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 (AE1 ex3)) and susceptibility to malaria infection, in the Wosera, a malaria holoendemic region, we tested the association between GPCex3 and susceptibility to malaria. While AE1ex3 has not been reported in residents of the malaria holoendemic Wosera region of Papua New Guinea, ovalocytic RBCs are common. Additional polymorphisms characterizing the human population in the Wosera include an exon 3 deletion of the integral membrane sialoglycoprotein glycophorin C (GPC). This deletion (GPCex3) changes serologic phenotypes of the Gerbich (Ge) blood group system in Melanesians. Because GPC is involved in maintaining the integral RBC membrane lattice, the association between GPCex3 and ovalocytosis was tested. Furthermore, because GPCex3 is distributed within a malaria holoendemic region, we tested the association between GPCex3 and susceptibility to malaria infection.

Study population and malaria

The study was conducted in the Wosera region of Papua New Guinea, where all 4 human Plasmodium species are transmitted year-round. Blood samples were collected monthly from permanent residents (median age, 17 years; range, 1-86 years) of 6 villages within the Wosera from July 1998 to January 1999. The human investigations institutional review boards of Case Western Reserve University, University Hospitals of Cleveland, and the Papua New Guinea Medical Research Advisory Committee approved all protocols.

Malaria and red blood cell morphology

Thick and thin films stained with 4% Giemsa were prepared at the time of blood collection (Figure 1A-D). Malaria parasites were identified by light microscopy. Parasite densities were recorded as the number of parasites per 200 leukocytes (average 8000 leukocytes per microliter).

Thin smears were examined by light microscopy for the proportion of ovalocytes (erythrocytes with length:width ratio more than 1:1) without knowledge of genotypic results. Because previous studies have shown that elliptocytes are rare in the Wosera, the distinction between ovalocytes and elliptocytes (RBC with length:width more than 2:1) was not made. Blood smears from North Americans were prepared using a modified Wright stain (Diff-Quick Stain Set, Dade-Behring, Newark, DE). One reader reviewed all of the blood smears. Two additional observers independently reviewed a subset of smears.

Genotyping for AE1 and GPC polymorphisms

Blood was collected in ethylenediaminetetraacetic acid vacutainer tubes and stored at −70°C until DNA extraction was performed with the QiAmp96 DNA blood kit (Qiagen, Valencia, CA). Genotyping of band 3 was performed as previously described. New polymerase chain reaction (PCR) genotyping strategies for GPC are described in Figure 1E-G.

Statistical analysis

Categorical variables were analyzed by the χ² test and continuous variables by the Wilcoxon or Kruskal-Wallis test. Statistical Analysis Systems version 8.1 software package (Cary, NC) was used.
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).10

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 GPCαex3 individuals (lane 3). The second (Figure 1F) and third reactions (Figure 1G) amplified GPC sequence within the wt or GPCαex3 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 GPCαex3 were 0.535 and 0.465, respectively. Genotyping showed 211 (28.4%) of 742 individuals as homozygous wt, 372 (50.1%) of 742 as heterozygous GPCαex3, and 159 (21.4%) of 742 as GPCαex3 homozygotes. This distribution is in Hardy-Weinberg equilibrium, indicating that GPCαex3 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.2

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 GPCαex3 (Kruskal-Wallis, \( P = .021 \)). Individual comparisons among the 3 genotypic groups showed significant differences by a one-sided Wilcoxon test (wt/wt vs wt/GPCαex3, \( P = .0392 \); wt/wt vs GPCαex3/GPCαex3, \( P = .0045 \)). These results suggest that GPCαex3 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 heterogeneous condition caused by additional unknown mutations in RBC membrane proteins, such as protein 4.1 and spectrin as well as environmental or nutritional factors.
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 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 sample of 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.

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## References

The association of the glycophorin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea

Sheral S. Patel, Rajeev K. Mehotra, William Kastens, Charles S. Mgone, James W. Kazura and Peter A. Zimmerman