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Protective immunity induced by daily bites from irradiated mosquitoes infected with *Plasmodium yoelii*

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Summary

Individuals in malaria endemic regions do not develop fully protective immune responses against *Plasmodium* liver stage infections. In high transmission areas, individuals can be exposed to more than two infective mosquito bites daily. Their exposure to *Plasmodium* sporozoites, therefore, is in the form of small and frequent doses. This is very different from individuals studied in controlled immunization trials where the delivery of large numbers of radiation-attenuated sporozoites in a limited number of doses can induce sterile protective immunity. Using irradiated mosquitoes infected with *Plasmodium yoelii* 17XNL, we tested whether daily bites from a few mosquitoes can induce a protective immune response in mice. This immunization strategy successfully induced a protective response, preventing the development of liver stages when mice were challenged with non-irradiated sporozoites. These results provide further support for the development of liver stage vaccines. They are also a call for further study into why fully protective responses against the liver stage are not seen in individuals from endemic regions.

Keywords

liver stage malaria; parasite-protozoan; immunization; irradiated sporozoite; mosquito; *Plasmodium yoelii*

Introduction

Immunization with radiation-attenuated *Plasmodium* sporozoites provides complete protection against infection with non-irradiated sporozoites (1,2). Ever since this discovery, there has been a fervent search for a vaccine against malaria, a disease that kills one person every 30 seconds. Although the subunit vaccines against malaria are the furthest along in their clinical trials (3), there is an active effort to develop an attenuated sporozoite-based vaccine, as the protective immunity induced by attenuated sporozoites is stronger and more consistent than that induced by subunit immunization (1,4).

Although it is known that vaccination with large amounts of attenuated sporozoites can induce a sterile protective response, in endemic areas individuals suffer continuous re-infections throughout life, indicating that they lack sterile liver stage immunity. Even if some degree of liver stage immunity is acquired in endemic areas (5), the question still remains: why are individuals in the field not being fully protected against *Plasmodium* liver stages (6), especially

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when they can receive as many as two infective bites per day in areas of high transmission (7,8)?. Some studies have indicated that the development of *Plasmodium* blood-stage infection that follows after the liver infection interferes with the generation of adequate immune responses against *Plasmodium* liver stage (9-11)

Continuous deposition of small quantities of antigens into the skin leads to antigen-specific tolerance probably by regulating the balance between Th2 and Treg cells (12). *Plasmodium* sporozoites are deposited into the skin (13) where many of them remain and never reach the liver (14-16). In endemic areas, daily bites from infected mosquitoes are common and therefore, subjects must be exposed to continuous deposition of small quantities of antigens into the skin. We wanted to test whether continuous delivery of sporozoites may potentially induce tolerance to sporozoite antigens, similarly how subcutaneous allergen immunotherapy builds tolerance by providing low doses of the allergen under the skin (12). Using two different immunization strategies, we compared a three-dose regimen vaccination versus a daily immunization schedule with irradiated *P. yoelii*-infected mosquitoes. We found that both immunization protocols induce effective immune responses and protection.

Materials and Methods

Parasites, mosquitoes and mouse immunizations

Anopheles stephensi mosquitoes were maintained as described (17) and infected with *P. yoelii* 17XNL. Irradiated mosquitoes were generated by exposure to 12 krad (120 Gy) of γ -irradiation (MDS Nordion Gammacell 1000 Elite). Female BALB/c (NIH, Bethesda MD) were used for all experiments. For three-dose immunization regimen, each mouse was anesthetized using a cocktail of ketamine and xylazine, and 28–30 irradiated mosquitoes were allowed to feed for 15 minutes, with a repositioning of the mouse halfway through the feeding session. For daily immunizations, each mouse had two mosquitoes feed on her tail for 5 minutes. This was repeated daily for 5 weeks.

ELISPOT assay

Determination of individual IFN- γ -secreting T cells specific for the *H-2K^d* CD8 epitope of the circumsporozoite (CS) protein SYVPSAEQI of *P. yoelii* was done by ELISPOT (18). Spleen cells were harvested and erythrocytes were lysed using an ammonium-chloride-potassium lysing buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.3). Starting at 10⁶ cells per well, three-fold dilutions of the splenocytes were plated in ELISPOT plates (Millipore) in triplicate. To each well, 10⁵ A20.2J cells, which had been preincubated with the CS peptide, were added as antigen presenting cells for the splenocytes. The plates were then incubated at 37°C for 48 h before processing. The numbers of antigen-specific T cells were calculated by subtracting the mean spot numbers in triplicate control wells where splenocytes are incubated with A20.2J cells without peptide. Purified anti-mouse IFN- γ (R4) and biotinylated anti-mouse IFN- γ (XMG1.2) were obtained from BD Pharmingen. Plates were counted on a CTL ImmunoSpot Plate Reader (Series 3).

