Minimal Role for the Circumsporozoite Protein in the Induction of Sterile Immunity by Vaccination with Live Rodent Malaria Sporozoites

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Immunization with live Plasmodium sporozoites under chloroquine prophylaxis (Spz plus CQ) induces sterile immunity against sporozoite challenge in rodents and, more importantly, in humans. Full protection is obtained with substantially fewer parasites than with the classic immunization with radiation-attenuated sporozoites. The sterile protection observed comprised a massive reduction in the hepatic parasite load and an additional effect at the blood stage level. Differences in the immune responses induced by the two protocols occur but are as yet little characterized. We have previously demonstrated that in mice immunized with irradiated sporozoites, immune responses against the circumsporozoite protein (CSP), the major component of the sporozoite’s surface and the leading malaria vaccine candidate, were not essential for sterile protection. Here, we have employed transgenic Plasmodium berghei parasites in which the endogenous CSP was replaced by that of Plasmodium yoelii, another rodent malaria species, to assess the role of CSP in the sterile protection induced by the Spz-plus-CQ protocol. The data demonstrated that this role was minor because sterile immunity was obtained irrespective of the origin of CSP expressed by the parasites in this model of protection. The immunity was obtained through a single transient exposure of the host to the immunizing parasites (preerythrocytic and erythrocytic), a dose much smaller than that required for immunization with radiation-attenuated sporozoites.

Plasmodium species undergo an obligatory initial developmental stage in the liver that leads to the pathogenic erythrocytic phase of the infection. Effective inhibition of sporozoites (Spz) and hepatic parasites, the preerythrocytic (PE) stages, prevents blood infection and consequently disease and transmission. However, immunity against PE parasites must be total, because productive infection of a single hepatocyte can lead to a patent blood infection. Three vaccination protocols have been shown to confer sterile immunity (absence of blood stage parasites after challenge with sporozoites) against PE stages. First, immunization with radiation-attenuated sporozoites (RAS) has long been the gold standard for the induction of sterile immunity in rodents, monkeys, and humans (12, 19). Second, immunization with a smaller number of live sporozoites under chloroquine prophylaxis (Spz plus CQ) equally conferred sterile protection against sporozoite challenge in mice (2, 3). Recently, full protection was also obtained in human volunteers immunized by the bites of 15 Plasmodium falciparum-infected mosquitoes while under chloroquine prophylaxis (23). Finally, immunization with genetically attenuated sporozoites (GAS) was shown to be effective at fully protecting mice from sporozoite infections (16, 31). In hosts immunized with irradiated sporozoites, persistence of developmentally arrested early liver stages is thought to be important, because primaquine treatment abolishes protection (25). For vaccination with live sporozoites under drug cover, few if any hepatic parasites are expected to persist, but still, the liver parasites are necessary for the induction of immunity (3). In vaccination with GAS, liver parasite numbers waned rapidly, becoming undetectable 4 to 5 days postinoculation (16). In all three models of protection, antigens expressed during the liver stages appeared to be crucial for the induction and maintenance of sterile immunity. The circumsporozoite protein (CSP), a protein expressed by sporozoites and early liver forms, has long been considered the antigen that contributes most to the protective responses induced, justifying its status as the major vaccine candidate (reviewed in reference 18). Nonetheless, two recent studies demonstrated that protection in-
duced by RAS could be obtained in the absence of significant responses against CSP (10, 15). We wished to characterize the immune responses induced against CSP in mice immunized by Spz plus CQ and to ascertain whether they played an important role in the sterile protection obtained. In order to do so, we used two rodent malaria parasite species, *Plasmodium berghei* and *Plasmodium yoelii*, and a transgenic line of *P. berghei* in which the endogenous csp gene had been replaced by csp of *P. yoelii* (*P. berghei*PYC5)). In this manner, the contribution of CSP alone to protection could be assessed.

**MATERIALS AND METHODS**

*Plasmodium* sporozoites. BALB/c J female mice were purchased from Harlan Laboratories (Gannat, France) and were housed in a pathogen-free rodent facility. All experiments and procedures involving mice were approved by the Direction Départementale des Services Vétérinaires de Paris, France (authorization no. 75-129) and performed in compliance with the regulations of the French Ministry of Agriculture for animal experimentation (1997). *P. berghei* ANKA cloned line was used to derive *P. berghei*PYC5 (29) and the line of *P. berghei* ANKA expressing the green fluorescent protein line used in this study was submitted to the same selection procedures that were used to obtain the *P. berghei*PYC5 parasites used (7). The infectivity and development of *P. berghei*PYC5 sporozoites were similar to those of *P. berghei* sporozoites in the mosquito and in the mouse, as described previously (29). *Anopheles stephensi* mosquitoes were fed on infected mice and maintained for 15 days at 24°C in the case of the *P. yoelii* yoelii 17XL clone 1.1 line and for 21 days at 21°C for *P. berghei* strains before dissection of the salivary glands to isolate the sporozoites.

