INFECTION AND IMMUNITY, Mar. 2008, p. 873–878 Vol. 76, No. 3
0019-9567/08/$08.00+0 doi:10.1128/IAI.00431-07

Cross-Species Immunity in Malaria Vaccine Development: Two, Three, or Even Four for the Price of One?▽

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For millennia, malaria has been one of the most deadly human infectious diseases in the world, and it is still the cause of about 2 million deaths per year, mainly among young children and pregnant women. Malaria is caused by protozoan parasites of the genus *Plasmodium*, four species of which are infectious for humans: *Plasmodium falciparum* (the most deadly species, responsible for 80% of infections worldwide and 90% of malaria-related deaths), *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* (56).

The "gold standard" for full and sterile immunity against malaria—immunization with *Plasmodium* whole-organism, radiation-attenuated sporozoites (RAS)—was described for mice, monkeys, and humans over three decades ago (12, 14, 45a). The original RAS immunization strategy, however, usually required either the bites of many irradiated mosquitoes on multiple occasions or intravenous inoculation of sporozoites, both of which were considered impractical for mass vaccination campaigns (36, 45a). The advent of recombinant DNA technology in the 1970s led many researchers to believe that the development of vaccines for the majority of known diseases, including malaria, was imminent (45). Although significant achievements in vaccinology have indeed been made using this technology (e.g., the pertussis vaccine [47]), the quest for a malaria vaccine remains unfulfilled. Several potential candidate vaccines have progressed to clinical trials recently (http://www.who.int/vaccine_research/documents/RainbowTable_ClinicalTrials_December2006.pdf), and many others have been the subject of preclinical assessments (24, 42; http://www.who.int/vaccine_research/documents/RainbowTablePreclinical_December2006.pdf). Disappointing results with either DNA- or subunit protein-based vaccines for malaria have led recently to a renewed effort focusing on scaled-up production of *P. falciparum* RAS and optimization of the immunization regimen for mass vaccination using this attenuated whole-organism-based approach (36; http://www.sanaria.com). In the latter context there have been other exciting recent developments. In rodent malaria models, both *Plasmodium berghei* genetically attenuated parasites (PbGAP) and *Plasmodium yoelii* GAP and *P. yoelii* sporozoites inoculated under cover of chloroquine (PyLUCS) have been shown to confer similar degrees of protection (2, 31, 44, 44b, 64). Together, these different types of whole-organism, live attenuated sporozoites constitute a powerful tool that should help to unravel the host mechanisms leading to protection, thus providing knowledge that may be crucial for development of the long-awaited vaccine. Intriguingly, however, the immunity mediated in mice by RAS and PbGAP (*p36p*$^-$ type) is not plasmodial species specific but in both cases confers protection against challenge infection with heterologous rodent plasmodial species (19, 45b).

Four different *Plasmodium* species are infectious for humans (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*). The first hint that sporozoites may induce cross-species immunity came from medical observations made in the middle of last century. At that time, inoculation of plasmodial parasites, either by injection of infected blood or by allowing sporozoite-infected mosquitoes to bite patients, was investigated as a treatment for neurosyphilis in Europe and the United States (11, 15, 27, 66). Reports of such treatments indicate that inoculation of *P. malariae* (either blood from infected donors or sporozoites), but not inoculation of *P. vivax* or *P. ovale*, resulted in reduction of parasitemia levels and the frequency of fever following subsequent infection with *P. falciparum* (15, 27). Based on these observations, Collins and Jeffery proposed that *P. malariae* might share common antigens with *P. falciparum* that elicit an immune response strong enough to affect the development of *P. falciparum* (15). Developing a vaccine that could induce simultaneous protection against *P. falciparum* and *P. vivax* or even more human plasmodial species would have enormous economic, safety, and manufacturing advantages. However, the issue of cross-species immunity in malaria, specifically in the context of live attenuated whole-organism-based protection, has been largely ignored in the last few decades. Here, we provide an overview of the available information concerning *Plasmodium* cross-species immunity and discuss the potential application of this information to the development of a vaccine for malaria.

CROSS-SPECIES IMMUNITY IN LIVER-STAGE MALARIA

Once injected into the mammalian host by an infected anopheline mosquito, plasmodial sporozoites make the journey via the blood vasculature to their target organ, the liver (1, 43, 44, 56). Once they are there, development proceeds within hepatocytes, with each sporozoite giving rise to a schizont that

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▽ Published ahead of print on 3 December 2007.

progressively matures until tens of thousands merozoites are formed (56). This intrahepatocytic schizont development is not associated with clinical symptoms.

