INTRODUCTION

Plasmodium falciparum malaria has exerted a profound effect on the evolution of the human genome. A wide variety of genetic variants in populations historically exposed to *P. falciparum* malaria have been associated with resistance to severe clinical forms of malaria, including sickle cell trait, hereditary ovalocytosis, glucose-6-phosphate dehydrogenase deficiency, and polymorphisms in a number of immune response genes.\(^1\) There is some evidence that specific HLA alleles may also be associated with resistance or susceptibility to malaria. In a case-control study of severe malaria in children in The Gambia,\(^2\) HLA-B*5301 and HLA-DRB1*1302 were independently associated with resistance to severe malaria. These findings were not reproduced in a similarly designed study in Western Kenya; instead, a new association between HLA-DRB1*04 and severe malaria was reported.\(^3\) A case-control study of severe malaria in children in Gabon identified associations between DRB1*04 and DBP1*1701 and severe malaria;\(^4\) when the children enrolled in this study were followed prospectively, HLA-DQB1*0501 was associated with decreased risk of reinfection and anemia.\(^4\) There are several possible explanations for the lack of consistency in malaria/HLA association studies in different populations. Non-HLA genetic background may affect the disease susceptibility phenotype differently in different ethnic groups. The protective effect of HLA-B53 was suggested to be linked to effective presentation of a specific T-cell epitope on *P. falciparum* liver stage antigen \(^1\); it is possible that allelic variation of this epitope, or others, in different sites affects the expression of the resistant phenotype. Different transmission intensities or other epidemiologic characteristics of the different sites may affect the ability of modest effects of susceptibility or resistance genes to be observed. Finally, even in the absence of bias, studies attempting to identify small genetic effects on complex disease phenotypes are very prone to false-positive associations; it is possible that none of the reported HLA associations for resistance or susceptibility to malaria is biologically important. We therefore attempted to confirm previously reported malaria/HLA associations or to identify new ones in a large case-control study of severe malaria in Northern Ghana.

MATERIALS AND METHODS

Study area. The study was carried out in the Kassena-Nankana district (KND) of the Upper East region of Northern Ghana. KND is located in the Sahelian Savannah. There are three ecological zones within the district: irrigated zone, lowland zone, and rocky highland zone. Malaria transmission in the KND is intense and seasonal with an overall peak entomologic inoculation rates (EIRs) of >400 infective bites/person-year during the June–November rainy season;\(^6\) EIRs are consistently higher in the irrigated zone compared with the non-irrigated zones. The malarial epidemiology at this site has been extensively characterized.\(^7\)--11 One hospital, the Navrongo War Memorial Hospital (NWMH), and four outpatient clinics serve the KND with a population of ~150,000. The population is approximately equally divided between two major ethnic groups: the Kassem and the Nankam.

Study design and subjects. This was a frequency-matched case-control study. Cases consisted of children 6–60 months who presented to the NWMH with severe malaria from 20 August 2002 to 20 December 2002 and from 15 April 2003 to 20 January 2004. Criteria for diagnosis of severe malaria were those included in the standard World Health Organization (WHO) definition: 1) cerebral malaria (CM), unrousable coma, or Blantyre Coma Score of 3 or less; coma persists for >30 minutes after fits have ceased and normal cerebrospinal fluid findings; 2) repeated or prolonged generalized convulsions, generalized convulsions lasting >30 minutes, or more than two convulsions in 24 hours despite cooling; 3) severe malarial anemia (SMA), hemoglobin <5 g/dL; 4) respiratory distress, presence of alar flaring, intercostals or subcostal chest recession, use of accessory muscles of respiration, or abnormally deep respiration; 5) hypoglycemia, blood glucose <2.2 mmol/L; 6) circumulatory collapse, systolic blood pressure <50 mm of Hg; 7) renal failure, urine output <12 mL/kg/24 h or serum creatinine >3.0 mg/dL; 8) malarial hemoglobinuria; 9) hyperparasitemia, parasitemia >100,000/µL; 10) hypopryrexia, rectal temperature ≥40°C; or 11) impaired consciousness.

