Various PfRH5 polymorphisms can support *Plasmodium falciparum* invasion into the erythrocytes of owl monkeys and rats

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Abstract

Aotus nancymaae, the owl monkey, provides a useful laboratory model for research to develop drugs and vaccines against human falciparum malaria; however, many *Plasmodium falciparum* parasites are unable to invade *A. nancymaae* erythrocytes, rendering the parasites noninfective to the monkeys. In previous work, we identified a key polymorphism that determined the inheritance of erythrocyte invasion in a genetic cross of two *P. falciparum* clones that were virulent (GB4) or noninfective (7G8) to *A. nancymaae*. This polymorphism, an isoleucine-to-lysine polymorphism at position 204 (I204K) of the GB4 erythrocyte binding protein PfRH5, was nevertheless not found in several other *P. falciparum* lines that could also invade *A. nancymaae* erythrocytes. Alternative PfRH5 polymorphisms occur at different positions in these virulent parasites, and additional polymorphisms are found in *P. falciparum* parasites that cannot infect *A. nancymaae*. By allelic replacement methods, we have introduced the polymorphisms of these *A. nancymaae*-virulent or noninfective parasites at codons 204, 347, 358, 362, 410, and 429 of the endogenous *PfRH5* gene in the noninfective 7G8 line. 7G8 transformants expressing the polymorphisms of the *A. nancymaae*-virulent parasites show neuraminidase-sensitive (sialic acid-dependent) invasion into the monkey erythrocytes, whereas 7G8 transformants expressing the *PfRH5* alleles of noninfective parasites show little or no invasion of these erythrocytes. Parasites harboring PfRH5 polymorphisms 204K or 204R are also able to invade rat erythrocytes and are differentially sensitive to the removal of surface sialic acids by neuraminidase. These studies offer insights into the PfRH5 receptor-binding domain and interactions that support the invasion of various primate and rodent erythrocytes by *P. falciparum*.

Keywords

1. Introduction

Invasion of erythrocytes by malaria parasites is a multistep, complex process that involves a number of diverse and perhaps functionally redundant receptor-ligand interactions [1, 2]. Invasion is initiated when a merozoite attaches to an erythrocyte [3–5]. The parasite

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repositions so the apical end is in direct contact with the erythrocyte surface and then secretes the contents of the rhoptries and micronemes, specialized apical organelles that store and release the molecules necessary for host cell invasion [6]. Parasite ligands in this released material bind to erythrocyte molecules, forming a tight junction between the host and parasite membrane surfaces. The tight junction develops into an annulus that supports entry of the parasite into the erythrocyte as the annulus is translocated by an actin-myosin motor from the apical to posterior ends of the merozoite [7]. After entry of the merozoite, the erythrocyte membrane seals, completing the process of invasion.

In *Plasmodium falciparum*, a number of important erythrocyte binding ligands occur in two families, the erythrocyte binding-like (EBL) molecules and the reticulocyte binding-like (RBL) molecules [1, 2, 8]. Members of the EBL family are orthologs of the Duffy binding protein of *Plasmodium vivax*, appear to be stored in the micronemes, and include erythrocyte binding antigen 175 (EBA-175), EBA-140 (also known as BAEBL), EBA-181 (or JESEBL) and EBL-1 [9–16]. *P. falciparum* members of the RBL family include five proteins of the rhoptries: PfRH1 (PfNBP1), PfRH2a, PfRH2b, PfRH4 and PfRH5 [17–25]. These proteins were originally identified by their homology to RBL members of other *Plasmodium* species, including the *P. vivax* reticulocyte binding proteins PvRBP1 and PvRBP [26] and Py235 proteins of the rodent malaria parasite *Plasmodium yoelii* that mediate erythrocyte selection and virulence [27].

Despite knowledge of several known ligand-erythrocyte receptor interactions, much about these interactions in erythrocyte invasion pathways remains to be understood. In *P. falciparum*, many interactions appear to be redundant and individually dispensable, offering an advantage that may promote parasite survival when particular ligands or receptors are compromised, for example by host immune defenses or polymorphisms of the erythrocytes [28–30]. In some cases, members of the EBL and RBL families may function in cooperation [31]. Some but not all erythrocyte receptors for *P. falciparum* ligands have been identified: in the EBL family, EBA-175 binds sialic acid residues on the integral membrane protein glycophorin A [9, 32, 33], EBA-140 binds sialic acids on glycophorin C [12, 34, 35], and EBL-1 binds sialic acids on glycophorin B [16]. In studies of the RBL family, complement receptor 1 (CR1) and an isoform of basigin (BSG-S, the Ok blood group antigen, CD147) have been identified as human erythrocyte receptors for PfRH4 and PfRH5, respectively [36, 37], and the binding domains of PfRH1, PfRH4 and PfRH5 have been mapped to N-terminal regions of their sequences [23–25, 38, 39].

Various *P. falciparum* lines are able to invade erythrocytes that lack receptors for specific PfEBL- or PfRH-proteins (because of mutations or after enzyme treatment in the laboratory). Conversely, knock-out parasites that no longer express individual PfEBL or PfRH proteins generally retain their ability to invade erythrocytes [2]. An important exception, however, has been the unsuccessful attempt in at least two laboratories to knock out the gene encoding PfRH5 [2, 23]. Together with more recent evidence that invasion of human erythrocytes can be inhibited by a soluble pentamerized form of basigin, by anti-basigin monoclonal antibodies, or by reduction of basigin levels on the erythrocyte surface [37], these findings suggest that *P. falciparum* depends upon the PfRH5-basigin interaction for effective invasion of human erythrocytes. Unlike other members of the PfRH family, PfRH5 lacks a transmembrane domain; it instead interacts with a processed EGF-like protein (PfRipr) to form a complex that associates with an unidentified partner on the merozoite membrane (attempts to disrupt the *PfRipr* gene were also unsuccessful) [40].

The New World monkey *Aotus nancymaae* (owl monkey) is one of a limited number of primates susceptible to infection with human malaria parasites and available as a model for vaccine and drug development research. However, only some *P. falciparum* parasite lines

are able to invade the erythrocytes of these monkeys and produce infections *in vivo*. Recently, using a laboratory genetic cross between the GB4 and 7G8 *P. falciparum* clones from Ghana and Brazil, we identified an I→K substitution at position 204 (I204K) of PfRH5 as an important GB4 determinant for the invasion of *A. nancymaae* red blood cells [23]. Further, in contrast to the finding that engineered absence of all glycans from human basigin did not alter PfRH5 binding [37], removal (by neuraminidase) of sialic acids from the *Aotus* erythrocytes abrogated the binding of I204K-containing PfRH5 as well as invasion of the GB4 parent and I204K-containing progeny [23]. Certain other *P. falciparum* parasites that carry PfRH5 polymorphisms different from I204K are also able to infect *A. nancymaae* [23–25, 41]. Here, we report on investigations of these PfRH5 polymorphisms and interactions that support the invasion of various primate and rodent erythrocytes by *P. falciparum*.