**Immunization and challenge.** Mice were injected intravenously (i.v.) with one dose of 20,000 sporozoites. One hundred microliters of an 8-μg/ml chloroquine hydrochloride (CQ) (Sigma) solution in phosphate-buffered saline (PBS) was injected intraperitoneally for 10 consecutive days, starting the same day as sporozoite inoculation, into both immunized and control mice. This regimen induced sterile immunity in 80 to 100% of the mice (3). Control and immunized mice were challenged intravenously with 100 *P. yoelii* Spz or 5,000 *P. berghei* Spz at least 15 days after the last injection of CQ. Because of differences in the infectivities of sporozoites from these *Plasmodium* species, the doses were chosen to induce infection in all control mice. Successful infection was determined by the presence of parasites in Giemsa-stained blood smears prepared daily from days 4 to 10 postchallenge, and parasitemia was determined by counting the number of infected red blood cells (RBC) per 1,000 erythrocytes.

**Peptides.** All peptides were obtained from NeoSystems (Strasbourg, France) and were produced according to the amino acid sequence shown in Fig. S1 in the supplemental material. Peptides Py3 ([QGPGAP]3) and Pb2 ([DPPPPNPN]2), corresponding, respectively, to the repeat regions of CSPs of *P. yoelii* and *P. berghei* (PYC5 and PbCSP) were used in enzyme-linked immunosorbent assays (ELISAs) and ELISPOT assays (ELISPOTs) (18). The following long peptides corresponding to NH2-terminal (Nt) and COOH-terminal (Ct) parts of the other two CSPs (kindly given by Giampietro Corradin, Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland) were used in ELISA and enzyme-linked immunosorbent (ELISPOT) assays: PyC5 long peptides, PyNT (N-terminal region, amino acid segment 20 to 138: PYGQGKSKVQARNLNYLNNHSNGKI NRVNVRLLGDANGKPEEKDKDPKDGNKDLPKKEEKDPNDKPPKDPPKNDPPKND) and PyCt (C-terminal region, amino acid segment 277 to 345: NEDYVYPSAEQIEFLVKQISSTLEWQQETSCVSGGVVRKK KNVKMPNLELTDIEDTCKDKCS); PbC5 long peptides, PbNT (amino acid segment 21 to 91: YQGGKNQSSAQARNLNYLNNHSNGKI NGKPYRNTVLRALLAFADEPQKKKEKKRNEKKL) and PbCt (amino acid segment 242 to 310: NDSDYISAEKIELFVKQIRDITSEWQQCNVTGSQGVKKRKGSNNKAATDDTDITEDTCKDKCS) (14). Lyophilized material was resuspended in sterile distilled water at 10 mg/ml, aliquoted, and stored at −20°C until it was used.

**ELISA.** Antibodies to *P. yoelii* and *P. berghei* CSP peptides (PyC5 and PbC5) were detected by ELISA, as previously described (10). Briefly, 96-well flat-bottom plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 1 μg/ml of peptide in PBS, pH 7.8, by overnight incubation at 4°C. After extensive washing, the wells were incubated with 1:1,000 dilution of mouse sera diluted 1/100 in PBS-Tween-BSA. After two washes, the wells were incubated for 45 min at room temperature, either with goat IgG anti-mouse IgM (Invitrogen) or with a biotinylated goat anti-mouse IgG (Jackson Immuno-Research) diluted in PBS-Tween. The wells containing the goat IgG anti-IgM antibody were washed and further incubated with a biotinylated rabbit anti-goat IgG (Sigma-Aldrich) diluted in PBS-Tween for 45 min at room temperature. The wells were then washed and incubated with extravidin-coupled alkaline phosphatase (Sigma-Aldrich) as a substrate, and the fluorescence at 550/460 nm was measured using a spectrophotometer (Victor 1420; Wallac Oy). Immunofluorescence antibody test (IFAT). Pooled sera from each group of mice immunized with Spz plus CQ were individually tested by immunofluorescence on wet sporozoites from the different *Plasmodium* lines to detect surface antigens, as previously described (21).

