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Infectivity of *Plasmodium falciparum* in Malaria-Naive Individuals Is Related to Knob Expression and Cytoadherence of the Parasite

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***Plasmodium falciparum* is the most virulent human malaria parasite because of its ability to cytoadhere in the microvasculature. Nonhuman primate studies demonstrated relationships among knob expression, cytoadherence, and infectivity. This has not been examined in humans. Cultured clinical-grade *P. falciparum* parasites (NF54, 7G8, and 3D7B) and *ex vivo*-derived cell banks were characterized. Knob and knob-associated histidine-rich protein expression, CD36 adhesion, and antibody recognition of parasitized erythrocytes (PEs) were evaluated. Parasites from the cell banks were administered to malaria-naive human volunteers to explore infectivity. For the NF54 and 3D7B cell banks, blood was collected from the study participants for *in vitro* characterization. All parasites were infective *in vivo*. However, infectivity of NF54 was dramatically reduced. *In vitro* characterization revealed that unlike other cell bank parasites, NF54 PEs lacked knobs and did not cytoadhere. Recognition of NF54 PEs by immune sera was observed, suggesting *P. falciparum* erythrocyte membrane protein 1 expression. Subsequent recovery of knob expression and CD36-mediated adhesion were observed in PEs derived from participants infected with NF54. Knobless cell bank parasites have a dramatic reduction in infectivity and the ability to adhere to CD36. Subsequent infection of malaria-naive volunteers restored knob expression and CD36-mediated cytoadherence, thereby showing that the human environment can modulate virulence.**

Plasmodium falciparum is the most virulent of the six *Plasmodium* sp. parasites that infect humans. Its ability to cytoadhere and sequester itself in the microvasculature can result in obstruction of blood flow and organ dysfunction, and these are key processes in the development of severe falciparum malaria (1).

Parasite cytoadherence and sequestration are facilitated by parasite-encoded knoblike structures that first appear on early trophozoite-stage parasites and are formed beneath the plasma membrane of parasitized erythrocytes (PEs) (2). Cytoadherence to receptors in the deep vasculature prevents removal and destruction of the parasite by the mononuclear phagocytic system, especially the spleen. Higher knob densities have been reported on PEs collected directly from patients than on cultured PEs infected *in vitro* (3). Following *in vitro* cultivation of *P. falciparum*, knob formation on PEs varies in an isolate-dependent manner (3–5), ranging from a mild reduction in density to complete ablation. The major structural protein in knobs is the knob-associated histidine-rich protein (KAHRP) (2, 6, 7). Deletion of the gene encoding KAHRP results in the loss of knobs (8, 9).

P. falciparum isolates can lose the ability to adhere to tissue receptors *in vitro* (10). Loss of adherence is isolate dependent and can occur independently of whether the knob phenotype is retained (10). Cytoadherence involves an interaction between parasite ligands and tissue receptors, including CD36, on the vascular endothelium (11, 12). The principal parasite adhesin is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (13), an antigenically variant product of the *var* gene family that is expressed on the surface of late-stage PEs and is concentrated on knobs (14–16). Knobless parasites continue to express PfEMP1 (17, 18) and have been shown to cytoadhere in static assays (17, 19, 20). However, under physiologic flow conditions, the ability of knob- and KAHRP-negative parasites to bind to tissue receptors is significantly reduced (7). A reduction in the amount of PfEMP1 displayed on the surface of knobless PEs *in vitro* has been reported (21).

Knobby and knobless parasites have been examined in nonhuman primates (22–24). Knobby clones were more virulent than knobless clones in nonsplenectomized monkeys (22, 23). Knobless *P. falciparum* parasites were rapidly cleared from the circulation, unlike knob-expressing parasites, an observation attribut-

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able to the parasite's ability to sequester itself and replicate without clearance (23). In contrast, in splenectomized monkeys, this knobless clone showed virulence and did not sequester. A knob-positive phenotype was stable *in vivo* and was not affected by the presence of the spleen (23). Infection of splenectomized monkeys with knobby *P. falciparum* parasites can result in either loss of knobs (22) or loss of cytoadherence in the presence of knobs (25). Cytoadherence could be restored when parasites from splenectomized animals were subsequently transferred into animals with intact spleens (25). The effect of the spleen on cytoadherence may reflect a fitness cost to the parasite. This is supported by the observation that sequential passage of *P. knowlesi* in splenectomized monkeys resulted in loss of agglutinability of PEs (26) and a reduction in variant surface antigen expression (24). Together, these simian studies demonstrate that knob expression and the ability to cytoadhere can be variable and affect parasite infectivity.

