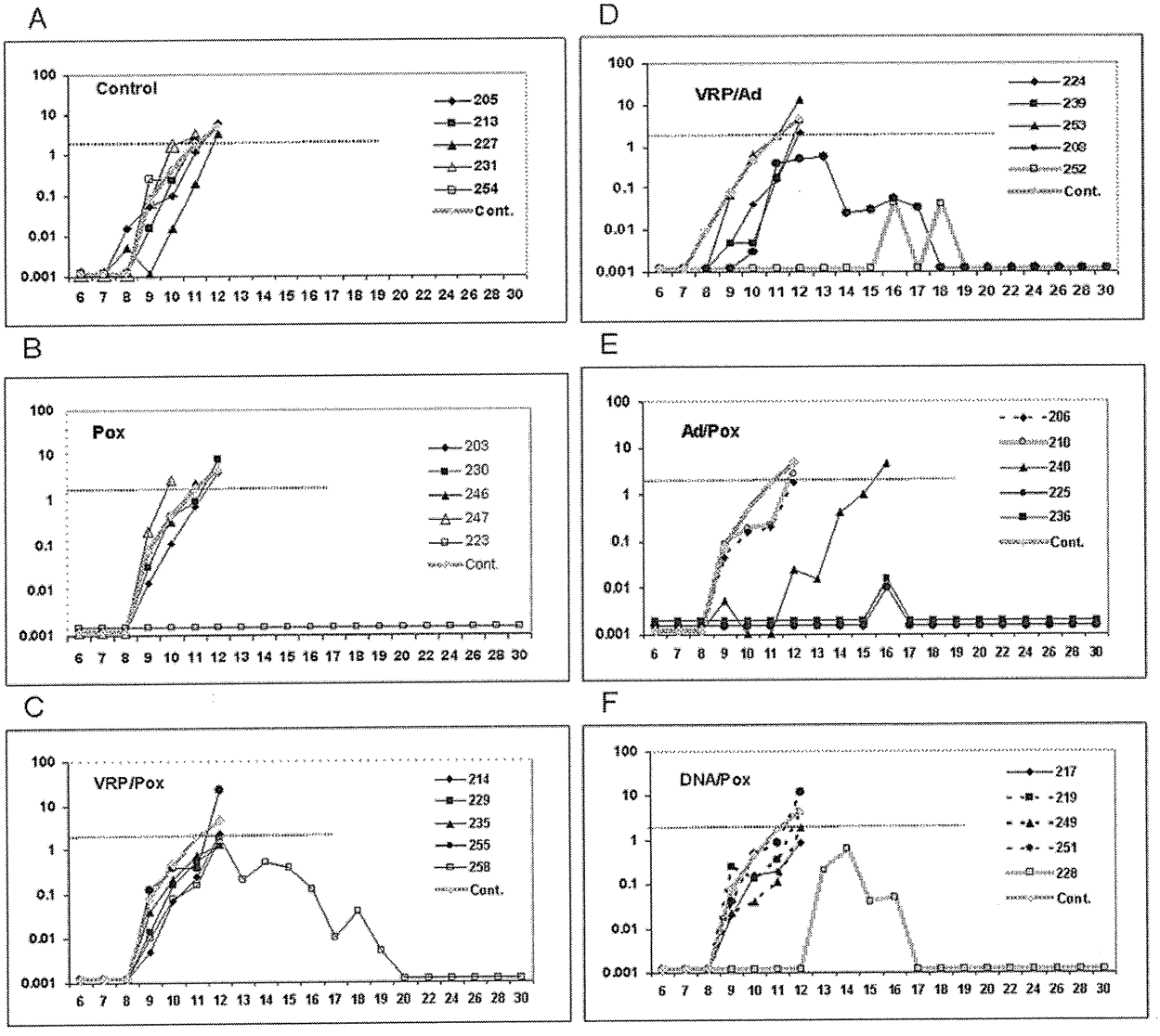

Figure 5. Daily parasitemias from individual monkeys after the second sporozoite challenge. Panel A, Control group: average parasitemia levels of 5 individual animals was presented as a thick grey line (Cont.) and is included in all 6 panels for comparison; Panel B, Pox group; Panel C, VRP/Pox group; Panel D, VRP/Ad group; Panel E, Ad/Pox group; Panel F, DNA/Pox group; The dotted line in each panel shows the 2% parasitemia level at which we treated animals with anti-malaria drugs. One monkey (223) in Panel C had no detectable parasitemia is shown as a horizontal line. The four monkeys which had no detectable parasitemias after the first challenge all developed parasites in the second challenge and are graphed with black interrupted lines. The three monkeys which self-cured their parasitemias after the first challenge are graphed with thick stippled lines.

doi:10.1371/journal.pone.0006559.g005

boost vaccine in the first challenge. Three of five monkeys (60%) never developed parasitemia after sporozoite challenge, and of the two monkeys that did become infected, one cured its parasitemia without the need for drug treatment. The fact that this protection was achieved using a DNA/poxvirus vaccine regimen suitable for humans is especially encouraging. In our previous four challenge experiments (Table 4), a total of 3/30 monkeys were sterilely protected by the Pk4 DNA/Pox vaccine, with protection ranging from 0 to 18%. Comparing the present study with the pooled results of our previous studies yields an Odds Ratio of 0.074 (95% CI 0.008, 0.636). Thus it is not likely that the improved protection is a random fluctuation due to the small number of experimental animals. Our hypothesis is that the increased protection may be due in part to the long intervals between vaccine doses used in this study, as has been seen in rodent malaria vaccine studies [32].

However there are several caveats to be considered when comparing the present study with our previously published results. Because we have been working over a period of years, different

Table 4. Summary of Sterile Protection in Five Pk4 DNA/Pox Vaccine Studies.

Trial								N =	Sterile #	Sterile %
a	DNA1	DNA2		DNA3			Pox	5	3	60
	day 0	28		96			420			
b	DNA1	DNA2	DNA3		DNA4	Pox		11	2	18
	day 0	30	60		280	310				
c	DNA1	DNA2	DNA3		Pox			5	0	0
	day 0	30	60		207					
d	DNA1	DNA2	DNA3		Pox			10	1	10
	day 0	30	60		156					
e	DNA1	DNA2	DNA3	Pox				4	0	0
	day 0	30	60	108						

Summary of five published vaccine studies in rhesus monkeys using the Pk4 DNA/Pox vaccine and challenge with 100 Pk sporozoites IV. Trial a is the present experiment. Trial b is from Rogers (18). Trials c and e are from experiment 3 in Weiss (19), Trial d is from experiments 1 and 2 in Weiss (19). N gives the number of animals receiving the Pk4 DNA/Pox vaccine, and Sterile gives the number of animals which did not develop parasites in the blood. Longer regimens give higher proportion of sterilely protected animals.

doi:10.1371/journal.pone.0006559.t004

production batches of plasmid and viral vaccines have been used. There are also some differences in the vaccine regimens, with the present study spreading the three priming DNA vaccinations over 4 months, and the study from Rogers et al. [18] including a fourth DNA dose before poxvirus boosting. Also, although challenge has always been with 100 P. knowlesi sporozoites the infectivity of those sporozoites no doubt varied between experiments. Finally, the rhesus monkeys in our studies have been obtained from several sources. In our previous studies using the Pk4 vaccine, we have used rhesus monkeys from breeding colonies founded with rhesus monkeys of Indian origin. In this study, we used rhesus monkeys of Chinese origin because Indian origin rhesus were not available at our institution. Although the immune responses to vaccines of genetic subgroups of rhesus monkeys may differ [33], in the absence of an immune correlate of protection we cannot know if genetic differences are responsible for the improved protection seen in this experiment. Because of all these concerns, the concept of longer vaccinations leading to better protection remains a conjecture which must be directly tested in a future experiment.

We were not able to identify an immune correlate of protection in this study. The two vaccines, DNA/Pox and Ad/Pox, which induced the most consistent immune responses to the P. knowlesi CSP were also the only two vaccines which sterilely protected monkeys (Table 2). From this, one might expect that the blood of protected monkeys would have higher antibody or T cell responses to CSP than non-protected monkeys. However, this was not the case (Table 3). One explanation for this seeming contradiction is that immune responses in the blood do not reflect protective immune responses in tissues. In mice, it has been shown that the immune responses that correlate with pre-erythrocytic malaria immunity occur within the liver tissue itself [34]. We think it likely that similar tissue specific liver immunity is occurring with pre-erythrocytic immunity in primates and humans as well, and that these immune events may not be easy to detect in the peripheral blood. We are undertaking studies of immune responses in the monkey liver to examine this concept.

Using flow cytometry, we were able to measure antigen specific responses from CD4+ T cells but we did not detect antigen specific CD8+ T cell responses. This is consistent with our previous studies of the Pk4 DNA/Pox vaccine [19,28]. We had hoped that the VRPs or Ad5 viruses would be able to induce CD8+ T cell responses but this was not the case. CD8+ T cells are important immune effectors against liver stages of malaria in mice [35–37] and monkeys (Weiss, unpublished data) protectively immunized with radiation-attenuated malaria sporozoites. We believe that a vaccine which induces CD8+ T cell effectors may have increased efficacy against malaria liver stages.

The biggest failing of the P. knowlesi vaccines has been the short duration of protection: no sterilely protected animals in the first sporozoite challenge were sterilely protected in the second challenge four months later. This has also been the case in all of our previous studies. Lacking an immune correlate of protection, our vaccine development strategy is to improve the magnitude and longevity of all immune responses to malaria vaccine antigens, and to induce CD8+ effector T cells. Our next plan is to replace DNA priming with recombinant malaria proteins in novel adjuvants [38,39]. We hope these next generation priming vaccines will allow stronger and longer lasting immune responses after boosting with recombinant viral vaccines, and a corresponding lengthening of vaccine efficacy.

Acknowledgments

We thank the Department of Animal Medicine at WRAIR/NMRC, and Charles White for statistical advice. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

Author Contributions

Conceived and designed the experiments: GJ KIK JR JFS JB CRK TLR WRW. Performed the experiments: GJ SC NR GB HG AV PS JCA KL WRW. Analyzed the data: GJ MS DLD TLR WRW. Contributed reagents/materials/analysis tools: KIK JR JFS JB CRK TT ST YE. Wrote the paper: GJ TLR WRW.

