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Protective T Cell Immunity against Malaria Liver Stage after Vaccination with Live Sporozoites under Chloroquine Treatment

Elodie Belnoue,2,3* Fabio T. M. Costa,2α† Tobias Frankenberg,* Ana Margarida Vigário,4* Tatiana Voza,5 Nicolas Leroy,* Mauricio M. Rodrigues,6 Irène Landau,7 Georges Snounou,¶ and Laurent Rénia5*

In this study we present the first systematic analysis of the immunity induced by normal Plasmodium yoelii sporozoites in mice. Immunization with sporozoites, which was conducted under chloroquine treatment to minimize the influence of blood stage parasites, induced a strong protection against a subsequent sporozoite and, to a lesser extent, against infected RBC challenges. The protection induced by this immunization protocol proved to be very effective. Induction of this protective immunity depended on the presence of liver stage parasites, as primaquine treatment concurrent with sporozoite immunization abrogated protection. Protection was not found to be mediated by the Abs elicited against pre-erythrocytic and blood stage parasites, as demonstrated by inhibition assays of sporozoite penetration or development in vitro and in vivo assays of sporozoite infectivity or blood stage parasite development. CD4+ and CD8+ T cells were, however, responsible for the protection through the induction of IFN-γ and NO. The Journal of Immunology, 2004, 172: 2487–2495.

Malaria infection is initiated by the bite of an infected mosquito, which injects sporozoites (spz)2 into the host’s circulation. Spz rapidly invade hepatocytes and develop to give rise to thousands of merozoites, which invade erythrocytes after release in the bloodstream, thus initiating the pathogenic asexual erythrocytic cycle. The spz and the infected hepatocyte are known as the pre-erythrocytic (PE) stages of the malaria parasite.

To date, the experimental induction of a sterile immunity against a spz challenge, whereby a patent blood-stage infection is prevented, has only been achieved through immunization with irradiation-attenuated spz (reviewed by Nussenzweig and Nardin (1)). In humans, repeated inoculations with hundreds of irradiated infected mosquitoes are required for subsequent protection. These observations initiated extensive programs for the development of vaccines targeting the PE stages of the infection, and provided a model to investigate immune mechanisms specific of these stages (reviewed by Druilhe et al. in Ref. 2). Although the irradiated spz (irr.spz) is the form inoculated, the induction of protective responses depends on the presence of viable and developmentally arrested liver stage (LS) parasites, generally as trophozoites or very early schizonts (3–7).

In some initial and subsequent studies of irr.spz in rodents, it was observed that normal spz under CQ treatment can also confer sterile protection (8–10). However, the protective immune mechanisms thus induced were not further investigated. Such investigations are of interest for two reasons: immunization by normal spz represent the natural course of exposure in endemic populations, and the LS antigenic repertoire exposed to the immune system in natural infection is likely to be broader than that of irr.spz.

In this type of investigation it is crucial to dispose of an antimalarial drug that does not influence LS parasites, but efficiently eliminates blood stage parasites as an acute blood stage infection is known to suppress immune response to PE parasites (11, 12). In this study we validated the use of chloroquine (CQ) as a specific inhibitor of blood stage parasites in P. yoelii-infected mice. We then developed a model of spz immunization in the presence of CQ to investigate the parasitological and immunological effects of exposure to normal spz.

Materials and Methods

Mice and parasites

BALB/c female mice were purchased from Harlan Laboratories (Gannat, France). WT and IFN-γR−/− mice (a gift from Dr. M. Aguet, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) (13) were bred in specific pathogen-free animal facilities. Mice used in each experiment were matched for age and sex. All experiments and procedures were performed in compliance with French Ministry
of Agriculture regulations for animal experimentation (1987). Spz of the uncloned line of the 265BY strain of *P. yoelii yoelii* were obtained by dissection of the salivary glands of infected *Anopheles stephensi* mosquitoes 16–21 days after an infected blood meal. This parasite species was initially isolated from a naturally infected *Thamnomyia gazellea* captured in the wild and maintained in our laboratory (14), and the 265 BY line has been maintained by cyclical passage between Swiss mice and *Anopheles* mosquitoes. The broad characteristics of the infection do not differ substantially from those of the frequently used 17X strain-derived lines, except that the minimum numbers of sporozoites needed to obtain patent blood stage infection is higher (1000 instead of 10). In one experiment, spz irradiated at 12,000 rad were used. After this irradiation dose the spz are capable of invading hepatocytes, where development is arrested at the uninucleated trophozoite stage (15). Infected RBC (rRBC) were obtained from *P. yoelii*-infected mice on days 8–12 after spz injection, using heparin (Sigma-Chemie, Saint Quentin-l’Arbresles, France) as anticoagulant. These infected blood samples were then diluted 5–10 times in PBS and washed twice in PBS. The percentage of parasitemia was determined by counting the number of iRBC per 1000 RBC on Giemsa-stained blood smears, and the absolute number of iRBC was calculated using total RBC number determined with a Malassez chamber.

