NF135.C10: A New \textit{Plasmodium falciparum} Clone for Controlled Human Malaria Infections

Anne C. Teirlinck,1,a Meta Roestenberg,1,a,b Marga van de Vegte-Bolmer,1 Anja Scholzen,1 Moniek J. L. Heinrichs,1 Rianne Siebelink-Stoter,1 Wouter Graumans,1 Geert-Jan van Gemert,1 Karina Teelen,1 Martijn W. Vos,1 Krystelle Ngonou-Makamdop,1 Steffen Bormann,1,b Yolanda P. A. Rozier,2 Marianne A. A. Erkens,2 Adrian J. F. Luty,3,b Cornelis C. Hermens,1 B. Kim Lee Sim,6 Lisette van Lieshout,2,3 Stephen L. Hoffman,4 Leo G. Visser,1, and Robert W. Sauerwein1

1Department of Medical Microbiology, Radboud University Nijmegen Medical Center, and Departments of 2Medical Microbiology (Clinical Microbiology Laboratory); 3Parasitology, and 4Infectious Diseases, Leiden University Medical Center, the Netherlands; 5Department of Infectious Diseases, Heidelberg University School of Medicine, Germany; and 6Sanaria Inc, Rockville, Maryland

We established a new field clone of \textit{Plasmodium falciparum} for use in controlled human malaria infections and vaccine studies to complement the current small portfolio of \textit{P. falciparum} strains, primarily based on NF54. The Cambodian clone NF135.C10 consistently produced gametocytes and generated substantial numbers of sporozoites in \textit{Anopheles} mosquitoes and diverged from NF54 parasites by genetic markers. In a controlled human malaria infection trial, 3 of 5 volunteers challenged by mosquitoes infected with NF135.C10 and 4 of 5 challenged with NF54 developed parasitemia as detected with microscopy. The 2 strains induced similar clinical signs and symptoms as well as cellular immunological responses.

\textit{Clinical Trials Registration.} NCT01002833.

\textbf{Keywords.} Malaria; parasite culture; parasite strain; parasite clone; field strain; cellular immunology; clinical trial; controlled human malaria infection.

Malaria caused an estimated 216 million cases and approximately 1 million deaths in 2010 [1], mainly in sub-Saharan Africa where most cases are caused by \textit{Plasmodium falciparum}. Development of vaccines and new drugs and better understanding of immunological processes are essential to tackling this immense problem. Controlled human malaria infection (CHMI), in which healthy volunteers are exposed to bites of \textit{P. falciparum}-infected mosquitoes, is a powerful tool to address questions regarding \textit{P. falciparum} drug and vaccine efficacy, clinical signs and symptoms, parasite kinetics, and human immunology. Since the first CHMI by mosquitoes fed on cultures of \textit{P. falciparum}, >1300 healthy volunteers have been exposed to CHMI with mainly the Nijmegen falciparum strain NF54 or its clone 3D7 [2]. Strain/parasite line NF54 stably produces sexual stages required for production of infectious mosquitoes. Parasites have been adapted to laboratory conditions by continuous in vitro culture for >3 decades. In the field, \textit{P. falciparum} displays a wide genetic diversity, which is currently not represented by the available laboratory strains for CHMI. Other strains, including the South American 7G8 \textit{P. falciparum} clone of the Brazilian strain IMTM22, have been sporadically used in limited number of volunteers [3–5]. We therefore aimed to identify, clone, and test an additional \textit{P. falciparum} strain that can be used in CHMIs, and we developed several qualification criteria: The clone (a) must consistently produce gametocytes and sporozoites, (b) should be cloned to create a single genetically homogeneous parasite population, (c) should be sensitive to commonly administered antimalarials, and (d) should be of non-African origin to be geographically and genetically distinct from the NF54 strain, an airport strain that probably originates from Africa [6].

Here we report the generation, characterization, and first CHMI for NF135.C10, a new Cambodian clone; findings include drug sensitivity, microsatellite profile, kinetics of parasitemia, and clinical and immunological properties in a direct comparison with NF54.