**ELISPOT assay.** Polysinuldena difluoride (PVDF) microplates (Millipore, Bedford, MA) were coated overnight at 4°C with 15 μg/ml of an anti-mouse gamma interferon (IFN-γ) rat monoclonal antibody (MAB) (clone AN18; Mabtech AB, Sophia Antipolis, France) diluted in PBS. After extensive washes and 2 h of incubation at 37°C with RPMI medium containing 10% fetal calf serum, 3 × 105 spleen cells isolated at least 15 days after the last CQ injection were incubated overnight with different peptides (final concentration, 10 μg/ml) and with 30 U/ml of recombinant human interleukin 2 (IL-2). The plates were then washed, incubated with 2 μg/ml of biotinylated anti-mouse IFN-γ rat monoclonal antibody (clone R4-6A2; Mabtech AB) diluted in PBS containing 0.5% bovine serum albumin for 2 h at 37°C and then overnight at 4°C. The plates were subsequently incubated with extravidin-coupled alkaline phosphatase (Sigma-Aldrich) diluted in PBS. After the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NBT) substrate (Sigma-Aldrich) was added, IFN-γ spot-forming cells were counted under a stereomicroscope and expressed as the number of spots per million tested cells.

**Quantification of the parasite loads in the livers of sporozoite-infected mice.** Quantification of the parasite loads in the livers of sporozoite-infected mice was performed as described previously (15). Briefly, mice were injected i.v. with 40,000 to 60,000 sporozoites at least 15 days after the last chloroquine injection. DNA was extracted from liver biopsy specimens collected 42 to 44 h after the sporozoite injection, and 50 to 100 ng of each DNA sample was used as a template in a real-time quantitative PCR assay, using specific primers for *P. yoelii* and *P. berghei* as described previously (15).

**Standard curves** were generated using a 10-fold dilution series (from 10−1 to 1 parasite nuclei/μl) of DNA solution purified from *P. yoelii* or *P. berghei* blood stages obtained from a sample in which the number of parasite nuclei per micrometer was determined accurately by microscopy examination of Giemsa-stained blood smears and calculation of the number of RBC/μl of blood. Genomic DNA, rather than a plasmid bearing a single-stranded rRNA (ssrRNA) gene, was used to generate the standard curve, because it reflects more accurately the multiple targets amplified, since there are more than 5 different ssrRNA genes in the genome of *Plasmodium*. One liver parasite load unit corresponds to the log number of parasite nuclei/μg of liver DNA. Log numbers were deduced from the linear portion of the standard calibration curve derived using incremental 10-fold amounts of genomic DNA. Statistical analyses of data were performed using a one-way analysis of variance (ANOVA) test, followed by Bonferroni’s posttest. Inhibition of liver stages was expressed as a percentage derived from the actual numbers of parasite nuclei/μg of liver DNA calculated from the log values.

**RESULTS**

The specificity of the Spz-plus-CQ immunization protocol was initially demonstrated using the rodent parasite *P. yoelii* 265 BY in BALB/c mice (3). The transgenic parasites we generated were *P. berghlei* ANKA parasites expressing the *P. yoelii* 1.1 CSP. In preliminary experiments, we established that full protection (80 to 100%) was also obtained in BALB/c mice after a single Spz-plus-CQ inoculation with 20,000 sporozoites of *P. berghlei* (data not shown) or of *P. yoelii* (3).

**Characterization of immune responses to CSP and to whole sporozoites.** PyC5 and PbC5 display significant homology outside the B-cell immunodominant central repeat region (see Fig. S1 in the supplemental material). Therefore, it was important to assess cross-reactive responses between the two heterologous CSPs, as well as the responses induced against
the homologous CSP by Spz-plus-CQ immunization. Three groups of mice (n = 5) were immunized with Spz plus CQ using either P. berghei (Pb), P. berghei[PyCS], or P. yoelii (Py) Spz plus CQ parasites. The resulting humoral responses were assessed by IFAT on whole sporozoites and by ELISA using CSP species-specific long peptides, one spanning the N terminus (Nt) and the other the C terminus (Ct), and short peptides representing the repeat region. T-cell responses were assessed in an IFN-γ ELISPOT assay using splenocytes restimulated by CSP long peptides containing all potential CD4+ and CD8+ epitopes (10). ELISA showed that the IgG responses were mainly directed against the CSP repeat region and, to a lesser extent, against the C terminus, whereas they were barely detectable against the CSP N terminus (Fig. 1). It was interesting that the responses against the PyCSP repeat region were higher in mice immunized with PbCSP than in those immunized with PyCSP. Some cross-reactive responses were found against the PbCSP C terminus in sera from mice immunized with a parasite expressing PyCSP, but cross-reactivity was not detected against PyCSP in the sera from P. berghei-immunized mice (Fig. 1, right). A similar profile was observed when IgM responses were assayed, except that in this case only low levels of antibodies reactive with the repeat region were found (see Fig. S2 in the supplemental material).

