific protection against several microorganisms (21). Plasmodium spp. Hsp60s are expressed in all the parasite development stages that occur in the vertebrate host (6, 24, 30). γδ T cells obtained from irradiated sporozoite-vaccinated mice that proliferate in the presence of Mycobacterium tuberculosis Hsp60 have been shown to protect naive mice against sporozoite challenge (33), and γδ T cells elicited during a P. yoelii infection respond by in vitro proliferation to Plasmodium falciparum Hsp60 (PiHsp60) and Hsp70 (15).

We recently cloned and sequenced the gene encoding P. yoelii heat shock protein 60 (PyHsp60) (24). Here we describe the evaluation of the immunogenicity and protective efficacy of a DNA vaccine that contains the PyHsp60 alone or in combination with a plasmid expressing murine GM-CSF.

**MATERIALS AND METHODS**

Experiments reported here were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, National Institutes of Health, publication no. 86-23).

**Mice and parasites.** The mice used in this study were 5- to 6-week-old female BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine). In all the experiments P. yoelii (17XNL nonlethal strain, clone 1.1) parasites were used (34). For immunization with irradiated sporozoites, sporozoites were obtained by the discontinuous gradient technique from infected mosquitoes that had been irradiated at 10 kilorads (137Ce) 14 days after an infectious blood meal (23). For challenges, sporozoites were obtained by hand dissection of infected mosquito glands in M199 medium containing 5% normal mouse serum.

**Construction and immunogenicity of DNA plasmids.** In a previous report (24) we described the cloning and complete sequence of a PyHsp60 cDNA clone. Based on this sequence, a primer containing the 5′ end of the coding region with a BamHI site and a primer containing the 3′ end and a BglII site were used to amplify the PyHsp60 gene from the cDNA clone, using the Gene Amp PCR reagent kit (Perkin-Elmer, Norwalk, Conn.). The PCR product was cloned into the pCR-Script Amp SK (+) cloning vector (Stratagene, La Jolla, Calif.), was sequenced using the ABI PRISM dye terminator cycle sequencing Ready Reaction (BRL, Life Technologies, Gaithersburg, Md.), and the success of cloning was confirmed by single-color fluorescence-activated cell sorting using the FACScan (Fox 400 Royal, Beckton Dickinson Immunochemistry Systems, San Jose, Calif.).

**Sporozoite challenge and protection against blood-stage infection.** Two weeks after the last immunization, mice were challenged by injection of 50 sporozoites of the nonlethal strain of P. yoelii (17XNL clone 1.1). Sporozoites were injected i.v. by tail vein in a volume of 200 μl. Blood smears were obtained every day from day 4 until day 14 after challenge and were examined for the presence of parasites (Giems stain). Complete protection was defined as the absence of P. yoelii parasites in the blood on all days including day 14. In order to determine whether immunization of mice with the PyHsp60 DNA vaccine had any effect on the course of blood-stage infection, percent parasitemias of mice were obtained by counting the number of infected red blood cells among 5,000 erythrocytes in Giemsa-stained blood smears.

**Sporozoite challenge and protection against liver-stage infection.** To assess protection against liver-stage infection, the parasite burden in the liver was measured using the Taqman (Applied Biosystems, Foster City, Calif.) automated real-time PCR system using the P. yoelii-specific 18S rRNA and the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as target sequences (37). Briefly, 2 weeks after the last immunization mice were challenged with 5 × 103 sporozoites purified by the discontinuous gradient technique from mosquitoes 14 days after an infectious blood meal. Forty-two hours after challenge, livers were recovered from each mouse, were stored in RNAlater solution (Ambion Inc., Austin, Tex.), and were kept at 4°C for a maximum of 1 week. Livers were disrupted by a polytron homogenizer (Omni International), and total RNA was purified using Trizol LS reagent (Life Technologies, Gaithersburg, Md.). Purified RNA was reverse transcribed, and target sequences of the P. yoelii 18S rRNA and mouse GAPDH were amplified simultaneously using specific primers and fluorescence-labeled probes. The threshold cycle, defined as the cycle at which the fluorescence exceeds 10 standard deviations above the starting fluorescence in the system, was converted to DNA equivalents by the amplification of the respective target sequences cloned into plasmids DNA and standard curves generated by 10-fold serial dilutions of these plasmids. The parasite burden was determined by calculating the ratio of the equivalence measures of the cloned target sequences obtained for the P. yoelii rRNA over the measures obtained for the GAPDH.

