Southeast Asian ovalocytosis is associated with increased expression of Duffy antigen receptor for chemokines (DARC)

I.J. Woolley, P. Hutchinson, J.C. Reeder, J.W. Kazura, and A. Cortés

The Duffy antigen receptor for chemokines (DARC or Fy glycoprotein) carries antigens that are important in blood transfusion and is the main receptor used by Plasmodium vivax to invade reticulocytes. Southeast Asian ovalocytosis (SAO) results from an alteration in RBC membrane protein band 3 and is thought to mitigate susceptibility to falciparum malaria. Expression of some RBC antigens is suppressed by SAO, and we hypothesized that SAO may also reduce Fy expression, potentially leading to reduced susceptibility to vivax malaria. Blood samples were collected from individuals living in the Madang Province of Papua New Guinea. Samples were assayed using a flow cytometry assay for expression of Fy on the surface of RBC and reticulocytes by measuring the attachment of a phycoerythrin-labeled Fy6 antibody. Reticulocytes were detected using thiazole orange. The presence of the SAO mutation was confirmed by PCR. There was a small (approximately 10%) but statistically significant (p=0.049, Mann-Whitney U test) increase in Fy expression on SAO RBC compared with RBC from individuals without this polymorphism: mean Fy expression (mean fluorescence intensity [MFI]) was 10.12 ± 1.22 for SAO heterozygotes versus an MFI of 8.95 ± 1.1 for individuals without SAO. For reticulocytes the MFI values were 27.61 ± 19.12 for SAO heterozygotes and 16.47 ± 3.81 for controls. SAO is associated with increased and not decreased Fy6 expression so that susceptibility to P. vivax infection is unlikely to be affected.


Key Words: Duffy, Southeast Asian ovalocytosis, antigen expression

In 1949 Haldane proposed that malaria had a dramatic influence on the human genome, by selecting for genetic polymorphisms that were known or suspected to protect against malaria. Although some of these polymorphisms can be detrimental when individuals are homozygous, e.g., sickle cell anemia, their apparent selection in human populations living in malaria-endemic areas is evidence that they offer some protection against death from malaria, an infectious disease that continues to kill more than 1 million persons annually. Critical to this theory is the concept of heterozygote advantage. That is, carriage of the mutant gene on one member of a chromosomal pair is beneficial whereas homozygosity is deleterious. An extreme example of heterozygote advantage in Melanesian populations is Southeast Asian ovalocytosis (SAO), a hereditary form of ovalocytosis that is caused by a 27-base pair deletion in the gene encoding the major integral RBC membrane protein band 3 (AE1, SLC4A1), leading to a distinctive change in RBC morphology. SAO is widespread in several populations of Papua New Guinea (PNG), where its prevalence correlates with malaria endemicity and altitude. In these populations, homozygosity is apparently incompatible with full development of the fetus inasmuch as no persons homozygous for the band 3 mutation have yet been described, but heterozygosity confers strong protection against cerebral malaria. Although early studies had suggested that the SAO trait may afford some protection against vivax or falciparum parasitemia, other studies did not support this hypothesis. The mechanism of protection against cerebral malaria is not completely understood, but the specific protection against cerebral malaria suggests that it may operate by means of alterations in sequestration of infected RBC. However, SAO confers limited or no protection against placental malaria, which is also linked to sequestration of infected RBCs.

Individuals whose RBCs are negative for expression of Duffy antigens illustrate homozygote advantage for vivax malaria in that their RBCs are completely refractory to invasion by Plasmodium vivax. Historic serologic data suggest expression of some antigens on human RBCs was suppressed by the SAO trait, but expression of Duffy antigens did not appear suppressed as determined by the less sensitive serologic instruments available at that time. In those studies, antibodies of varying specificities were placed in tubes using serial dilution, and results were read macroscopically with the aid of an eyepiece using a standardized scoring system. In this study, we used molecular and cytometric techniques to diagnose SAO and to reexamine whether there was a difference in expression of Duffy antigen receptor for chemokines (DARC) between SAO and control individuals in the context of possible differential susceptibility to infection with different Plasmodium species.

Materials and Methods

Blood was collected by venipuncture in CPD anticoagulant or by finger prick using a BD Microtainer (Becton Dickinson, San Jose, CA) with EDTA anticoagulant from healthy volunteers from two villages, Sempi and Bemlon, on the north coast of the Madang Province, PNG. Oral informed consent was obtained after approval for the study by the Medical Research Advisory Committee of the PNG...
Ministry of Health. After collection, samples were kept on ice or refrigerated at 4°C. Within 1 week, flow cytometric experiments were undertaken with antibody to Fy6 (kindly provided by John Barnwell, CDC, Atlanta, GA). This is a mouse monoclonal antibody that attaches to the first extracellular domain of the Fy glycoprotein, in an area thought to correspond to the binding site of P. vivax merozoites. The antibody was conjugated directly to a phycoerythrin (PE) label (Prozyme, San Leandro, CA) according to the manufacturer’s instructions. A 5-µL aliquot of blood was washed three times in 50 µL of PBS and pelleted. RBCs were incubated with 50 µL of a 1 in 50 dilution of Fy6 antibody for 15 minutes at 37°C. Cells were then washed twice in PBS and resuspended in thiazole orange (TO) solution according to the manufacturer’s specifications (Becton Dickinson). The samples were analyzed on a flow cytometer (Mo-Flo, DaKoCyntomation, Fort Collins, CO) equipped with a 70-mW air-cooled argon ion laser at 488 nm. TO fluorescence was read with a 525-nm bandpass optical filter and PE fluorescence with a 570-nm bandpass filter. Compensation was applied, and negative fluorescence signals were adjusted until they were orthogonal. Forward-scatter, side-scatter, and fluorescence data were analyzed. A count of 5000 TO-positive cells was taken per sample. This was approximately 1 to 2 percent of the total number of cells assayed for each individual. Beads of known immunofluorescence (Immuno-Brite, Coulter, Puerto Rico) were used to standardize experiments undertaken at different times. Detection of the SAO mutation was undertaken using previously described PCR methods. Experienced microscopists from the PNG Institute of Medical Research performed light microscopic inspection of Giemsa-stained blood films for malaria parasites. Statistical analysis was undertaken by using SPSS (Statview 4.51, Abacus Concepts, Berkeley, CA). We have used nonparametric testing because we do not have evidence for normal distribution of DARC abundance in the surface of SAO erythrocytes.