**Immunization schedule**

Mice were injected once or twice with either one or two doses of 4,000–20,000 spz. For the double immunization, spz were administered at least 10 days apart. Chloroquine hydrochloride (CQ; Sigma-Chemie) was diluted in PBS to a concentration of 8 mg/ml. Starting the same day as spz inoculation, immunized and control animals were injected i.p. with 100 μl of the CQ solution for 10 consecutive days. Mice immunized with two doses of spz received an additional 10-day regimen of CQ treatment after the second spz injection. During (every 2 days) and at the end of the CQ treatment, the absence of parasitemia in the immunized mice was confirmed by examination of Giemsa-stained slides of tail blood. The CQ regimen used for immunization derived from a previous report where it was shown that normal sporozoite inoculation(s) of *P. berghei* under this CQ treatment was able to confer protection against a live spz challenge (10). In one experiment, mice immunized with 20,000 live spz were treated with a single i.p. dose of 0.3 mg of primaquine diphosphate (PQ; Sigma-Chemie) on the day of immunization with and with a daily dose of 0.8 mg of CQ for 10 days starting on the day of immunization. This PQ treatment was shown to eliminate ~90% of LS parasites (data not shown). In another set of experiments, groups of mice were immunized with one dose of 25,000 iR.spz, two doses of 25,000 iR.spz 15 days apart, or one dose of 75,000 followed by two booster doses of 25,000 spz, 15 and 7 days apart.

**Challenge and protection assessment**

Mice immunized with live spz and treated with CQ were rested for a minimum of 15 days after the last injection of CQ to allow complete elimination of the drug. The mice were then challenged i.v. with 4000 spz or 106 or 107 IRBC. In one experiment, mice were challenged with the bites of five or five *P. yoelii*-infected mosquitoes. The presence of spz in the mosquitoes that had fed was verified by microscopic observation of their dissected salivary glands. Control mice and mice immunized with iR.spz were challenged i.v. with 4000 spz. Blood stage infection was determined by the presence of parasites in Giemsa-stained blood smears prepared daily from days 4–10 postchallenge, and parasitemia was determined by counting the number of iRBC per 1000 erythrocytes.

Detection of parasites in the blood and quantification of parasite load in the liver of animals were performed according to the method described by Hulier et al. (16) with minor modifications. For detection of parasite in the blood, 100 μl of blood was mixed with 0.5 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, and 0.5% SDS). For LS parasite quantification, infected mice were bled at 12,000 spz, and 42 h later a liver biopsy was taken, frozen in liquid nitrogen, and crushed in 2 ml of digestion buffer before incubation overnight at 37°C in the presence of proteinase K (0.1 mg/ml; Roche, Paris, France). DNA used for PCR was purified by phenol/chloroform extraction. A first amplification of a β-actin gene fragment was used to verify whether all the samples contained similar amounts of genomic DNA. The DNA was then used as a template in duplicate PCR reactions using oligonucleotides specific for the small subunit ribosomal RNA gene of the parasite. A standard curve corresponding to DNA extracted from equal quantity of erythrocytes in samples containing 10-fold dilutions of a known number of *P. yoelii* parasite nuclei was made. The PCR products were analyzed on a 2% agarose gel and quantified by scanning densitometry. Liver parasite load units correspond to the log number of parasite nuclei per milligram of liver DNA.