\textbf{METHODS}

Blood collected from patients for diagnosis of malaria was cultured in Roswell Park Memorial Institute 1640 medium containing 10% human serum at 5% hematocrit in a semiautomated
Table 1. NF135.C10 and NF54 Culture Characteristics

|                          | NF135.C10 | NF54 |
|--------------------------|-----------|------|
| Restarted cultures until CHMI | 7         | 306  |
| Country of origin        | Cambodia  | West Africa |
| Year of isolation        | 1993      | 1979 |
| Period 2009–2010          |           |      |
| Infection, %             | 74 (62–87)| 86 (78–94) |
| Oocysts                  | 12 (7.3–16)| 27 (22–33) |
| Sporozoites/mosquito, ×10^3 | 39 (18–60) | 99 (74–124) |
| CHMI (April 2010)        |           |      |
| Infection, %             | 100       | 100  |
| Oocysts                  | 5.6       | 17   |
| Sporozoites/mosquito, ×10^3 | 12.5     | 69   |
| Gametocyte male-female ratio | 1.5    | 1.3  |
| Drug sensitivity, mean IC_{50} (SD)^b | 3.4 (1.8) | 9.9 (6.0) |
| Dihydroartemisinin, nmol/L | 89 (26)  | 78 (9.7)  |
| Lumefantrine, nmol/L     | 21 (3.3)  | 27 (4.0)  |
| Proguanil, µmol/L        | 0.3 (0.1) | 0.6 (0.3) |
| Chloroquine, nmol/L      | 201 (45) | 24 (1.1) |

Abbreviation: CHMI, controlled human malaria infection; IC_{50}, half-inhibitory concentration; SD, standard deviation.

Mosquito infection and drug sensitivity profiles of NF135.C10 and NF54 in the period 2009–2010 and for the specific batches used in this CHMI.

Data for the period 2009–2010 represent mean findings (95% confidence intervals) after 26 and 39 standard dissections, for NF135.C10 and NF54 respectively, from 10 mosquitoes per dissection.

The drug sensitivities of NF135.C10 and NF54 were tested by the malaria SYBR Green I-based fluorescence assay in triplicate experiments; values represent means from 3 independent experiments.

RESULTS

Plasmodium falciparum strains obtained from 74 patients with malaria were adapted to culture; 21 strains produced gametocytes, and 16 were able to infect mosquitoes. Based on gametocyte production, exflagellation, and transmission to mosquitoes,
7 strains were cloned. Two of these clones produced at least 5 oocysts and 30,000 sporozoites in >70% of mosquitoes. The drug sensitivity profile of NF135.C10 is similar to that of NF54 for atovaquone, proguanil, dihydroartemisinin, and lumefantrine, but NF135.C10 is >8-fold less sensitive to chloroquine than NF54. The culture characteristics and drug sensitivity of NF135.C10 and NF54 are shown in Table 1. Comparison of NF135.C10 and NF54 genotypes using PCR and rifin microsatellite mapping showed distinct genetic profiles (Supplementary Figure 1).

Three of 5 volunteers infected with NF135.C10 and 4 of 5 infected with NF54 parasites had a positive thick smear during follow-up. The remaining 3 smear-negative volunteers were qPCR negative for *P. falciparum* for 21 days. In *P. falciparum*-positive volunteers, kinetics of parasitemia for both strains were comparable to those in historical controls (*n* = 48; Figure 1A [9]). Patent parasitemia for NF135.C10 occurred slightly earlier than for NF54, as measured by both thick smear (median [range], 7.0 [7.0–9.0] vs 10.6 [10.6–11] days after infection; *P* = .05, Mann–Whitney *U* test) and qPCR (median [range], 7.0 [6.3–7.0] vs 7.3 [7.0–7.3] days after infection; *P* = .1, Mann–Whitney *U* test). In addition, the peak of the first cycle seemed higher in infected volunteers for NF135.C10 (geometric mean [GM], 1.2 [95% confidence interval (CI), .61–2.4] parasites/µL) than for NF54 (GM [95% CI], 0.16 [0.55–4.6] parasites/µL; *P* = .06, Mann–Whitney *U* test). In the same 2 groups of volunteers, the GMs (95% CIs) for peak parasitemia were 11 (1.8–73) and 30 (7.7–120) parasites/µL, respectively (*P* = .4). All parasites were cleared from the blood of all volunteers during follow-up, with slopes that were similar for both strains (Figure 1B). The PCR identities of both strains were confirmed by culture of smear-positive samples from several randomly selected infected volunteers.

