Erythrocyte polymorphisms, including ovalocytosis, have been associated with protection against malaria. This study in the Wosera, a malaria holoendemic region of Papua New Guinea, examined the genetic basis of ovalocytosis and its influence on susceptibility to malaria infection. Whereas previous studies showed significant associations between Southeast Asian ovalocytosis (caused by a 27-base pair deletion in the anion exchanger 1 protein gene) and protection from cerebral malaria, this mutation was observed in only 1 of 1019 individuals in the Wosera. Polymerase chain reaction strategies were developed to genotype individuals for the glycophorin C exon 3 deletion associated with Melanesian Gerbich negativity (GPCΔex3). This polymorphism was commonly observed in the study population (GPCΔex3 frequency = 0.465, n = 742). Although GPCΔex3 was significantly associated with increased ovalocytosis, it was not associated with differences in either Plasmodium falciparum or P vivax infection measured over the 7-month study period. Future case-control studies will determine if GPCΔex3 reduces susceptibility to malaria morbidity. (Blood. 2001;98:3489-3491)
were performed as previously described. To assess the presence of exons 2 and 3, homologous, 2 reactions are needed to delineate all 3 genotypes. All PCR amplifications were performed as previously described. 

5' CAGATCTCTGCCTCTGGATCAAG-3' and GPCdn 5' TCAAAAC-CACCTGTGAGGAGG-3' annealing to conserved sequence around exons 2 and 3 were used (thermocycling program: 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds [× 40]). PCR products were subjected to electrophoresis on a 4% 5:1 GTG NuSieve:LE agarose gel (FMC Bioproducts, Rockland, ME) gel. A 264-bp band (exon 2) is detected in wt/wt, wt/Δ, and Δ/Δ. A 240 bp band (exon 3) is detected in only wt/wt and wt/Δ. The heteroduplex product is created by hybridization between complementary strands of exon 2 and 3 amplicons. (F) To identify individuals with the wt allele, primers annealing to a 51-bp intron 2 insert (GenBank AF342984) and downstream intron 2 polymorphism (GenBank M24627) (GPC349up 5' GGAAACTGCCGTGACTTCAGA-3' and 1679dn 5' CATGTTCTGGAAAGTTGCG-3') were used to amplify a 1376-bp product (thermocycling program: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds [× 40]). PCR products were subjected to electrophoresis on a 1.5% 5:1 GTG NuSieve:LE agarose gel. (G) To identify the GPCex3 allele and differentiate between wt/wt and wt/Δ, primers annealing to a 51-bp intron 2 insert and downstream intron 3 polymorphism (GenBank M24628) (GPC349up and GPC1680dn 5' AGTTTAACATACATACCCAGG-3') were used. Because the 3.4-kb deletion of the GPC gene includes exon 3, this PCR produces a 1377-bp band. To ensure that absence of the 1376-bp (D) and 1377-bp (E) bands was not caused by heterogeneity in PCR master mix, an additional primer, GPCdn, along with GPC349up, amplified conserved regions downstream of exon 2. The 155-bp product produced in all genotypes by GPC349up and GPCdn is not shown. All gels were stained with a 1:10 000 dilution of SYBR Gold (Molecular Probes, Eugene, OR), and products were visualized using a Storm 860 (Molecular Dynamics, Sunnyvale, CA).

Results and discussion

Ovalocytosis in the Wosera

To assess the relationship between ovalocytosis and Melanesian ancestry, 13 North Americans and 199 individuals from the Wosera were compared. The frequency of ovalocytes per 1000 RBCs was significantly higher in residents of the Wosera than North Americans (median, 292 ± 131.4; median, 24 ± 22.6, respectively, Wilcoxon, P < .0001). Representative blood smears from a North American Caucasian and 3 Melanesians with different GPC and band 3 genotypes are illustrated in Figure 1A-D.

AE1Δ27

Previous studies reported an absence of AE1Δ27 in the Wosera (n = 216). Consistent with these findings, only 1 of 1019 residents from the Wosera had this mutation (Figure 1B). The PCR product from this individual was cloned and sequenced, verifying that this individual carried AE1Δ27 (not shown).

GPC genotype in the Wosera

GPC genotyping was performed on 742 individuals (Figure 1E-G). The first reaction, screening for the presence or absence of the wild-type (wt) GPC allele where exons 2 and 3 are both present (Figure 1E), identified homozygous GPCex3 individuals (lane 3). The second (Figure 1F) and third reactions (Figure 1G) amplified GPC sequence within the wt or GPCex3 alleles, respectively, and allowed homozygous wt individuals (Figure 1F-G, lanes 1 and 4) to be distinguished from heterozygous individuals (Figure 1F-G, lane 2). Allele frequencies for GPC wt and GPCex3 were 0.535 and 0.465, respectively. Genotyping showed 211 (28.4%) of 742 individuals as homozygous wt, 372 (50.1%) of 742 as heterozygotes, and 159 (21.4%) of 742 as GPCex3 homozygotes. This distribution is in Hardy-Weinberg equilibrium, indicating that GPCex3 does not confer a selective disadvantage. This is in contrast to AE1Δ27, a balanced polymorphism, where the disadvantage of lethality in the homozygous form is outweighed by the selective advantage against severe malaria for heterozygotes.