2. Materials and methods

2.1. Parasites

The 7G8 *P. falciparum* clone, 7G8 × GB4 progeny clone LC12, and their transformants containing substitutions at PfRH5 positions 204 and 408, specifically 7G8$^{204I/407I}$ (7G8^{II} control transformant), 7G8$^{204K/407V}$ (7G8^{KV}), 7G8$^{204I/407V}$ (7G8^{IV}), LC12$^{204K/407V}$ (LC12^{KV} control transformant), LC12$^{204I/407I}$ (LC12^{II}) and LC12$^{204K/407I}$ (LC12^{KI}) have been previously described [23]. The original 7G8 clone [42] does not invade *A. nancymaae* erythrocytes and is not infective to *A. nancymaae* owl monkeys, whereas the LC12 parasite progeny clone invades *A. nancymaae* erythrocytes and is highly virulent to owl monkeys [23]. *P. falciparum* parasites were cultivated using standard techniques [43, 44]. Donor O$^+$ human blood was obtained from the Interstate Blood Bank (Memphis, TN).

2.2. Plasmid constructs

Using strategies similar to those for generation of the 7G8$^{204I/407I}$, 7G8$^{204K/407V}$, 7G8$^{204I/407V}$, LC12$^{204K/407V}$, LC12$^{204I/407I}$, LC12$^{204K/407I}$ transformed lines [23], six constructs were designed to generate additional codon changes in the endogenous *PfRH5* gene of the 7G8 clone (Table 1). For this purpose, 1396-bp fragments of *PfRH5* were amplified by PCR from the genomic DNA of *P. falciparum* Dd2, Malayan Camp (MalCamp), Santa Lucia, and FVO parasites. Primers 5′ (T<u>GCGGCCGC</u>ATGAAGACTATAAAAATGTGG) and 3′ (A<u>CTGCAG</u>ATGCTTTGTCTAATTAGAG) provided a *Not*I or *Pst*I site, respectively, for cloning into the pHD22Y vector [45]. The amplified fragments were incorporated into plasmids pHD22Y-rh5^{Dd2}, pHD22Y-rh5^{MalCamp}, pHD22Y-rh5^{SantaLucia}, and pHD22Y-rh5^{FVO}. A fifth construct, pHD22Y-rh5^{429N}, was generated using the site-directed mutagenesis kit QuikChange II, according to the manufacturer's instructions (Stratagene, La Jolla, CA).

During the course of our experiments, we noted that some plasmids consistently integrated into the target chromosome by single crossover events toward the 3′ of the 7G8 *RH5* gene, so that transfectants expressing all of the desired codon changes were difficult to obtain. This proved to be a particular problem for the case of the Palo Alto I204R polymorphism [23]. We therefore designed a sixth construct to express the amino acid sequence of the Palo Alto parasite but with codon adjustments, so that all codons downstream of I204R were with the nucleotide preferences of *Escherichia coli* instead of *P. falciparum* (pHD22Y-rh5^{PaloAlto}; GENEART, Toronto, Canada). Because the AT-rich *PfRH5* 3′ flanking sequence in the original design proved difficult to synthesize, this region of pHD22Y-rh5^{PaloAlto} sequence was also modified so that it contains the 3′ flanking region of *Plasmodium berghei* dihydrofolate reductase-thymidilate synthase [46]. The resulting synthetic I204R *PfRH5*

sequence, along with the dihydrofolate reductase-thymidilate synthase terminator, provided a 1.7-kb fragment that was cloned into pHD22Y using the *Not*I and *Pst*I sites. All other constructs in this study utilized the *PfRH5* endogenous 3′ flanking region. The plasmid constructs were confirmed by DNA sequencing.

2.3. Parasite transfections

Uninfected human erythrocytes were loaded with purified plasmid DNA by electroporation and mixed with mature-stage 7G8 parasites as described [47]. After 2 days, 5 nM WR99210 was applied to the cultures to select parasites carrying the transfected plasmids. After ~60 days of WR99210 pressure, homologous integration into endogenous *PfRH5* was detected by PCR. The transformed parasites were cryopreserved, confirmed by Southern blotting, and the presence of the desired codon polymorphisms in the allelic exchanges was verified by polymerase chain reaction (PCR) amplification and DNA sequencing.

2.4. Invasion assays

Mature-stage parasites were purified using the percoll/sorbitol method [48], washed three times, and adjusted to a final concentration of $1.0–1.5 \times 10^7$ parasitized erythrocytes/ml. Parasite suspension (100 μl) was added to 100 μl of target erythrocytes at 2×10^8 cells/ml in complete medium in a flat-bottomed 96-well plate, for an introduced parasitemia of approximately 5.0–7.5% [30]. The parasite suspension was also added to wells containing human or rhesus (*Macaca mulatta*) erythrocytes, which were included as positive and negative controls, respectively. Plates were incubated at 37°C for 18–24 h. The number of ring forms in at least 1,000 erythrocytes was counted for each determination. The invasion rate was calculated as a percentage from the observed frequency of ring-stage parasites in target cells relative to the observed frequency in control human erythrocytes. Any inoculation of erythrocytes from the purified parasite suspension was corrected by subtraction of the frequency of ring-stage parasites in control wells of rhesus erythrocytes, since rhesus erythrocytes are completely resistant to *P. falciparum* invasion and ring-stages can develop only in the inoculated cells [49, 50]. Each assay was performed in triplicate on at least three independent occasions. Blood from donor animals was obtained in compliance with National Institutes of Health guidelines under relevant Animal Care and Use Committee-approved protocols.

2.5. Sialic acid removal from erythrocytes by neuraminidase treatment

For the removal of erythrocyte surface sialic acids, 1×10^8 cells/ml were treated with 0.025 U/ml of *Arthrobacter ureafaciens* α2-3,6,8,9 neuraminidase (EMD Biosciences, Inc, San Diego, CA) in RPMI at 37°C for 1 h with gentle agitation and washed three times before use. For determination of sialic acid content, 1×10^8 erythrocytes were washed three times in phosphate buffered saline (PBS; 10 mM phosphate, 140 mM NaCl, pH 7.4), lysed in 400 μl of 5-mM KH_2PO_4 containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and incubated on ice for 10 min. After centrifugation at $10,000 \times g$ for 10 min at 4°C, the erythrocyte ghosts were gently removed and washed three times with PBS containing protease inhibitor cocktail. The ghost membranes were treated with 0.25 U/ml *Ar. ureafaciens* neuraminidase in PBS at 37°C for 1 h with gentle agitation. Supernatants containing cleaved sialic acids were collected, and residual sialic acid remaining on the ghost membranes was hydrolyzed by acid treatment (2 M acetic acid at 80°C for 3 h). The sialic acids released by neuraminidase treatment alone or by acid hydrolysis post neuraminidase treatment were derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB) and analyzed by reverse-phase fluorometric high-performance liquid chromatography (HPLC) at the Glycotechnology Core Resource at the University of California, San Diego as described [51].