In humans, knobby and knobless cytoadherent *P. falciparum* parasites have been derived from splenectomized patients (27, 28). However, the relationship between knob expression, an adherent phenotype, and infectivity of *P. falciparum* has not been investigated. Controlled human malaria infection (CHMI) of malaria-naive human volunteers provides an opportunity to investigate this. CHMI can be undertaken by three means: allowing laboratory-reared *Plasmodium*-infected mosquitoes to feed on study participants (29–32), injecting cryopreserved sporozoites (33–36), or injecting PEs (37–40). We have recently undertaken the current good manufacturing practice production of clinical-grade cultured *P. falciparum* blood-stage cell banks (41). While evaluating the safety and infectivity of these cell banks in malaria-naive volunteers, we demonstrated that although knobless parasites have the ability to grow normally *in vitro*, they have a dramatic reduction in infectivity *in vivo*, mirroring a similar reduction in the ability to cytoadhere to CD36 *in vitro*. Of note, *in vivo* infection in malaria-naive volunteers restored the expression of knobs and cytoadherence, thus demonstrating a major role for the human environment in the modulation and reprogramming of parasite virulence.

MATERIALS AND METHODS

Clinical studies. Studies 1 and 2 were conducted at the Gold Coast Hospital, Southport, QLD, Australia; study 3 was conducted at Griffith University, Southport, QLD, Australia; and studies 4 and 5 were conducted at Q-Pharm, Brisbane, QLD, Australia. Study participants were healthy male Caucasians 18 to 45 years old. Key eligibility criteria are listed for each study at the Australian New Zealand Clinical Trials Registry (www.anzctr.org.au; study reference numbers are provided below). Two volunteers were enrolled in each study with a delay of >2 days between the inoculations of participants 1 and 2 in each group.

If clinical or parasitological evidence of malaria (identification of ≥2 malaria parasites on a malaria thick film, a platelet count of $<100 \times 10^9$/liter, or the onset of clinical features of malaria) was found or the parasitemia threshold defined in the study protocol was reached, antimalaria treatment was initiated. For study 1, antimalaria treatment was initiated as stipulated by the study protocol, despite the lack of parasite growth. Treatment of malaria entailed the administration of artemether-lumefantrine (A/L) according to its approved dosing schedule.

Adverse events (abnormal laboratory values, clinical signs or symptoms) were monitored either via telephone or at the clinical sites and graded in severity by experienced clinicians.

Ethics statement. Studies 1 to 3 were approved by the Gold Coast Hospital and Health Services District Human Research Ethics Committee (HREC) and/or the Griffith University HREC. Studies 4 and 5 were approved by the Queensland Institute of Medical Research (QIMR) HREC. The studies were registered at the Australian New Zealand Clinical Trials Registry (study 1, ACTRN12612001153808; study 2, ACTRN12613000615785; study 3: ACTRN12613001187730; study 4, ACTRN12613000669796 and study 5, ACTRN12612000824864). Written informed consent was obtained from all of the participants prior to the commencement of this study.

***P. falciparum* malaria blood-stage cell banks.** For studies 1 to 3, cultured *P. falciparum* NF54 (studies 1 and 2) and 7G8 (study 3) malaria blood-stage cell banks were manufactured at the Institute for Glycomics, Griffith University, as previously described (41). The *P. falciparum* 3D7B cultured malaria blood-stage cell bank (study 4) was manufactured at the QIMR Berghofer Medical Research Institute as previously described (41); it was derived from a vial of an *ex vivo P. falciparum* 3D7 cell bank that has been used in previous clinical studies (37–40, 42).

The *ex vivo P. falciparum* HMP02 cell bank (study 5) was derived from an Australian resident who contracted malaria in Ghana. Parasitized red blood cells were cryopreserved at the QIMR Berghofer Medical Research Institute as previously described (43).

Using methodology that has been previously described (43), *P. falciparum* NF54 blood-stage malaria cell banks (NF54-S01 and NF54-S02) were prepared from the peripheral blood of both study participants infected with *P. falciparum* NF54 (study 2) just prior to the commencement of A/L treatment.