**Statistical analysis.** The χ2 test or two-tailed Fisher's exact test was used to evaluate if there was any significant protection in the immunized animals compared with control groups. (Epi Info, version 6.04B, Centers for Disease Control and Prevention, Atlanta, Ga.). Mean parasitemias in groups of mice over several days of follow-up were compared using repeated-measure analysis of variance with the Scheffe test for multiple post hoc comparisons (SPSS for Windows, version 8.0; SPSS Inc., Chicago, Ill.).
mice were evaluated by IFAT against VR1020 DNA vaccines in BALB/c mice, sera from immunized the immunogenicity of the pPyHsp60-VR1012 and pPyHsp60-VR1020 DNA constructs expressing the PyHsp60 gene. Several sources led us to conclude that the vaccine plasmids produced antibodies that recognized Hsp60 from several sources (24) and obtained previous to the immunizations were negative by action with the untransfected cells. Sera from mice vaccinated with the unmodified VR1012 vector as well as sera from mice immunized with pVR1020 alone showed strong reactivity against P. yoelii sporozoite and blood-stage parasites. Sera obtained from mice before the injection of DNA vaccines (data not shown). The minimum identity of the sequences observed between any two proteins of the Hsp60 family is about 40%. It is likely that cross-reaction of the anti-PyHsp60 antibodies with human Hsp60 accounts for the reactivity against Mycobacterium Hsp60 and recombinant PfHsp60, a band of around 60 kDa in sporozoites and blood-stage parasites (data not shown). To determine whether the antibodies induced in mice inoculated with the PyHsp60 DNA vaccine in combination with pmurGM-CSF plasmid were able to inhibit the invasion of sporozoites into the hepatocytes, sera from mice immunized with pPyHsp60 alone or in combination with pmurGM-CSF were tested by ILSDA. In this assay the MAb NYS1 that recognizes the PyCSP on the surface of sporozoites (3, 4) and the MAb NYSL3 (5) that reacts with PyHep17, which is located on the parasitophorous vacuole membrane of the liver and blood stages of the parasite life cycle, consistently inhibit invasion or development of the parasite in hepatocytes by greater than 90%. Indeed, in the experiment conducted in the present study, NYS1 showed a 93% inhibition of sporozoite invasion. In contrast, none of the sera obtained from mice immunized with the PyHsp60-based DNA vaccines alone or in combination with pmurGM-CSF were able to inhibit invasion of sporozoites into the hepatocyte at the dilutions tested (Fig. 1).

**RESULTS**

**Characterization of PyHsp60 constructs.** The full-length sequences of the PyHsp60 insert and the cloning junctions were verified. The PCR-amplified product in the PCR-Script vector as well as the junctions at the cloning site of the mammalian expression vector showed the expected sequences. Characterization of antiserum obtained from CD-1 mice immunized with the pPyHsp60-VR1012 construct has been described (24). In Western blot analyses of lysates of untransfected as well as pPyHsp60-VR1012-transfected UM449 cells, these anti-PyHsp60 antisera reacted with a band of approximately 60 kDa (data not shown). The minimum identity of the sequences observed between any two proteins of the Hsp60 family is about 40%. It is likely that cross-reaction of the anti-PyHsp60 polyclonal antibodies with human Hsp60 accounts for the reactivity with the untransfected cells. Sera from mice vaccinated with unmodified VR1012 vector as well as sera from mice obtained previous to the immunizations were negative by IFAT and Western blot (data not shown). These results and the observation that mice immunized with the pPyHsp60 DNA construct produced antibodies that recognized Hsp60 from several sources (24) led us to conclude that the vaccine plasmids were expressing the PyHsp60 gene.

**Antibody titers (IFAT) and ILSDA.** In order to determine the immunogenicity of the pPyHsp60-VR1012 and pPyHsp60-VR1020 DNA vaccines in BALB/c mice, sera from immunized mice were evaluated by IFAT against P. yoelii sporozoite and blood-stage parasites. Sera obtained from mice before the injection of DNA vaccines (data not shown) or from mice immunized with the DNA plasmid control (groups 1.H and 1.I, Table 1) did not show any significant reaction by IFAT against P. yoelii sporozoite or blood-stage parasites at serum dilutions of 1:20. Immunization of mice with the plasmids pPyHsp60-VR1012 and pPyHsp60-VR1020 induced the production of antibodies that reacted by IFAT with sporozoites and blood-stage parasites after the second and third immunizations (groups 1.A, 1.B, 1.C, 1.D, and 1.E, Table 1). The magnitude of this immune response against sporozoites was less than that induced by immunization with the positive control vaccine (PyCSP and PyHep17 plasmid mixture) (group 1.G, Table 1). The PyHsp60 vaccine-elicited antibodies also showed strong reactivity against P. yoelii-infected red blood cells. The addition of GM-CSF plasmid to the PyHsp60 DNA vaccines induced only a modest increase in the antibody titers as observed by IFAT (groups 1.B, 1.C, and 1.D) after the third immunization.