3. Results

3.1. Various PfRH5 polymorphisms can support sialic acid-dependent parasite invasion of *A. nancymaae* erythrocytes

PfRH5 codon changes introduced into the 7G8 and LC12 lines allowed us to explore the effects of various amino acid polymorphisms on the ability of *P. falciparum* to invade monkey and rodent erythrocytes (Table 1). The 7G8 and LC12 parasites from the 7G8×GB4 cross and six transformants encoding polymorphisms at PfRH5 positions 204 and 407 ($7G8^{204I/407I}$, $7G8^{204K/407V}$, $7G8^{204I/407V}$, $LC12^{204K/407V}$, $LC12^{204I/407I}$, $LC12^{204K/407I}$) have been described [23]. For the present study, we generated an additional six transformants of the 7G8 parasite by allelic exchange (Fig. 1). These included five transformants containing polymorphisms from four *P. falciparum* lines that efficiently invade *A. nancymaae* erythrocytes: PfRH5 204R from the Palo Alto line; 347D from the Santa Lucia line; 347Y and 429N from the Malayan Camp line; 358F and 362D from the FVO line; and 429N alone from the Malayan Camp line (transformants identified as $7G8^{204R}$, $7G8^{347D}$, $7G8^{347Y/429N}$, $7G8^{358F/362D}$, and $7G8^{429N}$ in Table 1). A sixth transformant, $7G8^{410M}$, was also designed with the PfRH5 410M polymorphism of the Dd2 parasite that, like 7G8, is unable to invade *Aotus* erythrocytes [23]. All plasmids for these exchanges were constructed with the native *PfRH5* nucleotide sequence except $7G8^{204R}$, which required a plasmid construct carrying a codon-adjusted open reading frame with reduced homology downstream of codon 204.

Relative invasion rates into *A. nancymaae* erythrocytes were determined for each of the transformed 7G8 parasite lines (Table 1). Transformants expressing *PfRH5* alleles with the 204R codon of Palo Alto, the 347D codon of Santa Lucia, the 347Y/429N codons of Malayan Camp (MalCamp), or the 358F/362D codons of FVO had gained the ability to invade *A. nancymaae* erythrocytes. The 7G8 transformant expressing 358F and 362D codons (FVO allele) exhibited the highest *A. nancymaae* erythrocyte invasion rate (71.6 ± 5.7%), whereas the 204R (Palo Alto), 347D (Santa Lucia) and 347Y/429N (Malayan Camp) transformants invaded at lower rates of 29.4 ± 6.6%, 16.8 ± 2.6% and 26.8 ± 2.0%, respectively. These rates compare to 38.7 ± 7.6% for the previously reported $7G8^{204K/407V}$ transformant and to 55.4± 4.2%, 43.2 ± 7.3% and 34.6 ± 5.4% for the LC12 progeny clone and its $LC12^{204K/407V}$ and $LC12^{204K/407I}$ transformants, all of which contain PfRH5 204K (Table 1). The replacement of 204K by 204I in the $LC12^{204I/407I}$ transformant was associated with dramatically reduced invasion of *A. nancymaae* erythrocytes, as expected (4.6 ± 1.0%; Table 1).

In contrast to the *A. nancymaae* erythrocyte invasion rate of 26.8 ± 2.0% for the $7G8^{347Y/429N}$ transformant, little or no increased invasion was observed for the $7G8^{429N}$ line containing the single PfRH5 429N polymorphism (2.2 ± 1.4% *vs.* 0.4 ± 0.2% for the untransformed 7G8 line or 2.1 ± 0.4% for control 204I/407I-transformed 7G8 parasites; Table 1). These data suggest that the PfRH5 347Y amino acid substitution is a key determinant of *A. nancymaae* invasion by 7G8 transformants expressing the Malayan Camp allele. Also, the 347Y amino acid change occurs at exactly the same position as the 347D substitution that enables invasion of the Santa Lucia-transformed parasites. 7G8 transformants expressing the 410M polymorphism of the noninfective Dd2 parasite showed no substantial change from the noninvasive phenotype of untransformed 7G8 or control 204I/407I-transformed-7G8 parasites (2.0 ± 0.2% *vs.* 0.4 ± 0.2% or 2.1 ± 0.4%, respectively; Table 1).

In previous work with neuraminidase-treated cells, we found that the I204K polymorphism was linked to sialic acid-sensitive binding of PfRH5 at the surface of *A. nancymaae* as well as human erythrocytes [23]; neuraminidase treatment also abrogated the invasion of *A.*

nancymaae (but not human) erythrocytes by the LC12 and I204K-transformed 7G8 parasites. To test the sialic acid dependence of additional PfRH5 polymorphisms listed in Table 1, we compared the invasion rates of the corresponding 7G8 transformants into *A. nancymaae* erythrocytes treated with neuraminidase. Results (Fig. 2) showed that the neuraminidase-treated *A. nancymaae* erythrocytes were in all cases resistant to the otherwise invasive transformants: 7G8^{204R}, 7G8^{347D}, 7G8$^{347Y/429N}$, and 7G8$^{358F/362D}$.

3.2. A positive charge mutation at PfRH5 position 204 supports parasite invasion of rat erythrocytes

Previous studies noted that Malayan Camp but not 7G8 parasites could invade rat as well as *A. nancymaae* erythrocytes [50]. To investigate whether an ability to invade rat erythrocytes was PfRH5-dependent, we tested the invasion of rat erythrocytes by the 7G8 transformants listed in Table 1. Of the *A. nancymaae*-invasive transformants, only LC12 and 7G8 transformants that expressed a *PfRH5* allele encoding 204K or 204R invaded rat erythrocytes (invasion rates of $23.5 \pm 2.3\%$ for 7G8$^{204K/407V}$, $20.5 \pm 3.6\%$ for LC12$^{204K/407V}$, $20.4 \pm 2.7\%$ for LC12$^{204K/407I}$, and $26.7 \pm 4.0\%$ for 7G8^{204R}). The other *A. nancymaae*-invasive transformants that retained 204I and expressed PfRH5 polymorphisms at different positions did not invade rat erythrocytes, including the highly *A. nancymaae*-invasive 7G8$^{358F/362D}$ (FVO) transformant. A potential paradox was that the 7G8$^{347Y/427N}$ (Malayan Camp) transformant was among our lines that did not invade rat erythrocytes, in contrast to the previous report that Malayan Camp parasites could invade rat erythrocytes [50]. We therefore confirmed the PfRH5 sequence of our untransformed Malayan Camp parasites, tested their invasion rates and found that their invasion of rat erythrocytes was negligible ($1.7 \pm 0.3\%$). These data suggest that our Malayan Camp parasites and their PfRH5 sequence are not the same as those of Klotz *et al.* [50], perhaps because of a change during the long laboratory history of Malayan Camp and its use for various experimental purposes including Aotus monkey challenges.