PCR. Sample preparation, DNA extraction, and parasitemia measurement by quantitative PCR (qPCR) were done as previously described (44), with the following modifications. A standard curve was prepared from a lyophilized WHO *P. falciparum* international standard (National Institute for Biological Standards and Control code 04/176) (45) that was reconstituted in 500 μl of nuclease-free water and diluted in a 1:1 solution with 1× phosphate-buffered saline (PBS; Gibco). DNA was isolated from 500 μl of this solution at a concentration of 5×10^8 IU/ml. Blood samples from study participants and standards were tested in triplicate. Established modified calculations (46) were used to equate international units per milliliter to parasites per milliliter, with 1 IU/ml equivalent to 0.5 parasite/ml. The number of parasites per milliliter was calculated with the CFX96 Touch Real Time detection system software (Bio-Rad, Australia).

Measurement of antibodies to the surface of *P. falciparum* PEs by flow cytometry. Testing for IgG binding to the surface of PEs by flow cytometry was performed as described previously (47), with some minor modifications (see the supplemental material).

Preparation and administration of the parasite inoculum. Inocula were prepared as previously described, by taking an aliquot of the relevant cell bank and thawing, washing, and diluting it to the appropriate dose and volume with 0.9% saline for injection (42). The number of parasites present in the inoculum was verified retrospectively by qPCR assay of surplus material. The inoculums were dispensed into as many 2-ml syringes as required for administration to the study participants who were inoculated by intravenous injection.

***P. falciparum* adhesion assays.** Adhesion assays were performed as previously described (48, 49), with *P. falciparum* trophozoite-stage PEs. Incubation was at 37°C for 30 min, and washing steps were performed with RPMI. Bound cells were fixed in 2% glutaraldehyde in PBS, stained with Giemsa, and counted by microscopy. Adhesion to CD36 at 20 μg/ml (rhCD36/Fc Chimera; R&D Systems) was tested in triplicate.

Scanning electron microscopy. Scanning electron microscopy was performed with *P. falciparum* trophozoite-stage PEs as outlined in the supplemental material.

***kahrp* expression assay.** The level of *kahrp* expression in the cell bank parasites and participant blood was quantified by reverse transcription-qPCR as outlined in the supplemental material.

Statistical analysis. The R package was used to calculate the Pearson correlation coefficients for the relative *kahrp* gene copy number and the average binding data to CD36.