**Culture of malaria LSs**

BALB/c hepatocytes were prepared as previously described, with minor modifications (17). Cells were isolated by collagenase perfusion (Roche) of liver fragments and were further purified over a 60% Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Hepatocyte purity and viability were >95% as assessed by trypan blue dye exclusion. Cells (60,000) were cultured in eight-chamber plastic Lab-Tek slides (Nunc, Naperville, IL) in William’s E medium (Life Technologies, Edinburgh, Scotland) supplemented with 5% FCS (Life Technologies) and 100 IU/ml penicillin-streptomycin (Life Technologies) and incubated at 37°C in 3.5% CO2 for 24 h. After removal of medium from the culture chambers, 40,000 spz were added in 100 μl of fresh supplemented medium. Three and 24 h later, the medium was replaced by fresh complete medium. Cultures were stopped 48 h after spz infection, fixed with cold methanol, and processed for Ab titration.

**AB titration assays**

Sera from immunized mice were collected 15 days after the last CQ injection. AB titers were determined by indirect immunofluorescence assay (IFAT) using methanol-fixed sporozoites, LS, or iRBC as previously described (17).

**Effects of sera on parasite development in vitro and in vivo**

Mice were added to the hepatitis cultures at a dilution of 1/10 at the time of spz addition. Cultures were then processed as described above. Schizont numbers were assessed in triplicate cultures by IFAT using hyperimmune sera recognizing *P. yoelii* LS. The percent inhibition was calculated by comparing the numbers of parasites in the experimental cultures with those in control wells.

Serum neutralization assay was performed by incubating 40,000 spz in a 100-μl volume of PBS containing the test sera at a final dilution of 1/10 for 15 min at 37°C. This solution was further diluted in PBS to obtain a final concentration of 40,000 spz/ml. Naive BALB/c mice were then challenged with 100 μl of this solution, corresponding to 4,000 spz. Blood stage patency was monitored during the 10 days after challenge.

Sera (200 μl) obtained from mice immunized with 20,000 spz under CQ or from control mice treated with CQ alone were passively transferred twice to groups of five recipient BALB/c mice 40 and 60 h after challenge with 4,000 spz. Parasitemia was then monitored.

**In vivo depletions**

Purified control rat Abs were purchased from Sigma-Chemie. Rat IgG2a anti-CD4 mAb (clone GK1.5, ATCC TIB 207; American Type Culture Collection, Manassas, VA) and rat IgG2b anti-CD8 mAb (clone 2.43, ATCC TIB 210; American Type Culture Collection) were used. The rat IgG2a anti-IL-12 mAb (clone C17.8.20) cell line was provided by Dr. P. Trinchieri (Wistar Institute, Philadelphia, PA) (18). The rat IgGl anti-IL-6 mAb (clone MP5-20F3), the rat IgG2a anti-IFN-γ (clone XMG-1) (19) cell lines were supplied by Dr. G. Minoprio (Institut Pasteur, Paris, France). Abs were purified from culture supernatant by ammonium sulfate precipitation. Immunized mice were injected i.p. with 500 μg of anti-CD8, anti-CD4 mAbs, or control rat IgG on days 1 and 0 before challenge. More than 98% of blood CD8+ or CD4+ T cells were depleted by this procedure, as verified by cytofluorometry (FACScan; BD Biosciences, Mountain View, CA) using anti-CD4 (clone H12-19; Sigma-Chemie) and anti-CD8 (clone 53-6-7; BD PharMingen, San Diego, CA) mAbs that recognized epitopes different from those recognized by the depleting mAbs.

**IFN-γ neutralization**

On days 2, 1, and 0 before and days 1 and 2 after challenge, mice received a single i.p. dose of 2 mg of the anti-IFN-γ mAb XMG-1. The efficacy of the IFN-γ Abs was verified in an experiment in which anti-IFN-γ mAb treatment was able to reverse protection in mice immunized with three injections of iR.spz. In the anti-IFN-γ mAb-treated group, 80% (four of five) of the mice developed patent parasitemia compared with none (of five) in the control group.

**IL-12 neutralization**

At 12 h before and 3 h after challenge, mice received a single i.p. dose of 1 mg of the anti-IL-12 mAb. Efficacy was verified by testing the ability of this mAb (1 mg i.p.) to prevent mortality in mice treated with an i.v. injection of 100 μg of LPS (18). In the anti-IL-12 mAb-treated group, no
mouse died (zero of five) compared with the control group, in which 60% of the mice died (three of five).

