EXPERIMENTAL TEST FOR PREMUNITION IN A LIZARD MALARIA PARASITE (*PLASMODIUM MEXICANUM*)

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ABSTRACT: Premunition in *Plasmodium* spp. is the prevention of superinfection by novel genotypes entering an already established infection in a vertebrate host. Evidence for premunition was sought for the lizard malaria parasite, *P. mexicanum*, in its natural host, the fence lizard, *Sceloporus occidentalis*. Clonal diversity (= alleles for the haploid parasite) was determined with the use of 3 microsatellite markers. Both naturally infected lizards (N = 25) and previously noninfected lizards (N = 78) were inoculated intraperitoneally (IP) with blood from donor infections and followed over a 3-mo period. Compared to the success of clonal establishment in all the naive lizards (78/78 successful), clones entering preexisting infections had a significant disadvantage (9/25 successful). The number of preexisting clones (1–2 vs. 3–4) within recipient infections had no effect on the success of superinfection. Infections that excluded entering novel clones did not have higher initial asexual parasitemia, but had a higher initial density of gametocytes, suggesting they were older. Infections allowing superinfection experienced a higher final parasitemia.

Parasites could reduce the cost of intraspecific competition within a host by excluding entry of additional conspecific parasite larvae (helminths) or genotypes/clones (protists). This process may be an adaptive manipulation of the host's immune response, or simply an incidental byproduct of normal host immune function (Brown and Grenfell, 2001). The effect is best known in the concomitant immunity of *Schistosoma* spp. infections (Smithers and Terry, 1967; Brown and Grenfell, 2001), but also seems likely in other helminths, such as filarial worms (MacDonald et al., 2002), and species of the protists, *Leishmania* (Anderson et al., 2005), *Toxoplasma* (Cesbron-Delauw et al., 1989), and *Plasmodium* (Smith et al., 1999).

In the case of *Plasmodium* spp., the blocking of superinfection by genotypes that are novel to a chronic infection is a more limited version of concomitant immunity and is termed *premunition* (Sergent and Parrott, 1935). Premunition has now been detected in an experimental study of the rodent parasite, *P. chabaudi*, in a mouse model system (de Roode et al., 2005) such that clones entering a mouse first have a competitive advantage over those arriving later in the experimental manipulations. The experimental protocol introduced parasites via infected blood into the host's circulation, indicating *P. chabaudi* premunition is, at least in part, a result of immune system response against blood stages (an additional response could attack incoming sporozoites or the forms in the liver). Chronic infections of *P. relictum*, with very low-level parasitemia, are also refractory to challenge with a novel strain even years after establishment of the original experimental infection, suggesting premunition is occurring for avian malaria parasites (Atkinson et al., 2001).

No studies have sought evidence for premunition in the malaria parasites of reptiles (approximately half the known *Plasmodium* species infect lizard hosts; Schall, 1996). Until recently, studies on the genetic diversity of reptile malaria parasites have not been possible because of the lack of known variable genetic markers to score clonal identity. Here, we test for premunition in the lizard malaria parasite, *P. mexicanum*, using recently described variable microsatellite markers for this parasite (Schall and Vardo, 2006). Identifying these markers allowed us to introduce novel genotypes of parasites into established, natural infections *of P. mexicanum*.

MATERIALS AND METHODS

We conducted the study at the University of California Hopland Research and Extension Center, near the town of Hopland in Mendocino County, California. Since 1978, a long-term study at the Hopland site has examined the biology of *P. mexicanum* and its hosts, the western fence lizard, *Sceloporus occidentalis*, and the sandfly vectors, *Lutzomyia vexator* and *L. stewardi* (Schall and Marghoob, 1995; Schall, 1996, 2002).

Lizards were collected by noosing, and a drop of blood was taken from each animal to make a thin blood smear for staining with Giemsa. Another drop of blood was dried on filter paper for later genetic studies. We scanned the blood smears and selected naturally infected lizards for use in the experiments. We determined parasitemia in these infections by counting the number of asexual cells (trophozoites and schizonts) and gametocytes in 1,000 erythrocytes, taking care to count cells and parasites from areas throughout the smear. The clonal diversity and identity of clones for all naturally infected lizards in the study were scored with the use of 3 microsatellite markers (Pmx306, 732, and 747; Schall and Vardo, 2006). DNA from a dried blood sample was extracted with the use of the DNeasy kit (Qiagen, Valencia, California), and the 3 loci amplified by PCR with the use of 1 florescent labeled primer for each locus. Primers and PCR conditions are given in Schall and Vardo (2006). Fragment sizes (alleles) were detected with an ABI 3130xl Genetic Analyzer and scored with the use of GeneMapper v3.7 software (ABI).

