Abstract. Red blood cells (RBCs) infected with Plasmodium falciparum are protected from complement-mediated lysis by surface membrane glycosyl-phosphatidylinositol (GPI)−anchored proteins, which include decay accelerating factor (DAF or CD55) and CD59. To determine if P. falciparum avoids or replicates less efficiently in GPI protein-deficient cells at a higher risk for complement-mediated lysis, we compared P. falciparum infectivity among control RBCs with those from subjects with paroxysmal nocturnal hemoglobinuria (PNH), a condition in which RBCs express variable levels of DAF (negative and positive) and CD59 (negative [−], intermediate [I], and high [H]). Co-cultures of 19 matched samples of control and PNH RBCs were infected with P. falciparum to directly compare parasitic invasion. Each PNH RBC sample was then assessed for P. falciparum infectivity across the spectrum of GPI protein deficiency. Identification methods included biotin-streptavidin for RBC populations, fluorescein isothiocyanate−labeled antibodies to DAF and CD59, hydroethidine for parasite DNA, and flow cytometry. The mean ± SD parasitemias in co-cultured PNH and control RBCs were 24.7 ± 6.9% versus 21.0 ± 5.9% (P = 0.12). For individual PNH samples, parasitemias were significantly higher in DAF (−) cells versus DAF (+) cells (25.0 ± 8.9% versus 19.1 ± 8.7%; P < 0.001) and in CD59 (−) cells versus I/H cells (22.5 ± 6.4% versus 17.6 ± 4.2%; P = 0.0003). Across the CD59 spectrum, mean parasitemias were highest in CD59 (−) cells (24.5 ± 6.4%), followed by CD59-H cells (19.5 ± 5.4%), and CD59-I cells (16.4 ± 4.8%). Expression of DAF in 12 (63%) of 19 infected PNH samples was reduced. Thus, P. falciparum does not selectively avoid RBCs with fewer GPI proteins and parasite replication in PNH cells is at least as robust as in normal RBCs.

INTRODUCTION

Decay accelerating factor (DAF or CD55) and membrane inhibitor of reactive lysis (MIRL or CD59) are glycosyl-phosphatidylinositol (GPI)−anchored, complement regulatory red blood cell (RBC) surface proteins that protect cells from the hemolytic effects of complement activation.1 In paroxysmal nocturnal hemoglobinuria (PNH), a condition associated with deficient GPI protein expression, RBCs are rendered susceptible to complement-mediated lysis.2 In malaria, complement activation causes destruction of infected and uninfected RBCs in a process modulated by GPI protein expression.3,4 Since RBCs are non-endocytic, malaria parasites invade through a specific, multiple-step process involving interactions between surface receptors and parasite ligands.5,6 The GPI proteins are probably unnecessary for invasion, but recent evidence suggests that GPI proteins, especially CD59, confer resistance against complement-mediated lysis of Plasmodium falciparum infected RBCs, and probably uninfected RBCs.1,3,4,7 Given this, we propose that as a survival strategy, P. falciparum might selectively avoid RBCs with reduced expression of GPI protein. In this study, we infected co-cultured PNH and control RBCs, which is an established method to depict parasite preference and reduce inherent variability in P. falciparum infectivity experiments.8,9 We then assessed individual PNH samples for P. falciparum infectivity across the GPI protein-deficient spectrum. The RBC populations, DAF, CD59, and parasite DNA were identified by specific staining and flow cytometry. Co-culture experiments indicated comparable infectivity among PNH and controls, whereas within individual PNH samples, there was greater infectivity in GPI protein (−) versus (+) cells. Despite the higher risk of host cell complement-mediated lysis accompanied by interruption of parasite replication, P. falciparum does not selectively avoid GPI protein-deficient RBCs, and replication is at least as robust as in normal RBC.