**TABLE 1. Experiment no. 1—immunization regimen, antibody titers, and protective efficacy of PyHsp60 DNA vaccines**

<table>
<thead>
<tr>
<th>Group</th>
<th>DNA vaccinea</th>
<th>No. of mice per group</th>
<th>Antibody titer from IFAT:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2nd imm.</td>
</tr>
<tr>
<td>1.A</td>
<td>pPyHsp60-VR1012</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>1.B</td>
<td>pPyHsp60-VR1012 + GM-CSF</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>1.C</td>
<td>pPyHsp60-VR1012 + GM-CSF (CD8 depleted)</td>
<td>10</td>
<td>Neg</td>
</tr>
<tr>
<td>1.D</td>
<td>pPyHsp60-VR1020</td>
<td>10</td>
<td>Neg</td>
</tr>
<tr>
<td>1.E</td>
<td>pPyHsp60-VR1020 + GM-CSF</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>1.F</td>
<td>pPyCSP-VR1020 + pPyHep-17-VR1012 + GM-CSF</td>
<td>10</td>
<td>5,120</td>
</tr>
<tr>
<td>1.G</td>
<td>Irradiated sporozoites</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>1.H</td>
<td>pVR1020 alone</td>
<td>10</td>
<td>Neg</td>
</tr>
<tr>
<td>1.I</td>
<td>pVR1020 + GM-CSF</td>
<td>10</td>
<td>Neg</td>
</tr>
</tbody>
</table>

a 100 µg of pPyHsp60-VR1012 or pPyHsp60-VR1020 DNA vaccines and 30 µg of pmurGM-CSF were given to the experimental groups. For group 1.F, a single dose consisting of 100 µg of pPyCSP-VR1020 + 100 µg of pPyHep17 + 30 µg of pmurGM-CSF. In all groups DNA was injected i.m. at 0, 3, and 9 weeks.

b IFATs against sporozoites and P. yoelii-infected erythrocytes were conducted with sera collected 2 weeks after the second and third immunizations. Antibody titers in pooled sera are the last dilution at which fluorescence was considered positive. imm., immunization; Neg, negative; ND, not determined.

c Statistically significant protection (two-tailed Fisher’s exact test: P = 0.031, 1.B versus 1.H + 1.I).

d Protection was defined as the complete absence of parasites on Giemsa-stained blood smears on day 14 postchallenge.
1. H that were immunized with unmodified pVR1020 DNA alone (Table 1) and all the mice in the negative control group 1.I immunized with VR1020 in combination with pmurGM-CSF developed parasitemia by day 14 postchallenge. Because there did not appear to be a significant difference in the behavior of the two negative control groups, in subsequent analysis we pooled them. In contrast, 40% of mice immunized with the combination of pPyHsp60-VR1012 and pmurGM-CSF did not develop parasitemia during the 14 days postchallenge (group 1.B, Table 1). Only this group had statistically significant protection on day 14 as compared with the pooled controls (two-tailed Fisher’s exact test: \( P = 0.031 \), group 1.B versus group 1.H + group 1.I [Table 1]). Only 10% of mice in groups 1.A, 1.C, and 1.D that were immunized with the other PyHsp60-based DNA vaccine alone or in combination with pmurGM-CSF plasmid did not develop parasitemia. Only 10% of mice that were depleted of CD8+ T cells did not develop parasitemia. However, this difference was not statistically significant when compared with immunized undepleted mice (Fisher’s exact test: \( P > 0.05 \), group 1.B versus group 1.C [Table 1]).