Two categories of control subjects were frequency matched to cases on the basis of age category (6–24 or 25–60 months), sex, and ecological zone (irrigated or non-irrigated). Mild ma-
laria controls were identified in the KND outpatient clinics and consisted of subjects with axillary temperature > 37.5°C, parasitemia > 2,500 parasites/µL, and no other apparent cause of fever. Recruiting teams for mild malaria controls visited each of the four free-standing public outpatient clinics in the KND and the NWMH outpatient department on sequential days to recruit controls matched for age, sex, and ecological zone corresponding to cases recruited within the previous 5 days. Asymptomatic community controls were selected by generating random lists of potential subjects matched for age, sex, and ecological zone with the cases enrolled during the previous 5 days. Lists were generated using the database maintained by the Navrongo Demographic Surveillance System (NDSS), which conducts a complete census of the KND every quarter. Potential community controls were eligible for enrollment if their primary caregiver reported that they were feeling well. Subjects enrolled in the study once remained eligible to be enrolled again. A total of 821 severe malaria cases, 1,642 outpatient mild malaria cases, and 1,674 asymptomatic community controls were enrolled, of which 12% represented repeated enrollment of the same individual on two occasions.

The use of human subjects in these studies was approved by scientific and ethical review boards of the Noguchi Memorial Institute for Medical Research, the Navrongo Health Research Center, the Ghanaiian Ministry of Health, and US Naval Medical Research Unit #3, and was conducted in accordance with regulations governing the protection of human subjects in medical research. Informed consent was obtained from all adult subjects and from the parent or legal guardians of minors.

**HLA typing.** Blood from all subjects was collected by venipuncture and blotted onto filter paper. Genomic DNA was extracted from filter paper blood blots as described by Kain and Lanar. Low to moderate resolution, polymerase chain reaction (PCR)-based, HLA typing was performed with the AB/DR SSP Uni-Tray (Dynal Biotech, Milwaukee, WI) for HLA-A, -B, and -DR typing; DR4T SSP Uni-Trays (Dynal Biotech) for HLA-DR typing, and Ambisolve primer PMR007G (Dynal Biotech) to identify the presence or absence of HLA-DRB1*04, according to the manufacturer’s instructions. Overall, 97% (4,032/4,137) of samples from the study subjects were successfully typed.

**Statistical analysis.** Odds ratios (ORs) and exact confidence intervals (CIs) were calculated using SPSS 10.0.1 (standard version) and StataSE (Version 8.0). For stratified analysis, ORs and CIs were calculated for each stratum, and the ORs in different strata were compared using the Breslow-Day χ² test of homogeneity. Pooled, frequency-weighted (Mantel-Haenszel) ORs and ORs adjusted for ethnicity were calculated when the ORs were homogeneous across strata. Subjects who were enrolled as both cases and controls at different times were included in the analysis as both cases and controls.

**RESULTS AND DISCUSSION**

HLA disease association studies are liable to false-positive associations arising from the large number of possible associations tested simultaneously. To avoid such false-positive associations, we conducted our analysis in three stages. In the first stage, we typed ~10% of study subjects for HLA-A, -B, and -DR. We selected the three strongest associations detected in this preliminary experiment and typed an additional 10% of study subjects to attempt to confirm any or all of the three possible associations. Finally, we tested the remaining 80% of subjects for the presence or absence of the HLA allele involved in the most promising association of the three tested in the second round.

Table 1 shows the demographic characteristics of the study subjects. Most severe malaria occurred in very young children and infants. Approximately three quarters of the severe malaria cases occurred in children in the 6- to 24-month age range, and > 90% of severe cases occurred in children < 36 months old. The detailed clinical characteristics of the severe malaria cases will be described elsewhere; in brief, ~5% of the severe malaria cases suffered from CM, 45% from severe malarial anemia, and 50% from a mixture of the remaining WHO severe malaria criteria including respiratory distress, hypoglycemia, lactic acidosis, and hyperparasitemia. The early peak in the age distribution of severe malaria is consistent with the previously described intense transmission of malaria in the KND. Members of the Kassem ethnic group were slightly under-represented in the severe malaria group (54.7%) compared with the mild malaria (64.8%) and asymptomatic control (62.4%) groups. In principle, this might represent a reduced susceptibility to severe malaria in the Kassem compared with the Nankam (OR = 0.79, 95% CI = 0.71–0.89), and such interethnic differences in malaria susceptibility have been previously reported. Nonetheless, it is very difficult to exclude selection biases in a case-control study, and a prospective cohort study may be needed to determine whether there is in fact differential susceptibility to severe malaria in the two groups.