Immunofluorescence titration of serum antibodies

For titration of *P. yoelii*-specific antibody levels, 10⁵ salivary-gland sporozoites in each well were air-dried on glass multiwell IFA slides. Mouse serum was titrated and primary antibody bound to sporozoites was detected using FITC-labeled anti-mouse IgG (Sigma). A monoclonal CS-specific antibody (2F6) was used as a positive control.

Challenge and quantitative real-time PCR

Challenge of mice was performed in groups of three mice by i.v. injection of 10^5 (non-irradiated) *P. yoelii* sporozoites freshly dissected from mosquito salivary glands. Forty hours later, livers were harvested, total RNA was isolated, and malaria infection was quantified using reverse transcription followed by real-time PCR with primers that recognize *P. yoelii*-specific sequences within the 18S rRNA as previously described (19). Ten-fold dilutions of a plasmid construct containing the *P. yoelii* 18S rRNA gene were used to create a standard curve.

Statistical analysis

Data were analyzed using Prism (v. 4.0c, GraphPad). ANOVA or Student's t-tests were performed as mentioned. Statistics were considered significant if $P < 0.05$.

Results and Discussion

We compared the responses of mice to two different immunization strategies, both of which involved the delivery of sporozoites via mosquito bite. Using an immunization strategy previously shown to provide protection (20), the first group received three-dose immunizations, each two weeks apart, using 28–30 γ -irradiated mosquitoes infected with the non-lethal parasite *P. yoelii* 17XNL. For each round of immunization in this group, 28–30 irradiated mosquitoes were allowed to feed for 15 minutes on each mouse. In parallel, the second group received a immunization regimen of daily bites from two irradiated mosquitoes to mimic an entomological inoculation rate found in regions of very high transmission (7,8). For this group, each mouse had two mosquitoes feed on her tail for 5 minutes. This was repeated daily for 5 weeks (Fig. 1). In summary, both groups received the same number of infected mosquito bites. The use of irradiated mosquitoes was necessary to avoid the interference of blood-stage infection in the generated immune response (9).

A well-established correlate of protection against *Plasmodium* liver stage infections is the expansion of IFN- γ -expressing parasite-specific CD8 T cells in the spleens of protected mice (21,22). We therefore determined the number of IFN- γ -secreting CD8 T cells specific for the circumsporozoite (CS) protein of *P. yoelii* via an ELISPOT assay (18,23,24). As expected, the three-dose immunization regimen induced the splenic expansion of CD8 T cells specific for the CS epitope. Interestingly, mice that had received daily immunizations had an equally robust response (Fig. 2A). This result indicates that daily immunizations with low doses of irradiated sporozoites can indeed induce an immune response in mice.

Parasite specific antibodies are an indicator of a successful immunization against sporozoites (25). We therefore looked at the serum antibody titers elicited in the two groups of mice. We determined antibody titers through indirect immunofluorescence of *P. yoelii* sporozoites (26). Both groups of immunized mice had high serum antibody titers against *P. yoelii* sporozoites. Serum from naive mice, in contrast, failed to stain sporozoites even at the lowest dilution (Fig. 2B). A difference with low statistical significance was found between the two groups of immunized mice.

In order to correlate these cellular and humoral readouts against protection in our mice, we challenged a subset of the immunized mice with non-irradiated sporozoites and then determined the parasite load present in the liver. Three-dose regimen or daily-immunized mice were injected intravenously with 10^5 non-irradiated *P. yoelii* sporozoites isolated from the salivary glands of infected mosquitoes. The experiment was conducted twice, in the first experiment animals were challenged one day after completion of the immunization protocol and in the second experiment they were challenged five weeks after completion of immunization. Naive mice were also challenged, and their liver parasite load represented the

maximum level of sporozoite infection. Forty hours after injection, their livers were harvested and assayed for their parasite load through quantitative real-time PCR (19).