All volunteers, including the smear-negative volunteers, reported solicited adverse events that were considered possibly or probably related to the trial procedures (Table 2), particularly headache, fatigue, myalgia, and nausea, without apparent differences between the 2 groups. One volunteer infected with NF54 reported severe malaise, headache, and vomiting. One infected volunteer in the NF135.C10 group had a decreased platelet count of 146 × 10^9/L at day 3 after treatment (cutoff, 150 × 10^9/L), which returned to normal values at routine examination on day 28. Levels of D-dimers did not increase in any of the volunteers before thick smear positivity. Highly sensitive troponin T values were always <0.05 µg/L.

T lymphocytes of volunteers successfully infected with either NF135.C10 or NF54 showed similarly increased IFN-γ, tumor necrosis factor, and interleukin 2 recall responses 35 days after infection and the same kinetics for both homologous and heterologous stimulation (Supplementary Figure 2A–I). IFN-γ–producing cells were found in both the innate compartment (γδ-T, natural killer, natural killer–T) and the adaptive compartment (CD4 and CD8) with an effector memory phenotype which was generally consistent over time and in both groups (Supplementary Figure 2J and 2K and data not shown).

**DISCUSSION**

We identified and characterized NF135.C10 as the first *P. falciparum* clone of Asian origin for successful infection of malaria-naive human volunteers by CHMI. Clone NF135.C10 consistently produced gametocytes in culture and was able to generate infections in laboratory-reared mosquitoes with high yields of sporozoites. NF135.C10 parasites were clearly distinct from NF54 parasites by genetic marker profiles and were sensitive to the most commonly used antimalarials. Clinical presentation after CHMI and characteristics of *P. falciparum* RBC-specific recall (T-)lymphocyte responses in vitro were similar to those in NF54.

For manufacturing purposes, cultures should ideally produce gametocytes that consistently infect ≥75% of the mosquitoes with ≥10 oocysts, resulting in 10,000–30,000 sporozoites per mosquito. Selection, identification, and cloning of *P. falciparum* field strains that meet those criteria pose technical difficulties. Only after extensive efforts on >70 strains were we able to identify a parasite clone, NF135.C10, that met these
criteria and which is geographically and molecularly distinct from NF54. We consider NF135.C10 closely related to its original field strain because of the limited restarts of the culture. We showed that clinical signs and symptoms after infection with NF135.C10 or NF54 were similar despite a shorter prepatent period in NF135.C10-infected volunteers. The observed difference in the prepatent period may represent a true difference in infectivity or may be due to coincidental distribution within the previously observed variation related to the limited number of volunteers.

Notably, not all volunteers exposed to NF54 infected mosquitoes became parasitemic, in contrast to findings in 22 previous CHMI trials infecting 128 naive volunteers with NF54 parasites [10]. Unsuccessful infection after bites from 5 mosquitoes, although rare, has been described elsewhere for 3D7 [11, 12]. Although the exact reason for this low infectivity is unclear, it might be due to a technical disturbance in our cultures leading to unusually low NF135.C10 and NF54 oocyst and sporozoite counts in this particular trial, although the relation of these parameters to infectivity has never been formally established [13]. Surprisingly, all 3 unsuccessfully infected volunteers reported adverse events that were considered possibly or probably related to the trial procedures, which might have been the result of overreporting in an intense follow-up schedule. More studies are required to determine whether 100% infection rates can be achieved and to fully establish NF135.C10 as a heterologous field clone to complement the current CHMI portfolio of *P. falciparum* parasites. We have established master and working cell banks required to produce aseptic, purified, cryopreserved *P. falciparum* sporozoites using NF135.C10 parasites (B. K. L. S. et al, unpublished data), enabling the potential future needle and syringe inoculation of a stable number of sporozoites, in analogy to NF54 [14].