We tested whether the invasion rates of rat erythrocytes are sialic acid dependent. Various 7G8 transformants containing 204K or 204R were assayed with rat erythrocytes that had been treated with neuraminidase. The 204K- or 204R-containing transformants showed different degrees of sialic acid sensitivity, invading neuraminidase-treated rat erythrocytes with invasion rates of $19.1 \pm 5.4\%$ for 7G8$^{204K/407V}$, $13.8 \pm 2.4\%$ for LC12$^{204K/407V}$, $4.5 \pm 1.0\%$ for 7G8^{204R} and $16.3 \pm 0.6\%$ for LC12$^{204K/407I}$ (Fig. 3). Compared to the rates of invasion into untreated rat erythrocytes, these results demonstrate greater dependence of the 204R-containing than 204K-containing PfRH5 transformants on the presence of rat erythrocyte sialic acid (Fig. 3).

3.3. PfRH5-transformed 7G8 and LC12 parasites exhibit sialic acid-sensitive invasion of mouse erythrocytes

The 7G8 and Malayan Camp parasites were previously reported to invade mouse erythrocytes [50]. In our evaluations of the 7G8 and LC12 transformants, no lines failed to invade normal mouse erythrocytes; invasion rates ranged from $22.8 \pm 0.5\%$ for the 7G8$^{358F/362D}$ transformant to $58.4 \pm 29.4\%$ for 7G8$^{204I/407I}$ (Table 1). Neuraminidase treatment of the mouse cells greatly decreased invasion rates and eliminated invasion by the 7G8$^{358F/362D}$ transformant (Fig. 4).

3.4. *A. nancymaae* human, rat and mouse erythrocytes release sialic acid to different degrees upon neuraminidase treatment

Sialic acids are N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone, and are typically found as the outermost units of cell surface and secreted glycans such as the glycophorins. Substitutions at the 4, 5, 7, 8, and 9-carbon

positions, with different α-glycosidic linkages at the 2-carbon position, result in over 50 variations [52, 53]. These sialic acids constitute the majority of the negative charge in a glycocalyx at the erythrocyte surface, supplying a repelling force that helps to prevent adherence between erythrocytes and of erythrocytes to blood vessels, and they also provide important surface attachments and entry functions for a variety of host-pathogen interactions [54]. In addition to the role of sialylated erythrocyte surface receptors recognized by *P. falciparum* merozoites [12, 14, 23, 33, 38], examples include the binding of influenza hemagglutinin to host cells and of *Helicobacter pylori* hemagglutinin to gastric mucins [55, 56].

In view of the various degrees of complete or partial reduction of invasion into neuraminidase-treated *A. nancymaae*, mouse or rat erythrocytes by the PfRH5-transformed parasites, we investigated the extent of sialic acid removal from erythrocyte membranes by neuraminidase cleavage. *A. nancymaae*, human, rat and mouse erythrocyte ghosts were treated with neuraminidase and the released sialic acids were collected, DMB-derivatized and quantitatively analyzed by HPLC. After neuraminidase treatment, the erythrocyte ghost membranes were subjected to acid hydrolysis to release residual uncleaved sialic acids, which were also collected, DMB-derivatized and analyzed by HPLC. As expected, the large proportion of sialic acid released from human and *A. nancymaae* erythrocytes was 5-*N*-acetlyneuraminic acid (Neu5Ac) [51]: only 10–20% of this sialic acid remained on the human and *A. nancymaae* membranes after neuraminidase treatment (Table 2). Neu5Ac release from rat and mouse erythrocytes was less efficient: 25–35% remained on the membranes after neuraminidase treatment (Table 2).

5-*N*-glycolylyneuraminic acid (Neu5Gc) was released from rat and mouse erythrocyte membranes in smaller amounts and was less sensitive to neuraminidase treatment than Neu5Ac (65–70% remaining after treatment; Table 2). Very low levels of Neu5Gc were obtained from *A. nancymaae* and human erythrocytes as these species carry a mutated, non-functional DMP-Neu5Ac hydroxylase gene that is incapable of Neu5Gc synthesis; the small amounts of Neu5Gc on the erythrocytes of these species probably originate from dietary sources [51].

4. Discussion

Polymorphisms of PfRH5 can confer different erythrocyte invasion abilities to *P. falciparum* parasites. In the present and previous studies [23], we have shown that the inability of 7G8 parasites to invade *A. nancymaae* erythrocytes can be transformed to a highly invasive phenotype by the introduction of polymorphisms from *P. falciparum* lines such as Santa Lucia (204R), GB4 (204K/407V), Palo Alto (347D), Malayan Camp (347Y/429N), or FVO (358F/362D). Further, PfRH5 polymorphisms 204K and 204R enable the 7G8 transformants to invade rat erythrocytes whereas the other polymorphisms fail to promote the invasion of rat erythrocytes at all. A positive charge at PfRH5 position 204 may therefore serve as an important enabler for the invasion of rat erythrocytes by 7G8 transformants. All transformants showed little or no ability to invade neuraminidase-treated *A. nancymaae* erythrocytes, suggesting that the 204K, 204R, 347D, 347Y/429N, and 358F/362D polymorphisms may support the binding of PfRH5 to neuraminidaseremovable sialic acid residues on proteins at the *A. nancymaae* erythrocyte surface.

Neuraminidase treatment of rat erythrocytes also reduced the invasion rates of 7G8[204K] and 7G8[204R] PfRH5 transformants but to different extents, producing a particularly strong effect on the invasion ability of the 7G8[204R] transformant. It is not yet clear how these different levels of reduction relate to incomplete removal of Neu5Ac and Neu5Gc by the action of neuraminidase. Neuraminidase stripped less Neu5Ac from rat than from *A. nancymaae*

erythrocytes (~67% *vs.* 83% removed; Table 3), and rat erythrocytes carried levels of Neu5Gc that *A. nancymaae* could not synthesize. One possibility is that the Neu5Ac and Neu5Gc residues remaining after neuraminidase treatment were sufficient to support the observed levels of 7G8[204K] and 7G8[204R] invasion. In this scenario, lower invasion of neuraminidase-treated rat erythrocytes by the 7G8[204R] *vs.* 7G8[204K] transformants might have resulted from differential ability of the modified PfRH5 molecules to bind residual sialic acids on the erythrocyte surface. A similar explanation might account for the reduced invasion rates of transformed 7G8 and LC12 lines into neuraminidase-treated mouse erythrocytes, including complete inability of 7G8[358F/362D] parasites to invade the treated mouse erythrocytes.