**TNF-α neutralization**

At the time of challenge, mice received a single i.p. dose of 1 mg of the anti-TNF-α mAb. Neutralization efficacy was verified by testing the ability of this mAb (1 mg i.p.) to prevent mortality in mice treated with an i.p. injection of 0.1 μg of LPS together with 20 mg of galactosamine (20). In the anti-TNF-α mAb-treated group, no mouse died (zero of five), whereas in the control group three of five mice died.

**Inhibition of NO production**

On days 0, 1, and 2 after spz challenge, mice received a single i.p. dose of 100 μg of LPS together with 20 mg of galactosamine (20). In the anti-TNF-α mAb-treated group, no mouse died (zero of five), whereas in the control group three of five mice died.

**Inhibition of free radicals**

On days 0, 1, and 2, mice received a single i.p. dose of 100 μg of N-acetylcysteine (NAC) (22).

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**Table I. Immunization with live spz during CQ treatment protected against sporozoite challenge**

<table>
<thead>
<tr>
<th>Sporozoites Status</th>
<th>Inoculations (n)</th>
<th>Challenge Infected/injected (%)</th>
<th>Protection (%)</th>
<th>Rechallenge Infected/injected</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>0/10</td>
<td>100*</td>
<td>0/5</td>
<td>100*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1/10</td>
<td>90*</td>
<td>2/5</td>
<td>60*</td>
</tr>
<tr>
<td></td>
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<td>1/10</td>
<td>90*</td>
<td>0/5</td>
<td>100*</td>
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<tr>
<td></td>
<td>10</td>
<td>4/18</td>
<td>78*</td>
<td>1/3</td>
<td>67</td>
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<tr>
<td></td>
<td>4</td>
<td>3/5</td>
<td>40</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5/5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20/20</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Irr.spz</td>
<td>75, 25, 25</td>
<td>0/5</td>
<td>100*</td>
<td>0/5</td>
<td>100*</td>
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<tr>
<td></td>
<td>25</td>
<td>5/5</td>
<td>0</td>
<td>ND</td>
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<tr>
<td></td>
<td>25</td>
<td>5/5</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mice were challenged i.v. with 4000 spz at least 15 days after CQ treatment. The data represent pooled results from four experiments (with four to five mice per group in each experiment).
* Mice were rechallenged i.v. with 4000 spz 2–4 mo after the first challenge.
* Mice were treated with CQ from days 0 to 10. Control mice received CQ alone.
* p < 0.05 between immunized and control mice.
* ND, not done.

**ELISA detection of IFN-γ**

Ninety-six-well plates (Nunc, Naperville, IL) coated with anti-IFN-γ (clone R4-6A2; BD PharMingen) mAb were incubated overnight at 4°C, then the sera were added, and the plates were incubated overnight at 4°C. Detection was made after adding second biotinylated Ab (clone XMG1.2; BD PharMingen), followed by incubation for 45 min at room temperature. Extravidin-phosphatase alkaline was then added, and plates were incubated for 1 h at room temperature. The phosphatase activity was measured by adding 4-methylumbelliferyl phosphate (Sigma-Chemi) as substrate and reading fluorescence at 360/460 nm using a spectrophotometer. Dilutions of known quantities of IFN-γ were used for the standard curve. The detection limit was 50 pg/ml.

**Statistical analysis**

To determine differences in liver parasite load between two groups or between multiple groups of mice, the Mann-Whitney U test or one-way ANOVA followed by Tukey’s multiple comparison tests were used, respectively. Calculations were performed using PRISM software version 3.02 (GraphPad, San Diego, CA).

**Results**

**CQ treatment does not inhibit LS development**

CQ, a potent inhibitor of blood stage parasites (23), does not prevent the emergence of blood stage parasites when applied only during the PE stages (24, 25). It is thus considered to have no effect on LS parasites, although no formal demonstration of this was obtained. We therefore first confirmed that CQ treatment during the 45-h LS (on the day on spz inoculation and the next day) does not inhibit *P. yoelii* LS development in vivo after a spz challenge of normal mice (Fig. 1). As expected, mice (n = 20) treated for 10 days with CQ starting on the day of sporozoite inoculation did not develop patent parasitemia, whereas all nontreated control mice (n = 20) did.