GPC genotype and ovalocytosis

The association between ovalocytosis and GPC genotype was evaluated in 134 individuals who did not carry AE1Δ27. The wt individuals (n = 32) had the lowest proportion of ovalocytes per 1000 RBCs (median, 238 ± 115.1). Heterozygous individuals (n = 52) had a higher proportion of ovalocytes (median, 297 ± 103.8), while homozygotes (n = 49) had the highest of all 3 genotypes (median, 312 ± 145.9). In a comparison of all 3 genotypes, the proportion of ovalocytes was significantly associated with GPCex3 (Kruskal-Wallis, P = .021). Individual comparisons among the 3 genotypic groups showed significant differences by a one-sided Wilcoxon test (wt/wt vs wt/GPCex3, P = .0392; wt/wt vs GPCex3/GPCex3, P = .0045). These results suggest that GPCex3 contributes to ovalocytosis in the Wosera. When erythrocyte morphology was compared between homozygous wt individuals from the Wosera and North Americans, the former had a significantly increased ovalocyte frequency (Wilcoxon, P < .0001). This suggests that altered RBC morphology in the Wosera is a heterogenous condition caused by additional unknown mutations in RBC membrane proteins, such as protein 4.1 and spectrin as well as environmental or nutritional factors.
Table 1. Glycophorin C genotype and infections over time

<table>
<thead>
<tr>
<th>Month</th>
<th>No.§</th>
<th>Prevalence (%)</th>
<th>Parasitemia (% 95% CI)</th>
<th>Prevalence (%)</th>
<th>Parasitemia (% 95% CI)</th>
<th>Prevalence (%)</th>
<th>Parasitemia (% 95% CI)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>July</td>
<td>696</td>
<td>35/200 (17.0)**</td>
<td>2.11 (2.02-2.20)</td>
<td>63/345 (18.3)</td>
<td>2.21 (2.20-2.48)</td>
<td>26/151 (17.2)</td>
<td>2.38 (2.27-2.78)</td>
<td>.953</td>
</tr>
<tr>
<td>Aug</td>
<td>528</td>
<td>30/139 (21.6)‡‡</td>
<td>2.08 (1.95-2.21)</td>
<td>52/264 (19.7)</td>
<td>1.91 (1.88-2.25)</td>
<td>29/125 (23.2)</td>
<td>2.08 (1.96-2.28)</td>
<td>.663</td>
</tr>
<tr>
<td>Sep</td>
<td>415</td>
<td>21/121 (17.4)‡‡</td>
<td>2.08 (1.93-2.22)</td>
<td>38/199 (19.1)</td>
<td>1.91 (1.86-2.28)</td>
<td>20/95 (21.1)</td>
<td>2.15 (2.00-2.44)</td>
<td>.789</td>
</tr>
<tr>
<td>Oct</td>
<td>466</td>
<td>35/136 (25.7)‡‡</td>
<td>2.08 (2.02-2.30)</td>
<td>52/235 (22.1)</td>
<td>2.08 (2.05-2.25)</td>
<td>26/95 (27.4)</td>
<td>2.30 (2.13-2.62)</td>
<td>.537</td>
</tr>
<tr>
<td>Nov</td>
<td>495</td>
<td>23/130 (26.9)**</td>
<td>2.08 (2.02-2.30)</td>
<td>41/235 (17.5)</td>
<td>2.30 (2.29-2.70)</td>
<td>29/125 (23.2)</td>
<td>2.08 (1.96-2.28)</td>
<td>.663</td>
</tr>
<tr>
<td>Dec</td>
<td>331</td>
<td>9/55 (19.1)**</td>
<td>2.08 (1.93-2.22)</td>
<td>41/235 (17.5)</td>
<td>2.08 (2.02-2.30)</td>
<td>21/171 (12.3)</td>
<td>2.21 (1.96-2.52)</td>
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<tr>
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<td>8/55 (19.1)**</td>
<td>2.08 (1.93-2.22)</td>
<td>14/162 (17.4)</td>
<td>2.11 (2.00-2.20)</td>
<td>14/162 (17.4)</td>
<td>2.21 (2.00-2.20)</td>
<td>.995</td>
</tr>
</tbody>
</table>

CI indicates confidence interval.
*Homozygous wild-type individuals.
†Heterozygotes for glycophorin C exon 3 deletion.
‡Homozygotes for glycophorin C exon 3 deletion.
§Total number of individuals examined each month.
¶Median parasite density in log10 parasites/μL.
††t test.
¶¶Kruskal-Wallis test.
**Number of individuals with P falciparum detected on blood smear.
†††Number of individuals with P vivax detected on blood smear.

GPC genotype and infection status

The prevalence of infection with P falciparum or P vivax determined by blood smear has been examined in relation to serologic Ge antigen status in one published study of 266 people.11 This study observed a lower combined smear positive rate for P falciparum and/or P vivax infection in Ge-negative individuals, suggesting that Ge antigen negativity protects against infection.11 To examine the relationship between GPC genotype and susceptibility to malaria infection more rigorously, we studied a larger population over 7 months. Results for genotype and infection status were available for 325 to 696 individuals at each of 7 monthly intervals. This analysis showed that the prevalence or density of P falciparum and P vivax infection was not significantly different for individuals in the 3 GPC genotypic groups at any time (Table 1). These results parallel findings of other RBC polymorphisms, such as AE1Δ27, where genotypic differences are associated with reduced susceptibility to severe malaria morbidity with no effect on susceptibility to infection.3 The relationship of GPCΔex3 to malaria morbidity in young children, the age group most susceptible to the clinical phenotype, requires further study.

Acknowledgments

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References

1. Miller LH, Good MF, Milton G. Malaria pathogene-
7. Serjeantson SW, White BS, Bhatia K, Trent RJ. A 3.5 kb deletion in the glycophorin C gene ac-
miology of malaria in the Wosera area, East Sepik Province, Papua New Guinea, in prepara-
The association of the glycophorin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea

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