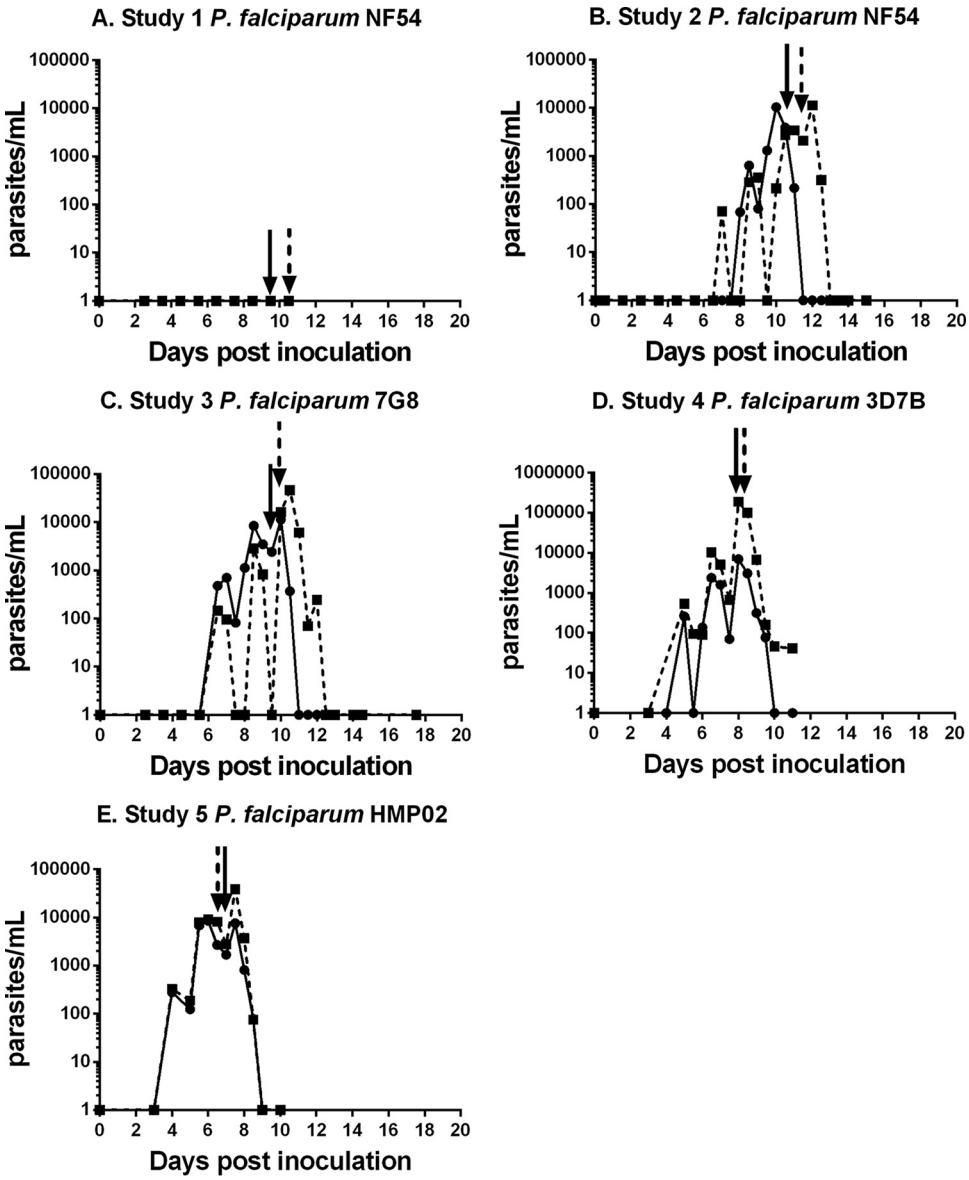

FIG 1 Course of parasitemia in study participants inoculated with *P. falciparum* cell banks. Shown are the parasite levels in study participants following inoculation with the different *P. falciparum* cell bank parasites. (A) Study 1, *P. falciparum* NF54 (cultured cell bank). (B) Study 2, *P. falciparum* NF54 (cultured cell bank). (C) Study 3, *P. falciparum* 7G8 (cultured cell bank). (D) Study 4, *P. falciparum* 3D7B (cultured cell bank). (E) Study 5, *P. falciparum* HMP02 (*ex vivo* cell bank). Arrows indicate the times of administration of drug treatment. Note the different *y*-axis scale for *P. falciparum* 3D7B. In each study, $n = 2$.

RESULTS

To test the infectivity of the NF54 strain of *P. falciparum*, two volunteers were inoculated with 1,800 PEs. This number had been previously used for CHMI studies with volunteers infected with an *ex vivo* bank of the 3D7 clone of NF54 (38). Parasites were not detected by qPCR in the blood of volunteers for up to 10 days (Fig. 1A), at which time participants commenced antimalaria treatment with A/L, in accordance with the study protocol. Six months later, the same volunteers were reinoculated with 30,000 NF54 PEs. We observed parasites in both individuals on day 6 (Fig. 1B). Parasite growth kinetics were similar to those previously reported for *ex vivo*-initiated infections with 1,800 PEs (38). A/L treatment was initiated on days 10 and 11. We then tested the infectivity of three other parasite lines with a dose of 1,800 PEs: a cultured 7G8 line (41), a 3D7 line referred to as 3D7B (41) that had only recently been cultured from the *ex vivo* 3D7 bank (43), and a novel *ex vivo* parasite, HMP02. The growth curves are shown in Fig. 1C to E. The parasite growth kinetics were similar to those of 30,000 NF54 PEs (Fig. 1B). No serious adverse events were observed in any volunteers. Minor adverse events and laboratory abnormalities are listed in Tables S1 and S2 in the supplemental material.

During the infectivity studies, new parasite isolates were derived directly *ex vivo* from the peripheral blood of the volunteers who received 30,000 NF54 PEs (NF54-S01 and NF54-S02) and from one of the volunteers who received 1,800 3D7B PEs (3D7-S102). These were used for further studies (described below) that were performed after six or seven cycles of *in vitro* culture.