References

1. World Malaria Report 2008. World Health Organization.
2. Bojang KA (2006) RTS,S/AS02A for malaria. Expert Rev Vaccines 5: 611–615.
3. Pombo D, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, et al. (2002) Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. Lancet 360: 610.
4. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, et al. (2002) Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. J Infect Dis 185: 1155–1164.
5. Tarun AS, Dumpit RF, Camargo N, Labaied M, Liu P, et al. (2007) Protracted sterile protection with Plasmodium yoelii pre-erythrocytic genetically attenuated parasite malaria vaccines is independent of significant liver-stage persistence and is mediated by CD8+ T cells. J Infect Dis 196: 608–616.
6. Wykes M, Good MF (2007) A case for whole-parasite malaria vaccines. Int J Parasitol 37: 705–712.
7. Mueller AK, Deckert M, Heiss K, Goetz K, Matuschewski K, et al. (2007) Genetically attenuated Plasmodium berghei liver stages persist and elicit sterile protection primarily via CD8 T cells. Am J Pathol 171: 107–115.
8. Druilhe P, Barnwell JW (2007) Pre-erythrocytic stage malaria vaccines: time for a change in path. Curr Opin Microbiol 10: 371–378.
9. Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, et al. (2006) The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. Nature 444: 937–940.
10. Perlaza BL, Valencia AZ, Zapata C, Castellanos A, Sauzet JP, et al. (2008) Protection against Plasmodium falciparum challenge induced in Aotus monkeys by liver-stage antigen-3-derived long synthetic peptides. Eur J Immunol 38: 2610–2615.
11. Perlaza BL, Zapata C, Valencia AZ, Hurtado S, Quintero G, et al. (2003) Immunogenicity and protective efficacy of Plasmodium falciparum liver-stage Ag-3 in Aotus lemurinus griseimembra monkeys. Eur J Immunol 33: 1321–1327.
12. Zapata JC, Perlaza BL, Hurtado S, Quintero GE, Jurado D, et al. (2002) Reproducible infection of intact Aotus lemurinus griseimembra monkeys by Plasmodium falciparum sporozoite inoculation. J Parasitol 88: 723–729.
13. G.Robert Coatney WEC, Mc WilsonWarren, Peter GContacos (1971) The Primate Malarias. Bethesda: U.S. DEPARMENT OF HEALTH, EDUCATION, AND WELFARE. 366 p.
14. Vythilingam I, Tan CH, Asmad M, Chan ST, Lee KS, et al. (2006) Natural transmission of Plasmodium knowlesi to humans by Anopheles latens in Sarawak, Malaysia. Trans R Soc Trop Med Hyg 100: 1087–1088.
15. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, et al. (2008) Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis 46: 165–171.
16. Barnwell JW, Howard RJ, Coon HG, Miller LH (1983) Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned Plasmodium knowlesi malaria. Infect Immun 40: 985–994.
17. Rogers WO, Baird JK, Kumar A, Tine JA, Weiss W, et al. (2001) Multistage multiantigen heterologous prime boost vaccine for Plasmodium knowlesi malaria provides partial protection in rhesus macaques. Infect Immun 69: 5565–5572.
18. Rogers WO, Weiss WR, Kumar A, Aguiar JC, Tine JA, et al. (2002) Protection of rhesus macaques against lethal Plasmodium knowlesi malaria by a heterologous DNA priming and poxvirus boosting immunization regimen. Infect Immun 70: 4329–4335.
19. Weiss WR, Kumar A, Jiang G, Williams J, Bostick A, et al. (2007) Protection of rhesus monkeys by a DNA prime/poxvirus boost malaria vaccine depends on optimal DNA priming and inclusion of blood stage antigens. PLoS ONE 2: e1063.
20. Rayner JO, Dryga SA, Kamrud KI (2002) Alphavirus vectors and vaccination. Rev Med Virol 12: 279–296.
21. Hubby B, Talarico T, Maughan M, Reap EA, Berglund P, et al. (2007) Development and preclinical evaluation of an alphavirus replicon vaccine for influenza. Vaccine 25: 8180–8189.
22. Li S, Locke E, Bruder J, Clarke D, Doolan DL, et al. (2007) Viral vectors for malaria vaccine development. Vaccine 25: 2567–2574.
23. Earle WC, Perez M (1932) Enumeration of parasites in the blood of malarial patients. JLabClinMed 17: 1124–1130.
24. Charoenvit Y, Leef ML, Yuan LF, Sedegah M, Beaudoin RL (1987) Characterization of *Plasmodium yoelii* monoclonal antibodies directed against stage-specific sporozoite antigens. Infect Immun 55: 604–608.
25. Madin K, Sawasaki T, Ogasawara T, Endo Y (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. Proc Natl Acad Sci U S A 97: 559–564.
26. Sawasaki T, Hasegawa Y, Tsuchimochi M, Kamura N, Ogasawara T, et al. (2002) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. FEBS Lett 514: 102–105.
27. Sawasaki T, Gouda MD, Kawasaki T, Tsuboi T, Tozawa Y, et al. (2005) The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information. Methods Mol Biol 310: 131–144.
28. Kumar A, Weiss W, Tine JA, Hoffman SL, Rogers WO (2001) ELISPOT assay for detection of peptide specific interferon-gamma secreting cells in rhesus macaques. J Immunol Methods 247: 49–60.
29. Jiang G, Charoenvit Y, Moreno A, Baraceros MF, Banania G, et al. (2007) Induction of multi-antigen multi-stage immune responses against Plasmodium falciparum in rhesus monkeys, in the absence of antigen interference, with heterologous DNA prime/poxvirus boost immunization. Malar J 6: 135.
30. Voller A, Rossan RN (1969) Immunological studies on simian malaria. III. Immunity to challenge and antigenic variation in P. knowlesi. Trans R Soc Trop Med Hyg 63: 507–523.
31. Silvie O, Franetich JF, Charrin S, Mueller MS, Siau A, et al. (2004) A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. J Biol Chem 279: 9490–9496.
32. Brice GT, Dobano C, Sedegah M, Stefaniak M, Graber NL, et al. (2007) Extended immunization intervals enhance the immunogenicity and protective efficacy of plasmid DNA vaccines. Microbes Infect 9: 1439–1446.
33. Stahl-Hennig C, Suh YS, Park KS, Sauermann U, Kim KS, et al. (2007) Immunogenicity of a DNA prime and recombinant adenovirus boost regime significantly varies between rhesus macaques of Chinese and Indian origins. J Med Primatol 36: 195–205.
34. Krzych U, Schwenk J (2005) The dissection of CD8 T cells during liver-stage infection. Curr Top Microbiol Immunol 297: 1–24.
35. Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig RS, et al. (1987) Gamma-interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. Nature 330: 664–666.
36. Weiss WR, Sedegah M, Beaudoin RL, Miller LH, Good MF (1988) $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. Proc Natl Acad Sci USA 85: 573–576.
37. Jobe O, Lumsden J, Mueller AK, Williams J, Silva-Rivera H, et al. (2007) Genetically attenuated Plasmodium berghei liver stages induce sterile protracted protection that is mediated by major histocompatibility complex Class I-dependent interferon-gamma-producing CD8+ T cells. J Infect Dis 196: 599–607.
38. Stewart VA, McGrath SM, Dubois PM, Pau MG, Mettens P, et al. (2007) Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to Plasmodium falciparum CS compared to that with either malaria vaccine alone. Infect Immun 75: 2283–2290.
39. Wille-Reece U, Wu CY, Flynn BJ, Kedl RM, Seder RA (2005) Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. J Immunol 174: 7676–7683.

Evidence for the Transmission of *Plasmodium vivax* in the Republic of the Congo, West Central Africa

Richard Culleton,[1,3] Mathieu Ndounga,[5] Fadile Yildiz Zeyrek,[6] Cevayir Coban,[2] Prisca Nadine Casimiro,[5] Satoru Takeo,[4] Takafumi Tsuboi,[4] Anjali Yadava,[8] Richard Carter,[7] and Kazuyuki Tanabe[1]

[1]Laboratory of Malariology, International Research Centre of Infectious Diseases, Research Institute of Microbial Diseases, and [2]Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Osaka, [3]Department of Protozoology, Institute of Tropical Medicine (NEKKEN) and the Global Centers of Excellence Program, Nagasaki University, Nagasaki, and [4]Cell-Free Science and Technology Research Centre, Ehime University, Ehime, Japan; [5]Centre d'Etudes sur les Resources Vegetales, Brazzaville, Republic of the Congo; [6]Department of Microbiology, Medical Faculty, Harran University, Sanliurfa, Turkey; [7]Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, United Kingdom; [8]Division of Malaria Vaccine Development, Walter Reed Army Institute of Research, Silver Spring, Maryland

***Plasmodium vivax* is not thought to be transmitted in western and central Africa, because of the very high prevalence of the red blood cell Duffy-negative phenotype in local populations, a condition which is thought to confer complete resistance against blood infection with *P. vivax*. There are, however, persistent reports of travelers returning from this region with *P. vivax* infections. To investigate whether transmission occurs in this region, the presence of antibodies specific to *P. vivax* preerythrocytic-stage antigens was assessed in individuals from the Republic of the Congo. A total of 55 (13%) of 409 samples tested by enzyme-linked immunosorbent assay had antibodies to *P. vivax*–specific antigens.**

Transmission of *Plasmodium vivax* is not generally thought to occur in western or central continental Africa, where 95%–99% of the human population is refractory to *P. vivax* blood infection because of the protective effect of the red blood cell (RBC) Duffy-negative condition [1, 2]. Despite this, reports of Duffy-positive nonimmune travelers returning from these areas with infections diagnosed as being due to *P. vivax* are common and have persisted over many years of surveillance [3]. Furthermore, a recent report has implied that *P. vivax* transmission may occur in a population consisting of very high percentages of Duffy-negative individuals, with the presence of *P. vivax*–specific proteins reported in 0.65% of mosquitoes from an area of western Kenya [4]. An additional study reported evidence of *P. vivax* infections in 2 Duffy-negative individuals in Brazil [5]. Some investigators have interpreted such findings as implying that the parasite may be in the process of evolving the ability to infect Duffy-negative individuals [6]. However, we have argued elsewhere [3] that *P. vivax* transmission can be expected in populations with high levels of RBC Duffy negativity and in which malaria transmission intensities are sufficiently high, as is the case in many areas of western and central Africa. Notwithstanding this expectation, a recent polymerase chain reaction (PCR)–based parasite species–typing survey of 2588 blood samples obtained from patients in 9 western and central African countries failed to find any *P. vivax* parasites, except on the island of Sao Tome, where *P. vivax* transmission is known to occur [3].

In the present study, we used serological testing to search for evidence of *P. vivax* transmission in Pointe-Noire, a city on the west coast of the Republic of the Congo, where >95% of the population is expected to be RBC Duffy negative and, thus, refractory to *P. vivax* blood infection. In September 2007, we collected blood samples from 415 Pointe-Noire residents and searched for the presence of antibodies to the *P. vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) and *P. vivax* merozoite surface protein 1 (PvMSP1). Both antigens are expressed in liver-stage parasites and induce antibodies even in the absence of *P. vivax* blood infection [7]. Detection of antibodies to these *P. vivax*–specific antigens in a largely Duffy-negative human population could be evidence of its transmission there.

Materials and methods. By means of passive case detection, 415 samples were collected from the Mbota health center in Pointe-Noire, located on the west coast of the Republic of

Received 16 March 2009; accepted 10 June 2009; electronically published 2 October 2009.

Reprints or correspondence: Dr. Richard Culleton, Dept. of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852–8523, Japan (richard@nagasaki-u.ac.jp); or Dr. Kazuyuki Tanabe, Laboratory of Malariology, International Research Centre of Infectious Diseases, Research Institute of Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka, Japan (ktanabe@biken.osaka-u.ac.jp).

The Journal of Infectious Diseases 2009;200:1465–9

0022-1899/2009/20009-0015$15.00
DOI: 10.1086/644510

Potential conflicts of interest: none reported.

Presented in part: 17th International Conference for Tropical Medicine and Malaria, Jeju Island, South Korea, 1 October 2008 (abstract LB-P15); British Society for Parasitology Malaria Meeting, Edinburgh, United Kingdom, 6–8 April 2009 (abstract M8).

Financial support: Japanese Ministry of Education, Culture, Sports, Science, and Technology (grant-in-aid 18073013 for scientific research on priority areas); Japan Society for the Promotion of Science (grants-in-aid 17-05495 and 20390120 for scientific research).

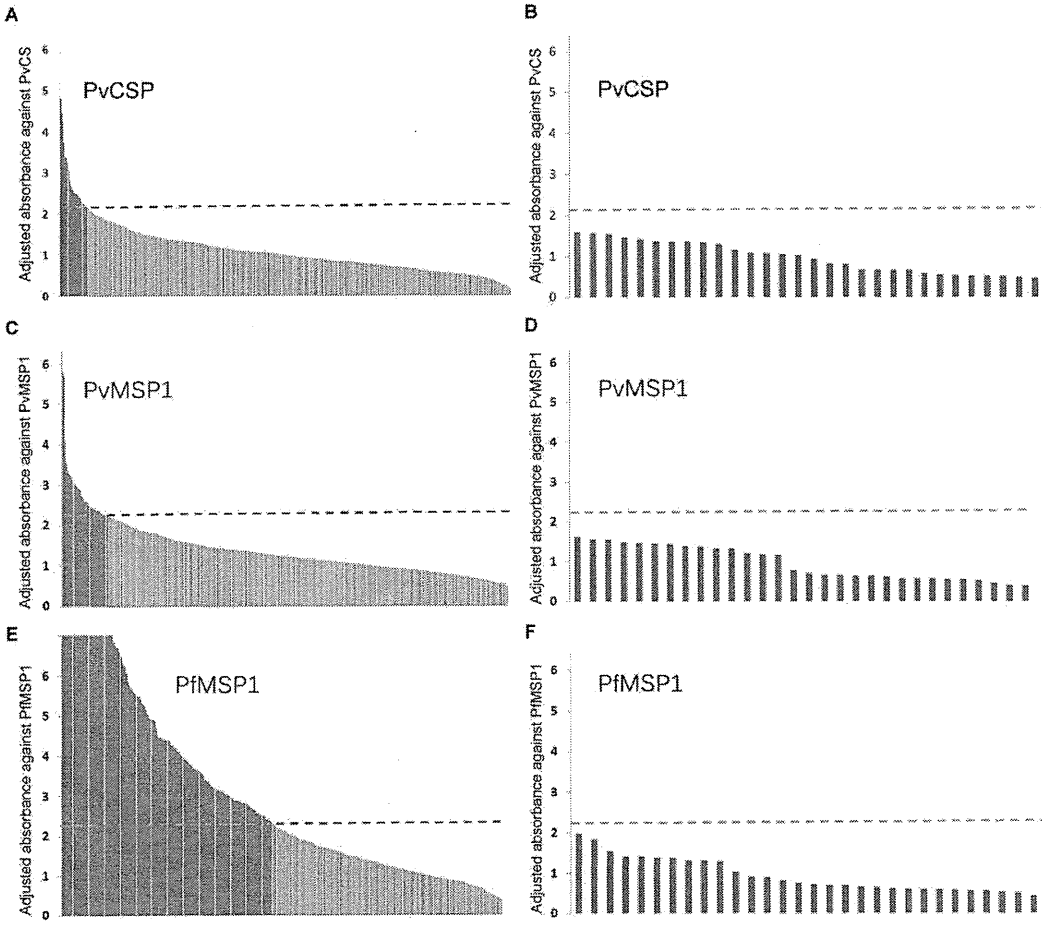

Figure 1. Adjusted absorbances against *Plasmodium vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) *(A), P. vivax* merozoite surface protein 1 (PvMSP1) *(C),* and the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) *(E)* for 409 individuals from Pointe-Noire, Republic of the Congo. *B, D,* and *F,* Corresponding adjusted absorbances for nonexposed individuals (from Japan and the United Kingdom). Cutoff values are denoted by horizontal dashed lines, and positive individuals are denoted by the areas shaded dark gray. The cutoff value was calculated as the mean value (+3 standard deviations) of the adjusted absorbances of 30 nonexposed individuals. All absorbances were measured at 405 nm.

the Congo, during September 2007. No age restrictions were applied to individuals from whom samples were obtained. The samples were collected on Whatman 31ETCHR filter paper. Travel histories were obtained from individuals before sample collection, and those who had traveled outside of the Republic of the Congo were excluded from the study ($n = 6$). Approval of the sample collection was obtained from the ethics committee at the Research Institute of Microbial Diseases, Osaka University (Osaka, Japan), and sampling was authorized by the administrative authority of the Ministry for Research and the Ministry for Health in the Republic of the Congo. Written informed consent was obtained from individual patients, and antimalarial treatment was provided when appropriate. An additional 10 blood samples were collected from *P. vivax*–infected patients from Siverek-Sanliurfa in the southeast of Turkey, for use as positive controls, and from 30 individuals from Japan and the United Kingdom with no previous exposure to *P. vivax* (ie, nonexposed individuals), for use as negative control samples (for collection details, see the description of supplementary methods in the Appendix, which appears only in the electronic version of the *Journal*).