We collected male *S. occidentalis* not infected with *P. mexicanum* (N = 78) from areas of the field site where the parasite has been absent in the lizards throughout the long-term study (Schall and Marghoob, 1995; J. Schall, unpubl. obs.). Infections so weak that parasites are not seen during a 6 min scan of a smear are rare at Hopland (Perkins et al., 1998). However, we confirmed that these lizards were not infected by using a PCR-based method that can detect extremely low parasitemia infections (Vardo et al., 2005). Each noninfected lizard received an inoculation of infected whole blood IP containing approximately 200 × 10^3 parasites in 0.02 ml of blood/PBS (Osgood and Schall, 2003). Five infected donor lizards chosen as a source of blood for these inoculations harbored 1–3 clones (alleles) of parasites (alleles varied among both loci and donors).

A second group of naturally infected lizards was chosen to be experimental recipients (N = 25) and donors (N = 3). The 3 donors for these inoculations contained infections with 1, 1, and 3 clones over all loci.

Donor lizards were assigned to experimental lizards depending upon multilocus genotypes, with each donor having at least 1 unique allele compared to its recipient lizard. The experimental naturally infected lizards were inoculated in the same manner as the noninfected lizards, with approximately 200 × 10^3 asexual parasites in 0.02 ml blood/PBS injected IP into each infected recipient. Differing alleles in donor and
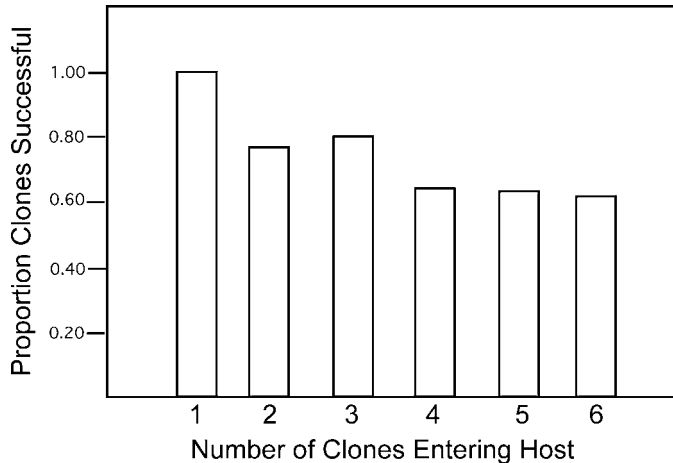
FIGURE 1.   Transmission success of genotypes (clones) of *Plasmodium mexicanum* resulting from experimental inoculation of whole blood from infected fence lizards, *Sceloporus occidentalis*, into previously noninfected lizards. Infections within the 5 donor lizards were genotyped for 3 microsatellite loci, and contained from 1 to 6 alleles (depending on infection and locus). The proportion of entering clones that became established (averaged over all loci) in 78 previously noninfected lizards is presented.

recipient lizards allowed us to determine if the challenging clones became established.

After inoculation, all recipient lizards were maintained in large outdoor cages, and fed daily with mealworms and crickets. Blood to make blood smears and dried blood dots for later genotyping were made every 10 days from toe clips. All collecting and experimental procedures followed a protocol approved by the University of Vermont Animal Care and Use Committee.

RESULTS

All 78 noninfected lizards that were inoculated with infected blood became infected after 1 mo (determined by examination of blood smears). Donors with single-clone infections (at all loci) established infections in all recipients and most clones in multiclone donor blood were also successful in becoming established in the recipients (Fig. 1). In contrast, transmission was successful for only 9 of 25 natural infections that were challenged with entry of novel genetic clones. Thus, transmission was far more likely to be successful if the lizard was not already infected with *P. mexicanum* ($\chi^2 = 59.1$, $P < 0.0001$). Recipients already infected with 1 or 2 clones (across all 3 loci) were compared with those with 3–4 clones; we found no difference in the ability of challenging novel clones to become infected (4/13 for recipients with 1 or 2 preexisting clones; 5/12 for recipients with 3–4 preexisting clones: Fisher exact test, $P = 0.688$).