Very little is known about the capacity of live attenuated human plasmodial sporozoites to induce cross-species immunity. In immunization studies with either *P. vivax* or *P. falciparum* RAS performed with human volunteers, no cross-species protection was reported (25). However, this conclusion relied on results for a single volunteer who was immunized six times via the bites of *P. falciparum* RAS-infected mosquitoes. This individual was then shown to be fully protected against challenge with intact *P. falciparum* sporozoites on three separate occasions, but not against a single challenge infection performed later with intact *P. vivax* sporozoites. Due to the obvious difficulties in conducting research tests with human volunteers, this type of experiment was not repeated. Given these conditions, it is therefore difficult to draw any firm conclusions concerning the existence of cross-species protection induced by *P. falciparum* and *P. vivax* RAS. Malarial sporozoites that infect other nonhuman mammalian hosts do, however, confer protection against challenge infection with sporozoites of a heterologous species. Mice inoculated with *P. falciparum* sporozoites, for example, were shown to be protected against subsequent challenge with *P. berghei* sporozoites (57). This is clear evidence that cross-species effects can be mediated by common malarial antigens; i.e., plasmodial proteins that share a high degree of homology can induce cross-reactive immune responses. The effects observed in the study cited above may have been due to sporozoite-specific antigens since *P. falciparum* sporozoites are unable to infect murine hepatocytes (54). Obviously, not all the antigens expressed by one species contribute to such a cross-species effect in different hosts. Sera of mice immunized with peptides comprising B-cell epitopes present in the central immunodominant region of circumsporozoite protein (CSP) of *P. yoelii*, an antigen that stimulates both humoral and cellular responses, were unable to inhibit *P. falciparum* intrahepatic development in human primary hepatocytes, for example (21).

The vast majority of our knowledge concerning cross-species immunity and immunization with live attenuated plasmodial sporozoites was derived from experiments performed with murine models. Almost 40 years ago, Nussenzweig and colleagues showed that *P. berghei* RAS-immunized mice were fully protected against challenge with either *Plasmodium chabaudi* or *Plasmodium vinckei* sporozoites (45b). Mice immunized with *P. chabaudi* RAS were also fully protected against challenge with *P. berghei* sporozoites (45c). The protection observed seemed to be partially dependent on a blood factor, most probably antibodies (45b). Our recent results showed that the immune response elicited by both *P. berghei* RAS and GAP (*p36p*$^-$) sporozoites provided partial protection against infection with *P. yoelii* (19). When two different mouse strains that were previously immunized with live attenuated *P. berghei* parasites were challenged with different doses of *P. yoelii* sporozoites, there were significant reductions in the parasite burdens in the livers of 99.9 and 94.6% of RAS- and *p36p*$^-$-immunized mice, respectively. Moreover, these immunized mice had longer prepatent periods (which increased from 0.7 to 3 days) and significantly lower peak parasitemias (which were more than 50% lower), and most remarkably, the majority of the RAS-immunized mice (but fewer *p36p*$^-$-immunized mice) displayed complete sterile protection. The absence of full protection in all mice contrasts with the findings of Nussenzweig and colleagues, but the discrepancy may reflect the different immunization and challenge doses, protocols, and mouse strains used (19, 45b, 45c). To the best of our knowledge, no investigations of the capacity of other GAP to induce cross-species immunity have been performed yet. In mice immunized with live attenuated *P. yoelii* parasites (PyLUCS) and challenged with *P. berghei*, the levels of parasitemia were similar to those of the control mice (50; L. Rénia, submitted for publication). Nevertheless, a small delay in the onset of blood-stage parasitemia was observed, possibly indicating low-level cross-species protection. Thus, cross-species protection can indeed be demonstrated in different murine malaria models, but it appears to depend on the experimental conditions used.

MECHANISMS OF SPOROZOITE-INDUCED CROSS-SPECIES IMMUNITY

In the absence of relevant detailed information, one can only speculate about the mechanisms that mediate cross-species protection, based on observations of experimental malaria in mouse models. It seems quite plausible that in the case of liver stages, CD8$^+$ T cells play a key role in this type of immunity. Together with other factors, these cells are essential for induction of RAS-, *uis3*$^-$ PbGAP-, and PyLUCS-induced homologous protection (2, 3, 17, 18, 29, 44c) and most probably also other GAP-induced immunity, although the latter remains to be confirmed. A cytotoxic T-cell clone obtained from a mouse immunized with *P. yoelii* RAS recognized an epitope on both *P. yoelii* and *P. berghei* CSP (67). This clone conferred protection to naïve mice against a challenge infection with either *P. berghei* or *P. yoelii* sporozoites, supporting the belief that cross-species immunity elicited by RAS indeed relies on CD8$^+$ T cells. In contrast, T-cell clones obtained from mice immunized with *P. yoelii* synthetic peptides corresponding to segments of CSP do not affect *P. berghei* sporozoite infection (49, 51, 53). However, immunization with whole-parasite RAS leads to a broader and more effective immune response than immunization with subunit vaccines, as discussed above. Most probably, the quantity and quality of T cells stimulated by RAS are enhanced, and therefore the cells are able to exert the species-transcending effect observed by Weiss and colleagues (67).