MATERIALS AND METHODS

Blood samples. After informed consent was obtained from each volunteer, 5 mL of venous blood was obtained, preserved in sterile citrate dextrose solution, and used within one week. Blood samples were collected from 19 otherwise healthy PNH patients attending the Hematology Clinic at Siriraj Hospital in Bangkok, Thailand. For each PNH blood donor, a group O positive blood sample was obtained the same day from a healthy control volunteer. Diagnostic criteria for PNH included intravascular hemolysis, a positive result in a Ham test, and deficient complement regulatory proteins.

Parasite and culture conditions. Culture of Thai strain TM267TR of P. falciparum were maintained as described by Trager and Jansen10 in normal group O+ RBCs in RPMI 1640 medium (Gibco, Grand Island, NY). The culture was supplemented with 10% heat-inactivated AB+ serum with 2 mM L-glutamine (Flow Laboratories, Herts, United Kingdom) and 25 mM HEPES buffer (Calbiochem, San Diego, CA) in an atmosphere of 5% CO2, 5% O2, and 90% N2. Synchronous parasite and culture conditions. Culture and PNH RBC co-culture experiments. We used a modified co-culture system whereby parasites were simultaneously introduced into a mixture of PNH and control RBCs.8,9 Briefly, a synchronous collection of parasites at the
90% ring stage in either normal control or PNH RBCs was used for the starting culture. The percentage of RBCs containing parasite DNA (parasitemia) at the beginning of incubation was 1% and was nearly equal in all experimental and control cultures. In this system, 1% parasitemia normally yielded a 15–30-fold increase in parasitemia after 1–2 schizogonic cycles. Aliquots containing equal numbers of infected RBCs and infected PNH RBCs were mixed and added to 1 mL of a co-culture containing 100 × 10⁶ uninfected RBCs and 100 × 10⁶ PNH RBCs. One of the two RBC populations was pre-labeled with biotin (sulfosuccinimidyl 6-biotinamido hexanoate; Pierce & Warriner, Ltd., Rockford, United Kingdom) at a concentration of 0.03 pg/cell. Three replicates of 200 µL each of RBC co-culture were transferred into 96-well Costar (Corning, Inc., Corning, NY) flat-bottomed microtiter plates. Aliquots of 5 × 10⁶ cultured RBCs were removed from the co-cultures at the end of the second schizogonic cycle and incubated with 10 µL of titrated streptavidin–fluorescein isothiocyanate (FITC) (Amersham, Arlington Heights, IL) for 30 minutes at 4°C in the dark. The cells were washed twice in cold phosphate-buffered saline (PBS), and the cell pellet was mixed with the vital DNA stain hydroethidine (Poly-science, Inc., Warrington, PA) at a concentration of 5 µg/mL in PBS for at least 30 minutes at 37°C before analysis by flow cytometry.

Parasitemias within individual PNH RBC samples. Individual PNH samples were infected with *P. falciparum* as described earlier in this report and cultured for 48 hours. Two million infected RBCs in 50 µL of culture medium were then washed once with PBS containing 1% bovine serum albumin (BSA). Cell pellets were mixed with 20 µL of biotinylated monoclonal antibodies to DAF and CD59 (PharMingen, San Diego, CA), followed by 10 µL of titrated streptavidin–FITC and incubated at room temperature for 30 minutes. An FITC-conjugated mouse IgG, was used as an isotopic control. The cells were then washed twice with PBS/BSA and mixed with hydroethidine at a concentration of 5 µg/mL in PBS. Stained cells were incubated at 37°C for at least 30 minutes prior to analysis by flow cytometry. Simultaneous, parallel control experiments were conducted with control RBCs.

Flow cytometric analysis. Analysis of cells for surface immunofluorescence or biotin-streptavidin–FITC and parasite DNA content was by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The cells were excited with 488 nm light from a 15-mW argon ion laser. Logarithmic green and red fluorescences of FITC and ethidine were measured through 530/30 and 585/42 band pass filters, respectively. The RBCs were gated on the basis of their logarithmic amplification of the forward scatter and 90° light scatter signals. A total of 50,000 RBCs in replicate wells were analyzed for each sample. Premixed, two-color fluorescent Calibrate beads (Becton Dickinson) together with FITC-labeled fluorescent beads (Flow Cytometry Standards, San Juan, PR) were used for calibrating alignment, compensation and sensitivity, and correcting daily shifts in fluorescent linearity.