To determine whether immunization with PyHsp60-based DNA vaccines had any effect on the course of erythrocytic stage infection in BALB/c mice, geometric mean parasitemias were obtained daily from day 4 until day 14 after challenge. Figure 2 shows geometric mean parasitemias calculated only in mice with positive Giemsa-stained films in all groups of the experiment described in Table 1. At day 5 postchallenge mice immunized with pPyHsp60-VR1012 plasmid in combination with pmurGM-CSF showed a lower level of parasitemia than the negative control groups (Fig. 2). By day 7, parasitemias were similar in all groups immunized with the PyHsp60 vaccines but were lower in the one mouse that received the PyCSP plus PyHEP17 vaccine. Mean parasitemias in groups of mice on each day were compared using repeated-measure analysis of variance. The subsequent post hoc analysis Scheffe test showed that differences in the mean parasitemias on the days 5, 7, 9, 11, and 13 among any of the groups immunized with the PyHsp60 DNA vaccines and negative control groups were not significant (\( P > 0.05 \), mean parasitemias of groups 1.A, 1.B, 1.C, 1.D, and 1.E versus mean parasitemias of groups 1.H and 1.I). A second set of experiments was undertaken in order to confirm the putative protective efficacy of the pPyHsp60-VR1012DNA vaccine in combination with the pmurGM-CSF plasmid. In this experiment all BALB/c mice received equal amounts of DNA. In mice immunized with pPyHsp60-

![FIG. 1. ILSDA. Sera with the highest titer of antibody from mice immunized with pPyHsp60-VR1012 DNA vaccine alone or in combination with pmurGM-CSF were pooled and tested in the assay. The percent inhibition was calculated using the mean of the schizonts counted on triplicate cultures and determined as follows: (mean of control – mean of test)/mean control × 100.](http://iai.asm.org/)

![FIG. 2. Geometric mean parasitemias of BALB/c mice immunized with the PyHsp60 DNA vaccines (experiment no. 1). As described in Table 1, mice were challenged with 50 P. yoelii 17X (NL) sporozoites. Percent parasitemia was calculated by counting the number of infected red blood cells among 5,000 erythrocytes on Giemsa-stained blood smears from each mouse. Only mice which developed parasitemia were included in the analysis.](http://iai.asm.org/)
VR1012, a single dose consisted of 100 μg of this DNA and 30 μg of unmodified VR1012 DNA. In mice immunized with the combination pPyHsp60-VR1012 and pmurGM-CSF, a single dose consisted of 100 μg of the Hsp60-based DNA plasmid and 30 μg of pmurGM-CSF DNA. The immunization schedule and conditions of the experiment were exactly as in the first experiment. Giemsa-stained blood smears were taken daily from day 4 until day 21 postchallenge. Table 2 shows the results of protective efficacy on days 4 and 14. Blood parasites were not observed at day 4 postchallenge in 40 to 50% of the mice vaccinated with pPyHsp60-VR1012 alone or with the same plasmid in combination with pmurGM-CSF. As there appeared to be no significant difference between groups immunized with or without pmurGM-CSF (e.g., 2.A and 2.C, 2.F and 2.G), these groups were pooled for subsequent analysis. Although the number of mice that did not have parasitemia was greater in the groups vaccinated with the PyHsp60-VR1012-based vaccine, the difference between the two groups immunized with this vaccine with and without GM-CSF plasmid and their controls did not quite reach the level of statistical significance (two-tailed χ² test: P = 0.0634, 2.A and 2.C versus 2.F and 2.G. No significant protection was observed in the other groups.

Table 2. Experiment no. 2—immunization regimen and protective efficacy of PyHsp60DNA vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>DNA vaccine</th>
<th>No. of mice per group</th>
<th>DNA single dose (μg)</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 4</td>
<td>Day 14</td>
</tr>
<tr>
<td>2.A</td>
<td>pPyHsp60-VR1012 + VR-1012</td>
<td>10</td>
<td>100 + 30</td>
<td>40%</td>
</tr>
<tr>
<td>2.B</td>
<td>pPyHsp60-VR1012 + VR1012 (CD8⁺ depleted)</td>
<td>10</td>
<td>100 + 30</td>
<td>50%</td>
</tr>
<tr>
<td>2.C</td>
<td>pPyHsp60-VR1012 + GM-CSF</td>
<td>10</td>
<td>100 + 30</td>
<td>50%</td>
</tr>
<tr>
<td>2.D</td>
<td>pPyHsp60-VR1012 + GM-CSF (CD8⁺ depleted)</td>
<td>10</td>
<td>100 + 30</td>
<td>30%</td>
</tr>
<tr>
<td>2.E</td>
<td>pPyCSP-VR1020 + pPyHep17-VR1012 + GM-CSF</td>
<td>10</td>
<td>50 + 50 + 30</td>
<td>100%</td>
</tr>
<tr>
<td>2.F</td>
<td>VR1012 + GM-CSF</td>
<td>10</td>
<td>130</td>
<td>10%</td>
</tr>
<tr>
<td>2.G</td>
<td>VR1012 + GM-CSF</td>
<td>10</td>
<td>100 + 30</td>
<td>20%</td>
</tr>
</tbody>
</table>