To understand why the infectivity of NF54 was lower, we used

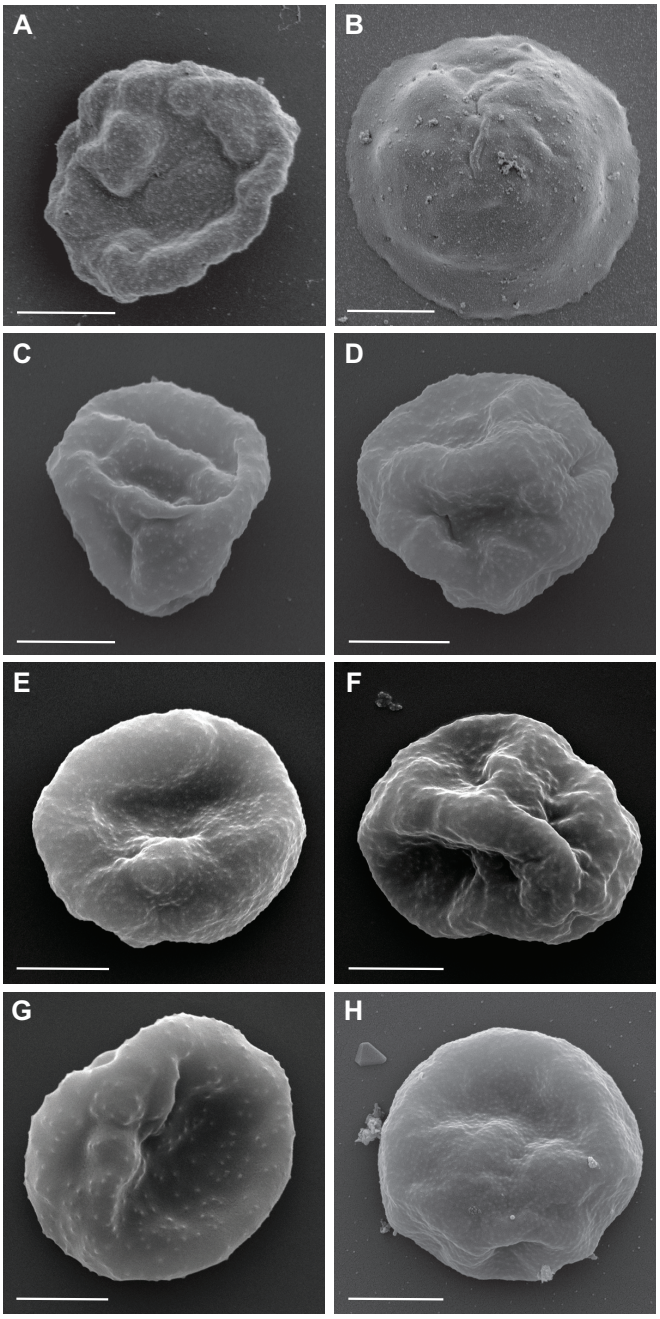

FIG 2 Surface characteristics of PEs from different *P. falciparum* parasite lines. Shown are scanning electron micrographs of the *P. falciparum* 3D7 control (A), *P. falciparum* NF54 (cultured cell bank) (B), *P. falciparum* NF54-S01 (derived *ex vivo* from S01 at the time of drug treatment) (C), *P. falciparum* NF54-S02 (derived *ex vivo* from S02 at the time of drug treatment) (D), *P. falciparum* 3D7B (cultured cell bank) (E), *P. falciparum* 3D7-S102 (derived *ex vivo* from S102 at the time of drug treatment) (F), *P. falciparum* 7G8 (cultured cell bank) (G), and *P. falciparum* HMP02 (*ex vivo* cell bank) (H). Representative images are shown, and the scale bars all represent 2 μm.

scanning electron microscopy to examine the surface composition of the PEs for the expression of knobs. A 3D7 parasite line that was regularly selected by gelatin flotation to maintain knob expression was used as a positive control and displayed numerous prominent knobs on the erythrocyte surface (50) (Fig. 2A). In comparison, the NF54 PEs exhibited a smooth surface, similar to what has been observed in *kahrp* knockout lines (18) (Fig. 2B). Knobs were present on the surface of the cultured 3D7B PEs (Fig. 2E), on the *P. falciparum* 7G8 PEs (Fig. 2G), on the *ex vivo* HMP02 PEs (Fig. 2H), and on PEs from the three *ex vivo* parasite lines (NF54-S01, NF54-S02, and 3D7-S102) derived from the study participants (Fig. 2C, D, and F). Thus, infection with NF54 PEs resulted in the selection of knob-expressing parasites in the two volunteers.

Knobs and PfEMP1 play an important role in parasite adhesion and virulence, with CD36 being an important receptor for PfEMP1. Thus, we performed static binding assays to examine the adhesion of PEs from the different parasite lines to CD36. Adhesion was evaluated relative to that of the same 3D7 control parasite used to characterize knob expression. No adhesion of *P. falciparum* NF54 PEs to CD36 was observed (Fig. 3), whereas adhesion comparable to that of the 3D7 control was observed in all of the other parasites. This included *ex vivo* parasite lines NF54-S01 and NF54-S02, indicating that *in vivo* infection with NF54 also altered the adhesive phenotype of the parasite.