Cellular responses in mice (n = 5) against both the N and C termini of the homologous PbCSP were induced by immunization with P. berghei, with levels considerably higher against the C terminus (Fig. 2, top). In contrast, responses against the heterologous PyCSP long peptides were very low. Cellular responses in mice (n = 5) immunized with P. yoelii against both homologous N- and C-terminal PyCSP long peptides were detected but were low (Fig. 2, middle). Although cross-reactive responses against the PbCt peptide were found in some mice immunized with P. yoelii Spz, they did not significantly differ from those against the homologous P. yoelii peptides. In mice immunized with P. berghei[PyCS], cellular re-
responses were induced against the homologous PyCSP long peptides, with those against the C terminus higher than those against the N terminus (Fig. 2, bottom). It is interesting that the responses against the PyCt were higher in these mice than in those immunized with P. yoelii sporozoites (P < 0.05; Kruskal-Wallis test). In the P. berghei[PyCS]-immunized mice, cross-reactive responses were detected against only the PbCt peptide. Overall, the cellular responses induced against CSP were mainly directed against the C terminus and were low.

Given that CSP covers the sporozoite surface, we assessed the abilities of the different sera to recognize whole sporozoites from the three parasite lines (Fig. 3). IgG antibody responses against the homologous sporozoite induced by immunization with P. yoelii or P. berghei[PyCS] had a 4- to 5-fold-lower titer (≤1/800) than those induced following P. berghei immunization (>1/3,000). Although IgM responses were substantially less pronounced, they showed a broadly similar profile of reactivities (Fig. 3, right). Cross-reactive responses against the sporozoite surface were minimal. The fact that reactivity to the sporozoite surface was correlated with the type of CSP expressed strongly indicated that the bulk of antibody reactivities to the sporozoite surface were directed against CSP. For example, pooled sera from P. berghei-immunized animals (n = 5) recognized the P. berghei sporozoites with high titers (>1/3,000), whereas pooled sera from P. berghei[PyCS]-immunized animals (n = 5) failed to react with the P. berghei sporozoites.

**Protection experiments.** Having established that the humoral responses induced in the homologous combinations were predominantly directed against CSP and that anti-CSP T-cell responses were present, at least for P. berghei-immunized animals, we wanted to measure their effects on the outcome of challenges with live sporozoites. If the CSP responses induced were central to the sterile protection seen in this model, we would expect the mice immunized/challenged with sporozoites bearing identical CSPs to be equally fully protected irrespective of the parasite species of the challenge sporozoites, whereas immunization/challenge using sporozoites expressing the heterologous CSP would not lead to full protection. When these experiments were carried out, protection from infection was observed equally in all animal groups (5 mice per group) immunized with P. berghei or P. berghei[PyCS] sporozoites whether they were challenged with P. berghei sporozoites expressing the homologous or the heterologous CSP (Fig. 4). When challenged with P. yoelii sporozoites, 20% or fewer of the mice immunized with P. berghei or P. berghei[PyCS] sporozoites were protected. Mice immunized with P. yoelii sporozoites were fully protected from challenge with homologous sporozoites, whereas only 10% to 20% of those challenged with P. berghei or P. berghei[PyCS] sporozoites were protected (Fig. 4). These patterns of protection cannot be accounted for by the pattern of CSP cross-reactive
humoral or cellular responses induced by immunization. For example, similar levels of cross-reactive cellular responses were observed against the PbCt in mice immunized with P. yoelii or P. berghei[PyCS] (Fig. 2), but on challenge with P. berghei sporozoites, little protection was observed in the former and full protection in the latter (Fig. 4). When P. yoelii was the challenge parasite, we observed no cross-species protection in P. berghei-immunized mice. In mice immunized with P. berghei[PyCS], that bore the homologous CSP, 20% were protected from P. berghei and 99% in P. berghei[PyCS]-immunized mice. (Right) Liver parasite loads in immunized mice challenged with 40,000 P. yoelii sporozoites. The percent reductions versus the control group were 99% in mice immunized with P. yoelii, 48% in P. berghei-immunized mice, and 66% in mice immunized with P. berghei[PyCS] Spz plus CQ. * P < 0.05 using one-way ANOVA followed by the Bonferroni posttest. BLD, below level of detection.