In previous work (ref [23] Fig. S3), we showed that the 204K form of PfRH5 expressed by LC12 parasites did not bind to neuraminidase-treated human cells in erythrocyte binding assays, even though these parasites were able to invade the neuraminidase-treated human erythrocytes at 60–70% of the rate they invaded untreated human erythrocytes. In comparison, the 7G8 form of PfRH5, which contains no 204K mutation, showed efficient binding of PfRH5 to the neuraminidase-treated erythrocytes and a 60–70% relative invasion rate similar to LC12. These findings are consistent with the observation that neuraminidase treatment of recombinant basigin or obliteration of its glycosylation motifs does not affect binding to PfRH5 from 3D7 parasites [37] (PfRH5 from 3D7 and 7G8 both contain the 204I residue [23]). Although the mutant 204K or 204R forms of PfRH5 expressed by LC12 and other *A. nancymaae*-invasive parasites remain to be tested for basigin binding ability, unsuccessful efforts in two laboratories to knock-out the PfRH5 gene from *P. falciparum* [23, 37], and the ability of LC12 to invade neuraminidase-treated human erythrocytes despite loss of PfRH5 binding in erythrocyte binding assays, support suggestions that the PfRH5-PfRipr complex may serve in an important role apart from or in addition to a mechanical attachment function [40, 57].

The PfRH5 polymorphisms reported here demonstrate the flexibility of *P. falciparum* parasites to invade the erythrocytes of primate and rodent species that present different surface receptors. Interestingly, a search of SNPs and alleles available in the MalariaGEN database [58] did not show prevalence in naturally-infected human populations of any of the *Aotus*-competent PfRH5 polymorphisms identified here (204K, 204R, 347D, 347Y, 358F or 362D). Other PfRH5 polymorphisms were instead reported from deep sequencing studies of *P. falciparum* samples from 227 patients in Africa, Asia and Oceania, including N88D, Y147H, H148D, S197Y, C203Y, A233E, I364I, H365N, V371I, I407V, I410M, Q477H AND I493V (http://www.malariagen.net/data) [58]. The specificity of particular PfRH5 polymorphisms to *Aotus*-infective parasites suggests that these polymorphisms were selected during adaptation of *P. falciparum* isolates to the monkeys. Deep sequencing data from additional surveys may be useful in further searches for the presence of *Aotus*-competent PfRH5 polymorphisms in human parasite populations. PfRH5 polymorphisms may also differ among the *P. falciparum*-like parasites that occur in apes [59–62]; indeed, a 204K polymorphism is present in the PrRH5 sequence of a single isolate of the chimpanzee malaria parasite *P. reichenowi* [23].

Interactions of PfRH5, PfRipr and basigin are thought to be essential to human erythrocyte invasion [37, 40]. The polymorphisms of PfRH5 at amino acid positions 204, 347 and 358/362 occur within a region proposed to recognition domain that binds erythrocytes [23, 25], and this region shares conserved features with the sialic acid-dependent binding region of PfRH1 as well as the sialic acid-independent binding region of PfRH4 [39]. PfRH1 binding may involve α-helices in coiled coils so that, following release from the rhoptries, they project out from the merozoite surface to interact with the red blood cell surface [38, 41]. Further characterization of the structure of PfRH5, the features of its binding to human

and non-human receptors, and the functional roles of its polymorphisms will advance our understanding of the events that underlie erythrocyte invasion and perhaps inform new strategies for molecular approaches against malaria.

Acknowledgments

Abbreviations

EBA	erythrocyte binding antigen
EBL	erythrocyte binding-like
RBL	reticulocyte binding-like

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Research Highlights

- Only some *P. falciparum* (*Pf*) lines are able to infect *Aotus nancymaae* (owl) monkeys

- Various *PfRH5* alleles in these lines support infection of monkey erythrocytes (RBC)

- Non-invasive *Pf* parasites (7G8) become invasive when transformed by these alleles