* Equal amounts of DNA were injected in all mice. When necessary the unmodified VR-1012 DNA plasmid was injected in order to obtain a total amount of 130 μg of DNA per single dose. In all mice i.m. injections of DNA were given at 0, 3, and 9 weeks.

† Protection was defined as the complete absence of parasites on Giemsa-stained blood smears on day 4 and day 14 postchallenge.

‡ Protection of pooled mice immunized with pPyHsp60-VR1012 as compared with pooled negative controls by the two-tailed χ² test: P = 0.0634, 2.A and 2.C versus 2.F and 2.G. No significant protection was observed in the other groups.

FIG. 3. Geometric mean parasitemias of BALB/c mice immunized with the PyHsp60 DNA vaccines (experiment no. 2). As described in Table 2, mice were challenged with 50 P. yoelii 17X (NL) sporozoites. Percent parasitemia was calculated by counting the number of infected red blood cells among 5,000 erythrocytes on Giemsa-stained blood smears from each mouse. Only mice which developed parasitemia were included in the analysis.
VR1012, and pmurGM-CSF on day 5 postchallenge with sporozoites. In mice vaccinated with the pPyHsp60-VR1012 plasmid, parasites were detected earlier (at day 4). Repeated-measure analysis of variance and the subsequent post hoc analysis Scheffe test showed that the differences between the mean parasitemias in the experimental versus the control groups of mice of this experiment were not statistically significant.

Boosting of pPyHsp60-VR1012 DNA with irradiated sporozoites and protection against liver-stage infection. We observed in the first of the two experiments described above that immunization with the pPyHsp60-VR1012 DNA vaccine used in combination with the plasmid pmurGM-CSF resulted in 40% protection. On the other hand, in the second experiment there was no protection on day 14 although there was a delay in the onset of parasitemia in the groups that had received the PyHsp60 plasmid DNA. It seemed from the first two experiments that a PyHsp60 vaccine administered as a DNA plasmid may have induced immune responses with protective activity against the preerythrocytic stages of \textit{P. yoelii} but that this immunity was not as protective as that elicited by immunization with the combination of PyCSP and PyHEP17 DNA vaccines with the plasmid expressing murine GM-CSF. Since mice immunized with PyHsp60 DNA vaccine produced antibodies against sporozoites, we speculated that the PyHsp60 DNA vaccine might prime for boosting with irradiated sporozoites. Furthermore, since protection against blood-stage parasitemia is an all or none phenomenon that does not allow us to detect 90% of protective activity against infected hepatocytes, we utilized a Taqman assay developed to detect liver-stage \textit{P. yoelii} ribosomal RNA as our endpoint measure (37). Table 3 shows the experimental design of this experiment. In Fig. 4 we report the mean ratios of the measures of amplified \textit{P. yoelii} 18S rRNA over amplified mouse GAPDH obtained from the livers of all immunized mice. Parasite rRNA was not detected in mice that received a first dose of the combination of pPyCSP-VR1020, pPyHep17-VR1012, and GM-CSF and were boosted with irradiated sporozoites. The maximum measurement of rRNA was obtained in the group of mice that received two doses of unmodified VR1012 control plasmid or in those that received two doses of pPyHsp60-VR1012 plasmid. A 75% reduction of parasite burden was observed in the mice that received either PyHsp60-VR1012 or VR1012 negative control plasmid as a first dose and irradiated sporozoites as the second

![Mean Ratio](https://example.com/fig4.png)

**FIG. 4.** Liver-stage infection burden. Mean ratios of DNA equivalent measures for the \textit{P. yoelii} 18S rRNA over the DNA equivalent for murine GAPDH are shown. Groups of mice were immunized with DNA vaccines and were boosted with irradiated sporozoites (as described in Table 3), and 2 weeks after the last immunization mice were challenged by intravenous injection of \(5 \times 10^3\) sporozoites. Forty-two hours later livers were recovered and RNA was extracted. The analysis included triplicate Taqman measures from livers recovered from three mice per group.