**Protective immunity is conferred by spz immunization under CQ in BALB/c mice**

We next assessed whether immunization with infective spz in the presence of CQ (henceforth abbreviated spz/CQ) was able to induce a protective immune response against an spz challenge made after CQ had been completely eliminated from the blood. Different regimens of spz/CQ (dose and frequency) were performed in BALB/c mice (Table I). For normal spz, the acquisition of strong protective immunity was observed when 10,000 or more spz were used (as further confirmed by a negative parasite-specific PCR on
blood samples taken 6 days after challenge). By contrast, even inoculation with two doses of 25,000 irr.spz failed to protect against spz challenge; sterile immunity was only obtained at substantially higher irr.spz doses (Table I). For both types of immunization, protection did not wane substantially with time, as determined by rechallenge 2–4 mo after the first challenge (Table I and data not shown). Furthermore, the protection conferred by spz/CQ immunization of BALB/c mice was equally effective when the challenge was performed naturally, i.e., by the bite of infected mosquitoes. None of the mice (zero of five) immunized with 20,000 spz/CQ developed patent parasitemia after challenge by four or five P. yoelii-infected mosquitoes, whereas all control mice (five of five) receiving CQ alone did.

Development of sporozoite into LS parasite is needed for protection

When PQ, an antimalarial drug that eliminates LS parasites (26), was administered concurrently with spz/CQ immunization, none of the BALB/c mice was protected from a subsequent spz challenge (Table II). The abrogation of protection by PQ treatment demonstrated that LS parasites are essential for the induction of protective immunity.

Protective immunity conferred by spz/CQ immunization in BALB/c mice is mainly directed against LS parasites

There are three possible targets for the LS-dependent protection elicited by spz/CQ immunization: the invading spz, the intracellular LS itself, and the blood stage parasites. Analysis of the Abs induced by our immunization protocol did not allow us to distinguish between these possibilities. Indeed, sera from the immunized animals reacted by IFAT with spz, LS, and blood stage parasites, and titers were correlated with the immunizing spz dose (Table III). This contrasts with the IFAT reactivity of sera obtained from irr.spz-immunized mice, in which spz and, to a lesser extent, LS parasites were labeled, but not blood stage parasites.

The sera from spz/CQ-immunized mice were then tested in three different functional assays. A strong inhibition of spz invasion and development in hepatocytes in vitro was obtained with sera from irr.spz-immunized mice; however, sera from the spz/CQ-immunized mice failed to do so; on the contrary, they produced an enhancement of spz invasion and development in some groups (Table III). The lack of an Ab effect on spz was confirmed by the spz infectivity neutralization in vivo assay, as sera from all spz/CQ-immunized mice failed to prevent infection (Table III). Finally, passive transfer of sera from mice immunized with 20,000 spz/CQ or mice receiving CQ alone to groups of five mice challenged with 4,000 spz at the end of LS development (40 h post-spz challenge) and at the beginning of blood stage development (60-h spz challenge) had no effect on the course of the subsequent blood stage infection.

The major consequence of spz/CQ immunization of BALB/c mice was observed when the hepatic parasite load was quantified by PCR after challenge with 30,000 spz (Fig. 2A). The reduction in LS parasite load increased with the immunizing dose and reached 99% for the most stringent immunization schedule (two injections of 20,000 spz), nearly equating the total elimination of LS parasites obtained in mice immunized with three injections of irr.spz (Fig. 2A).