All samples were screened by enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G antibodies to 3 *Plasmodium*-specific proteins. The first of these proteins was PvCSP recombinant protein. This *Escherichia coli*–expressed recombinant protein encompasses the N-terminal and C-terminal regions of PvCSP flanking a chimeric repeat region [8]. The second protein, PvMSP1 recombinant protein, was expressed using a wheat germ cell-free protein translation system [9] that encompasses N-terminal blocks 1 and 2 of

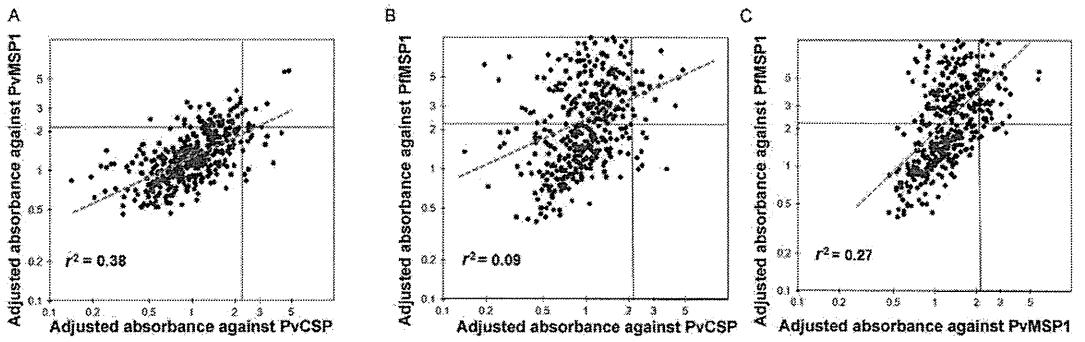

Figure 2. Linear regression analysis of the association between the adjusted absorbances against the *Plasmodium vivax* antigens *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. vivax* circumsporozoite protein (PvCSP) *(A)*, the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) and PvCSP *(B)*, and PfMSP1 and PvMSP1 *(C)* for 409 individuals from Pointe-Noire, Republic of the Congo. Coefficient of determination values (r^2) for each antigen pair are shown on the graph, and linear regression lines are denoted by dashed gray lines. The solid horizontal and vertical lines denote the positive cutoff values for each antigen.

PvMSP1 (Ser_{72} to His_{432} [based on the *SalI* sequence {GenBank accession number PVX_099980}]). The third protein, *Plasmodium falciparum* merozoite surface protein 1 (PfMSP1) recombinant protein, was an *E. coli*–expressed recombinant protein of N-terminal blocks 1–6 (M1/6) of the *P. falciparum* MAD20 sequence [10]. A detailed description of the ELISA protocol is provided in the Appendix.

To adjust for interplate variations, adjusted absorbances were obtained by dividing the mean optical density (OD) value of 2 repeats for each individual sample by the mean OD value for the same 4 nonexposed individuals assayed on the same 96-well plate (under identical conditions). Samples with adjusted OD values (+3 standard deviations) that were greater than the mean value for 30 nonexposed serum samples were considered to be positive for antibodies to the antigen tested. In the case of a positive result for PvCSP or PvMSP1, blood samples from the same patients were used for extraction of host and parasite DNA for parasite species typing and determination of host Duffy status.

Results. Figure 1 shows the results of ELISAs performed on the 409 samples collected from patients presenting to Mbota health center in Pointe-Noire, Republic of the Congo. For 25 (6%) of these samples, adjusted anti-PvCSP absorbance readings were greater than the mean value (+3 standard deviations) for 30 serum samples obtained from nonexposed individuals and were therefore considered to be positive for antibodies to this protein. For 39 (10%) of the samples, adjusted absorbance readings were greater than the cutoff value noted for PvMSP1. A total of 197 individuals (48%) were found to be positive for antibodies to PfMSP1, a *P. falciparum* antigen. All *P. vivax*–positive samples were independently tested twice more in duplicate, and the same positive results were obtained.

Of the 25 samples that were positive for PvCSP antibodies, 9 (36%) were also positive for antibodies to PvMSP1, and 16 (64%) were positive for antibodies to PfMSP1. Of the 39 samples that were positive for PvMSP1, 31 (79%) were also positive for PfMSP1. To investigate the possibility that there was cross-reactivity between antibodies to *P. falciparum* and *P. vivax* antigens, correlation and linear regression analyses were performed for the antigen pairs PvCSP/PvMSP1, PvMSP1/PfMSP1, and PvCSP/PfMSP1. Adjusted absorbance values were log transformed to meet the normality and homoscedasticity assumptions of the analysis, and coefficient of determination (r^2) values and linear regression lines were generated (Figure 2). There was a highly significant medium-strength positive correlation between antibody responses against PvCSP and PvMSP1 ($r^2 = 0.38$; 409 *df*; $P < .001$) but a much weaker, although still significant, low correlation between PvCSP and PfMSP1 ($r^2 = 0.09$; 409 *df*; $P < .001$). There was a stronger correlation between PvMSP1 and PfMSP1 ($r^2 = 0.27$; 409 *df*; $P < .001$), but this was also much weaker than the correlation between the 2 *P. vivax* antigens. Furthermore, serum antibody absorbance ELISA experiments performed with known positive serum samples incubated separately with *P. vivax* and *P. falciparum* MSP1 antigens showed no evidence of cross-reactivity between the respective antibodies (figure 3, which appears only in the electronic version of the *Journal*).

We assessed whether various factors (patient age or sex; pres-

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases.*

Figure 3. Enzyme-linked immunosorbent assay results for known *Plasmodium falciparum* antibody– and *Plasmodium vivax* antibody–positive serum samples incubated with *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. falciparum* merozoite protein surface protein 1 (PfMSP1) antigens and tested for antibody responses to PvMSP1 and PfMSP1 antigens.

Table 1. Descriptive Statistics for Adjusted Absorbencies against 3 Antigens for 409 Individuals from Pointe-Noire, Republic of the Congo

This table is available in its entirety in the online edition of *The Journal of Infectious Diseases.*

ence or absence of parasites; parasite species, if infected; and district of residence) were associated with an increased probability of seropositivity against *P. vivax* antigens. There was no association between the district of residence and the presence of antibodies to *P. vivax* or *P. falciparum* (Table 1, which appears only in the electronic version of the *Journal*). Similarly, there was no strong correlation between age and the presence of antibodies to PvCSP ($r^2 = 0.09$, 409 *df*; $P < .01$), PvMSP1 ($r^2 = 0.13$; 409 *df*; $P < .01$), and PfMSP1 ($r^2 = 0.16$; 409 *df*; $P < .01$), as determined by Spearman rank correlation tests. The presence or absence of parasites in blood, as detected by microscopy, as well as whether those parasites were *P. falciparum, Plasmodium malariae,* or *Plasmodium ovale,* was not correlated with the presence of antibodies to either *P. vivax* or *P. falciparum;* however, the numbers of *P. malariae*– and *P. ovale*–infected individuals were low ($n = 5$ and $n = 7$, respectively), precluding statistical analysis (Table 1). Interestingly, females were significantly more likely than males to be seropositive for *P. vivax* antibodies, with 45 (17%) of 269 females positive for antibodies to PvCSP or PvMSP1, or both, compared with 10 (7%) of 145 males (6.51, by χ^2 test; 1 *df*; $P = .01$). There was, however, no difference in seropositivity for *P. falciparum* antibodies between the sexes, with 137 (51%) of 269 females and 60 (41%) of 145 males having positive responses against PfMSP1 (1.81, by χ^2 test; 1 *df*; $P = .18$).

DNA was extracted from the 55 samples for which positive antibody responses against either of the 2 *P. vivax*–specific antigens were demonstrated by ELISA. *Plasmodium* species identification was performed by polymerase chain reaction (PCR), and *P. vivax* DNA was not detected in any samples. The Duffy genotype status of the 55 individuals was determined by PCR [11], and all these individuals were found to be homozygous carriers of the FY^*B^{null} allele and, thus, of the RBC Duffy-negative phenotype.

Discussion. We have shown that the serum samples from 55 (13%) of 409 individuals from Pointe Noire in the Republic of the Congo contained antibodies to the *P. vivax*–specific antigens PvCSP (25 samples [6%]), PvMSP1 (39 samples [9.5%]), or both (9 samples [2.2%]). These results suggest that *P. vivax* is transmitted in an area of west central Africa where the frequency of the Duffy-negative genotype is 95%–99% [1]. This finding goes against the current orthodoxy that *P. vivax* is not transmitted in western Africa and offers an explanation for the many cases of *P. vivax* contracted by Duffy-positive travelers in this region.

It has been established elsewhere [7] that Duffy-negative individuals who are refractory to the blood stages of *P. vivax* may develop antibodies to such antigens as CSP and MSP1, which are expressed in the preerythrocytic stages of this parasite in areas of endemicity. This finding is supported by evidence of the establishment of preerythrocytic immunity in individuals undergoing anti–blood-stage chemoprophylaxis for *P. falciparum* [12] and in mice with *Plasmodium yoelii* [13].

Although initial experiments indicated that there was no cross-reactivity between antibodies to the PvMSP1 and PfMSP1 antigens used in the present study, we did find a weak correlation between the antibody responses to the 2 species-specific versions of this antigen. There was also a very weak correlation between antibody responses to the PvCSP and PfMSP1 antigens. We do not consider, however, that these correlations are, in themselves, evidence for antigenic cross-reactivity between *P. vivax* and *P. falciparum* antigens. Indeed, if 2 species of malaria parasites are coendemic, this result is predicted from the fact that exposure to infection by one species of malaria parasite will be highly correlated with the risk of exposure to infection by other species.

Our data indicate that, in the region of study in western and central Africa, there is an endemic entity present that is inducing antibodies specific to the preerythrocytic stages of *P. vivax* in the RBC Duffy-negative human populations of the region. We suggest that this entity is most likely sporozoites of *P. vivax* itself, delivered by the local malaria vector mosquitoes. In conjunction with the frequent reports of travelers returning from western and central Africa with diagnosed *P. vivax* infections, these findings make a strong argument for the presence and continued transmission of *P. vivax* in this region. Given the very high malaria transmission intensity in this area, it is possible that the transmission of *P. vivax* is maintained within the local population by the ~1%–5% of Duffy-positive individuals who are presumed to be present in the local population.

Acknowledgments

We thank all the participants in this study who provided blood samples.