In both experiments all mice were protected (Fig. 3A). Though the mean level of protection provided by the daily immunization was slightly lower than that of the three-dose regimen group, this difference was not statistically significant. Notably, there were individual mice in both groups (all in the three-dose regimen group and one in the daily immunizations) that were completely protected, indicating that daily immunizations of small numbers of sporozoites have the potential to induce an equally successful protective response as the traditional three-dose immunization schedule. However, since we have not confirmed sterile protection by measuring pre-patent periods in these mice, we cannot exclude the possibility that very low levels of liver-stage replication that are not detected by real-time PCR could result in differences in the analysis of sterile protection between the two groups of immunized mice.

To see whether both immunization schedules had similar longevity in their protection capacity, we also performed a late protection assay, challenging a subset of mice with 10^5 non-irradiated sporozoites 5 weeks after the completion of their immunization regimen. As in the early challenge, both groups of immunized mice demonstrated effective protective responses to liver stage development that were not statistically different from each other (Fig. 3B).

In conclusion, daily immunization with small doses of sporozoites, delivered subcutaneously by mosquito bite, can provide a protective immune response similar to that provided by a three-dose immunization strategy. These results indicate that the continuous delivery of low doses of sporozoite antigens under the skin results in the generation of immunity, not tolerance, against *Plasmodium* liver stages.

Still, the cause of the low development of immunity against *Plasmodium* liver stages in the field is not fully understood. Some studies suggest that immunity induced by liver stages is later inhibited by the blood stage infection (9,10). Interestingly, it was found that in *P. vivax* endemic areas, individuals that do not develop blood stages because they do not have the Duffy antigen had significantly higher IFN- γ responses to sporozoite antigens, suggesting that blood-stage infections inhibit T cell responses to the liver stages (11). Though irradiated sporozoites reach the liver efficiently, they do not develop into blood-stage parasites and therefore this effect would not take place during our immunizations.

The importance of the skin as a location that determines antigen presentation and immunity against *Plasmodium* sporozoites has been recently recognized. After an infectious mosquito bite, a proportion of sporozoites stop at the proximal cutaneous lymph node (15,16) where dendritic cells prime the first CD8 T cells and launch the immune response (23). Our study indicates that the daily delivery of antigen under the skin does not induce tolerance against liver stages. This is relevant for the design of malaria vaccines, as it suggests that constant exposure to infected mosquito bites would not decrease immunity elicited by a vaccine against *Plasmodium* liver stages, but would rather enhance it.

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Abbreviations

CS, circumsporozoite; i.v., intravenous.

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Figure 1.

Immunization and experiment schedule. Mice were immunized by irradiated mosquito bite for 5 weeks, either through a three-dose or a daily immunization strategy. Splenocytes and serum were collected one week after the last dose or two days after the last daily immunization. Mice were challenged with non-irradiated sporozoites one day (early) or 5 weeks (late) after the last daily immunization and their livers were harvested 40 h after the challenge.

Figure 2.

Three-dose or daily immunizations with bites from infected irradiated mosquitoes induce comparable numbers of splenic IFN- γ -secreting parasite-specific CD8 T cells and parasite-specific antibodies. (A) ELISPOT results show IFN- γ -secreting CD8 T cells specific for the CD8 T cell *P. yoelii* CS epitope. Results are shown after subtracting the number of antigen-nonspecific IFN- γ spots. (B) Serum from immunized mice were serially diluted and used to stain *P. yoelii* sporozoites. The titers represent the inverse of the highest dilution at which sporozoites could still be detected (closed circles, •). Open circles (○) mark the lowest dilution tested, which still failed to stain sporozoites. Each circle represents an individual mouse. Error

bars represent standard deviation within groups of 3 mice (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when comparing naive and immunized mice using ANOVA).

Figure 3.

Daily immunizations with bites from infected irradiated mosquitoes can provide similar protection against the development of liver stages as a three-dose immunization strategy. Naive or immunized mice received 10^5 non-irradiated sporozoites one day (A) or 5 weeks (B) after the completion of their immunization schedules. The development of liver stages was quantified using real-time PCR for *P. yoelii* 18s rRNA on the livers of challenged mice. The data is presented as the percentage of protection provided by the immunization protocol when compared to the parasite load of challenged naive mice. Error bars represent standard deviation within groups of 3 mice (the differences between the two groups were statistically insignificant when using the Student's t-test).