Apoptosis may play a prominent role in cross-species protection. It is known that in order to survive, plasmodial sporozoites prevent host cell apoptosis during their intrahepatocytic development (34, 63). We have shown that the capacity of *p36p*$^-$ PbGAP to prevent infected hepatocyte apoptosis, in turn, is severely impaired; such cells are eliminated from the liver very early during parasite development (64). Host cell apoptosis has also been shown to occur during the early stages of RAS development (35), and we observed a similar but more widespread outcome during *p36p*$^-$ PbGAP intrahepatocytic growth (64). Apoptotic infected host cells can potentially provide a huge array of intracellular pathogen-derived antigens to antigen-presenting cells (e.g., dendritic cells), as has been observed for *Plasmodium*, *Mycobacterium tuberculosis*, and *Salmonella* (35, 68). Thus, a plausible conclusion is that the heterologous protection against *P. yoelii* mediated by *p36p*$^-$

PbGAP might be at least partially the result of apoptotic infected hepatocytes. The murine plasmodial species are considered to be phylogenetically distantly related (46); however, *P. berghei* has average protein identities of 88.2% with *P. yoelii* and 83.2% with *P. chabaudi* (23), suggesting that several antigens common to *P. berghei* and to the two other murine plasmodia could be presented to the immune system via such an apoptotic event. The cross-protective response elicited by one attenuated parasite species would therefore be strong enough to act against a different species. We speculate that higher doses of RAS or *p36p*$^-$ PbGAP per immunization would increase the possibility of species-common antigen presentation, leading to complete sterile protection against *P. yoelii* and other murine plasmodia.

Antibodies are also crucial for RAS-induced immunity (55a) and, most probably, for live attenuated sporozoite-based cross-species immunity. Passive transfer of serum from *P. berghei* RAS-immunized mice was able to fully protect 11% of the mice challenged with *P. vinckei* (45a). The serum contained antibodies with specificity for *P. berghei* antigens that seemed to have considerable cross-reactivity with *P. chabaudi* and *P. vinckei* antigens (45a). Since RAS-induced homologous immunity is dependent on both cellular and antibody-based responses (17, 55a), one could predict that these responses must also be crucial for RAS-induced cross-species protection. The fact that the PyLUCS-mediated protective effect does not rely on antibodies (2) may then explain the poor capacity of this attenuation approach to confer cross-species protection. PbGAP *uis3*$^-$-mediated protection, additionally, does not rely on antibodies (44c). Cross-species protection experiments using this GAP should definitively elucidate the role of antibodies in this type of immunity. The role of antibodies in *uis4*$^-$, *p36p*$^-$, and *p36*$^-$*/p36p*$^-$ GAP-based immunity (either homologous or heterologous protection) is currently unknown.

CROSS-SPECIES IMMUNITY TO ASEXUAL BLOOD-STAGE PARASITES

Cross-species interactions are also observed with plasmodial asexual blood stages, but these interactions are poorly understood and have been less well studied than the cross-species protection associated with exoerythrocytic stages discussed above. In fact, the existence of cross-species immunity is contested (9, 41, 52); for example, it is argued that parasites coexisting within the same host tend toward antigenic diversity in order to promote their own survival, leading to species-specific immunity. In this context, the low level of homology between some human *Plasmodium* species' surface proteins (e.g., CSP and merozoite surface protein-1) theoretically should be an obstacle to the establishment of cross-species protection. In fact, a monoclonal antibody raised against *P. malariae* CSP does recognize sporozoites of *Plasmodium brasilianum*, a nonhuman primate plasmodial parasite, but not sporozoites of other human plasmodia (13). Moreover, sera from patients infected with *P. falciparum* do not recognize other human plasmodia (32).

In most areas where malaria is endemic, two or more human plasmodial species coexist (7). Therefore, indigenous populations are exposed to infection with different plasmodia from birth onwards. Evidence suggests that previous exposure to *P. vivax* or *P. malariae* leads to lower-than-expected rates of morbidity and mortality following exposure to *P. falciparum* (7, 22, 37–39, 58). Maitland and colleagues suggested that these outcomes are related to cross-species immunity (38), so an immune response induced by *P. vivax* and/or *P. malariae* may somehow affect the course of subsequent infection with *P. falciparum*. In addition, studies in the 1930s showed that simultaneous or sequential inoculation of blood containing erythrocytes infected with *P. falciparum* and *P. vivax* into human volunteers led to suppression of *P. vivax* blood-stage parasitemia and an increase in relapsing episodes (4–6). Moreover, sera from patients exposed to *P. vivax* do recognize several proteins in *P. falciparum* asexual stages, but not in gametes or zygotes (30). Observations in Papua New Guinea, where different plasmodia coexist, suggested that immunity to *P. vivax* develops much more quickly than immunity to *P. falciparum* (41), an indication that each species elicits primarily non-cross-reactive immune responses. Other field-derived data from areas where two or more human plasmodia coexist are generally inconsistent and do not allow firm conclusions to be drawn concerning naturally acquired immunity and its effect on cross-species protection.