References

1. Cvalli-Sforza LL. Africa: the history and geography of human genes. Princeton, NJ: Princeton University Press, **1994**:158–94.
2. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks: the Duffy-blood-group genotype, FyFy. N Engl J Med **1976**; 295:302–4.
3. Culleton RL, Mita T, Ndounga M, et al. Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing. Malar J **2008**;7: 174.
4. Cavasini CE, Mattos LC, Couto AA, et al. *Plasmodium vivax* infection

among Duffy antigen–negative individuals from the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg **2007**; 101:1042–4.
5. Ryan JR, Stoute JA, Amon J, et al. Evidence for transmission of *Plasmodium vivax* among a Duffy antigen negative population in Western Kenya. Am J Trop Med Hyg **2006**; 75:575–81.
6. Rosenberg R. *Plasmodium vivax* in Africa: hidden in plain sight? Trends Parasitol **2007**; 23:193–6.
7. Herrera S, Gómez A, Vera O, et al. Antibody response to *Plasmodium vivax* antigens in Fy-negative individuals from the Colombian Pacific coast. Am J Trop Med Hyg **2005**; 73:44–9.
8. Yadava A, Sattabongkot J, Washington MA, et al. A novel chimeric *Plasmodium vivax* circumsporozoite protein induces biologically functional antibodies that recognize both VK210 and VK247 sporozoites. Infect Immun **2007**; 75:1177–85.
9. Tsuboi T, Takeo S, Iriko H, et al. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun **2008**; 76:1702–8.
10. Tolle R, Fruh K, Doumbo O, et al. A prospective study of the association between the human humoral immune response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections. Infect Immun **1993**; 61:40–7.
11. Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL. A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. Transfusion **1998**; 38:168–73.
12. Guerin-Marchand C, Druilhe P, Galey B, et al. A liver-stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. Nature **1987**; 329:164–7.
13. Belnoue E, Costa FT, Frankenberg T, et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. J Immunol **2004**; 172:2487–95.

OPEN ACCESS Freely available online

PLoS one

Identification of *Plasmodium malariae*, a Human Malaria Parasite, in Imported Chimpanzees

Toshiyuki Hayakawa[1]*, Nobuko Arisue[2], Toshifumi Udono[3], Hirohisa Hirai[4], Jetsumon Sattabongkot[5], Tomoko Toyama[1], Takafumi Tsuboi[6], Toshihiro Horii[2], Kazuyuki Tanabe[1]*

1 Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan, **2** Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan, **3** The Chimpanzee Sanctuary Uto, Sanwa Kagaku Kenkyusho, CO., LTD, Uki, Kumamoto, Japan, **4** Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan, **5** Department of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, **6** Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Ehime, Japan

Abstract

It is widely believed that human malaria parasites infect only man as a natural host. However, earlier morphological observations suggest that great apes are likely to be natural reservoirs as well. To identify malaria parasites in great apes, we screened 60 chimpanzees imported into Japan. Using the sequences of small subunit rRNA and the mitochondrial genome, we identified infection of *Plasmodium malariae*, a human malaria parasite, in two chimpanzees that were imported about thirty years ago. The chimpanzees have been asymptomatic to the present. In Japan, indigenous malaria disappeared more than fifty years ago; and thus, it is most likely inferred that the chimpanzees were infected in Africa, and *P. malariae* isolates were brought into Japan from Africa with their hosts, suggesting persistence of parasites at low level for thirty years. Such a long term latent infection is a unique feature of *P. malariae* infection in humans. To our knowledge, this is the first to report *P. malariae* infection in chimpanzees and a human malaria parasite from nonhuman primates imported to a nonendemic country.

Citation: Hayakawa T, Arisue N, Udono T, Hirai H, Sattabongkot J, et al. (2009) Identification of *Plasmodium malariae*, a Human Malaria Parasite, in Imported Chimpanzees. PLoS ONE 4(10): e7412. doi:10.1371/journal.pone.0007412

Editor: Gordon Langsley, INSERM U567, Institut Cochin, France

Received July 29, 2009; **Accepted** September 14, 2009; **Published** October 12, 2009

Funding: This research was supported by the Ministry of Education, Culture, Sports, Science and Technology grant (19790306 and 21790404 to T. Hayakawa; 20390120, 18073013, and 18GS03140013 to K.T.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: hayakawa@biken.osaka-u.ac.jp (TH); kztanabe@biken.osaka-u.ac.jp (KT)

Introduction

Malaria is a major infectious disease prevalent in most tropical and subtropical areas in the world. Malaria parasites, genus *Plasmodium*, infect all classes of terrestrial vertebrates (i.e. mammals, birds, and reptiles)[1]. Of them, the four classical human malaria parasites, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, are widely believed to infect only man as a natural host. However, earlier studies described several human malaria parasite-like species from great apes: *Plasmodium reichenowi*, a *P. falciparum*-like parasite in chimpanzees, *Plasmodium schwetzi*, a *P. vivax*/*P. ovale*-like parasite in chimpanzees and gorillas, and *Plasmodium rodhaini*, a *P. malariae*-like parasite in chimpanzees [2,3]. Determination of host specificity or host range of human malaria parasites is of great importance not only for further understanding the parasite biology but also for better malaria control. Surveys of malaria parasites in great apes are thus required. Besides, the investigation of malaria infection in great apes should be helpful for the primates' health and biodiversity conservation efforts.

The *Plasmodium* species reportedly identified as great ape malaria parasites were described in the early 20th century [3]. These previous studies were ambiguous as to whether natural infections of great apes are due to human malaria parasite-like species or to human malaria parasites. Recently, evidence of human infections of *Plasmodium knowlesi*, an Asian simian malaria parasite, is accumulating with the aid of current molecular diagnostic tools [4–7]. Prior studies on great ape malaria parasites may consequently be supported or disputed by new surveys using molecular diagnosis. Of the great ape parasites, *P. reichenowi* is the only species that has been confirmed to be close to but independent from *P. falciparum* at the molecular level [8]. Other malaria parasites from great apes await species identification using molecular analysis and phylogenetic relationship to human malaria parasites.

Recently, a new species, *Plasmodium gaboni*, has been identified from chimpanzees, and defined as a close relative of *P. falciparum* [9]. *P. ovale* has also been identified in chimpanzees in Africa, suggesting that *P ovale* can infect chimpanzees as a natural host [10].

Here, we report malaria parasites in two chimpanzees imported into Japan thirty years ago. The parasites isolated from these chimpanzees were identified as *P. malariae* based on two gene markers. The infections have been asymptomatic to the present, and have persisted for about thirty years. This study also indicates that human malaria parasite has been maintained in nonhuman primates in a nonendemic country, which has significance to public health issues.

Results

Blood samples of 60 chimpanzees (*Pan troglodytes*) imported into Japan were examined. First, molecular diagnosis for the presence

of malaria parasites was carried out using polymerase chain reactions (PCRs) that specifically amplify the mitochondrial genome and nuclear-encoded small subunit (SSU) rRNA gene of all known malaria parasites. PCR diagnosis yielded malaria positives in two chimpanzees, Takaboh and Oumu.

Takaboh is a male chimpanzee (*Pan troglodytes verus*), assumed to have been born in 1978. He was imported into Japan from the Republic of Sierra Leone in April 1980. His blood samples were collected during routine health examinations in January 2003 and in September 2008. PCR diagnosis was positive on both occasions. Oumu is a female chimpanzee (*Pan troglodytes verus*), assumed to have been born in 1976. She was imported into Japan from Africa in March 1977. There is no record of her country of origin. It is likely that Oumu came from West Africa because she belongs to *P. t. verus*, a subspecies living in the western part of Africa. Her blood was obtained during routine health examination in June 2003. Importantly, Takaboh and Oumu have shown no symptoms of malaria to the present while in Japan.

Microscopic observations of Giemsa-stained thin blood smears were done for specimens available for Takaboh and Oumu. Blood smears of Takaboh were prepared in August 2000, January and June 2003, and September 2008, and those of Oumu were made in October 1986, October 1996, and June 2003. We were able to detect, at one instance, one erythrocyte infected with malaria parasite in a Takaboh specimen obtained in September 2008 (Figure 1), but detection was unsuccessful from the other samples. The parasite shows the morphology of an immature schizont having irregular masses of chromatin, resembling *P. malariae*. The typical band-form of *P. malariae* could not be seen (Figure 1).

To identify the malaria parasite species from Takaboh and Oumu, the complete nucleotide sequence of the mitochondrial genome and the near-complete SSU rRNA gene sequence were obtained from these chimpanzee parasites. We also obtained an additional sequence of the mitochondrial genome from human *P. malariae* (Thailand isolate) because only one sequence (Uganda I isolate; GenBank accession number AB354570) was available. The sequences obtained were used for the construction of phylogenetic trees together with those from diverse malaria parasite species including primate, rodent, bird, and reptile parasites (Figure 2). In the phylogenetic tree of the SSU rRNA gene, both parasites of Takaboh and Oumu are clearly located within the cluster of human *P. malariae* isolates. This indicates that the two chimpanzee parasites belong to *P. malariae* (Figure 2A). Likewise, the phylogenetic tree of the mitochondrial genome represents clustering of the chimpanzee parasites with human *P. malariae* isolates. A closer look shows that the parasites of Takaboh and

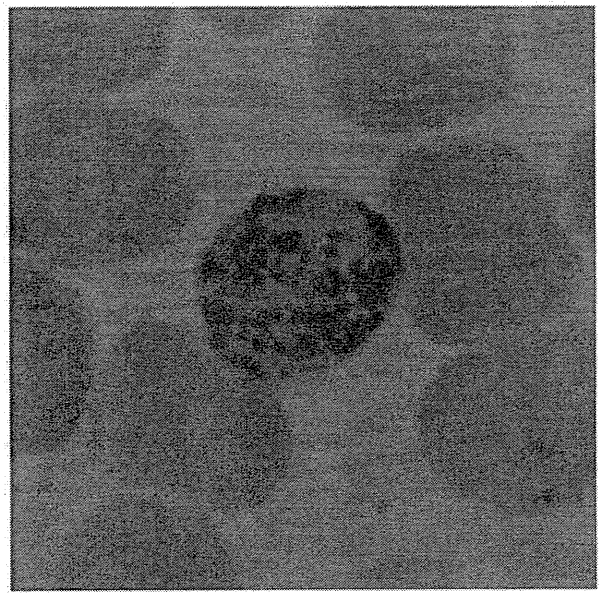

Figure 1. Giemsa-stained thin blood smear showing an immature schizont.
doi:10.1371/journal.pone.0007412.g001

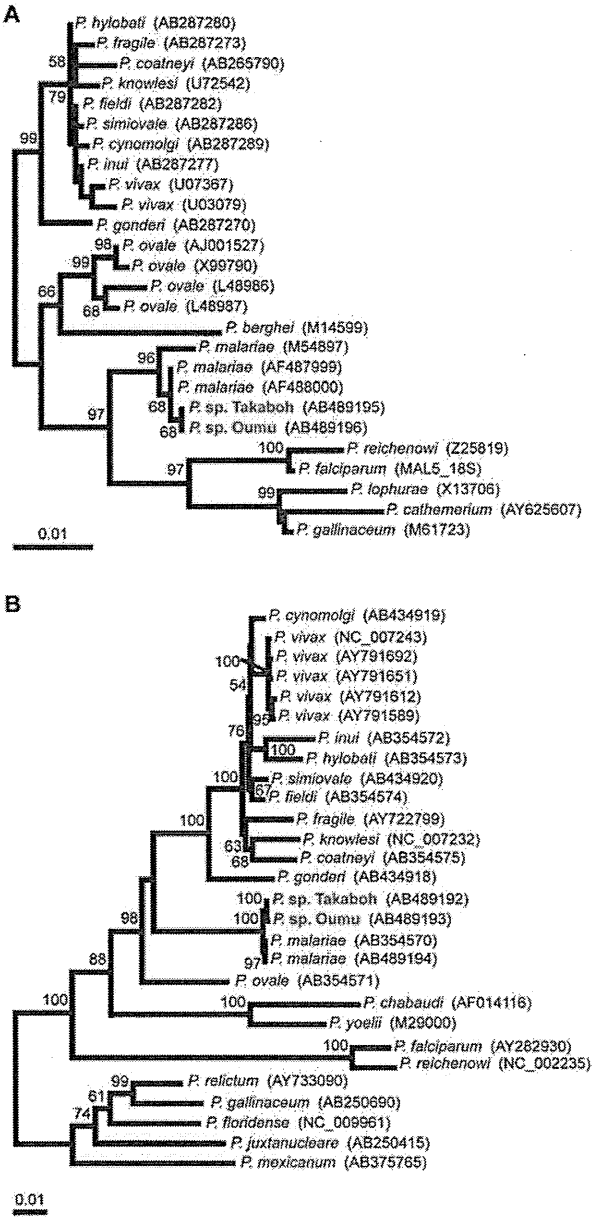

Figure 2. Phylogenetic trees of SSU rRNA gene (A) and mitochondrial genome (B). The numbers on the phylogenetic tree represent bootstrap values based on 1000 replications. GenBank accession numbers are in brackets.
doi:10.1371/journal.pone.0007412.g002

Oumu make a small sub-cluster and seem to be separated from the human *P. malariae* isolates (Figure 2B). This separation, however, does not indicate that the chimpanzee parasite is an independent species different from *P. malariae*. The genetic difference of the mitochondrial genome between the chimpanzee parasites and human *P. malariae* isolates should be regarded as an intraspecific variation (polymorphism): that is, the p-distances (the proportion of nucleotide sites at which two sequences being compared are different) between the parasites of Takaboh and Oumu and the human *P. malariae* isolates are at most 0.0023, smaller than the p-distance between the most distantly related isolates of *P. vivax* (at least 0.0026 is the observable polymorphism range in *P. vivax* populations; the calculation was based on the sequences in ref. 14; see also Figure 2B). We, therefore, conclude that the parasites from Takaboh and Oumu are isolates of *P. malariae*.