Data were analyzed with CellQuest software (Becton Dickinson) and expressed as the percentage parasitized RBCs (parasitemia). Two parameter cytograms depicted unbiotinylated and biotinylated RBCs containing parasite DNA within co-cultures, and for individual PNH samples, GPI protein expression and parasite DNA. For individual PNH samples, parasitemias were compared according to the following PNH spectrum: 1) DAF (−) versus DAF (+), 2) CD59 (−) versus CD59 (+) (intermediate [I]/high [H]) and, 3) CD59 (−) versus CD59 I versus CD59 H. The Mann-Whitney U test was used for statistical analyses and *P* values ≤ 0.05 were considered significant.

RESULTS

Expression of DAF (CD55) and CD59 on normal and PNH RBCs. Representative flow cytometric histograms of RBC DAF and CD59 expression from PNH and control samples are shown in Figure 1. Control RBCs were DAF (+) and CD59 (+) (Figure 1A–C), whereas PNH RBCs were variably deficient in each (Table 1). Expression of DAF in PNH patients had two peaks (Figure 1E, H, and K), whereas CD59 from most PNH patients showed three peaks (Figure 1F), which is consistent with the PNH phenotype of CD59 (−) (type III), CD59-intermediate (type II), and CD59-high (type I).12–14 Of 19 PNH samples, one showed a single population of GPI protein (−) RBCs (sample 19, Figure 1M, N, and O). The remainder showed co-existence of GPI protein (−) and (+) RBCs (Figure 1 D–L).

Parasitemias in co-cultured PNH and control RBCs. Normal intraerythrocytic development of *P. falciparum* malaria proceeds through ring, trophozoite, and schizont stages. The immature ring stages consist of a single parasite nucleus that undergoes asexual schizogony to produce a trophozoite, and then a mature schizont that contains 8–32 daughter merozoites. To confirm the progress of parasitemia in control and PNH RBCs, parasitized RBCs representing the three stages from synchronous cultures were stained with biotin-streptavidin–FITC and hydroethidine, and analyzed by flow cytometry. Control and PNH RBCs had normal forward and side light scatter flow cytometric patterns without cell aggregation. Cell morphology was preserved and immunofluorescence and parasite DNA staining had negligible nonspecific labeling. Intraerythrocytic parasite development in PNH RBCs was identical to that in control RBCs. The majority of parasitized RBCs in the synchronous cul-
ture experiments were trophozoites or schizonts (Figure 2). Although some ring stage parasites appeared in the mature stages, resulting in a peak of fluorescence from approximately 20 to 105, intraerythrocytic development of parasites and parasitemias was unaffected. As expected, the peak of fluorescence for the trophozoite and schizont stages, with higher DNA concentrations, occurred at higher signal intensities than the rings. The fluorescence peaks for the trophozoite and schizont stages were tight, with channels ranging from approximately 480 to 900 and 900 to 1,200, respectively. The percent parasitemia assessed by hydroethidine was consistent with that obtained by microscopic examination.

The in vitro co-culture system, with biotin-streptavidin-FITC and hydroethidine labeling to assess parasitemias in control and PNH RBCs, is shown in Figure 2. A representative flow cytogram comparison of unbiotinylated control RBCs with biotinylated PNH RBCs after three schizogonic cycles is shown in Figure 2A. Nineteen matched samples with the mean parasitemias slightly higher in the PNH versus control cells (mean ± SD = 24.7 ± 6.9% versus 21.0 ± 5.9%; P = 0.12) are shown in Figure 2B. For PNH sample 19 (severe GPI protein deficiency) and a matched control, parasitemias were 33.2% and 28.4%, respectively.