Cross-protection against one plasmodial species induced by asexual blood stages of another has been observed in both avian (48, 60) and murine (16, 26, 33, 40) models. Primary infection with *P. chabaudi chabaudi AS*-infected erythrocytes led to protection against mortality and lower parasitemia levels after a challenge infection with a lethal strain of *P. yoelii* (33). Mice infected with blood-stage *P. berghei* parasites and later reinfected with *P. yoelii*-, *P. vinckei*-, or *P. chabaudi*-parasitized erythrocytes were partially protected against mortality (26, 40). Coinfection with *P. berghei* ANKA and *P. yoelii* (but not *P. vinckei*) led to inhibition of development of cerebral malaria and to abolition of CD8$^+$ T cells in brain blood vessels (65). As seen with exoerythrocytic stages, cross-species protection with plasmodial parasites that infect different hosts has also been observed (10, 69). Sera from mice highly infected with *P. chabaudi* (parasitemia, ≥50%), for example, inhibit the growth of the asexual blood stages of *P. falciparum* in vitro (10). Recently, a family of proteins with domains conserved between *P. falciparum* and *P. yoelii*, called merozoite released soluble particles, was described (69). These antigens induce a high level of cross-species reactivity, and mice immunized with *P. falciparum* merozoite released soluble particles exhibit a ~50-fold decrease in peak parasitemia when they are challenged with *P. yoelii*.

CONCLUSIONS

Past observations based on experimental (16, 26, 33, 40, 48) and clinical studies (4–6, 11, 15, 27) led us to conclude that there is at least a degree of cross-species immunity within a host coinfected by two or more plasmodial species. Field data, however, are inconclusive on this issue (22, 37–39, 41). The fact that such immunity can indeed be induced experimentally via immunization with live attenuated parasites has been demonstrated with *P. berghei* RAS and *p36p*$^-$ GAP (19, 45b).

Developing a vaccine for malaria is one of the most sought-after goals in biomedical research. So far, protein subunit- and/or DNA-based vaccines have had only limited

success (24, 42), while immunization strategies using *P. falciparum* RAS have, until very recently at least, been considered a practical impossibility due to technical, clinical, and logistical hurdles (36). The reproducibility and safety of the irradiation process are considered two of the most important and contentious issues, since an overdose kills the sporozoites, with a resulting failure to generate protective immunity, while underirradiated parasites may remain infective (59, 62). The possibility that these hurdles may yet be overcome is evident from the large quantities of aseptic, metabolically active, nonreplicating *P. falciparum* RAS already produced (36; http://www.sanaria.com). This RAS-based vaccine, produced under good manufacturing practice, is due to enter clinical trials in 2008 (S. L. Hoffman, personal communication). The possibility of replacing RAS with GAP potentially represents a significant step in enhancing the safety of such a live whole-organism vaccine. Orthologues of the different GAP (*p36*$^-$, *p36p*$^-$, *uis3*$^-$, *uis4*$^-$) in *P. falciparum* and *P. vivax* have already been described (28, 39a, 61), suggesting that it should be possible to perform this type of genetic modification in human plasmodia, thus avoiding the known hazards of radiation. Another way to circumvent this safety issue might be to use this RAS-based technology not with *P. falciparum* but with a less virulent species, such as *P. vivax* or even *P. malariae*. One of the safety issues would thus be overcome (since any disease-related aspects would be of much less consequence than those with *P. falciparum*); clearly, if a vaccine based on any one of these species can be shown to be effective against any or all of the others, there would be enormous economic advantages. Manufacturing costs would be lower, since for the price of production of a vaccine for one species, vaccines for the other species could be developed. If a vaccine based on cross-species immunity does not prevent *P. falciparum* infection per se but nevertheless proves to be effective in preventing mortality, morbidity, and disease-related symptoms, it would still represent a significant achievement that should be strongly encouraged. The argument that the human plasmodial species are phylogenetically distantly related (20) and that therefore no degree of cross-species protection can be expected, can be countered by evidence from rodent models using live attenuated sporozoites of plasmodia that are also considered to be distantly related (46) but that are nevertheless still able to induce cross-species protection (19, 45b). Several advances in in vitro culture methods have been reported recently (30, 55). These advances may allow evaluation of such vaccines in vitro at an earlier stage than is currently possible, with potential savings in both time and resources for development decisions. It has been suggested that a single-species vaccine for *P. falciparum* could encourage infection and disease due to other malarial parasites that are usually suppressed by *P. falciparum* (7). In the past, during malaria control programs for example, reduction of *P. falciparum* infections was found to be followed by an increase in *P. malariae* cases (8). The development of a vaccine that is effective against multiple species would obviously be expected to preclude such issues, since it should act against all plasmodia infecting a given individual.

In conclusion, then, we consider it strategically important that the possibility of induction of cross-species immunity should be considered and evaluated using the whole-organism approach to malaria vaccine design. With this in mind, further research on the immunological mechanisms involved in induction and maintenance of heterologous antiplasmodial protection by attenuated parasites is clearly warranted.