<table>
<thead>
<tr>
<th>Group</th>
<th>First immunization</th>
<th>Second immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.B</td>
<td>pPyHsp60-VR1012 + GM-CSF</td>
<td>Irradiated sporozoites</td>
</tr>
<tr>
<td>3.C</td>
<td>VR1012 + GM-CSF</td>
<td>VR1012 + GM-CSF</td>
</tr>
<tr>
<td>3.D</td>
<td>VR1012 + GM-CSF</td>
<td>Irradiated sporozoites</td>
</tr>
<tr>
<td>3.E</td>
<td>pPyCSP-VR1020 + pPyHep17-VR1012 + GM-CSF</td>
<td>Irradiated sporozoites</td>
</tr>
</tbody>
</table>

* Each group consisted of three mice.
* A single dose consisted of 100 \(\mu\)g of pPyHsp60 DNA vaccine and 30 \(\mu\)g of pmurGM-CSF plasmid. Group 3.E received 50 \(\mu\)g of pPyCSP-VR1020, 50 \(\mu\)g of pPyHep17-VR1012, and 30 \(\mu\)g of pmurGM-CSF. All mice were immunized i.m. at 0 and 2 weeks.
* When indicated, mice boosted with irradiated sporozoites were injected i.v. with \(2 \times 10^5\) sporozoites purified from irradiated mosquitoes.
Dose. The results show that in contrast to immunization with the combination pPyCSP-VR1020, pPyHep17-VR1012, and pmurGM-CSF, immunization with pPyHsp60-VR1012 DNA vaccine neither diminished the parasite burden in the liver on its own nor primed immunized mice for a greater protective response to a single dose of irradiated sporozoites.

**DISCUSSION**

In the present study we evaluated the immunogenicity and protective efficacy of pPyHsp60-VR1012 and pPyHsp60-VR1020 DNA vaccines alone or in combination with pmurGM-CSF plasmid and compared them with the protection against sporozoite challenge afforded by immunization with the combination pPyCSP-VR1020, pPyHep17-VR1012, and pmurGM-CSF. As a negative control in all experiments, we used mice injected with the unmodified DNA vaccine vector alone or in combination with the pmurGM-CSF plasmid. Both pPyHsp60-VR1012 and pPyHsp60-VR1020 DNA vaccines were immunogenic in BALB/c mice, inducing antibodies that recognized *P. yoelii* parasites by IFAT. The levels of antibodies elicited against blood-stage parasites were higher than those induced against sporozoites. It has been shown that expression of PfHsp70 is not observed (16) or is observed at a very low level in *P. falciparum* sporozoites (32). The low antispore antibody titer observed in mice immunized with the PyHsp60 DNA vaccines may reflect a lower level of expression of this protein in this stage of the parasite compared with the blood stage. An intriguing result from the present study is the suggestion that immunization with PyHsp60 may be capable of inducing a degree of protection against sporozoite challenge, although the findings are inconsistent between different challenge experiments. Immunization of mice with the plasmid pPyHsp60-VR1012 in combination with pmurGM-CSF revealed a statistically significant 40% protection of BALB/c mice in one of the experiments (two-tailed Fisher's exact test: *P* = 0.031, group 1.B versus groups 1.H + 1.1 [Table 1]) and only a delay in the onset of blood-stage parasites in 40 to 50% of the mice immunized with the PyHsp60-VR1012 DNA vaccine in the second experiment (two-tailed *χ*² test: *P* = 0.0634, groups 2.A + 2.C versus 2.F + 2.G, day 4 [Table 2]). Some of the best protection obtained against challenge with *P. yoelii* sporozoites in BALB/c mice is conferred by immunization with PyCSP in combination with pmurGM-CSF for the first dose and boosting 3 weeks later with recombinant vaccinia expressing PyCSP (27). Under these conditions approximately 80% of the mice are protected. On the other hand, protection of BALB/c mice against sporozoite challenge ranging from 25 to 75% has been consistently obtained by immunizing with the PyCSP DNA vaccine alone (9, 26, 35). An attractive explanation for the variability in the level of protection from experiment to experiment may be the variability in sporozoite viability and/or infectivity between sporozoites isolated from different batches of infected mosquitoes. As demonstrated by McKenna et al. (18), the liver parasite burden in groups of mice infected with *P. yoelii* sporozoites purified from different batches of infected mosquitoes varies dramatically. One approach to asking whether there has been experiment-to-experiment variation in the infectivity of sporozoites is to consider the level of protection achieved with the positive control immunization regimen of PyCSP, PyHEP17, and pmurGM-CSF. Indeed, in the first experiment this regimen provided 100% protection, while in the second experiment it provided only 40% protection, suggesting that the challenge sporozoites in the second experiment were more infective.