Results

Samples from 19 SAO heterozygotes and 20 non-SAO donors were examined. Mean age of donors did not differ significantly between the two groups (mean age, 29.32 ± 13.70 years for SAO heterozygotes versus 26.91 ± 11.80 years for non-SAO individuals). Four individuals had malaria infection based on inspection of blood smears. One SAO and one non-SAO individual had Plasmodium malariae, and two non-SAO donors had Plasmodium falciparum. SAO RBC showed increased expression of Fy glycoprotein relative to non-SAO RBC as measured by binding of the Fy6 monoclonal antibody (Table 1). The level of Fy6 antigen was also higher in SAO reticulocytes compared with control reticulocytes, but the difference was not statistically significant (Table 1). Subsequent analysis of the data did not suggest the differences were caused by a systemic bias attributable to sex, village of origin, or method of obtaining blood (data not shown).

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<th>All Subjects (Reticulocytes)</th>
<th>All Subjects (Erythrocytes)</th>
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<tr>
<td>SAO heterozygotes (N = 19)</td>
<td>27.61 ± 19.12</td>
<td>10.12 ± 1.22</td>
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<tr>
<td>Non-SAO individuals (N = 20)</td>
<td>16.47 ± 3.81</td>
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<td>P value for difference (Mann-Whitney U test)</td>
<td>0.65</td>
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Discussion

The main finding of this study was that the SAO trait does not result in reduced expression of Fy antigen. In fact, we observed a 10 percent increase (p=0.049) in Fy antigen expression by SAO RBCs when compared with RBCs from non-SAO individuals from the same area. This difference was significant when expression by all RBC populations was evaluated but not when only SAO reticulocytes were compared with non-SAO reticulocytes (although a suggestive trend existed). As anticipated, reticulocyte expression of Fy6 antigen exceeded RBC expression. The reasons for the increased RBC Fy6 antigen expression in SAO are unclear at present, but it may reflect either a decreased rate of loss of Fy6 antigen from the surface of SAO RBC relative to control RBC, perhaps in relation to the increased rigidity of the SAO cell wall, or differences in the surface area of ovalocytes versus non-SAO RBC. Because there was only a small increase in Fy6 antigen expression on SAO RBC and there was no statistically significant increase in the reticulocyte subpopulation, which is the only subpopulation susceptible to infection by P. vivax, it is unlikely that this alteration affects susceptibility to this parasite, although there is other in vitro evidence that alterations in numbers of Fy antigens expressed may alter that susceptibility. It is not surprising that the difference in Fy levels was not shown in the paper of Booth et al. because they were using less sensitive serologic methods or because Fy6 antigen is more available or more expressed in SAO ovalocytosis over the antigens tested in the previous study. However, their findings with respect to other antigens, including Rh polypeptides, have subsequently been confirmed by molecular techniques.

Fy antigen negativity caused by homozygosity for a promoter mutation that silences transcription of the FY gene is observed in many ethnic groups in sub-Saharan Africa, and presumably accounts for the relative lack of vivax malaria in this part of the world. The apparently independent emergence of homozygosity for this same promoter mutation has also been described in the Wosera area of PNG, where SAO is rare or nonexistent. There is a need for further evaluation of whether other common RBC polymorphisms affect Fy antigen expression, especially those that are associated with changes in RBC shape and surface area. For example, α-thalassemia is common in PNG, and in many areas, including the Madang Province, nearly all individuals have the typical α-globin mutation seen in Melanesians.
seems unlikely that this common polymorphism would act as a confounding factor in our study on the grounds of biologic plausibility, low likelihood of linkage disequilibrium, and very high prevalence in the region (assuming therefore more or less equal distribution between SAO and control individuals within the population studied). Finally, it will be important to determine whether environmental variables extant in malaria-endemic populations might change Fy antigen expression. Another example would be iron deficiency, which is prevalent in PNG and many other malaria-endemic regions of the world.

There is evidence that the SAO trait affects several steps of the biologic cycle of \textit{P. falciparum} that may be relevant for the selective advantage conferred by this trait against malaria. In particular, SAO RBCs are partly resistant to invasion in culture by merozoites of \textit{Plasmodium knowlesi} and several lines of \textit{P. falciparum}, and the SAO trait also results in altered adhesion of infected RBCs to CD36. As with ABO, it is likely that the evolutionary pressure exerted by survival with SAO is related to survival of children from cerebral malaria before reproductive age. Although Fy expression probably affects \textit{P. vivax} infection, this evolutionary pressure is almost certainly caused by \textit{P. falciparum} through its relationship to RBC adhesion.

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**References**


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