ACKNOWLEDGMENTS

We thank Stephen L. Hoffman and Laurent Rénia for sharing their unpublished data and for their advice.

M.M.M. was a recipient of a EURYI Award from the European Science Foundation and is a Howard Hughes Medical Institute International Scholar.

REFERENCES

1. **Amino, R., S. Thiberge, B. Martin, S. Celli, S. Shorte, F. Frischknecht, and R. Ménard.** 2006. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat. Med. **12:**220–224.
2. **Belnoue, E., F. T. Costa, T. Frankenberg, A. M. Vigário, T. Voza, N. Leroy, M. M. Rodrigues, I. Landau, G. Snounou, and L. Rénia.** 2004. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J. Immunol. **172:**2487–2495.
3. **Berenzon, D., R. J. Schwenk, L. Letellier, M. Guebre-Xabier, J. Williams, and U. Krzych.** 2003. Protracted protection to *Plasmodium berghei* malaria is linked to functionally and phenotypically heterogeneous liver memory CD8$^+$ T cells. J. Immunol. **171:**2024–2034.
4. **Boyd, M., and S. F. Kitchen.** 1937. Recurring clinical activity in infections with the McCoy strain of *Plasmodium vivax.* Am. J. Trop. Med. **17:**833–843.
5. **Boyd, M., and S. F. Kitchen.** 1937. Simultaneous inoculation with *Plasmodium vivax* and *Plasmodium falciparum.* Am. J. Trop. Med. **17:**855–861.
6. **Boyd, M., and S. F. Kitchen.** 1938. Vernal vivax activity in persons simultaneously inoculated with *Plasmodium vivax* and *Plasmodium falciparum.* Am. J. Trop. Med. **18:**505–514.
7. **Bruce, M. C., and K. P. Day.** 2003. Cross-species regulation of *Plasmodium* parasitemia in semi-immune children from Papua New Guinea. Trends Parasitol. **19:**271–277.
8. **Bruce-Chwatt, L.** 1963. A longitudinal survey of natural malaria infection in a group of West African adults. West Afr. Med. J. **12:**199–217.
9. **Butcher, G.** 1998. Cross-species immunity in malaria. Parasitol. Today **14:** 166.
10. **Butcher, G. A., and I. A. Clark.** 1990. The inhibition of Plasmodium falciparum growth in vitro by sera from mice infected with malaria or treated with TNF. Parasitology **101:**321–326.
11. **Ciuca, M., A. Soflete, E. Teriteanu, P. Constantinescu, and P. Cortez.** 1953. Effect of combined chloroquine and plasmocid therapy on various *Plasmodium* species inoculated for therapeutic purposes. Bul. Stiint. Sect. Stiinte Med. Acad. Repub. Pop. Romane **5:**381–392.
12. **Clyde, D. F., H. Most, V. C. McCarthy, and J. Vanderberg.** 1973. Immunization of man against sporozoite-induced falciparum malaria. Am. J. Med. Sci. **266:**398–403.
13. **Cochrane, A. H., W. E. Collins, and R. S. Nussenzweig.** 1984. Monoclonal antibody identifies circumsporozoite protein of *Plasmodium malariae* and detects a common epitope on *Plasmodium brasilianum* sporozoites. Infect. Immun. **45:**592–595.
14. **Collins, W. E., and P. G. Contacos.** 1972. Immunization of monkeys against Plasmodium cynomolgi by X-irradiated sporozoites. Nat. New Biol. **236:**176–177.
15. **Collins, W. E., and G. M. Jeffery.** 1999. A retrospective examination of sporozoite- and trophozoite-induced infections with *Plasmodium falciparum* in patients previously infected with heterologous species of Plasmodium: effect on development of parasitologic and clinical immunity. Am. J. Trop. Med. Hyg. **61:**36–43.
16. **Cox, F. E.** 1970. Protective immunity between malaria parasites and piroplasms in mice. Bull. W. H. O. **43:**325–336.
17. **Doolan, D. L., and S. L. Hoffman.** 2000. The complexity of protective immunity against liver stage malaria. J. Immunol. **165:**1453–1462.
18. **Doolan, D. L., and S. L. Hoffman.** 1999. IL-12 and NK cells are required for antigen-specific adaptive immunity against malaria initiated by CD8$^+$ T cells in the *Plasmodium yoelii* model. J. Immunol. **2:**884–892.
19. **Douradinha, B., M. R. van Dijk, R. Ataide, G.-J. van Gemert, J. Thompson, J.-F. Franetich, D. Mazier, A. J. F. Luty, R. Sauerwein, C. J. Janse, A. P. Waters, and M. M. Mota.** 2007. Genetically attenuated P36p-deficient Plasmodium berghei sporozoites confer long-lasting and partial cross-species protection. Int. J. for Parasitol. **37:**1511–1519.
20. **Escalante, A., and F. Ayala.** 1994. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proc. Natl. Acad. Sci. USA **91:**11373–11377.