A minor effect on blood stages of spz immunization during CQ treatment could additionally be inferred. In groups of mice immunized with 20,000 spz/CQ, LS parasite load reduction was not complete (48–77%; Fig. 2A). However, in groups immunized in the same way, only a few (10%) of the spz-challenged mice developed patent parasitemia (Table I). Two sets of experiments were conducted to explore the occurrence of blood stage immunity. Groups of five BALB/c mice immunized with 20,000 live spz/CQ were challenged with 4,000 spz or with two different doses of iRBC. Sterile protection was observed in four of five spz-challenged mice, and only one of five developed parasitemia that peaked at lower levels and cleared faster than controls (Fig. 2B). Sterile protection was observed only in one of five or two of five spz/CQ-immunized mice after challenge with 100 or 105 iRBC, respectively (Table IV). The course of infection in the mice challenged with 106 iRBC was monitored for 3 wk postchallenge. Peak parasitemias and time to clearance were only lower in some of the

<p>| Table II. Primaquine treatment abrogated protection conferred by immunization with spz during CQ treatment |
|-------------------------------------------------|------------------------|------------------------|------------------------|</p>
<table>
<thead>
<tr>
<th>Sporozoite Dose (a)</th>
<th>Treatment</th>
<th>Infected/Injected</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20,000</td>
<td>CQ</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td>20,000</td>
<td>PQ</td>
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</tr>
<tr>
<td>20,000</td>
<td>PQ</td>
<td>5/5</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Mice were immunized with 20,000 spz and were treated with CQ from days 0 to 10. Control mice received CQ alone.
(b) Mice were challenged i.v. with spz 15 days after the last CQ injection.
(c) Mice were treated with a single i.p. dose of 0.3 mg of primaquine on the day of spz immunization.

*p = 0.05 between mice treated with CQ and control mice.

| Table III. Absence of inhibitory activity of Abs induced by immunization with live spz during CQ treatment |
|-------------------------------------------------|------------------------|------------------------|------------------------|
| Spz | IFAT | SNA |
| Dose (×10^3) | Injections (n) | Spz (mean range) | LS parasites | iRBC (mean range) | ILSDA (%) | SNA | Infected/Injected |
| Live spz | 2 | 3200 (400–6400) | 1200 | 3840 (3200–6400) | 8 ± 4 | 5/5 |
| 20 | 1 | 960 (200–1600) | 1600 | 3520 (1600–6400) | 109 ± 7 | 5/5 |
| 10 | 2 | 800 (400–1600) | 800 | 1600 | 30 ± 12.5 | 5/5 |
| 10 | 1 | 360 (0–800) | 1600 | 960 (0–6400) | ND | ND |
| Irr.spz | None | 3200 (1600–6400) | 800 | 0 | 82 ± 1.7 | 0/5 |
| 75, 25, 25 | 0 | 0 | 0 | 4 ± 7.5 | 5/5 |

(a) Ab titers against the different parasite stages were assessed by immunofluorescence assay.
(b) ILSDA, inhibition of liver stage development assay. The ability of Abs to inhibit liver stage parasites was tested using pooled sera (1/10 dilution) from five BALB/c mice per group obtained just before challenge. The number of schizonts in control wells incubated without serum was 174.5 ± 8.6.
(c) SNA, serum neutralization assay. Four thousand live spz were mixed with serum (final dilution, 1/10) and incubated 30 min at 37°C. They were injected i.v. into naive reporter mice.
(d) Mice were immunized with different doses of live spz or with irradiated sporozoites and treated with CQ from days 0–10. Control mice received CQ only.
(e) ND, Not done.
immunized mice (Fig. 2C). These observations indicate that a minor anti-blood stage component operates after the major elimination of LS parasites resulting from spz/CQ immunization.

**Protective immunity conferred by spz/CQ immunization can be induced in mice with a different genetic background**

129P2Sv/ev mice have a different genetic background from BALB/c mice and express a different MHC haplotype, H2^b^ rather than H2^d^.

Preliminary experiments established that 129P2Sv/ev mice are less receptive to spz from *P. yoelii* 265 BY, as patent blood parasitemia could only be obtained reproducibly after challenge with a minimum of 7,500 spz (n = 15 129P2Sv/ev mice/group in three experiments) compared with 4,000 spz for BALB/c mice. A strong protection (60–100%) was induced against spz challenge by immunization with two injections of 10,000 or 20,000 spz/CQ or one injection of 20,000 spz/CQ (Table V). This protection was also characterized by a statistically significant reduction in the LS parasite load (Fig. 3C).