Parasitemia within individual PNH samples. Pure cultures of control and PNH samples infected with P. falciparum are shown in Figure 3. Cells were labeled with antibodies to DAF and CD59, stained for DNA with hydroethidine, and the difference in parasitemia across the PNH spectrum was depicted by two-parameter dot plots. In PNH RBCs stained for DAF and gated for (−) versus (+) populations, parasitemias were significantly higher in DAF (−) cells than in DAF (+) cells (mean ± SD = 25.0 ± 8.9% versus 19.1 ± 8.7%; P < 0.001) (Figure 3A and B). For CD59, with cell gating for (−) versus (+) (I/H populations), parasitemias were significantly higher in CD59 (−) cells versus CD59 (+) cells (22.5 ± 6.4% versus 17.6 ± 4.2%; P < 0.0003) (Figure 3C and D).

In experiments with flow cytometric gating for CD59 (−), I, and H expression within individual PNH samples, parasitemia was highest in CD59 (−) cells (24.5 ± 6.4%), followed by CD59-H cells (19.5 ± 5.4%), and CD59-I cells (16.4 ± 4.8%) (Figure 4B). P values for the differences between CD59 (−) versus CD59-I and CD59 (−) versus CD59-H were 0.0001 and 0.01, respectively. The P value for the difference between parasitemia in CD59-I and CD59-H RBCs was 0.06.

Control RBCs infected with P. falciparum showed constant DAF and CD59 expression throughout the intraerythrocytic cycles, whereas in PNH RBCs, 12 (63%) of 19 samples had reduced DAF expression (Figure 5). Similar reductions in CD59 were not observed. The expression of GPI protein in control RBCs remained constant.

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* Values are the mean ± SD.

controls (n = 19) 98.5 ± 1.2 97.6 ± 1.4*

* Values are the mean ± SD.
DISCUSSION

Previous work shows that GPI protein-deficient PNH RBCs are efficiently infected by *P. falciparum*. Nonetheless, because some RBC abnormalities confer protection against malaria, and GPI protein expression inhibits complement-mediated lysis of parasitized RBCs, we proposed that reduced GPI protein expression, as occurs in PNH, might alter *P. falciparum* propagation. Indeed, PNH RBCs rendered more susceptible to complement-mediated lysis (with disrupted parasite replication) might be less attractive to *P. falciparum*. Here, we obtained 19 samples of PNH and matched control RBCs and conducted co-culture and individual population experiments, exploiting natural GPI protein deficiencies on PNH RBCs. Slightly increased parasitemias in co-cultured PNH versus control RBCs, coupled with significantly increased mean parasitemias among GPI (−) RBCs within each individual PNH sample, suggested that *P. falciparum* parasites do not selectively eschew GPI protein-deficient RBCs, and that *P. falciparum* replication is at least as robust in PNH RBCs as in normal RBCs. Our data also support the contentions that *P. falciparum* readily invades and replicates in PNH RBCs, and that GPI proteins are unnecessary for *P. falciparum* invasion.3,4,7

This is the first report of using a co-culture system to simultaneously compare *P. falciparum* infectivity among control and PNH RBCs, a technique that reduces inherent variability in comparative *P. falciparum* infectivity experiments.8,9 Similar to previous reports in which the model performed well, cultured RBCs did not aggregate and biotin labeling did not affect staining or flow cytometric assessments. Indeed, flow cytometric plot histograms indicated that biotin-streptavidin labeling readily identified RBC populations. Indeed, flow cytometric plot histograms indicated that biotin-streptavidin labeling readily identified RBC populations. Additionally, intermittent sampling of *P. falciparum*-infected PNH and control RBCs showed normal parasite propagation. Slightly increased mean parasitemias in PNH versus control RBCs provided further evidence that PNH RBCs are readily infected by *P. falciparum*, GPI proteins are uninvolved in *P. falciparum* invasion, and the PNH phenotype does not diminish parasite growth.3,4,7 Within the context of the co-culture, we argue that *P. falciparum* has equal affinity for PNH and control cells.