Discussion

The present finding of *P. malariae* isolates in two chimpanzees reinforces that *P. malariae* is able to infect chimpanzees as a natural host. Takaboh and Oumu were imported into Japan about thirty years ago at one or two years of age. In the mainland of Japan, there has been no indigenous malaria for more than 50 years [11]. These facts imply that the two chimpanzees were infected with *P. malariae* in Africa before their importation, and that *P. malariae* infection has persisted for about 30 years in the two chimpanzees. In addition, both Takaboh and Oumu have been asymptomatic to the present, and parasite densities were extremely low in their bloods. These circumstances are consistent with a long term latent infection, a unique feature of human *P. malariae* infection [12,13]. *P. malariae* therefore appears to have a similar course of infection in chimpanzees as in humans. A much larger survey in wild chimpanzees in Africa would present more details about *P. malariae* infection in chimpanzees.

It is known that, in humans, *P. malariae* infrequently recrudesces after tens of years of dormancy [13]. Takaboh and Oumu are currently under careful observation for such latency. One may argue that malaria parasites have been transmitted between chimpanzee hosts. But despite that Takaboh and Oumu currently live in the same facility, their parasites have distinct sequences of SSU rRNA and mitochondrial genome. Furthermore, since our PCR method can detect malaria parasites at very low parasite density (as few as 1 parasite/sample using PgeneralF3s and PgeneralR1s primers; see Materials and Methods; data not shown), more infection of other chimpanzees should have been detected. Thus, it is unlikely that the parasites have been transmitted among them. However, given the public health concern/issues, malaria monitoring may be proposed as a part of routine health examination for all captive chimpanzees.

P. rodhaini was previously described as a *P. malariae*-like quartan malaria parasite in chimpanzees [3,13]. Experimental transfer of *P. rodhaini* from chimpanzees to humans was successful by the inoculations of parasitized blood [3]. In addition, splenectomized chimpanzees are susceptible to *P. malariae* infection [3,13]. These experiments lead to note that *P. rodhaini* is synonymous with *P. malariae*. Our finding is consistent with this note. However, further findings from malaria survey in great apes are definitely needed to conclude that *P. rodhaini* is a variant of *P. malariae*.

Human malaria parasites have been widely supposed to be found only in humans; hence nonhuman primates are not included in the target for malaria control. The present finding necessitates a survey of human malaria parasites in nonhuman primates. In this context, it should be remembered that two New World monkey parasites, *Plasmodium brasilianum* and *Plasmodium simium*, are known as *P. malariae*-like and *P-vivax*-like parasites, respectively [3,13]. Evolutionary analysis represents that *P. brasilianum* and *P. simium* are very closely related to *P. malariae* and *P. vivax*, respectively [14,15]. Thus, both New World monkey parasites might be actually human parasites infected New World monkeys. In addition to chimpanzees, the New World monkey may therefore be a subject of malaria screening for public health.

Considering that great apes are endangered animals today [16], this study also has significance in their conservation biology. Further surveys may reveal the current situation of malaria infection in great apes, and help to assess risk of malaria in their life.

Materials and Methods

Chimpanzee samples

The chimpanzees are being kept in the Chimpanzee Sanctuary Uto, Kumamoto (n = 55) and Primate Research Institute, Kyoto University, Aichi (n = 5). During routine health examinations, chimpanzees were sedated with oral midazolam (1 mg/kg) or droperidol (0.2 mg/kg), and their bloods were collected under anesthesia by ketamine hydrochloride (7 mg/kg) or a combination of ketamine hydrochloride (3.5 mg/kg) and medetomidine hydrochloride (0.035 mg/kg). This study was approved by Research Institute for Microbial Diseases, Osaka University; Primate Research Institute, Kyoto University; and the Chimpanzee Sanctuary Uto. All animal work has been conducted according to the following guidelines: Guide for the Care and Use of Laboratory Primates, 2nd edn. (Primate Research Institute, Kyoto University), and Guideline for Care of Chimpanzees (the Chimpanzee Sanctuary Uto). Genomic DNA of blood samples was extracted using QIAamp DNA mini kit (Qiagen, Hilden, Germany).

SSU rRNA sequences

Genomic and nested PCRs using primers (PlaSSU5, PlaSSU3r, SSUF1, and SSUR1) were performed following methods reported in ref. 17. Briefly, genomic PCR conditions were as follows: denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, 68°C for 5 min, and extension at 68°C for 10 min, and the nested PCR conditions were as follows: denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 59.3°C for 30 s, 68°C for 5 min, and extension at 68°C for 10 min [17]. In addition, new primers [PmSSUF2 (5′-TCTC-AAAGATTAAGCCATGCAAGTG-3′), PmSSUR7 (5′-TTCAC-CGACGGAAACCTTGTTAC-3′), PmSSUF3 (5′-TTAAGC-CATGCAAGTGAAAGTATATG-3′), and PmSSUR2 (5′-TTA-AAAGATAGGATTTACGATTTTTC-3′)] were designed. PCR reactions using new primers (PmSSUF2 and PmSSUR7) were performed with 5 pmol of each primer and 1 μl of extracted genomic DNA solution in a total volume of 20 μl containing PrimeSTAR Max DNA polymerase (Takara, Otsu, Shiga, Japan). Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) was used to generate the following conditions: 35 cycles of 98°C for 10 s, 55°C for 5 s, and extension at 72°C for 3 min. The nested PCR were performed with 10 pmol of each new primer (PmSSUF3 and PmSSUR2) and 2 μl of PCR product in a total volume of 50 μl. The nested PCR conditions were as follows: 30 cycles of 98°C for 10 s, 55°C for 5 s, and extension at 72°C for 3 min. The PCR products obtained were cloned into pCR-Blunt II-TOPO vector according to the manufacture's instructions (Invitrogen, Carlsbad, CA, USA). The plasmids containing the SSU rRNA genes were prepared using QIAGEN Plasmid Mini kit (Qiagen), and then subjected to sequencing using an ABI 3130 genetic analyzer (Applied Biosystems).

Complete mitochondrial genome sequences

To amplify the mitochondrial genome sequences of chimpanzee parasites, the genomic PCRs using primers (PgeneralF2s, PgeneralR2, PgeneralF3s, and PgeneralR1s) were performed following methods reported in ref. 18. PCR conditions were as follows: denaturation at 93°C for 1 min followed by 40 cycles of 93°C for 20 s, 60°C for 1 min, 72°C for 3 or 5 min, and extension at 72°C for 10 min [18]. The nested PCRs were carried out using the nested primers [CP69F1 (5′-ATTTAGCGTGTATTGTTGCCTTG-TAC-3′), PgeneralR2 (ref. 18), PvmtF1001 (5′-CATGCAGGACG-GAGATTACCCGA-3′), and PgeneralR1s (ref. 18)] to obtain sufficient amount of PCR products for sequencing. The nested PCR reactions were performed with 10 pmol of each primer and 2 μl of PCR product in a total volume of 50 μl containing 400 μM dNTPs and 1 unit of LA-Taq DNA polymerase (Takara) in PCR buffer containing 2.5 mM $MgCl_2$. The nested PCR conditions were as follows: denaturation at 93°C for 1 min followed by 20 cycles of 93°C for 20 s, 60°C for 1 min, 72°C for 3 or 5 min, and extension at 72°C for 10 min.

In addition, we amplified mitochondrial genome sequences from one human *P. malariae* isolate (PVMS1229, Thailand). The genomic PCRs were performed with the primers [PgeneralF3s (ref. 18), PgeneralR1s (ref. 18), PmF1 (5′-CTAGCATGGGAC-TAAAAAATGTTATG-3′), PmR3 (5′-CTGTATCGTACCC-TAAAGGATTAG-3′), PmF3 (5′-AATTATGGAGTGGATGG-TGTTTTAG-3′), and PmR1 (5′-AGAAGTTAATATCTG-GAAGCGTCTG-3′)] and 2 μl of extracted genomic DNA solution under the following conditions: denaturation at 93°C for 1 min followed by 40 cycles of 93°C for 20 s, 59 or 60°C for 1 min, 72°C for 3 min, and extension at 72°C for 10 min. The nested PCRs were carried out using the nested primers [PvmtF1001 (5′-CATGCAGGACGGAGATTACCCGA-3′), PgeneralR1s (ref. 18), PmF2 (5′-TTAAGCCCTTTTTACCATA-CAAGAG-3′), PmR4 (5′-ATCTTTTTATAGTTGGATCACT-TACAG-3′), PmF4 (5′-TTACAGCTTTTATAGGTTATGTT-TTAC-3′), and PmR2 (5′-GTATCGTAAACGGTCCTAAGG-TAG-3′)] to obtain sufficient amount of PCR products for sequencing. The nested PCR conditions were as follows: denaturation at 93°C for 1 min followed by 20 cycles of 93°C for 20 s, 57 or 60°C for 1 min, 72°C for 3 min, and extension at 72°C for 10 min.

PCR products were purified using the QIA quick PCR purification Kit (Qiagen) and directly sequenced on an ABI 3130 genetic analyzer (Applied Biosystems).

Phylogenetic analysis

Maximum Likelihood trees of SSU rRNA gene and mitochondrial genome were inferred using PAUP 4.0 b [19] based on the GTR+Γ+I model from the selected 1419 sites and the GTR+Γ+I model from the selected 5837 sites, respectively. To construct trees, primate parasites (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium reichenowi*, *Plasmodium hylobati*, *Plasmodium knowlesi*, *Plasmodium cynomolgi*, *Plasmodium coatneyi*, *Plasmodium fieldi*, *Plasmodium fragile*, *Plasmodium gonderi*, *Plasmodium inui*, and *Plasmodium simiovale*), rodent parasites (*Plasmodium berghei*, *Plasmodium chabaudi*, and *Plasmodium yoelii*), bird parasites (*Plasmodium cathemerium*, *Plasmodium gallinaceum*, *Plasmodium juxtanucleare*, *Plasmodium lophurae*, and *Plasmodium relictum*), and reptile parasites (*Plasmodium floridense* and *Plasmodium mexicanum*) were used. The complete or nearly complete sequences of mitochondrial genome and SSU rRNA gene were obtained from the NCBI Web site (http://www.ncbi.nlm.nih.gov/; see also refs. 17 and 18).

Acknowledgments

We thank William E. Collins and John W. Barnwell at the CDC for reading this manuscript and constructive comments. We also thank Naoko Sakihama and Mayumi Fukui for technical assistance and Nirianne Marie Q. Palacpac for editorial assistance. Some chimpanzee blood samples were supplied by the Cooperative Research Program of Primate Research Institute, Kyoto University.

Author Contributions

Conceived and designed the experiments: TH KT. Performed the experiments: TH NA TT. Analyzed the data: TH NA. Contributed reagents/materials/analysis tools: TU HH JS TT. Wrote the paper: TH KT. Reviewed the paper: TH.

References

1. Levine ND (1988) The protozoan phylum Apicomplexa. Boca Raton, Florida: CRC Press.
2. Reichenow E (1920) Ueber das vorkommen der malariaparasiten des menschen bei den afrikanischen menschenaffen. Centralbl f Bakt I Abt Orig 85: 207–216.
3. Coatney GR, Collins WE, Warren M, Contacos PG (2003) CD-ROM. The primate malarias [original book published 1971] Atlanta, GA: CDC.
4. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, et al. (2004) A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet 363: 1017–1024.
5. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H (2004) Naturally acquired Plasmodium knowlesi malaria in human, Thailand. Emerg Infect Dis 10: 2211–2213.
6. Vythilingam I, Tan CH, Asmad M, Chan ST, Lee KS, et al. (2006) Natural transmission of Plasmodium knowlesi to humans by Anopheles latens in Sarawak, Malaysia. Trans R Soc Trop Med Hyg 100: 1087–1088.
7. Kantele A, Marti H, Felger I, Muller D, Jokiranta TS (2008) Monkey malaria in a European traveler returning from Malaysia. Emerg Infect Dis 14: 1434–1436.
8. Escalante AA, Ayala FJ (1994) Phylogeny of the malarial genus Plasmodium, derived from rRNA gene sequences. Proc Natl Acad Sci U S A 91: 11373–11377.
9. Ollomo B, Durand P, Prugnolle F, Douzery E, Arnathau C, et al. (2009) A new malaria agent in African hominids. PLoS Pathog 5: e1000446.
10. Duval L, Nerrienet E, Rousset D, Sadeuh Mba SA, Houze S, et al. (2009) Chimpanzee malaria parasites related to Plasmodium ovale in Africa. PLoS One 4: e5520.
11. Kano S, Kimura M (2004) Trends in malaria cases in Japan. Acta Trop 89: 271–278.
12. Vinetz JM, Li J, McCutchan TF, Kaslow DC (1998) Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. N Engl J Med 338: 367–371.
13. Collins WE, Jeffery GM (2007) Plasmodium malariae: parasite and disease. Clin Microbiol Rev 20: 579–592.
14. Mu J, Joy DA, Duan J, Huang Y, Carlton J, et al. (2005) Host switch leads to emergence of Plasmodium vivax malaria in humans. Mol Biol Evol 22: 1686–1693.
15. Escalante AA, Barrio E, Ayala FJ (1995) Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Mol Biol Evol 12: 616–626.
16. Jolly A (2005) The last great apes? Science 309: 1457.
17. Nishimoto Y, Arisue N, Kawai S, Escalante AA, Horii T, et al. (2008) Evolution and phylogeny of the heterogeneous cytosolic SSU rRNA genes in the genus Plasmodium. Mol Phylogenet Evol 47: 45–53.
18. Hayakawa T, Culleton R, Otani H, Horii T, Tanabe K (2008) Big bang in the evolution of extant malaria parasites. Mol Biol Evol 25: 2233–2239.
19. Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (and other methods). Sunderland, MA: Sinauer Associates.