**CD4^+ and CD8^+ T cells are involved in protection induced by spz/CQ immunization against LS parasites in BALB/c mice**

Having established that Abs did not seem to be implicated in the protection induced by spz/CQ immunization against LS parasites, we evaluated the role of CD4^+ and CD8^+ T cells in protection. BALB/c mice immunized with 20,000 spz/CQ were then treated with depleting anti-CD4 or anti-CD8 Abs before spz challenge. A partial reversal of protection against infection was obtained by depletion of CD8^+ or CD4^+ T cells (Fig. 3A). This reversal was
mirrored by a reduced in vivo elimination of LS parasites in the immunized depleted mice (Fig. 3B).

Role of IFN-γ in the protection induced by spz/CQ against LS parasites

Different cytokines have been shown to inhibit LS development in vivo and in vitro (27). The roles of these cytokines were tested by Ab neutralization just before and during spz challenge of BALB/c mice immunized with 20,000 spz/CQ. No reversal of protection could be observed when neutralizing Abs against IL-6, IL-12, and TNF-α were used, whereas injection of anti-IFN-γ Abs partially reversed the induced protection (Table VI). The importance of IFN-γ was underlined by the presence of high levels of this cytokine in the serum of immunized mice, but not in that of spz-challenged naive mice (Fig. 3C).

To confirm these results, 129P2Sv/ev mice deficient (KO) for the α-chain of the IFN-γR were used. A significant abrogation of protection was observed in the KO mice (Table V), and this was associated with a reversal of a reduction in the LS parasite load (Fig. 3D).

Protection is dependent on NO in BALB/c mice

Finally, we evaluated the role of toxic mediators in the protection induced against LS parasites. Treatment of spz-immunized mice under CQ with SMT, a potent and selective inhibitor of inducible NO synthase (21), at the time and 1 day after spz challenge completely abrogated protection (Table VI). By contrast, treatment with NAC, a potent inhibitor of oxygen radicals (22), had no effect on protection (Table VI).

Discussion

Experimental studies on the nature of naturally acquired immunity against PE stages in humans are precluded on ethical grounds.
Elegantly designed epidemiological investigations have provided indications that such immunity occurs (28–30). However, interpretations of observations in endemic populations are hampered by the difficulty in dissociating the effects of blood stage immunity from those of PE immunity. The study of the mechanisms of immunity against PE stages have therefore relied on the use of rodent models of malaria, but most of the investigations were concerned with an investigation of the protective mechanisms induced by immunization with irradiated sporozoites.

In this study we present the first systematic analysis of the immunity induced by normal P. yoelii spz in mice. In the rare previous related studies in which P. berghei infection of mice or rats was used (8–10), only gross protection against spz challenge was measured. In the present work we conducted immunization with spz during CQ treatment to minimize the influence of blood stage parasites. We demonstrated that inoculation of spz induced a strong protection against a subsequent sporozoite challenge. The induction of protective immunity depended on the presence of LS parasites, as primaquine treatment concurrent with spz immunization abrogated protection (Table II). Although it has not been formally demonstrated that LS parasites are required for the induction of protective responses after irr.spz immunization, the presence of developmentally arrested LS parasites is thought to be necessary for the maintenance of protection (31). The immunity induced by spz/CQ immunization is mainly directed at LS parasites, which is also the case for irr.spz immunization. However, we further uncovered a minor, albeit measurable, effect on blood stage parasite multiplication. As for the mechanisms that mediate protection, both the Abs against spz and the cellular responses against LS parasites induced by irr.spz play a role in the protection (2). Abs against spz, LS, and blood stage parasites were induced after spz/CQ immunization; however, functional tests revealed that they do not play an effector role in protection. Depletion experiments demonstrated that only T cell responses are responsible for the protection observed. Elimination of LS parasites was mediated by both CD4+ and CD8+ T cells (Fig. 3B). IFN-γ was also shown to play a role, as the levels of protection were reduced by a neutralizing Ab or when IFN-γR KO mice were used for immunization. The observation of some protection in the absence of IFN-γR signaling suggests that an alternative pathway is operating (Table V). This pathway, however, does not seem to involve other cytokines such as IL-12, IL-6, or TNF-α (Table VI), which have been shown previously to inhibit LS development (32–36). The protection induced by spz/CQ immunization was found to be strictly dependent on NO, as inhibition of inducible NO synthase by treatment with a highly specific inhibitor, S-methyl-thiourea (21) completely reversed protection (Table VI). Previous work has shown that LS parasites are susceptible to radical nitrogen intermediates induced by different cytokines (IFN-γ, IL-6, or TNF-α) or after immunization with irr.spz (32, 35–37). Thus, our results imply that in our immunization model, other cytokines may lead to NO-mediated killing. Identifying the cytokine(s) involved clearly deserves further studies.