- Invasion of owl monkey RBC by the 7G8 transformants is sialic acid-dependent

- Rat RBC can be invaded by 7G8 transformants expressing PfRH5 I204K or I204R
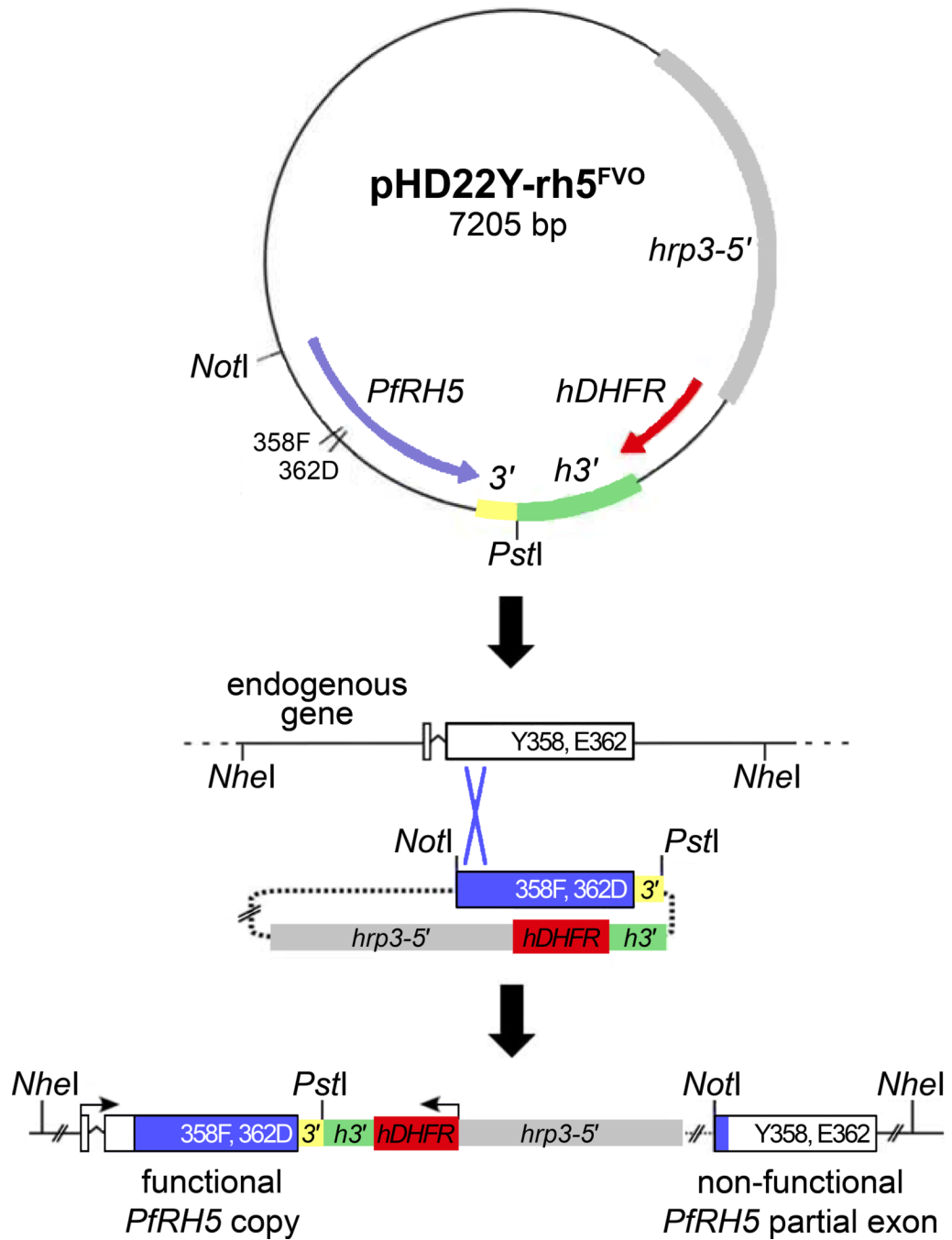
Fig 1.
Illustration of the strategy to generate *PfRH5*-modified 7G8 parasites by homologous recombination. A 1396-bp fragment of *PfRH5* containing codon polymorphisms of the FVO parasite was PCR-amplified and cloned into the pHD22Y vector [45]. Following transfection of 7G8 parasites with the desired construct, a portion of the FVO *PfRH5* gene along with the 0.2 kb 3′ flanking sequence spontaneously recombined by single-site crossover and replaced the endogenous 7G8 *PfRH5* sequence with a modified allele. The remainder of the construct, including the *hDHFR* drug selection marker, and a remnant of the endogenous 7G8 *PfRH5* sequence lie downstream of the modified *PfRH5* allele. Similar strategies were used for the other constructs employed in this study. 3′, 3′ terminal flanking