Expert Opinion

informa
healthcare

Wheat germ cell-free technology for accelerating the malaria vaccine research

Satoru Takeo, Thangavelu U Arumugam, Motomi Torii & Takafumi Tsuboi†
†*Ehime University, Cell-free Science and Technology Research Center, 3 Bunkyo-cho, Matsuyama, 790-8577, Japan*

Background: Malaria causes about 300 million illnesses and 1 million deaths annually. The likeliest scenario is the aggravation of this disease due to the re-emergence of drug-resistant parasites and insecticide-resistant mosquitoes. One of the promising solutions to this disease are vaccines. However, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts. Even the efficacy of RTS,S, the most advanced vaccine candidate in the pipeline of malaria vaccine development, is only around 50%. *Objective*: Against this backdrop, there is an urgency to rapidly enrich the pipeline of vaccine development with novel vaccine candidates that can be discovered by synthesizing and screening a multitude of malaria proteins. *Methods*: However, to achieve this objective, we require optimal technologies for high-throughput synthesis of quality malaria proteins. Among the various protein synthesis systems, the wheat germ cell-free protein synthesis system is advantageous and successful to this end. *Results/conclusion*: The wheat germ cell-free protein synthesis system is optimal for accelerating the decoding of malaria genome and hence characterization of malaria proteins and discovery of malaria vaccine candidates.

Keywords: malaria, *Plasmodium falciparum*, post-genome, vaccine candidate discovery, wheat germ cell-free protein synthesis

Expert Opin. Drug Discov. (2009) 4(11):1191-1199

1. Introduction

Malaria is a serious infectious disease caused by protozoan parasites of the genus *Plasmodium*. The most deadly form of the disease is caused by the inoculation of *Plasmodium falciparum* by infected mosquito bites. This disease causes deaths in millions of people annually and illness in about 300 million people and also puts the health of 3.3 billion people at risk [1]. The complex life cycle of the parasite starts when sporozoites are inoculated during the bite of female *Anopheles* sp. mosquito infected with *Plasmodium*. The sporozoites in the peripheral blood circulation then invade the liver and develop into schizonts containing thousands of merozoites. The schizonts in the hepatocytes then rupture and release into the blood stream the merozoites capable of invading red blood cells. This initiates an intraerythrocytic cycle which comprises the development of ring, trophozoite and schizont stage parasites, then rupture of schizonts, and release of free merozoites for reinvasion into the erythrocytes. This developmental cycle takes about 48 h. These asexual erythrocytic-stage parasites are responsible for the clinical manifestations and pathology of malaria, the most serious illnesses being anemia and cerebral malaria [2].

Despite the parasite's complex life cycle, high level of antigenic diversity and mechanism of immune evasion, naturally acquired immunity to malaria indeed develop after repeated exposure over a period of several years. Even though this natural protective immunity possibly never leads to sterile immunity as suggested by

10.1517/17460440903369813 © 2009 Informa UK Ltd ISSN 1746-0441

the fact that parasites can still be found in the circulation of adults living in high transmission settings, this immunity still confers protection against symptomatic disease, high-density parasitemia and death [3,4]. The naturally acquired immunity seems to predominantly target blood-stage parasites [5]. The importance of antibodies in passive immunization has been supported by the transfer of immunoglobulin conferring protection from immune individuals to non-immune individuals [6,7]. Furthermore, immunity against severe, life-threatening malaria is apparent after a few disease episodes, suggesting that the target antigens have limited diversity in parasite populations [8]. The immune effector mechanisms are poorly understood; however, it is widely understood that antibodies may have a role in preventing merozoite invasion, clearance of infected erythrocytes, prevention of adhesion and sequestration of parasitized infected erythrocytes in the vasculature, and prevention of schizont rupture. Functionally, antibodies could prevent erythrocyte invasion by opsonizing merozoites and facilitating phagocytosis by macrophages, activating complement-mediated damage of merozoites [3]. It is becoming increasingly clear, however, that cell-mediated immunity may also be a critical component of naturally acquired immunity to malaria [9]. In addition to the naturally acquired immunities mentioned above, there are encouraging experimental evidences that the sterile, long-lasting protective immunity was induced, after vaccination with radiation-attenuated sporozoites, in mice [10] and humans [11], or after monthly inoculation of sporozoites through mosquito bites to human volunteers under a prophylactic regimen of chloroquine which prevent only the growth of blood-stage parasites [12]. The above-mentioned evidences strongly support the strategy of malaria control through vaccination that elicits protective immunity.

2. Current situation on malaria vaccine development

Malaria is a leading cause of mortality and morbidity. The efficacy of malaria control through current interventions that use drugs and insecticides may not be sustained too long because they rely on too few compounds [4]. In fact, the disease is re-emerging mainly due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes [13]. Very recently, there is a disturbing report on the emergence of parasite's resistance even to artemisinin [14]. Therefore, we are now forced to endeavor to develop malaria vaccines as one of the essential components for the malaria eradication [15]. We should also keep in mind the fact that, until now, not even a single licensed malaria vaccine has been developed despite intensive efforts [16].

Malaria vaccines are classically categorized into three groups based on the stage of the parasite in which the vaccine works. In other words, the parasite presents different proteins as antigens in different stages of life cycle. Pre-erythrocytic vaccines, either based on subunit vaccines or live attenuated parasite vaccines, have been designed to prevent infection and development of diseases. Protection is, to some extent, mediated by antibodies that prevent sporozoite invasion into hepatocytes [17]. Currently, the RTS,S, a pre-erythrocytic vaccine candidate that uses a fragment of *P. falciparum* circumsporozoite protein fused to the HBV surface antigen [18], is undoubtedly the most advanced and promising vaccine candidate based on a number of clinical trials [19-22]. However, the efficacy reported in these trials was around only 50%, suggesting that there is an urgent need to develop new adjuvant systems, test new boosting strategies and enrich the pipeline of vaccine development with additional promising vaccine candidates.

Asexual blood-stage vaccines are aimed at reducing parasite multiplication and growth in order to protect against clinical symptoms and particularly severe disease, and not infection. They are designed to induce antibody responses against the asexual blood stage of the parasites. Asexual blood stage parasite development is a repeating cycle of the release of new merozoites from schizonts and invasion of merozoites into naive erythrocytes. The ability of the merozoite to specifically attach to and invade erythrocytes is essential for blood stage development; for example, *Plasmodium vivax* must bind to the Duffy antigen to invade into reticulocytes [23]. This and other findings have inspired the search for merozoite antigens that elicit antibodies that block parasite invasion of erythrocytes. Immunization with blood-stage antigens, mainly with different merozoite antigens, has been shown to be protective in a number of animal models [24-27].

For example, Combination B, a vaccine combining merozoite surface protein-1 (MSP1) and MSP2 and a part of the ring-infected erythrocyte surface antigen when tested in humans conferred some level of strain specific protective effect. This vaccine showed promising effect in reducing the severity of malaria episodes but the effect is specific only to 3D7 type allele of MSP2, not to FC27 type allele. Because of this, this vaccine subsequently became ineffective in controlling morbidity, due to the selection pressure of this vaccine and subsequent emergence of the parasite with FC27 type allele of MSP2. This fact strongly argues for the development of vaccines comprising multiple components covering all major allelic types [4]. At present, the leading blood-stage vaccine candidates are all merozoite proteins, either located on the surface or within the apical organelles of merozoites. However, none of the merozoite antigens that have been tested in humans, including MSP1, MSP3 and apical merozoite antigen-1 (AMA1), has yet been shown convincingly to confer high levels of protection in clinical trials [28]. Unlike *P. vivax*, *P. falciparum* uses multiple redundant pathways to invade erythrocytes, complicating the effort to develop anti-invasion vaccines against *P. falciparum* [29,30].

Blood stage immunity might also target variant proteins encoded by the parasite multi-gene families (e.g., *var* gene families). PfEMP1, the most famous protein encoded by the *var* gene family, is exported by parasites onto the surface of the

infected erythrocytes (IRBC) and this protein is involved in IRBC adhesion to endothelium and immunoevasion [31]. The best example of this type of immunity has been demonstrated during pregnancy. In pregnant women, parasites emerge that express IRBC surface proteins, allowing these IRBC to bind chondroitin sulfate A (CSA) and sequester in the placenta [32]. First-time mothers lack antibodies specific for the IRBC surface proteins of these parasites and are highly susceptible to infection and disease. Women become resistant over successive pregnancies as they acquire antibodies that block IRBC binding to CSA [33]. Placental parasites express distinct genes and proteins, including an IRBC variant surface protein called VAR2CSA that is required for adhesion to CSA. A program to develop a vaccine based on VAR2CSA is underway [17,34-38]. This fact strongly argues for the development of vaccines comprising conserved epitopes covering all major variant genes [4].

Recently, there has been a renewed call for the 'eradication' of malaria [39]. An effective vaccine will probably be needed to achieve eradication, in addition to other existing drugs and insecticides-based measures. To achieve malaria eradication, the emphasis is on approaches that reduce not only clinical illness in humans but also parasite transmission from humans to mosquitos. Therefore, vaccines that confer sterile immunity and/or transmission-reducing activity would be preferred. The role of asexual blood-stage vaccine as part of this malaria eradication strategy has been questioned, as this vaccine is generally believed to reduce parasitemia and prevent clinical illness but not affect transmission. However, it is also possible that, in addition to preventing clinical illness, an effective blood-stage vaccine may also contribute to the reduction in the efficiency of the transmission by interrupting the blood-stage life cycle in the human body [28].

Transmission-blocking vaccines are aimed at blocking malaria transmission by interrupting the parasite life cycle in the mosquito. These vaccines elicit antibodies against antigens that are expressed by the sexual stage of the parasite and, thus, stop their subsequent development in the mosquito midgut [40]. The target antigens of this vaccine were shown to be sexual-stage specific surface molecules (e.g., P230 and P48/45) that are involved in the process of fertilization of male and female gametes. Subsequently, other antigens (e.g., P25 and P28) that are uniquely expressed by zygotes and ookinetes in the mosquito midgut were shown to be equally good for induction of transmission-blocking immunity. Antibodies in experimental animals against Pfs25 and Pvs25 have been successful at preventing parasite transmission [41,42] and, therefore, these vaccine antigens, Pfs25 [43] and Pvs25 [44], were tried out in Phase I clinical trials. Such vaccines will not provide any immediate direct benefit to the vaccinated individual, but their widespread deployment will help reduce transmission of the parasite and thus protect both the vaccinated individuals and their community as a whole [45]. These transmission-blocking vaccines, if used in combination with pre-erythrocytic or erythrocytic vaccines, might also prevent the transmission of mutant parasites and parasites expressing variant alleles that emerge by evading and escaping human protective immune responses. When used in combination with vector control measures, the transmission-blocking vaccine could play a key role in finally breaking the transmission of parasites, leading to eradication of the diseases [46].

An effective malaria vaccine is expected to target several antigens expressed at different stages of parasite [47] and induce protective immune responses equivalent to, or better than, those provided by naturally acquired immunity or immunization with attenuated whole parasite [48]. In order to accelerate the discovery of such vaccine candidates, we need two things. One is the optimal system for high-throughput synthesis of malaria proteins and the other is the efficient post-genomic high-throughput approaches for screening and selecting potential vaccine candidates from these synthesized proteins.

3. How the malaria genome data can be exploited profitably for malaria vaccine candidate discovery

Decades of research in the pre-genomic era have identified only a handful of vaccine candidates [4]. With the recent completion of the genome projects of human malaria parasites, *P. falciparum* [48], *P. vivax* [49], zoonotic primate malaria, *Plasmodium knowlesi* [50] and rodent malaria parasite, *Plasmodium yoelii* [51], we are now in the post-genome era. Thousands of novel genes have been annotated, providing new opportunities for vaccine research. In order to successfully identify putative novel vaccine candidates for downstream vaccine research, we now need to thoroughly analyze these genomic data using at least two post-genomic high-throughput approaches. One is the functional approach (otherwise called reverse vaccinology [52]) and the other is the immunoscreening approach.