KAHRP is essential for knob formation, and deletion of the *kahrp* gene from one end of chromosome 2 has been observed in long-term-cultured isolates (8). Thus, we examined the levels of *kahrp* gene transcription in the parasite lines by qPCR, aiming to determine if the lack of knobs and CD36 receptor binding by NF54 PEs was due to deletion of the *kahrp* gene or downregulation of gene expression. Expression of *kahrp* was observed in all of the parasite lines, including *P. falciparum* NF54, which did not express knobs (Fig. 4). There was no relationship between the relative copy numbers of the *kahrp* gene present in the parasites and adhesion to CD36 ($r = -0.18$; $P = 0.7$).

Using serum samples from adults residing in a region of Papua New Guinea where malaria is endemic, we examined antibody recognition of the surface of PEs from the different parasite lines by flow cytometry. Antibodies to the surface of PEs predominantly recognize PfEMP1 (50). The 3D7 parasite was again used as a control. PEs from all of the parasite lines were recognized by the Papua New Guinea serum samples (Fig. 5; see Fig. S1 in the supplemental material), suggesting that the NF54 PEs continued to express PfEMP1 and other variant surface antigens. It was of interest that PEs from the *ex vivo*-derived lines (NF54-S01, NF54-S02, and 3D7-S102) were not as well recognized by antibodies in the serum as PEs from the parental parasites with which the volunteers were infected (NF54, 3D7B), perhaps reflecting a switch to the expression of a new PfEMP1-encoding gene during infection.

DISCUSSION

Here, using novel *P. falciparum* cultured and *ex vivo*-derived blood-stage cell banks (41), we have demonstrated, for the first time in malaria-naive individuals, a relationship between knob expression, cytoadhesion, and the *in vivo* infectivity of *P. falciparum*. This is also the first time that blood-stage parasites from clinical-grade *P. falciparum* cultured cell banks (41) have been administered to human volunteers to evaluate their safety and infectivity.

Following the administration of 1,800 NF54 PEs to two malaria-naive study participants, parasite growth was not detected in their blood up to day 10 postinoculation. We cannot exclude the possibility that if we had continued to monitor these individuals, parasites would have been detected. However, the study protocol required initiation of antimalaria treatment at that time. Limit-