21. **Franke, E. D., S. L. Hoffman, J. B. Sacci, Jr., R. Wang, Y. Charoenvit, E. Appella, R. Chesnut, J. Alexander, M.-F. del Guercio, and A. Sette.** 1999. Pan DR binding sequence provides T-cell help for induction of protective antibodies against Plasmodium yoelii sporozoites. Vaccine **17:**1201–1205.
22. **Gunewardena, D. M., R. Carter, and K. N. Mendis.** 1994. Patterns of acquired anti-malarial immunity in Sri Lanka. Mem. Inst. Oswaldo Cruz **89:** 63–65.
23. **Hall, N., M. Karras, J. D. Raine, J. M. Carlton, T. W. A. Kooij, M. Berriman, L. Florens, C. S. Janssen, A. Pain, G. K. Christophides, K. James, K. Rutherford, B. Harris, D. Harris, C. Churcher, M. A. Quail, D. Ormond, J. Doggett, H. E. Trueman, J. Mendoza, S. L. Bidwell, M.-A. Rajandream, D. J. Carucci, J. R. Yates III, F. C. Kafatos, C. J. Janse, B. Barrell, C. M. R. Turner, A. P. Waters, and R. E. Sinden.** 2005. A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. Science **307:**82–86.
24. **Hill, A. V.** 2006. Pre-erythrocytic malaria vaccines: towards greater efficacy. Nat. Rev. Immunol. **6:**21–32.
25. **Hoffman, S. L., L. M. Goh, T. C. Luke, I. Schneider, T. P. Le, D. L. Doolan, J. Sacci, P. de la Vega, M. Dowler, C. Paul, D. M. Gordon, J. A. Stoute, L. W. Church, M. Sedegah, D. G. Heppner, W. R. Ballou, and T. L. Richie.** 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J. Infect. Dis. **185:**1155–1164.
26. **Jarra, W., and K. N. Brown.** 1985. Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection. Parasite Immunol. **7:**595–606.
27. **Jeffery, G. M.** 1966. Epidemiological significance of repeated infections with homologous and heterologous strains and species of *Plasmodium.* Bull. W. H. O. **35:**873–882.
28. **Kaiser, K., K. Matuschewski, N. Camargo, J. Ross, and S. H. Kappe.** 2004. Differential transcriptome profiling identifies *Plasmodium* genes encoding pre-erythrocytic stage-specific proteins. Mol. Microbiol. **55:**1221–1232.
29. **Krzych, U., R. Schwenk, M. Guebre-Xabier, P. Sun, D. Palmer, K. White, and I. Chalom.** 2000. The role of intrahepatic lymphocytes in mediating protective immunity induced by attenuated *Plasmodium berghei* sporozoites. Immunol. Rev. **174:**123–134.
30. **Kumar, N., J. Perez Folgar, and P. Lubega.** 1992. Recognition of Plasmodium falciparum asexual stage antigens by antibodies in sera from people exposed to Plasmodium vivax. Am. J. Trop. Med. Hyg. **47:**422–428.
31. **Labaied, M., A. Harupa, R. F. Dumpit, I. Coppens, S. A. Mikolajczak, and S. H. I. Kappe.** 2007. *Plasmodium yoelii* sporozoites with simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against infection. Infect. Immun. **75:**3758–3768.
32. **Lee, M., and C. Lambros.** 1988. The Elisa-U: an enzyme-linked immunosorbent assay using urease as the enzyme marker for rapid detection of Plasmodium falciparum antibody in human serum. Am. J. Trop. Med. Hyg. **39:**421–426.
33. **Legorreta-Herrera, M., M. L. Ventura-Ayala, R. N. Licona-Chavez, I. Soto-Cruz, and F. F. Hernandez-Clemente.** 2004. Early treatment during a primary malaria infection modifies the development of cross immunity. Parasite Immunol. **26:**7–17.
34. **Leirião, P., S. S. Albuquerque, S. Corso, G. J. van Gemert, R. W. Sauerwein, A. Rodriguez, S. Giordana, and M. M. Mota.** 2005. HGF/MET signalling protects *Plasmodium*-infected host cells from apoptosis. Cell. Microbiol. **7:**603–609.
35. **Leirião, P., M. M. Mota, and A. Rodriguez.** 2005. Apoptotic *Plasmodium*-infected hepatocytes provide antigens to liver dendritic cells. J. Infect. Dis. **191:**1576–1581.
36. **Luke, T. C., and S. L. Hoffman.** 2003. Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated Plasmodium falciparum sporozoite vaccine. J. Exp. Biol. **206:**3803–3808.
37. **Maitland, K., T. N. Williams, S. Bennett, C. I. Newbold, T. E. Peto, J. Viji, R. Timothy, J. B. Clegg, D. J. Weaterall, and D. K. Bowden.** 1996. The interaction between *Plasmodium falciparum* and *P. vivax* in children on Espiritu Santo Island, Vanuatu. Trans. R. Soc. Trop. Med. Hyg. **90:**614–620.
38. **Maitland, K., T. N. Williams, and C. I. Newbold.** 1997. *Plasmodium vivax* and *P. falciparum*: biological interactions and the possibility of cross-species immunity. Parasitol. Today **13:**227–231.
39. **Maitland, K., T. N. Williams, T. E. Peto, K. P. Day, J. B. Clegg, D. J. Weaterall, and D. K. Bowden.** 1997. Absence of malaria-specific mortality in children in an area of hyperendemic malaria. Trans. R. Soc. Trop. Med. Hyg. **91:**562–566.