region of *PfRH5; hrp3-5′*, promoter region from *P. falciparum* histidine-rich protein 3; *hDHFR*, human dihydrofolate reductase coding segment; *h3′3′* regulatory region of *P. falciparum* histidine-rich protein 2.
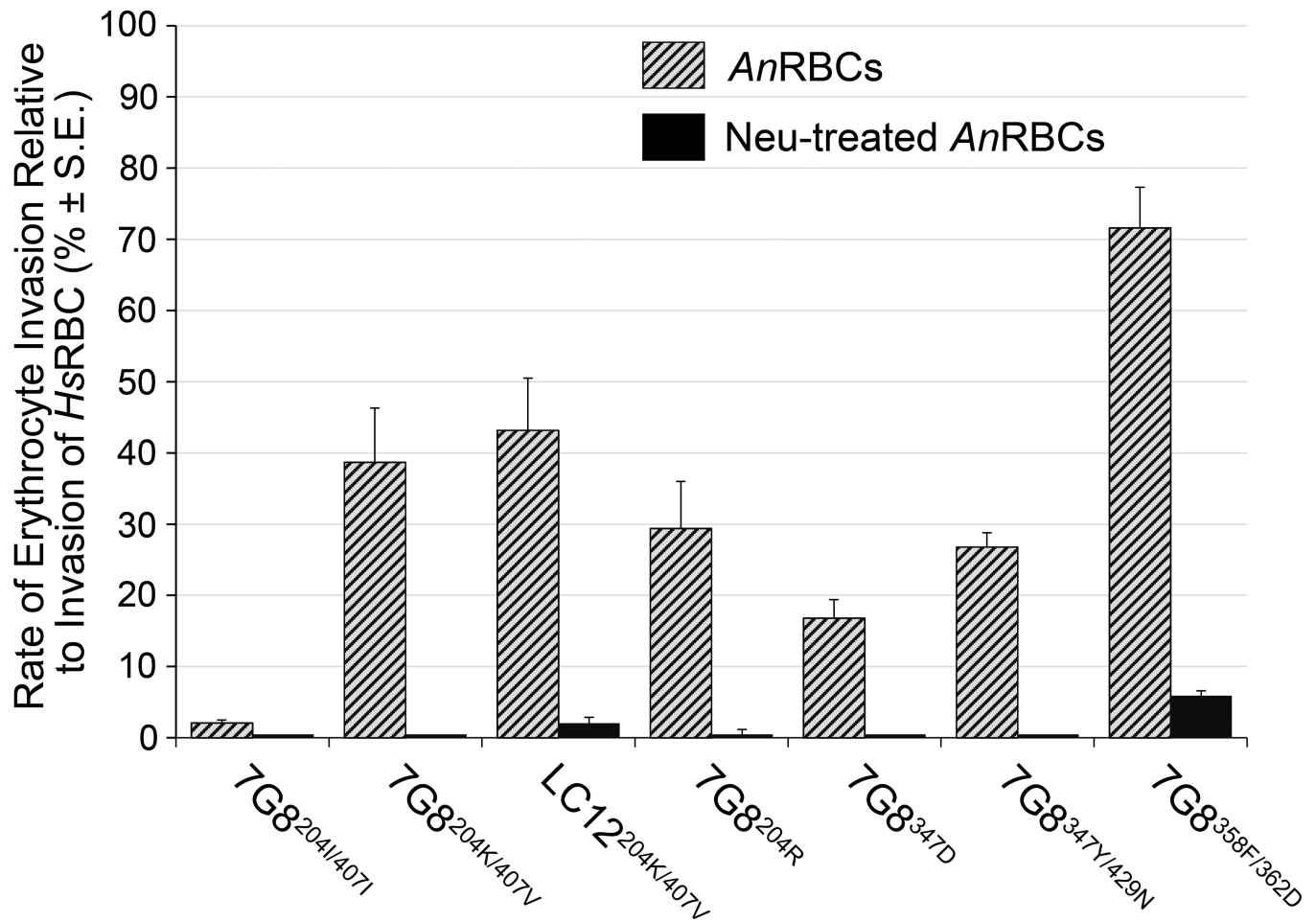
Fig. 2.
Relative invasion rates of *PfRH5* allele-modified *P. falciparum* lines into *A. nancymaae* erythrocytes and neuraminidase-treated *A. nancymaae* erythrocytes. *An*RBC, *A. nancymaae* erythrocytes; *Hs*RBC, human erythrocytes; Neu, neuraminidase.
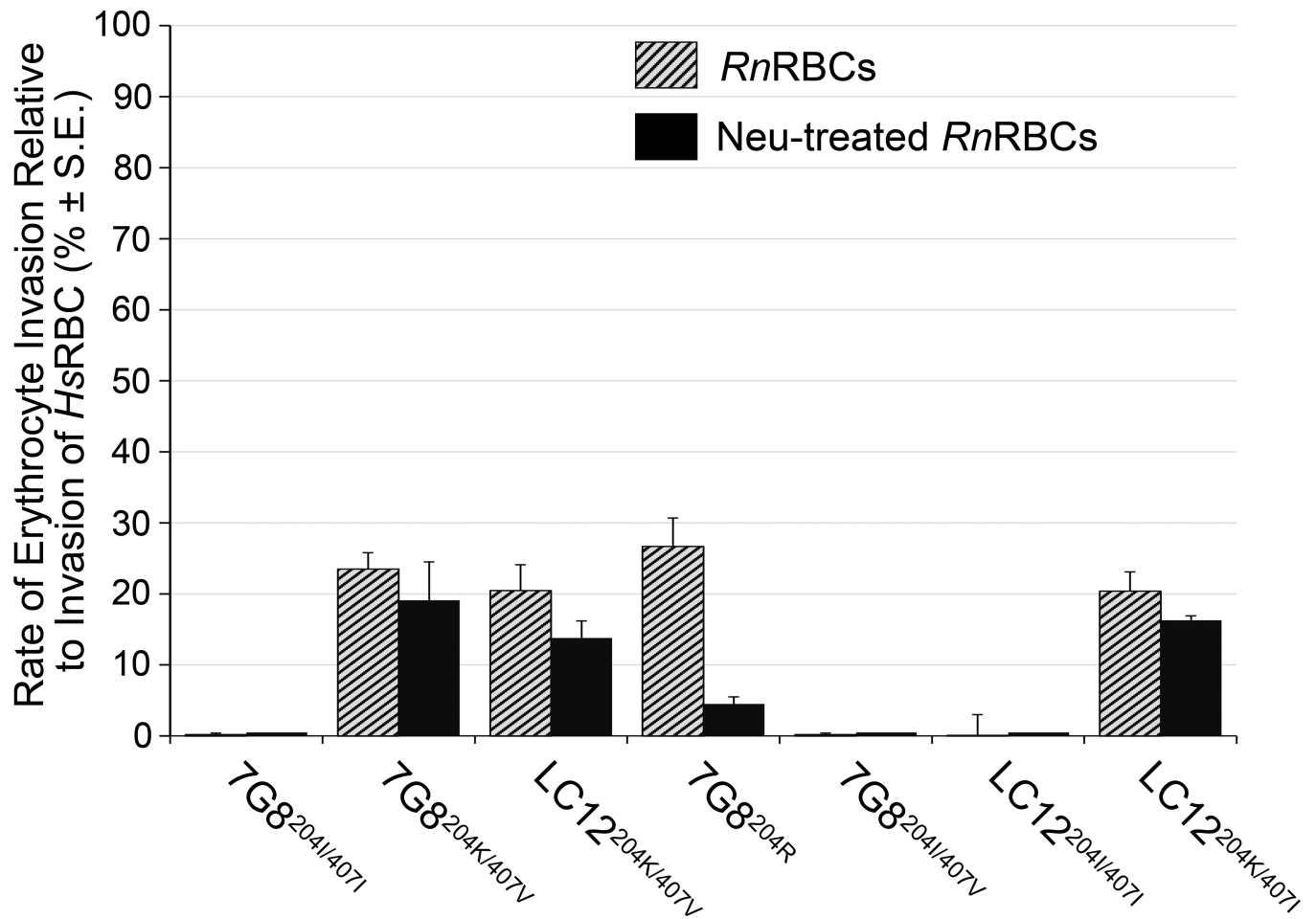
Fig. 3.
Relative invasion rates of *PfRH5* allele-modified *P. falciparum* lines into rat erythrocytes and neuraminidase-treated rat erythrocytes. *Rn*RBC, rat *(Rattus norvegicus)* erythrocytes; *Hs*RBC, human erythrocytes; Neu, neuraminidase.
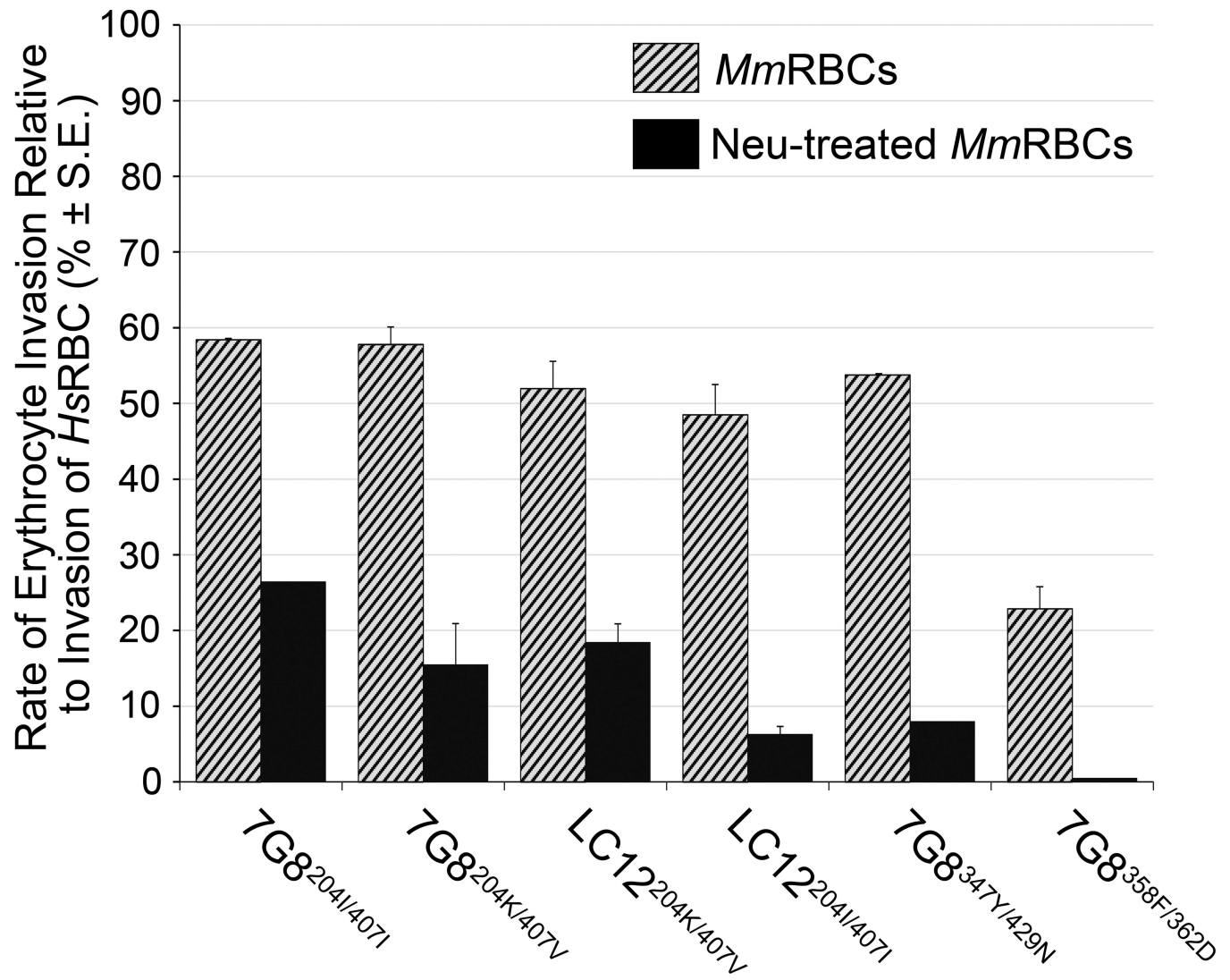
Fig. 4.
Relative invasion rates of *PfRH5* allele-modified *P. falciparum* lines into mouse
erythrocytes and neuraminidase-treated mouse erythrocytes. *Mm*RBC, mouse *(Mus
musculus)* erythrocytes; *Hs*RBC, human erythrocytes; Neu, neuraminidase.