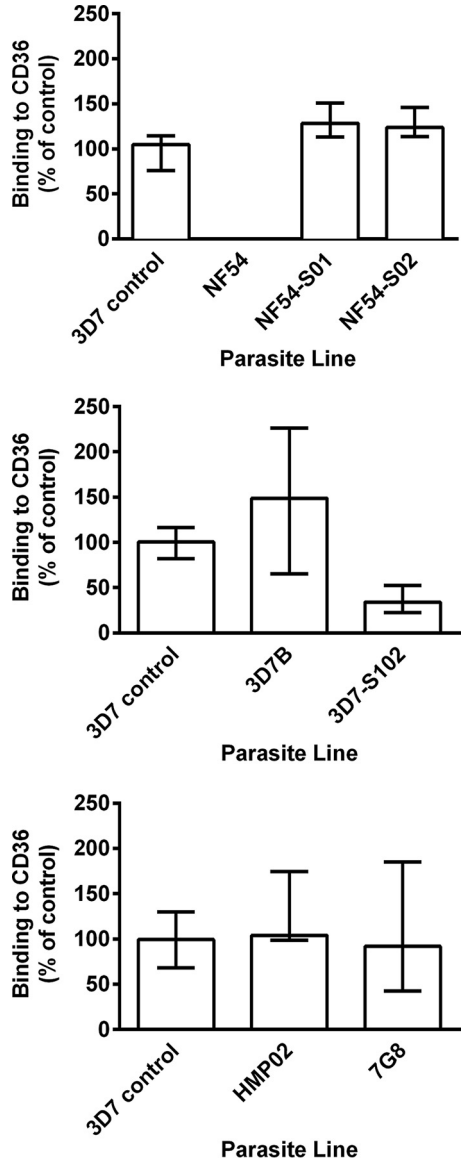

FIG 3 Binding of *P. falciparum* PEs from different parasite lines to CD36. Shown is the adhesion to recombinant CD36 of *P. falciparum* NF54 (cultured cell bank) and *P. falciparum* NF54-S01 and NF54-S02 (derived *ex vivo* from S01 and S02 at the time of drug treatment) (top), *P. falciparum* 3D7B (cultured cell bank) and *P. falciparum* 3D7-S102 (derived *ex vivo* from S102 at the time of drug treatment) (middle), and *P. falciparum* HMP02 (*ex vivo* cell bank) and *P. falciparum* 7G8 (cultured cell bank) (bottom). Values are expressed as percentages of the *P. falciparum* 3D7 control parasite binding to CD36. Assays were performed twice independently, and bars represent median values and interquartile ranges of experimental replicates of samples tested in triplicate.

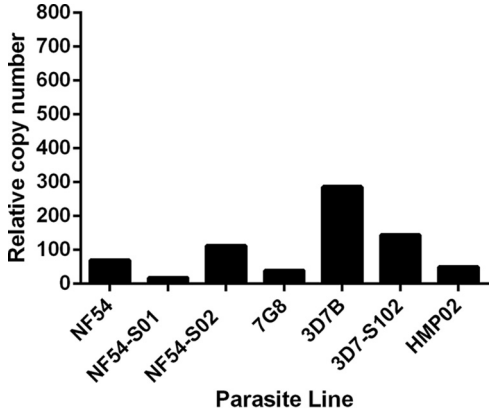

FIG 4 Expression of *kahrp* in different *P. falciparum* parasite lines. Shown are *kahrp* expression levels in *P. falciparum* cell bank and *ex vivo*-derived parasites relative to those of the single-copy fructose-bisphosphate aldolase gene.

ing-dilution analysis of this cell bank has demonstrated that the viability of these parasites is approximately 50% (41), thereby excluding the possibility that lack of parasite growth was due to unhealthy/dead parasites. We subsequently administered 30,000 NF54 PEs to the same volunteers and observed parasite growth. Infectivity was observed for all other cultured and *ex vivo*-derived cell banks. *In vitro* characterization of the NF54 PEs demonstrated that they lacked knobs and were unable to adhere to CD36 but were still recognized by antibodies present in the serum of malaria-exposed individuals, suggesting that PfEMP1 and/or other variant surface antigens were still being expressed. Previous studies have demonstrated that long-term culture can be associated with loss of knobs and loss of CD36-mediated cytoadhesion as measured *in vitro* (4, 10), as well as in *in vivo* studies in animal models. Furthermore, this phenotype is associated with attenuation of parasitemia in nonsplenectomized monkeys (23). We do not know when our NF54 cell bank became predominantly knobless PEs. Prior to drug treatment, new parasite isolates (NF54-S01 and NF54-S02) were derived *ex vivo* from the peripheral blood of the volunteers who received 30,000 NF54 PEs. Knob expression and CD36-mediated adhesion were observed in PEs from these two isolates. Recovery of the knobby, cytoadherent phenotype following administration to the study participants could reflect that the NF54 parasite line is not clonal, with a small proportion of PEs still expressing knobs and able to cytoadhere. With an increased inoculum dose of 30,000 PEs, it is possible that this minor population initiated infection by sequestering in the periphery and evading destruction by macrophages in the spleen. Transcription of the *kahrp* gene was detected in cell bank parasites, indicating that the lack of knob expression was not due to a gene deletion or transcriptional silencing. We cannot exclude the possibility that epigenetic reprogramming or deletion of other genes important in knob formation contributed. As infectivity was observed with the other cultured banks (7G8 and 3D7B) when 1,800 PEs were administered, and knob expression and adhesion were absent from only NF54, this phenomenon appears to be isolate dependent, as was previously described *in vitro* (10).

This is the first study to document the administration of blood-stage parasites from cultured *P. falciparum* malaria cell banks directly to human volunteers. Additionally, it is the first study to evaluate the safety and virulence of well-characterized, clinical-grade *P. falciparum* lines that may be used in CHMI studies to assess the efficacy of novel antimalaria drugs and vaccines. For all four of the cell banks tested, the inoculum was well tolerated by recipients. Until recently, the ability to conduct CHMI studies has relied on access to clinical-grade sporozoites or *P. falciparum*-infected erythrocytes derived directly *ex vivo* from malaria-infected individuals. Our recent production of clinical-grade, cultured *P. falciparum* blood-stage cell banks (41) offers an additional approach to CHMI. The use of cultured parasites in CHMI studies will accelerate the development of novel antimalaria drugs