Mildly increased parasitemias in PNH RBCs during co-culture were further borne out when individual PNH samples were infected and then assessed across the GPI protein expression spectrum. Here, mean parasitemias were approximately 30% higher in DAF (−) versus DAF (+) RBCs and approximately 22% higher in CD59 (−) versus CD59 (+) RBCs. The solid line in the middle of each box plot shows the mean, the upper and lower box edges depict the 25th and 75th percentiles, respectively, and the whiskers represent 10th and 90th percentiles. Dots show outlier values. Parasitemias among CD59 (−), I, and H populations were significantly different.
RBCs (combined CD59-I/H expressors). When CD59 expression was analyzed according to (−), I, and H levels, mean parasitemias were highest in CD59 (−) RBCs, followed by CD59-H RBCs, and then CD59-I RBCs. Despite a reversal in what might be expected if increased parasitemias were wholly related to GPI protein expression, whereby mean parasitemias in CD59-H were higher than CD59-I, all three experimental panels showed convincing increases in parasitized DAF (−) and CD59 (−) RBCs. The reversal in parasitemias among CD59-H and CD59-I may reflect culture variability, underscoring the value of the co-culture experiments.

The significance of increased parasitemias in GPI protein (−) versus (+) RBCs among our PNH samples is unclear, especially in light of studies showing that P. falciparum replication is similar among PNH GPI protein (−) and (+) RBCs.4,7 Our approach was similar to that of Samuel and others, whereby synchronized P. falciparum cultures were used to infect PNH RBCs, and then assessed by monoclonal antibodies, DNA staining, and flow cytometry.5 Unlike our study, however, whereby RBCs were infected with a 1% parasitemias, Samuel and others infected PNH RBCs with parasitemias ranging from 4.5% to 18.3%, and then used flow cytometry to detect early parasite stages (rings) to depict invasion, and late stage parasites (trophozoites, schizonts) with greater DNA loads to depict propagation. In contrast, we measured PNH RBC parasitemia at a time estimated to correlate with 2–3 cycles of schizogony, with most parasites being trophozoites or schizonts. We question whether higher parasitemias used by Samuel and others prohibited detection of subtle differences among PNH RBC infectivity that were apparent with lighter infective loads. For parasite DNA staining, Samuel and others added propidium iodide to formalin-fixed cells, whereas we used unfixed RBCs mixed with hydroethidium. Other considerations for the differences include ethnic variability in the PNH phenotype, as well as P. falciparum strain variability. Indeed, P. falciparum strains may bind different RBC receptors, and we speculate whether GPI protein deficiency, at least for our P. falciparum strain, reduced hindrances with improved access of parasites to relevant invasion receptors.4,6,13–17

In vitro reduction in the expression of DAF, but not CD59, in some PNH samples after infection with P. falciparum is a new finding. During in vivo P. falciparum infections of normal subjects, especially with severe anemia, DAF may be removed from uninfected (and probably infected) RBC surfaces through the transfer of immune complexes from the RBC surface to macrophages, partial cleavage by proteases or phospholipases released during schizont rupture, or accelerated RBC aging and oxidative damage as occurs in malaria.1,18–20 Indeed, genetic aberrations altering GPI expression might also render PNH RBCs more susceptible to oxidative stresses during P. falciparum infection. Infected RBCs with diminished DAF expression might be more susceptible to complement-mediated lysis, perhaps a host defense mechanism for reducing the risk of chronic malaria infections.3,18,20 However, if DAF reduction was a host response, it would likely occur on control RBCs, and involve CD59, the most abundant and active anti-complement GPI protein on RBC surfaces.3 Indeed, reduced DAF in some but not all PNH samples is suggestive of previously described CD59 variability, with diminished expression upon storage.18–20

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The replication of *Plasmodium falciparum* in paroxysmal nocturnal hemoglobinuria (PNH) red blood cells is a complex process that involves multiple factors. Several studies have explored the interaction between *P. falciparum* and PNH red blood cells (RBCs), highlighting the importance of understanding the molecular mechanisms involved in this interaction. Here, we summarize some key findings from recent studies that shed light on this topic.

1. **Dren with severe *Plasmodium falciparum* anemia.** *Blood* 95: 1481–1486.