We found similar kinetics and composition of IFN-γ recall responses with homologous and heterologous *Pf*54 and *Pf*135.C10 restimulation, possibly suggesting a role for conserved antigens in the induction and maintenance of heterologous memory responses against *P. falciparum* [15, 16]. Whether these cross-strain T-lymphocyte responses also translate into or represent cross-strain protective immunity in vivo remains to be investigated.

In conclusion, increasing the portfolio of new *P. falciparum* parasite strains, as achieved here for NF135.C10, will accelerate the evaluation of malaria vaccines candidates by facilitating the downstream selection process for further clinical vaccine development. Moreover, heterologous parasite clones may be a component of whole sporozoite combination vaccines in order to enhance cross-strain protection. Although more trials will be necessary to fine-tune the heterologous CHMI model with clone

---

**Table 2. Adverse Events in Volunteers**

| Adverse Event   | NF135.C10 (n = 3) | NF54 (n = 4) | Smear and PCR Negative (n = 3) |
|-----------------|-------------------|-------------|-------------------------------|
|                 | Events, No.       | Duration, Mean (SD), d | Events, No.       | Duration, Mean (SD), d | Events, No.       | Duration, Mean (SD), d |
| Abdominal pain  | 2 0 (0.0)         | 0 ...       | 0 ...                        | 0 ...                        |
| Arthralgia      | 0 ...            | 0 ...       | 0 ...                        | 0 ...                        |
| Chills          | 0 ...            | 0 ...       | 0 ...                        | 0 ...                        |
| Fatigue         | 3 2.4 (1.9)       | 1 3.0 (...) | 2 13.5 (9.3)                | 0 ...                        |
| Fever           | 1 0.2 (...)       | 2 0.7 (0.8) | 0 ...                        | 0 ...                        |
| Headache        | 3 1.5 (2.4)       | 4 2.3 (2.0) | 3 4.6 (3.8)                 | 0 ...                        |
| Itching         | 0 ...            | 4 3.1 (1.5) | 2 5.2 (0.3)                 | 0 ...                        |
| Malaise         | 0 ...            | 4 2.5 (3.3) | 0 ...                        | 0 ...                        |
| Myalgia         | 1 2.7 (...)       | 3 1.3 (1.4) | 2 2.7 (2.4)                 | 0 ...                        |
| Nausea          | 1 0.1 (...)       | 3 2 (2.0)   | 1 0.0 (...)                 | 0 ...                        |
| Vomiting        | 0 ...            | 1 0.4 (...) | 0 ...                        | 0 ...                        |
| Any             | 3 1.3 (1.7)       | 4 2.2 (1.9) | 3 5.7 (5.8)                 | 0 ...                        |

Grade 3 adverse event

| Headache  | 0 ... | 1 4.6 (...) | 0 ... |
| Malaise   | 0 ... | 1 0.4 (...) | 0 ... |
| Vomiting  | 0 ... | 1 0.4 (...) | 0 ... |
| Any       | 0 ... | 1 1.8 (2.4) | 0 ... |

Reported solicited adverse events, collected throughout the postinoculation period, that were considered possibly, probably, or definitely related to the trial procedures.