In the functional approach, several direct and indirect functional criteria are used to objectively select and prioritize putative vaccine candidates for further functional studies [53]. For example, the putative candidates for vaccine research can be selected based on their role in erythrocyte invasion [54], essentiality for the parasite's survival revealed by gene knockout studies [30], putative secretory function predicted based on presence of signal peptides and transmembrane regions, localization on the surface or in the apical organelles of the merozoite, or signatures of immune selection pressure revealed by polymorphisms and diversity studies [55].

In the immunoscreening approach, hundreds or thousands of malaria proteins as antigens are screened, using immunoassays such as enzyme-linked immunosorbent assay and so on, with a large number of human serum samples obtained from non-immune, semi-immune and immune individuals. This profiling of immune responses that develop in humans, after natural or experimental infection, or after vaccination with irradiated or genetically attenuated organisms, will facilitate

identifying immunoreactive and protective antigens [56]. Whatever may be the approach used for the discovery of novel vaccine candidates, we do need to have an optimal recombinant protein synthesis system not only for the synthesis and characterization of these vaccine candidates in the initial stage, but also for the mass production of vaccine antigens for vaccination purpose in the later stage.

The first and the foremost barrier to the rapid discovery of vaccine candidates is the lack of an efficient system to synthesize quality malaria proteins. *Plasmodium falciparum* genes are A/T rich (average A/T content per coding sequence is 76%) and a number of them encode repeated amino-acid motifs [48], and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional *Escherichia coli* cell-based systems [57-59]. Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses [60-62]. There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity, and requirement of preparation of large quantities of antigen for immunization. The above impediments are completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus or Chinese hamster ovary cell. But, fortunately, we learnt that the wheat germ cell-free system could surmount most of the above stumbling blocks in the path of post-genome malaria vaccine candidate discovery [63].

4. Wheat germ cell-free protein synthesis system

Recently, the wheat germ cell-free protein synthesis system was established for practical use in protein production [64] and is now commercially available as simple protein synthesis kits including *in vitro* transcription system and translation system (CFS Co., Ltd, Matsuyama, Japan). The system is especially powerful when used for the production of eukaryotic proteins because of its eukaryotic nature [65,66]. Basically, two wheat germ cell-free protocols were established for practical use [65]. The first one is for small scale synthesis of proteins. It can be used to produce small quantities of proteins from a large number of cDNAs, in parallel, for testing synthesis and solubility of proteins and also for genome-wide biochemical annotation of gene products [67]. The second protocol is for the production of large quantities of proteins [68]. In this protocol, genes of interest are cloned into the pEU wheat germ expression vectors [69,70] and the mRNA is transcribed. Then, the mRNA is translated either using the bilayer or discontinuous batch translation method [65]. The bilayer method can produce hundreds of micrograms of protein. The discontinuous batch translation method can produce around 150 mg of a control protein in a reaction volume of 50 ml in 5 h and gives room for scale up [68]. These two methods have already been acknowledged as advantageous to yield good quality proteins, in the fields of structural and functional genomics of eukaryotes [66,71]. If we could establish a good manufacturing practice facility for production of recombinant proteins for clinical studies such as vaccine research using this system, then this system may be suitable for mass production of vaccine. The fact that both the wheat germ system and the malaria parasite do not have glycosylation machinery is an advantage for expressing malaria proteins without any inadvertent glycosylation in wheat germ system. Taken together, this system is versatile for both small and large scale production of quality malaria proteins.

We found from our initial studies that the wheat germ cell-free system can surmount most of the above impediments and hence accelerate the discovery of malaria vaccine candidates [63]. Initially, in order to test the suitability of wheat germ cell-free system for high-throughput expression of the parasite genes, we tried to express 124 genes encoding asexual blood-stage parasite proteins, selected from the malaria genome database, PlasmoDB (http://plasmodb.org/plasmo/). Seventy-five percent of genes (93 out of 124 genes) yielded soluble protein products. However, the extent of solubility among these 93 soluble proteins was on an average 65% and ranged from 26 to 100% [63]. The average yield of expressed protein estimated for each full size product was 1.9 µg per 150 µl of reaction mixture, an amount sufficient for preliminary antigen discovery studies using hyper-immune serum. We found that there was significant inverse correlation between yield and molecular size of the protein; the greater the size, the lower the protein yield. Because of this reason, it will be difficult to synthesize the full size of high molecular mass vaccine candidates, such as PfEMP1. There was also an inverse correlation between the protein yield and the abundance of low-complexity regions. In addition, solubility was inversely correlated with the isoelectric point (pI) value [63]. Similar observations have been also documented in *E. coli* cell-based expression system [58,59]. Surprisingly, we did not see any correlation among yield and A/T content, pI value or the existence of a transmembrane domain [63]. From these pilot experiments, we found that the wheat germ system can be used successfully for the expression of *P. falciparum* genes.

Later on, from many of our and our collaborator's proof-of-principle studies, we confirmed that wheat germ cell-free protein synthesis system is advantageous when it comes to synthesizing biologically active malaria proteins by directly using the native A/T rich malaria genes without any codon optimization (summarized in **Table 1**). First, the system was able to produce active malaria enzymes, such as *P. falciparum* dihydrofolate reductase-thymidylate synthase [72] and chitinase of *P. vivax* [73]. Localization of pyruvate kinase type-II isozyme was also identified in apicoplast using antibody raised against recombinant protein produced in this system [74]. In fact, all the above mentioned three genes were recalcitrant to expression in other protein expression systems. Second, the wheat germ cell-free system was able to produce a sufficient amount of good quality proteins (an important prerequisite

Table 1. Proof-of-principle studies for the production of biologically active malaria proteins using the wheat germ cell-free expression system*.

Gene ID‡	Gene name	Species§	Results¶	Ref.
Enzymes				
PFD0830w	Bifunctional dihydrofolate reductase-thymidylate synthase	Pf	Enzyme activities (DHFR, TS)	[72]
PVX_087680	Chitinase	Pv	Enzyme activities	[73]
PF10_0363	Pyruvate kinase 2	Pf	Ab (WB, IFA)	[74]
Sporozoite antigens				
PFC0210c	Circumsporozoite protein	Pf	Ab (WB, IFA)	[63]
PFD0215c	P52	Pf	Ab (IFA)	[75]
Merozoite antigens				
PF11_0344	Apical membrane antigen 1	Pf	Ab (WB, IFA)	[63]
PF14_0495	Rhoptry neck protein 2	Pf	Ab (IP, WB, IFA, IEM)	[76]
PY04764	Erythrocyte binding ligand	Py	Ab (WB, IFA, IEM)	[77]
Ookinete antigen				
PF10_0303	Pfs25	Pf	Ab (WB, IFA, TBA)	[63]

*The individual articles may be consulted for further details.
‡Detailed information is available at the PlasmoDB website (http://plasmodb.org/plasmo/).
§*Plasmodium* species, *Pf*; *P. falciparum*, *Pv*; *P. vivax*, *Py*; *P. yoelii*.
¶Results obtained by the contribution of recombinant proteins synthesized by the wheat germ cell-free system.
Ab: Antibody; DHFR: Dihydrofolate reductase; IEM: Immunoelectron microscopy; IFA: Immunofluorescence microscopy; IP: Immunoprecipitation; TBA: Transmission-blocking activity; TS: Thymidylate synthase; WB: Western blot.

for the malaria vaccine candidate discovery), such as a repeat-rich molecule, circumsporozoite protein [63], a cysteine-rich molecule, P52 (one of the target molecules for the generation of genetically attenuated live-sporozoite vaccine) [75], RON2 (merozoite rhoptry neck protein involved in the erythrocyte invasion process) [76], PyEBL (orthologue of EBA175 in a rodent malaria parasite, *P. yoelii*, that is responsible for the merozoite invasion into erythrocyte) [77] and cysteine-rich Pfs25 (a promising transmission-blocking vaccine candidate) [63]. AMA1 requires synthetic codon optimized gene in order to be expressed in *E. coli* cell-based system and the synthesized proteins also need a series of labor-intensive and technically complex refolding processes, because of its cysteine-richness, to obtain quality proteins [78]. Even this recalcitrant AMA1 could be expressed easily without any codon optimization in the wheat germ cell-free system [63].

Recently, we attempted to express 567 of *P. falciparum* cDNA clones selected from sporozoite, merozoite and gametocyte stages in a high-throughput format by the wheat germ cell-free system. Out of 567 genes, 478 (i.e., around 84%) yielded soluble protein products (unpublished). Our biochemical, immunocytochemical and biological analyses have revealed that the recombinant malaria proteins synthesized by this system are of high quality and, therefore, amenable for vaccine candidate assessment.

5. Expert opinion

In order to establish high-throughput genome-wide functional or immunoscreening methods for malaria vaccine candidate discovery, the choice of the recombinant protein synthesis method is the most crucial factor. There were many leading trials to achieve genome-wide expression of *P. falciparum* genes using the conventional *E. coli*-based protein synthesis system. Aguiar *et al.*, using *E. coli* cells, were able to express only 39 out of 292 malaria genes cloned in GST-fusion vector [57]. Mehlin *et al.* carried out an even more challenging trial in which 1000 genes encoding relatively small (< 450 amino acids) malaria cytosolic proteins were expressed in *E. coli* [58]. In this study, only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9 – 406 mg of protein per liter of culture medium. Vedadi *et al.* used another approach that used an engineered *E. coli* strain with genetically supplemented tRNAs that allows reading of high number of A/U codons in malaria mRNA [59]. They found marginal improvement in protein solubility, that is, 38 (20.9%) out of 182 proteins tested were soluble. Mu *et al.* [55] and Doolan *et al.* [56] attempted to express malaria recombinant proteins using an *E. coli* cell-free expression system in order to find potential vaccine candidates. Mu *et al.* [55] could express around 60% of the malaria genes (65 out of 108 genes) that are considered to be under immune selection pressure. They verified the expression of proteins via western blot using antibodies to the histidine tags incorporated into the C-terminus of the expressed proteins [55]. Recently, Doolan *et al.* reported the construction of the *P. falciparum* protein microarrays using *E. coli* cell-free protein synthesis system for high-throughput immunoscreening for vaccine candidate discovery [56]. In their study, they obtained > 90% efficiency in their effort to express 250

P. falciparum genes using *E. coli* cell-free *in vitro* transcription and translation reactions. And they have attributed their high success rate in this system, at least in part, to the fact that the system is supplemented with rare transfer RNAs to help translate A/T-rich genes and that the proteins are printed directly without purification on to microarray slides. The above experiments showed better productivity and throughput only in the *E. coli* cell-free system and not in *E. coli* cell-based system. Nonetheless, because the results based on the *E. coli* cell-free system study [55,56] were obtained using a relatively small number of *P. falciparum* genes, they do not truly represent the capacity of *E. coli* cell-free system for genome-wide *P. falciparum* gene expression.

It has been suggested that the eukaryotic translation and folding machinery have been optimized through evolution to facilitate co-translational domain folding of a multi-domain protein. In fact, the rate of peptide growth on ribosomes differs considerably between eukaryotes and bacteria, being five to ten times slower in eukaryotes. This slow rate of peptide growth in eukaryotic protein expression system contributes greatly towards correct conformational folding of a multi-domain protein or its epitope and their solubility [79,80]. Although the *E. coli* system is known to support folding of prokaryotic and small eukaryotic proteins, because of its prokaryotic nature, the proteins with multiple domains that are commonly found in eukaryotes such as malaria parasite proteins, when expressed either cell free or cell-based *E. coli* system, tend to fold incorrectly, resulting in the formation of inclusion bodies [79]. After all, the solubility and the functionality of the correctly folded malaria proteins and/or their epitopes are indispensable and critical factors that directly affect the success of our genome-wide search for potential vaccine antigens. Therefore, from this point of view, the eukaryotic-based system is greatly advantageous over the *E. coli*-based system.

Other cell-free systems derived from eukaryotic sources, including rabbit reticulocytes, yeast cells, tumor cells and insect cell lines, have also been widely used to produce eukaryotic multidomain proteins in active forms, but all suffer from low productivity [65]. One of the most convenient and promising eukaryotic cell-free translation systems for achieving high productivity and high solubility of proteins is conceivably the one based on wheat germ embryos [65].