Although the results suggest that novel clones are indeed prevented from establishment in already infected lizards, the outcome could also be the result of donor effects. That is, some infections (donors) may simply be poor at becoming established in any lizard. Two donors containing a single parasite clone were successful at establishing the parasite in noninfected lizards (16/16). Success of donors with a single clone to establish in already infected lizards was only 8/18 ($\chi^2 = 12.6$, $P = 0.0004$). For donors harboring 2 clones, establishment was successful in 15/15 noninfected lizards (with both clones becoming

established in 12/15 recipients), but only 1/7 infected recipients, with only 1 of the clones becoming established in that single successful transfer ($\chi^2 = 9.673$, $P = 0.0019$). Thus, transmission was successful into noninfected lizards for all donor infections, and transmission was poor for all donors used to supply blood for the naturally infected recipients.

Comparison of parasitemia of preexisting infections prior to introduction of the novel clones revealed no difference in numbers of asexual stages (trophozoites and schizonts) per 1,000 erythrocytes for successful and failed introductions (*U*-test, $P > 0.05$). However, the initial number of gametocytes and proportion of gametocytes among all parasites was greater for those recipients in which the introduced clones failed to become established than for the recipients that accepted the introduced clones. For introductions that failed, the initial range of gametocyte density was 0–12/1,000 RBC and 17–75% gametocytes, and for introductions that succeeded, gametocyte density ranged from 0 to 5/1,000 RBC and from 0% to 42% gametocytes (*U*-test for gametocyte parasitemia, $P = 0.0012$, and proportion of gametocytes, $P = 0.0002$). Final parasitemia was also lower for infections in which the novel clones failed to become established (median = 5, range = 1–16/1,000 RBC) versus those with successful establishment of the introduced clones (median = 12, range = 5–244/1,000 RBC) (*U*-test, $P = 0.022$).

DISCUSSION

The hypothesis of premunition in *Plasmodium* spp. infections proposes that established infections in the vertebrate host will hinder or exclude newly arriving novel genotypes, and genetically complex infections (multiclonal) would be more effective at hindering incoming genotypes than single-clone infections (Sergent and Parrott, 1935; Smith et al., 1999; de Roode et al., 2005).

We found that transmission by inoculation of whole infected blood into noninfected lizards was highly successful in establishing infection (all of 78 recipients became infected). When the donor infection was multiclonal, most clones became established in the recipients. However, already established infections were successful in limiting transmission of novel incoming clones, with approximately two-thirds resisting establishment of novel parasite genotype. Our results differ from previous proposals, though, because 1–2 clone infections were just as efficient in hindering the incoming clones as genetically more complex infections.

The natural infections we used had an unknown history prior to use in the experiment. The density of asexual-state parasites in the blood prior to the manipulation was not related to the success of incoming clones. However, infections with higher numbers and proportions of gametocytes were less likely to allow establishment of experimentally introduced novel clones. We suspect that these infections may have been older, because in both natural infections followed over time (Bromwich and Schall, 1986) and experimentally induced infections (Eisen and Schall, 2000) proportion of gametocytes in *P. mexicanum* infections generally increases with the duration of the infection. Older infections may have had more time to induce a lasting immune response from the host, which may, in turn, limit the success of newly arrived parasites. At the end of the experiment, the parasitemia of infections in which introduced clones

became established was greater than the resistant infections, similar to the increase in asexual replication noted for human malaria infections (Smith et al., 1999). It was not possible to determine if the overall higher rate of increase of the infections with established new clones was a result simply of the sum of all clones or if there were changes in the reproductive strategy of the genetically more complex infections.

The present study and that of de Roode et al. (2005) demonstrate that premunition in *Plasmodium* spp. is a result, at least in part, of processes taking place in the blood circulation, and some interference with the blood stages of the parasite. This effect most likely involves the host immune response and, if so, the *P. mexicanum* experiment provides rare evidence that reptiles mount an immune response to infection with malaria parasites. The only other evidence is the rapid destruction of host erythrocytes and appearance of immature red blood cells in circulation of infected lizards (Schall, 1990). Whether premunition observed for *P. mexicanum* represents an adaptive interference competition in which established parasites manipulate the host immune system is unknown, but an interesting possibility. Most natural infections we genotyped contained more than 1 clone of *P. mexicanum* (only 2 of the naturally infected lizards used as recipients contained a single genotype of parasites at all loci). If the effect seen in the experimental study occurs during natural transmission by the vector, this suggests that multiclonal infections become established primarily by nearly simultaneous bites of infected vectors or bites by vectors carrying multiple genotypes of parasite. Thus, premunition must play a role in the transmission biology of *P. mexicanum* and establishment of multiclonal infections.

ACKNOWLEDGMENTS

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