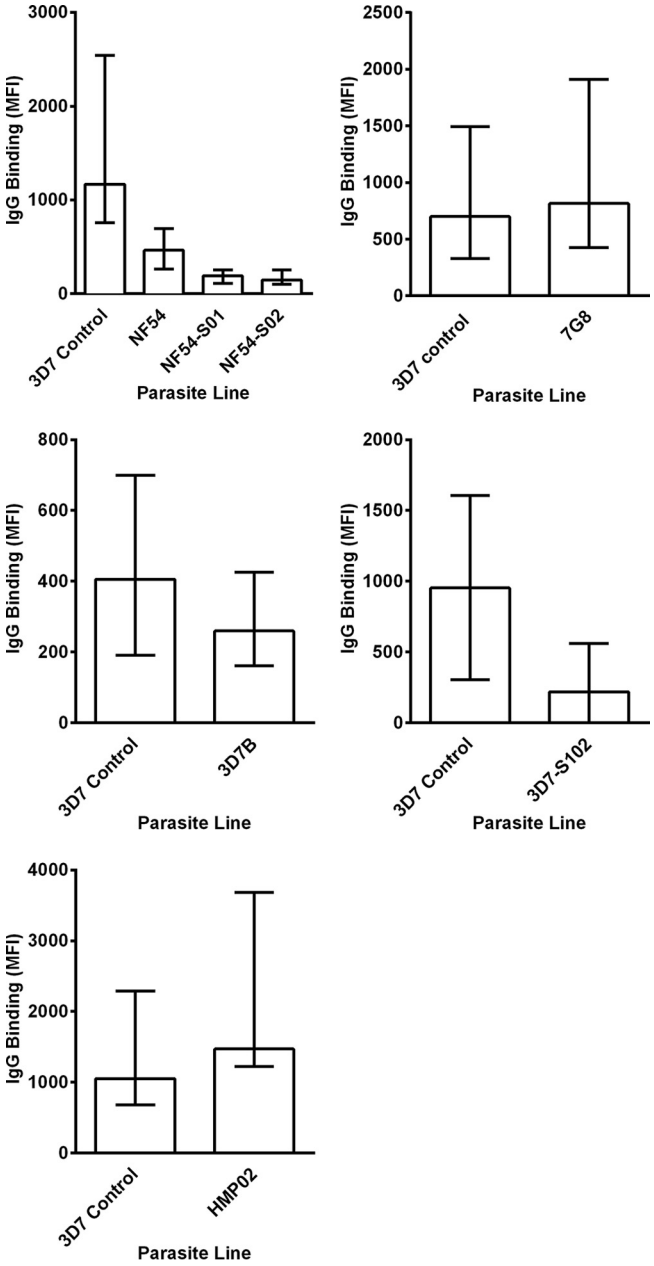

FIG 5 Antibody recognition of the surface of *P. falciparum* PEs from different parasite lines. Shown is the binding of antibodies in serum samples from Papua New Guinean adults to surface antigens expressed by PEs from *P. falciparum* NF54 (cultured cell bank), *P. falciparum* NF54-S01 and NF54-S02 (derived *ex vivo* from S01 and S02 at the time of drug treatment) (top left), *P. falciparum* 7G8 (cultured cell bank) (top right), *P. falciparum* 3D7B (cultured cell bank) (middle left), *P. falciparum* 3D7-S102 (derived *ex vivo* from S102 at the time of drug treatment) (middle right), and *P. falciparum* HMP02 (*ex vivo* cell bank) (bottom). The IgG binding level is expressed as the geometric mean fluorescence intensity (MFI) in all of the graphs, and the bars represent the median values and interquartile ranges of samples tested in duplicate ($n = 10$ for all *P. falciparum* cell banks). Minimal reactivity was observed among serum samples from nonexposed Melbourne controls.

and malaria vaccine candidates, provided the phenotype and infectivity of the parasites are known. We observed that NF54 infectivity is related to cytoadherence and knob expression. However, loss of these characteristics is isolate dependent.

In conclusion, by using a novel approach to CHMI, we have demonstrated that the human environment can directly modulate the virulence of *P. falciparum* by altering the surface phenotype of PEs. While we have studied a single virulence phenotype, it seems likely that other virulence phenotypes may also be under selective pressure from the human host. CHMI with cultured malaria parasites provides an exciting opportunity to study malaria pathogenesis in humans.

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