DISCUSSION

All immunization protocols based on live sporozoites (attenuated or otherwise) have led to the induction of sterile protection. These observations, initially made for RAS in mice more than 40 years ago (19) and more recently for Spz plus CQ (2, 3) and GAS (16, 31), gained in importance and relevance to vaccine development when they were repeated in humans in the 1970s for RAS (4, 12, 22) and last year for Spz plus CQ (23). Clinical trials to test the efficacy of immunization with recently produced P. falciparum GAS (30) are imminent. In RAS-immunized hosts, the immunodominance of the humoral responses to the major sporozoite surface protein CSP (13) and the crucial cellular responses (18) directed at the hepatic parasite in which CSP is also expressed during the early stages have justified the selection and subsequent concerted focus on CSP as a vaccine candidate. The immunodominance of CSP was also observed in mice immunized with GAS. Here, we show that humoral responses against CSP are also dominant in animals immunized by Spz plus CQ.

The immunodominance of CSP notwithstanding, we have previously demonstrated, using a parasite of one species made transgenic for CSP of another species, that immune responses specifically induced against the CS by RAS immunization in mice did not contribute to the consequent sterile protection (10, 15). Here, we have demonstrated that the full protection induced by Spz plus CQ in inbred BALB/c mice is obtained with a minimal role for responses against CSP. In a single experiment, where outbred CD1 mice (5 mice per group) were immunized with P. berghei or P. berghei[PyCS] Spz and challenged with Spz of P. berghei, substantial sterile protection (60%) was observed in both groups, suggesting that the mouse background did not influence the outcome. This supports the conclusion that CSP is minimally involved in the protection induced by immunization with Spz plus CQ.

Cross-reactive responses against the different CSPs were induced in some combinations, as expected from the relative homology between the conserved domains of the gene in the two parasite species, but their presence could not account for the protection observed. It must be noted that we did not detect cross-species sterile protection in the Spz-plus-CQ model, as observed for the models of RAS (15, 26) or GAS (6). This clearly suggests that the immune mechanisms involved in protection differ between the models but are nevertheless independent of CSP.

In the course of this work, we observed that the levels of immune responses against CSP depended in part on the parasite genetic background in which the antigen was presented (the same CSP gene in P. berghei sporozoites or P. yoelii sporozoites induced different levels of T-cell responses and antibody
This phenomenon, though of immunological interest, does not affect the conclusions discussed above.

The demonstration that induced responses to CSP do not lead to full protection should not be taken as inimical to efforts to develop vaccines based on CSP. Indeed, such vaccine formulations have invariably shown some protective efficacy, culminating in RTS, S, where the association of CSP-containing particles with a powerful new-generation adjuvant has significantly reduced malaria morbidity in African adults and children (1) despite short-lived maintenance of sterile immunity (27). By comparing the results of this study with our previously published data using the same parasites but the RAS immunization protocol (15), it appears that CSP plays a less important role in the Spz-plus-CQ immunization protocol than in that based on RAS. A factor that we consider most likely to account for this difference concerns the levels and duration of exposure of CSP to the immune system, particularly when in the liver. CSP is continuously expressed by the Spz, whether normal or radiation attenuated, and by liver stage parasites, but not by blood stage parasites. In contrast to Spz that develop normally, liver stages derived from radiation-attenuated sporozoites can persist for long periods, during which they continue to express CSP (25). We suggest that this allows the induction of responses to CSP higher than those induced by a shorter, transient expression of the antigen.

Ultimately, the main conclusion from this work and our previous observations using another immunization scheme that also induces sterile immunity against sporozoite infection (10, 15) is that one or more antigens other than CSP are actually responsible for the induction of sterile immunity against preerythrocytic malaria parasites. Although other preerythrocytic antigens have been studied (TRAP, STARP, SALSA, LSA1, and LSA3 for P. falciparum) (11), the identities and natures of most antigens expressed by the sporozoites and, possibly more pertinently, by the hepatic parasite have remained inaccessible to detailed investigations until recently (9, 24, 28, 32). The implication of other preerythrocytic antigens in our Spz-plus-CQ immunization model would be important to investigate, using an approach similar to ours and those of others (5, 17, 20). Nevertheless, studies of preerythrocytic parasites remain technically challenging, and knowledge of the biology and immunology of these stages has consequently lagged behind that of the erythrocytic stages.

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