Together with a major effect on LS, we observed a minor protective effect on blood stages. Passive transfer of Abs indicated that they do not a major role in this protection. This suggested that T cells, in particular, CD4+ T cells, may inhibit the subsequent blood stage through the action of IFN-γ. High levels of IFN-γ were indeed produced in normal spz-immunized mice after sporozoite challenge, and in a preliminary experiment we observed that CD4+ T cells were the main source of IFN-γ (data not shown). It has been shown previously that P. yoelii blood stages are susceptible to the action of this cytokine (38, 39), and evidence that infection by this parasite can be controlled by T cells and that they are high producers of IFN-γ has been obtained recently (40–42).

The level of protection induced by spz/CQ immunization is of particular biological and epidemiological interest. After the injection of irr.spz, the resulting LS forms persist for extended periods of times (weeks to months) (31), whereas the normal LS stages of P. yoelii mature to release merozoites starting 45 h after spz inoculation (43). Our observations imply that a very short exposure of the immune system to LS parasites could result in an efficient induction of protection. Two mutually nonexclusive parameters could also play a role. First, the antigenic repertoire of normal LS parasites is likely to be broader than that of the LS parasites derived from irr.spz, which rarely develop beyond the early schizont stages (4, 5, 7). This would suggest that Ags expressed by the maturing and mature LS are potent inducers of immunity. The fact that some Ags will be shared by the merozoite could explain the reproducible minor anti-blood stage component of the protection induced by spz immunization during CQ treatment. Second, LS development might occur at different rates after spz invasion of the hepatocyte, thus leading to a longer exposure of LS Ags to the immune system. Careful morphological observation in the natural host and experimental hosts of P. yoelii have provided evidence that LS development is asynchronous and might occur in waves (14, 44). Observations of the prepatency period in humans infected through mosquito bites are consistent with heterogeneous duration of the LS phase (45).

The observations made in this extensively used rodent model of malaria infections lend weight to the idea that PE stage immunity plays an important role in subjects living in endemic areas (28–30). The fact that the protection we obtained relied on the use of CQ to suppress the blood stage infection leads us to speculate on potential consequences of the widespread use of this drug over the last 50 years and, in particular, on the spread of parasites resistant to CQ. Our results would suggest that the levels of immunity to P. falciparum LS would be enhanced when CQ is employed as a causal prophylaxis. Although CQ was widely used during and after the malaria eradication campaign in the 1950s and 1960s, few data from which levels of immunity to LS in population exposed or not to CQ can be inferred could be found in the literature. In a study in India in which CQ treatment was administered at intervals of 3 mo, malaria incidence in adults was significantly diminished (46). As CQ has a terminal half-life of ~1 mo in humans (47), it was proposed that this regimen had facilitated the development of a protective immunity. In a more pertinent report, Gambian children were followed up for 6 mo after cessation of 12–24 mo of CQ.
prophylaxis. The prevalence of *P. falciparum* and *P. malariae* was substantially reduced in these children compared with children in an untreated control group (48). When a similar study was conducted with nonimmunized Indonesian adults, no influence was observed on the incidence of *falciparum* or *vivax* malaria (49). Interestingly, the parasites circulating in the study area proved to be highly CQ resistant (50, 51). When interpreted in the light of our results, these observations suggest another nefarious consequence to the spread of CQ resistance. When CQ-sensitive *P. falciparum* parasites predominate in hyperendemic areas, administration of CQ will prevent acute blood stage infections, and this could promote the acquisition of immunity against LS parasites. However, as resistance to CQ rises, the increased frequency of acute blood infections would increasingly suppress such an immunity.

Clearly, the results presented in this report reinforce the need to study immune responses against the neglected LS of malaria infection and, in particular, the search for Ags expressed by mature LS.

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References


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