39a. **Matuschewski, K., J. Ross, S. M. Brown, K. Kaiser, V. Nussenzweig, and S. H. Kappe.** 2002. Infectivity-associated changes in the transcriptional repertoire of the malaria parasite sporozoite stage. J. Biol. Chem. **277:**41948–41953.

40. **McColm, A. A., and L. Dalton.** 1983. Heterologous immunity in rodent malaria: comparison of the degree of cross-immunity generated by vaccination with that produced by exposure to live infection. Ann. Trop. Med. Parasitol. **77:**355–377.
41. **Michon, P., J. L. Cole-Tobian, E. Dabod, S. Schoepflin, J. Igu, M. Susapu, N. Tarongka, P. A. Zimmerman, J. C. Reeder, J. G. Beeson, L. Schofield, C. L. King, and I. V. O. Mueller.** 2007. The risk of malarial infections and disease in Papua New Guinean children. Am. J. Trop. Med. Hyg. **76:**997–1008.
42. **Moorthy, V. S., M. F. Good, and A. V. Hill.** 2004. Malaria vaccine developments. Lancet **363:**150–156.
43. **Mota, M. M., J. C. Hafalla, and A. Rodriguez.** 2002. Migration through host cells activates *Plasmodium* sporozoites for infection. Nat. Med. **8:**1318–1322.
44. **Mota, M. M., G. Pradel, J. P. Vanderberg, J. C. R. Hafalla, U. Frevert, R. S. Nussenzweig, V. Nussenzweig, and A. Rodriguez.** 2001. Migration of Plasmodium sporozoites through cells before infection. Science **291:**141–144.

44a. **Mueller, A. K., M. Labaied, S. H. Kappe, and K. Matuschewski.** 2005. Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. Nature **433:**164–167.

44b. **Mueller, A. K., N. Camargo, K. Kaiser, C. Andorfer, U. Frevert, K. Matuschewski, and S. H. Kappe.** 2005. Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface. Proc. Natl. Acad. Sci. USA **102:**3022–3027.

44c. **Mueller, A. K., M. Deckert, K. Heiss, K. Goetz, K. Matuschewski, and D. Schlüter.** 2007. Genetically attenuated Plasmodium berghei liver stages persist and elicit sterile protection primarily via CD8 T cells. Am. J. Pathol **171:**107–115.

45. **Nabel, G.** 2004. Genetic, cellular and immune approaches to disease therapy: past and future. Nat. Med. **10:**135–141.

45a. **Nussenzweig, R. S., J. Vanderberg, H. Most, and C. Orton.** 1967. Protective immunity produced by the injection of X-irradiated sporozoites of Plasmodium berghei. Nature **216:**160–162.

45b. **Nussenzweig, R. S., J. P. Vanderberg, H. Most, and C. Orton.** 1969. Specificity of protective immunity produced by X-irradiated Plasmodium berghei sporozoites. Nature **222:**488–489.

45c. **Nussenzweig, R. S., J. Vanderberg, G. L. Spitalny, C. I. O. Rivera, C. Orton, and H. Most.** 1972. Sporozoite-induced immunity in mammalian malaria. A review. Am. J. Trop. Med. Hyg. **21:**722–728.

46. **Perkins, S., I. Sarkar, and R. Carter.** 2007. The phylogeny of rodent malaria parasites: simultaneous analysis across three genomes. Infect. Genet. Evol **7:**74–83.
47. **Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, et al.** 1989. Mutants of pertussis toxin suitable for vaccine development. Science **246:**497–500.
48. **Redmond, W. B.** 1939. The cross-immune relationship of various strains of *Plasmodium cathemerium* and *P. relictum.* J. Infect. Dis. **64:**273–287.
49. **Renia, L., D. Grillot, M. Marussig, G. Corradin, F. Miltgen, P. H. Lambert, D. Mazier, and G. Del Giudice.** 1993. Effector functions of circumsporozoite peptide-primed $CD4^+$ T cell clones against Plasmodium yoelii liver stages. J. Immunol. **150:**1471–1478.
50. **Rénia, L., A. C. Gruner, M. Mauduit, and G. Snounou.** 2006. Vaccination against malaria with live parasites. Exp. Rev. Vaccines. **5:**473–481.
51. **Renia, L., M. S. Marussig, D. Grillot, S. Pied, G. Corradin, F. Miltgen, G. D. Giudice, and D. Mazier.** 1991. In vitro activity of $CD4^+$ and $CD8^+$ T lymphocytes from mice immunized with a synthetic malaria peptide. Proc. Natl. Acad. Sci. USA **88:**7963–7967.
52. **Richie, T. L.** 1988. Interactions between malaria parasites infecting the same vertebrate host. Parasitology **96:**607–639.
53. **Romero, P., J. L. Maryanski, G. Corradin, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala.** 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature **341:**323–326.
54. **Sacci, J. B., Jr., M. E. Schriefer, J. H. Resau, R. A. Wirtz, L. J. Detolla, Jr., R. B. Markham, and A. F. Azad.** 1992. Mouse model for exoerythrocytic stages of Plasmodium falciparum malaria parasite. Proc. Natl. Acad. Sci. USA **89:**3701–3705.
55. **Sattabongkot, J., N. Yimamnuaychoke, S. Leelaudomlipi, M. Rasameesoraj, R. Jenwithisuk, R. E. Coleman, R. Udomsangpetch, L. Cui, and T. G. Brewer.** 2006. Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites *Plasmodium falciparum* and *P. vivax.* Am. J. Trop. Med. Hyg. **74:**708–715.