Recently, Goshima *et al.* have again proved that wheat germ cell-free system is more advantageous than *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In their study, they have successfully expressed human proteins on a whole-proteome-scale [66]. In their study, initially, on a trial basis, they expressed 26 cDNA clones for soluble protein (molecular mass; MM 10.4 – 101.3 kDa) and 24 clones for membrane protein (MM 7.0 – 80.1 kDa) in various expression systems, such as wheat germ cell-free system, *E. coli* cell-free system, *E. coli* cell-based system, and MD2 and CHO cultured cell expression systems. In this expression study, they found that the wheat germ cell-free system is the best of all the methods for achieving high-throughput, high-quality and high-productivity synthesis of recombinant proteins, and ease of handling. Moreover, of the 75 phosphatases expressed by them in wheat germ cell-free system, 58 (77%) showed biological activity and several cytokines containing disulfide bonds were produced in active forms. They also expressed 13,277 human proteins by this system. The success rate of recombinant protein synthesis was almost 100%. Although the protein-synthesis system exhibits nearly perfect efficacy, the yields of the proteins were variable [66].

In order to discover novel vaccine candidates, the malaria genome has to cross a lot of barriers, such as protein synthesis, selection of protein purification tags, characterization of protein molecules, selection of *in vitro* functional assay systems (i.e., growth inhibition assay or antibody-dependent cellular inhibition assay) and selection of adjuvant. From our experience of expressing around 500 malaria genes using wheat germ cell-free system (unpublished), we learnt that the recombinant malaria proteins synthesized are of high quality and, therefore, amenable for vaccine candidate assessment. Therefore, we indeed believe that this system is a key tool to overcome the first and the foremost barrier, that is, decoding of the malaria genome. From a malaria vaccine perspective, wheat germ system will facilitate production of malaria proteins on a whole-proteome-scale and construction of microarrays of malaria proteins. These microarrays will be an indispensable tool for profiling immune responses developed in non-immune, semi-immune and immune individuals, and identifying immunoreactive antigens that correlate with protection. In fact, we are now focusing on the establishment of microarray of malaria proteins. No doubt, production of malaria proteins on a whole-proteome-scale using wheat germ cell-free protein synthesis system is achievable and microarray of malaria proteins will be soon available for facilitating post-genomic high-throughput identification of novel vaccine candidates.

Acknowledgements

The authors thank K Kato, A Tamai and M Tasaka for their technical assistance.

Declaration of interest

This work was supported in part by Grants-in-Aid for Scientific Research 19406009 (TT), Scientific Research on Priority Areas 21022034 (TT) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and, in part, by a Grant-in-Aid of the Ministry of Health, Labour, and Welfare (H21-Chikyukibo-ippan-005) (TT), Japan.

Bibliography

1. WHO. World Malaria Report 2008. Geneva, Switzerland: WHO Press; 2008
2. Miller LH, Baruch DI, Marsh K, et al. The pathogenic basis of malaria. Nature 2002;415:673-9
3. Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. Parasite Immunol 2006;28:51-60
4. Genton B. Malaria vaccines: a toy for travelers or a tool for eradication? Expert Rev Vaccines 2008;7:597-611
5. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. Clin Microbiol Rev 2009;22:13-36, Table of Contents
6. Cohen S, Mc GI, Carrington S. Gamma-globulin and acquired immunity to human malaria. Nature 1961;192:733-7
7. McGregor IA. The passive transfer of human malaria immunity. Am J Trop Med Hyg 1964;13(Suppl):237-9
8. Gupta S, Snow RW, Donnelly CA, et al. Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med 1999;5:340-3
9. Pombo DJ, Lawrence G, Hirunpetcharat C, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with *plasmodium falciparum*. Lancet 2002;360:610-7
10. Nussenzweig RS, Vanderberg J, Most H, et al. Protective immunity produced by the injection of x-irradiated sporozoites of *plasmodium berghei*. Nature 1967;216:160-2
11. Hoffman SL, Goh LM, Luke TC, et al. Protection of humans against malaria by immunization with radiation-attenuated *plasmodium falciparum* sporozoites. J Infect Dis 2002;185:1155-64
12. Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 2009;361:468-77
13. Greenwood B, Mutabingwa T. Malaria in 2002. Nature 2002;415:670-2
14. Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *plasmodium falciparum* malaria. N Engl J Med 2009;361:455-67
15. Greenwood B. Can malaria be eliminated? Trans R Soc Trop Med Hyg 2009;103(Suppl 1):S2-5
16. Richie TL, Saul A. Progress and challenges for malaria vaccines. Nature 2002;415:694-701
17. Greenwood BM, Fidock DA, Kyle DE, et al. Malaria: progress, perils, and prospects for eradication. J Clin Invest 2008;118:1266-76
18. Stoute JA, Slaoui M, Heppner DG, et al.; RTS,S Malaria Vaccine Evaluation Group. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *plasmodium falciparum* malaria. N Engl J Med 1997;336:86-91
19. Heppner DG Jr, Kester KE, Ockenhouse CF, et al. Towards an RTS, S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. Vaccine 2005;23:2243-50
20. Alonso PL, Sacarlal J, Aponte JJ, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet 2005;366:2012-8
21. Aponte JJ, Aide P, Renom M, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 2007;370:1543-51
22. Bejon P, Lusingu J, Olotu A, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 2008;359:2521-32
23. Miller LH, Mason SJ, Clyde DF, et al. The resistance factor to *plasmodium vivax* in blacks. The duffy-blood-group genotype, FyFy. N Engl J Med 1976;295:302-4
24. Ling IT, Ogun SA, Holder AA. Immunization against malaria with a recombinant protein. Parasite Immunol 1994;16:63-7
25. Perera KL, Handunnetti SM, Holm I, et al. Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human *plasmodium vivax* malaria. Infect Immun 1998;66:1500-6
26. Crewther PE, Matthew ML, Flegg RH, et al. Protective immune responses to apical membrane antigen 1 of *plasmodium chabaudi* involve recognition of strain-specific epitopes. Infect Immun 1996;64:3310-7
27. Collins WE, Pye D, Crewther PE, et al. Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of *plasmodium fragile*. Am J Trop Med Hyg 1994;51:711-9
28. Richards JS, Beeson JG. The future for blood-stage vaccines against malaria. Immunol Cell Biol 2009;87:377-90
29. Baum J, Maier AG, Good RT, et al. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. PLoS Pathog 2005;1:e37
30. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006;124:755-66
31. Maier AG, Cooke BM, Cowman AF, et al. Malaria parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol 2009;7:341-54
32. Fried M, Duffy PE. Adherence of *plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science 1996;272:1502-4
33. Fried M, Nosten F, Brockman A, et al. Maternal antibodies block malaria. Nature 1998;395:851-2
34. Francis SE, Malkov VA, Oleinikov AV, et al. Six genes are preferentially transcribed by the circulating and sequestered forms of *plasmodium falciparum* parasites that infect pregnant women. Infect Immun 2007;75:4838-50
35. Salanti A, Staalsoe T, Lavstsen T, et al. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *plasmodium falciparum* involved in pregnancy-associated malaria. Mol Microbiol 2003;49:179-91
36. Viebig NK, Gamain B, Scheidig C, et al. A single member of the *plasmodium falciparum* var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. EMBO Rep 2005;6:775-81
37. Gamain B, Trimnell AR, Scheidig C, et al. Identification of multiple chondroitin sulfate A (CSA)-binding domains in the var2CSA gene transcribed in CSA-binding parasites. J Infect Dis 2005;191:1010-3
38. Duffy PE. Plasmodium in the placenta: parasites, parity, protection, prevention and possibly preeclampsia. Parasitology 2007;134:1877-81

39. Feachem R, Sabot O. A new global malaria eradication strategy. Lancet 2008;371:1633-5

40. Carter R. Transmission blocking malaria vaccines. Vaccine 2001;19:2309-14

41. Hisaeda H, Stowers AW, Tsuboi T, et al. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *plasmodium vivax* to infect mosquitoes. Infect Immun 2000;68:6618-23

42. Wu Y, Przysiecki C, Flanagan E, et al. Sustained high-titer antibody responses induced by conjugating a malaria vaccine candidate to outer-membrane protein complex. Proc Natl Acad Sci USA 2006;103:18243-8

43. Wu Y, Ellis RD, Shaffer D, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS ONE 2008;3:e2636

44. Malkin EM, Durbin AP, Diemert DJ, et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for *plasmodium vivax* malaria. Vaccine 2005;23:3131-8

45. Smith TA, Leuenberger R, Lengeler C. Child mortality and malaria transmission intensity in Africa. Trends Parasitol 2001;17:145-9

46. Targett GA, Greenwood BM. Malaria vaccines and their potential role in the elimination of malaria. Malar J 2008;7(Suppl 1):S10

47. Lasonder E, Ishihama Y, Andersen JS, et al. Analysis of the *plasmodium falciparum* proteome by high-accuracy mass spectrometry. Nature 2002;419:537-42

48. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite *plasmodium falciparum*. Nature 2002;419:498-511

49. Carlton JM, Adams JH, Silva JC, et al. Comparative genomics of the neglected human malaria parasite *plasmodium vivax*. Nature 2008;455:757-63

50. Pain A, Bohme U, Berry AE, et al. The genome of the simian and human malaria parasite *plasmodium knowlesi*. Nature 2008;455:799-803

51. Carlton JM, Angiuoli SV, Suh BB, et al. Genome sequence and comparative analysis of the model rodent malaria parasite *plasmodium yoelii yoelii*. Nature 2002;419:512-9

52. Flower D. Bioinformatics for Vaccinology. Chichester, West Sussex, UK: John Wiley & Sons; 2008

53. Hall N, Karras M, Raine JD, et al. A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science 2005;307:82-6

54. Sim BK, Chitnis CE, Wasniowska K, et al. Receptor and ligand domains for invasion of erythrocytes by *plasmodium falciparum*. Science 1994;264:1941-4

55. Mu J, Awadalla P, Duan J, et al. Genome-wide variation and identification of vaccine targets in the *plasmodium falciparum* genome. Nat Genet 2007;39:126-30

56. Doolan DL, Mu Y, Unal B, et al. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. Proteomics 2008;8:4680-94

57. Aguiar JC, LaBaer J, Blair PL, et al. High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. Genome Res 2004;14:2076-82

58. Mehlin C, Boni E, Buckner FS, et al. Heterologous expression of proteins from *plasmodium falciparum*: results from 1000 genes. Mol Biochem Parasitol 2006;148:144-60

59. Vedadi M, Lew J, Artz J, et al. Genome-scale protein expression and structural biology of *plasmodium falciparum* and related apicomplexan organisms. Mol Biochem Parasitol 2007;151:100-10

60. Gowda DC, Davidson EA. Protein glycosylation in the malaria parasite. Parasitol Today 1999;15:147-52

61. Kedees MH, Azzouz N, Gerold P, et al. *Plasmodium falciparum*: glycosylation status of plasmodium falciparum circumsporozoite protein expressed in the baculovirus system. Exp Parasitol 2002;101:64-8

62. Samuelson J, Banerjee S, Magnelli P, et al. The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. Proc Natl Acad Sci USA 2005;102:1548-53

63. Tsuboi T, Takeo S, Iriko H, et al. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun 2008;76:1702-8

64. Madin K, Sawasaki T, Ogasawara T, et al. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. Proc Natl Acad Sci USA 2000;97:559-64

65. Endo Y, Sawasaki T. Cell-free expression systems for eukaryotic protein production. Curr Opin Biotechnol 2006;17:373-80

66. Goshima N, Kawamura Y, Fukumoto A, et al. Human protein factory for converting the transcriptome into an in vitro-expressed proteome. Nat Methods 2008;5:1011-7

67. Sawasaki T, Ogasawara T, Morishita R, et al. A cell-free protein synthesis system for high-throughput proteomics. Proc Natl Acad Sci USA 2002;99:14652-7

68. Sawasaki T, Endo Y. The wheat germ cell-free protein synthesis system. In: Spirin AS, Swartz JR, editors, Cell-free protein synthesis, methods and protocols. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2008. p. 111-39

69. Sawasaki T, Gouda MD, Kawasaki T, et al. The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information. Methods Mol Biol 2005;310:131-44

70. Kamura N, Sawasaki T, Kasahara Y, et al. Selection of 5′-untranslated sequences that enhance initiation of translation in a cell-free protein synthesis system from wheat embryos. Bioorg Med Chem Lett 2005;15:5402-6

71. Vinarov DA, Lytle BL, Peterson FC, et al. Cell-free protein production and labeling protocol for NMR-based structural proteomics. Nat Methods 2004;1:149-53

72. Mudeppa DG, Pang CK, Tsuboi T, et al. Cell-free production of functional *plasmodium falciparum* dihydrofolate reductase-thymidylate synthase. Mol Biochem Parasitol 2007;151:216-9

73. Takeo S, Hisamori D, Matsuda S, et al. Enzymatic characterization of the *plasmodium vivax* chitinase, a potential malaria transmission-blocking target. Parasitol Int 2009:58;243-8

74. Maeda T, Saito T, Harb OS, et al. Pyruvate kinase type-II isozyme in *plasmodium falciparum* localizes to the apicoplast. Parasitol Int 2009;58:101-5