In the third challenge study, immunizations with either the experimental vaccine or the protective DNA vaccine mix were boosted with irradiated sporozoites and the liver parasite burden was used as a read-out for the effect of the regimen on the intrahepatic growth of the parasites. Priming immunization with the PyHsp60 followed by boosting with irradiated sporozoites, however, did not have any effect on the liver parasite burden that was evaluated 42 h after the injection of 5 × 10⁴ sporozoites. In contrast, immunization with the protective combination in the first dose and boosting with irradiated sporozoites completely abolished the infection of parasites in the infected hepatocyte. In this case it seems likely that whatever potential protective effect of immunization with PyHsp60 was present was overwhelmed by the large challenge dose.

Since PyHsp60 is a protein expressed in all the parasite stages found in the vertebrate host, we assessed whether immune responses elicited by immunization with PyHsp60 DNA had antiparasite effects at different stages of the life cycle. Data from the LPSA indicated that the antibodies induced did not prevent sporozoite invasion of hepatocytes or inhibit the growth of parasites developing within hepatocytes. Assessment of the course of parasitemias in immunized mice provided no evidence for an anti-infected erythrocyte effect. However, the protection in the first experiment, the delay in onset of parasitemia in the second experiment, and the slightly lower parasitemias early in infection (Fig. 3 and 4) suggested a pre-erythrocytic stage effect. Since antibodies apparently had no effect, this suggests that T-cell responses against the infected hepatocyte may have been responsible for this effect. The suggestion in experiment no. 1 that CD8⁺ T-cell depletion reduced the limited protection observed, although not statistically significant, is consistent with a role for T-cell responses in protection.

In summary, we have suggestive evidence that immunization with PyHsp60 can induce a partially protective immune response against challenge with *P. yoelii* sporozoites. The magnitude of the response is small and it is easily overwhelmed by large doses of sporozoites and even by variation in the infectivity of different sporozoite preparations. Nonetheless, the findings suggest that PyHsp60 may still have relevance as a vaccine target. It is certainly possible that, as in the case of the PyCSP DNA vaccine, optimal protective efficacy will be obtained only by boosting the DNA vaccine immunization with recombinant poxvirus encoding PyHsp60. In order to fully evaluate the potential of PyHsp60 as a vaccine antigen it will be important to construct and characterize recombinant poxvirus expressing this protein and to evaluate it in prime-boost experiments.

In other animal models, it has been shown that Hsp60 is a prominent immunogen able to induce a variety of immune responses or act as a carrier when administered with other antigens (2, 22, 28). In the *P. yoelii*-mouse model, γδ T cells elicited by immunization with irradiated sporozoites or elicited by blood-stage infection proliferate in the presence of Hsp60 (15, 33). Furthermore, Hsp60-specific γδ⁺ T cells elicited by
immunization with irradiated sporozoites seem to contribute to the decrease of liver parasite burden in this model (33). In the present preliminary study we have produced DNA vaccines based on PyHsp60 that were immunogenic in BALB/c mice. We also demonstrated that this vaccine did seem to have an effect on the parasites in mice challenged with 50 sporozoites. Given the preexisting literature on the potential of Hsp60 as a vaccine target and our results suggesting a modest protective effect in the P. yoelii model, we conclude that PyHsp60 is an interesting candidate vaccine antigen and that further experiments using more immunogenic vaccination regimens, such as DNA-virus prime-boost approaches, should be carried out to further evaluate the usefulness of PyHsp60 as a vaccine candidate. Additionally, it has been demonstrated that immunization of mice with a recombinant Hsp60 in fusion with an influenza virus protein efficiently primes CD8+ cytotoxic T lymphocytes against this viral antigen (1). Our long-term goal in the design of a DNA vaccine against malaria is to induce different mechanisms of immunity in the heterogeneous population able to recognize and eliminate the different stages of the parasite life cycle (13). The potential of PyHsp60 DNA vaccines as an adjuvant for use in combination with other malaria antigens should also be explored.

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