Table 1

PfRH5 polymorphisms and invasion of native and transformed *P. falciparum* 7G8 and LC12 lines

Parasite line[b]	Transfection plasmid	PfRH5 polymorphisms							Erythrocyte invasion rate (% ± S.E.)[a]		
		204	347	358	362	407	410	429	*A. nancymaae*	Rat	Mouse
7G8	—	I	N	Y	E	I	I	K	0.4 ± 0.2[c]	0.2 ± 0.2	N.D.[d]
LC12	—	K	N	Y	E	V	I	K	55.4 ± 4.2[c]	25.7 ± 2.9	N.D.
7G8$^{204I/407I}$	pHD22Y-rh5^{7G8}	I	N	Y	E	I	I	K	2.1 ± 0.4	0.2 ± 0.2	58.4 ± 29.4
7G8$^{204K/407V}$	pHD22Y-rh5^{LC12}	**K**	N	Y	E	**V**	I	K	38.7 ± 7.6	23.5 ± 2.3	57.8 ± 9.9
7G8$^{204I/407V}$	pHD22Y-rh5^{LC12}	I	N	Y	E	**V**	I	K	4.9 ± 3.0	0.0 ± 0.0	N.D.
LC12$^{204K/407V}$	pHD22Y-rh5^{LC12}	K	N	Y	E	V	I	K	43.2 ± 7.3	20.5 ± 3.6	52.0 ± 2.9
LC12$^{204I/407I}$	pHD22Y-rh5^{7G8}	**I**	N	Y	E	**I**	I	K	4.6 ± 1.0	0.1 ± 2.9	48.5 ± 4.5
LC12$^{204K/407I}$	pHD22Y-rh5^{7G8}	K	N	Y	E	**I**	I	K	34.6 ± 5.4	20.4 ± 2.7	N.D.
7G8^{204R}	pHD22Y-rh5^{PaloAlto}	**R**	N	Y	E	I	I	K	29.4 ± 6.6	26.7 ± 4.0	N.D.
7G8^{347D}	pHD22Y-rh5^{SantaLucia}	I	**D**	Y	E	I	I	K	16.8 ± 2.6	0.0 ± 0.0	N.D.
7G8$^{347Y/429N}$	pHD22Y-rh5^{MalCamp}	I	**Y**	Y	E	I	I	**N**	26.8 ± 2.0	0.5 ± 0.5	53.8 ± 10.3
7G8$^{358F/362D}$	pHD22Y-rh5^{FVO}	I	N	**F**	**D**	I	I	K	71.6 ± 5.7	0.0 ± 0.0	22.8 ± 0.5
7G8^{410M}	pHD22Y-rh5^{Dd2}	I	N	Y	E	I	**M**	K	2.0 ± 0.2	0.0 ± 0.0	N.D.
7G8^{429N}	pHD22Y-rh5^{N429}	I	N	Y	E	I	I	**N**	2.2 ± 1.4	0.0 ± 0.0	41.9 ± 5.3

[a] Relative to rates of invasion into human erythrocytes.

[b] Parasite transfectants 7G8$^{204I/407I}$, 7G8$^{204K/407V}$, 7G8$^{204I/407V}$, 7G8$^{204K/407V}$, LC12$^{204K/407V}$, LC12$^{204I/407I}$ and LC12$^{204K/407I}$ previously reported as 7G8II, 7G8KV, 7G8IV, LC12KV, LC12II, and LC12KI, respectively [23].

[c] As reported in [23].

[d] N.D., not determined.

Table 2

Sialic acid release from *A. nancymaae*, human, rat, and mouse erythrocyte membranes

Erythrocyte membrane sample	Sialic acid release step[a]	Neu5Ac (pmol)	% remaining after neuraminidase treatment	Neu5Gc (pmol)	% remaining after neuraminidase treatment
A. nancymaae	Neuraminidase treatment	2876.2	16.9%	0.5	N.D.[b]
	Residual release by acid treatment	584.4		4.3	
Human	Neuraminidase treatment	2599.3	11.2%	0.8	N.D.
	Residual release by acid treatment	327.4		0.9	
Rat	Neuraminidase treatment	768.9	32.8%	16.8	65.7%
	Residual release by acid treatment	374.9		32.2	
Mouse	Neuraminidase treatment	664.6	26.8%	40.5	66.3%
	Residual release by acid treatment	243.9		79.7	

[a]Residual sialic acids remaining on the membranes after *Arthrobacter urealfaciens* neuraminidase treatment were released by exposure to 2 M acetic acid at 80°C for 3 h.

[b]N.D., not determined because of low signal levels. *A. nancymaae* and humans carry a mutated, non-functional DMPNeu5Ac hydroxylase gene that is incapable of Neu5Gc synthesis; the small amounts of Neu5Gc on the erythrocytes of these species probably originate from dietary sources [51].