55a. **Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig.** 1987. Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. Nature **330:**664–666.

56. **Sherman, I. W.** 1998. Malaria: parasite biology, pathogenesis, and protection. American Society for Microbiology Press, Washington, DC.
57. **Sina, B. J., V. E. do Rosário, G. Woollett, K. Sakhuja, and M. R. Hollindale.** 1993. *Plasmodium falciparum* sporozoite immunization protects against *Plasmodium berghei* sporozoite infection. Exp. Parasitol. **77:**129–135.
58. **Smith, T., B. Genton, K. Baea, N. Gibson, A. Narara, and M. P. Alpers.** 2001. Prospective risk of morbidity in relation to malaria infection in an area of high endemicity of multiple species of *Plasmodium.* Am. J. Trop. Med. Hyg. **64:**262–267.
59. **Suhrbier, A., L. A. Winger, E. Castellano, and R. E. Sinden.** 1990. Survival and antigenic profile of irradiated malarial sporozoites in infected liver cells. Infect. Immun. **58:**2834–2839.
60. **Taliaferro, W. H.** 1939. Immunity to the malaria infections, p. 941–942. *In* M. Boyd (ed.), Malariology. W. B. Saunders & Co., Philadelphia, PA.
61. **Thompson, J., C. J. Janse, and A. P. Waters.** 2001. Comparative genomics in

Plasmodium: a tool for the identification of genes and functional analysis. Mol. Biochem. Parasitol. **118:**147–154.
62. **Vanderberg, J. P., R. S. Nussenzweig, H. Most, and C. G. Orton.** 1968. Protective immunity produced by the injection of X-irradiated sporozoites of Plasmodium berghei. II. Effects of radiation on sporozoites. J. Parasitol. **54:**1175–1180.
63. **van de Sand, C., S. Horstmann, A. Schmidt, A. Sturm, S. Bolte, A. Krueger, M. Lutgehetmann, J. M. Pollok, C. Libert, and V. T. Volker.** 2005. The liver stage of *Plasmodium berghei* inhibits host cell apoptosis. Mol. Microbiol. **58:**731–742.
64. **van Dijk, M. R., B. Douradinha, B. Franke-Fayard, V. Heussler, M. W. van Dooren, B. van Schaijk, G. J. van Gemert, R. W. Sauerwein, M. M. Mota, A. P. Waters, and C. J. Janse.** 2005. Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. Proc. Natl. Acad. Sci. USA **102:**12194–12199.
65. **Voza, T., A. M. Vigário, E. Belnoue, A. C. Gruner, J. C. Deschemin, M. Kayibanda, F. Delmas, C. J. Janse, B. Franke-Fayard, A. P. Waters, I. Landau, G. Snounou, and L. Rénia.** 2005. Species-specific inhibition of cerebral malaria in mice coinfected with *Plasmodium* spp. Infect. Immun. **73:**4777–4786.
66. **Weijer, C.** 1999. Another Tuskegee? Am. J. Trop. Med. Hyg. **61:**1–3.
67. **Weiss, W. R., J. A. Berzofsky, R. A. Houghten, M. Sedegah, M. Hollindale, and S. L. Hoffman.** 1992. A T cell clone directed at the circumsporozoite protein which protects mice against both *Plasmodium yoelii* and *Plasmodium berghei.* J. Immunol. **149:**2103–2109.
68. **Winau, F., S. H. Kaufmann, and U. E. Schaible.** 2004. Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria. Cell. Microbiol. **6:**599–607.
69. **Xu, L., X. Pei, K. Berzins, and A. Chaudhuri.** 2007. Plasmodium yoelii: experimental evidences for the conserved epitopes between mouse and human malaria parasite, Plasmodium falciparum. Exp. Parasitol. **116:**214–224.

Editor: J. B. Kaper