**Abbreviations:** SD, standard deviation; PCR, polymerase chain reaction.
NF135.C10, the current results will boost the continued application of CHMIs as a crucial tool for malaria vaccine development.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the volunteers for their enthusiastic participation in this trial and Kitty Suijk for her nursing support. We thank Laura Pelser, Jolanda Klaassen, Astrid Poulwelsen, and Jacqueline Kuhnen for their work in the culturing and dissection of mosquitoes. We are indebted to all the slide readers in Leiden: Jan Kromhout, Jaco Verweij, Meriam Beljon, Jolanda van Schie, Jaqueline Schelfaut, Jeanette van der Slot, Heleen Gerritsma, Fons van der Sande, Eric Brienzen, and Els van Oorschot. We thank Adriana Ahumada at Protein Potential, Jianbing Mu and Xin-zhuan Su at the Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institute of Health, for the microsatellite mapping studies; Chris Janse, Shahid Khan, and the malaria team for their hospitality in their laboratory in Leiden; and safety monitors Sandra Arend and Mark de Boer and independent physician Frank Kroon for their continuing support.

Financial support. This work was supported by Top Institute Pharma (grant T4-102), the European Malaria Vaccine Development Association (A. C. T), a long-term EMBO fellowship (A. S.), and NWO Mozaiek (grant 017.005.011 to K. N.).

Potential conflicts of interest. B. K. L. S. is an employee of Sanaria Inc. S. L. H. is a major shareholder of Sanaria Inc. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. World Health Organization. World malaria report: 2011. World Health Organization, Geneva, Switzerland 2011.

2. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev 2011; 11:57–64.

3. 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.

4. Jeffery GM, Young MD, Burgess RW, Eyles DE. Early activity in sporozoite-induced Plasmodium falciparum infections. Annu Trop Parasitol 1959; 53:51–8.

5. Rieckmann KH, Carson PE, Beaudoin RL, Cassells JS, Sell KW. Letter: Sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparum. Trans R Soc Trop Med Hyg 1974; 68:258–9.

6. Drakeley CJ, Duraisingh MT, Povo M, Conway DJ, Targett GA, Baker DA. Geographical distribution of a variant epitope of Pf48/45, a Plasmodium falciparum transmission-blocking vaccine candidate. Mol Biochem Parasitol 1996; 81:253–7.

7. Ponnudurai T, Lensen AH, Van Gemert GJ, Bensink MP, Bolmer M, Meuwissen JH. Infection of cultured Plasmodium falciparum gametocytes to mosquitoes. Parasitology 1989; 98(Pt 2):165–73.

8. Hermens CC, Telgts DS, Linders EH, et al. Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR. Mol Biochem Parasitol 2001; 118:247–51.

9. Roestenberg M, de Vlas SJ, Nieman AE, Sauerwein RW, Hermens CC. Efficacy of pre-erythrocytic and blood-stage malaria vaccines can be assessed in small sporozoite challenge trials in human volunteers. J Infect Dis 2012; 206:319–23.

10. Roestenberg M, O’Hara GA, Duncan CJ, et al. Comparison of clinical and parasitological data from controlled human malaria infection trials. PLoS One 2012; 7:e38434.

11. Edelman R, Hoffman SL, Davis JR, et al. Long-term persistence of sterile immunity in a volunteer immunized with X-irradiated Plasmodium falciparum sporozoites. J Infect Dis 1993; 168:1066–70.

12. Kester KE, McKinney DA, Tornieporth N, et al. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental Plasmodium falciparum malaria. J Infect Dis 2003; 188:640–7.

13. Rickman LS, Jones TR, Long GW, et al. Plasmodium falciparum-infected Anopheles stephensi inconsistently transmit malaria to humans. Am J Trop Med Hyg 1990; 43:441–5.

14. Roestenberg M, Bijker EM, Sim BK, et al. Controlled human malaria infections by intradermal injection of cryopreserved Plasmodium falciparum sporozoites. Am J Trop Med Hyg 2012 [epub ahead of print].

15. Borrmann S, Matuschewski K. Protective immunity against malaria by ‘natural immunization’: a question of dose, parasite diversity, or both? Curr Opin Immunol 2011; 23:500–8.

16. Douradinha B, Mota MM, Luty AJ, Sauerwein RW. Cross-species immunity in malaria vaccine development: two, three, or even four for the price of one? Infect Immun 2008; 76:873–8.