We first performed low-to-moderate resolution HLA-A, -B, and -DR typing on 399 samples drawn from the subjects of the case-control study. Table 2 shows the frequencies of HLA-A, -B, and -DR types in this subset of study subjects. There were only minor differences in the assayed HLA antigen frequencies between the Kassem and Nankam ethnic groups (data not shown). We compared the antigen frequencies for 41 HLA Class I and 16 HLA Class II antigens in severe malaria cases, mild malaria controls, and asymptomatic community controls for a total of (41 + 16) × 3 = 171

**Table 1**

Demographic characteristics of subjects

<table>
<thead>
<tr>
<th>Clinical category</th>
<th>Male sex, N (%)</th>
<th>Age category, N (%)</th>
<th>Ethnicity, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6–24 month</td>
<td>&gt; 24 months</td>
</tr>
<tr>
<td>Severe malaria</td>
<td>454 (57.5)</td>
<td>598 (76.8)</td>
<td>181 (23.2)</td>
</tr>
<tr>
<td>Mild malaria</td>
<td>905 (56.2)</td>
<td>1,187 (75.5)</td>
<td>385 (24.5)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>929 (57.0)</td>
<td>1,215 (75.8)</td>
<td>388 (24.2)</td>
</tr>
<tr>
<td>Total</td>
<td>2,288 (56.7)</td>
<td>3,000 (75.9)</td>
<td>954 (24.1)</td>
</tr>
</tbody>
</table>
different comparisons. In this preliminary, hypothesis-
generating analysis, HLA-DRB1*11 was under-represented
in severe malaria cases compared with the mild controls
(OR = 0.56, 95% CI = 0.32, 0.96), HLA-DRB1*13 was over-
represented in severe malaria cases compared with the mild
controls (OR = 1.81, 95% CI = 1.06, 3.07), and HLA-
DRB1*04 was over-represented in severe malaria cases
compared with asymptomatic community controls (OR = 9.07,
95% CI = 1.22, 400.65). We found no evidence of the pre-
viously reported protective effect of HLA-B53.2,5 on severe ver-
sus mild malaria (OR = 1.29, 95% CI = 0.74, 2.27).

The large number of comparisons made it likely that spu-
rious associations would be detected in this first round anal-
ysis. We therefore performed low-to-moderate resolution
HLA-DRB typing on an additional subset of subjects from the
case-control study (N = 389) and analyzed possible asso-
ciations with HLA-DRB1*04, -DRB1*11, and -DRB1*13.
In this second round analysis, neither HLA-DRB1*11 nor
HLA-DRB1*13 was associated with resistance or susceptibil-
ity to severe malaria (data not shown). HLA-DRB1*04, how-
ever, remained over-represented in severe cases compared
with asymptomatic controls (OR = 2.71, 95% CI = 1.06,
8.04). To confirm the apparent association between HLA-
DRB1*04 and susceptibility to severe malaria, we assayed the
remaining 3,244 subjects from the case-control study for the
presence of HLA-DRB1*04. In this third round of analysis,
HLA-DRB1*04 remained associated with severe malaria
(OR = 2.14, 95% CI = 1.36, 3.36). Because HLA-DRB1*04
was associated with severe malaria in sequential analysis of
three independent subsets of the case-control study subjects
and this was the only association tested in the third analysis,
we consider that the association is unlikely to be an artifact of
multiple hypothesis testing. We therefore went on to analyze
the association in more detail, using the pooled data from all
study subjects.

Controls were enrolled based on matching to three criteria:
sex, age category (6-24 and 25-60 months), and residence in
an irrigated or non-irrigated area of the KND; there are
therefore eight strata in the study. Table 3 shows the effect
measure (OR) of HLA-DRB1*04 for risk of severe malaria
within each of the four age–sex strata; similar results were
obtained for the eight age–sex–irrigation strata, but the num-
er of HLA-DRB1*04 subjects in some strata was so low that
very wide 95% CIs were obtained. The effect measures did
not differ significantly between strata, and the pooled, fre-
quency-weighted estimate of the OR (2.42; 95% CI
9.07, 9.13) was not different from the crude estimate (2.41;
95% CI = 1.60–3.64). Because we found that members of the Kas-
sem ethnic group were under-represented in the severe cases
compared with the two control groups, it was possible that the
HLA-DRB1*04 association was confounded by ethnicity.
There was, however, no significant difference between strata,
and the pooled, frequency-weighted estimate of the OR (2.42;
95% CI = 1.63–3.58) was not different from the crude estimate (2.41;
95% CI = 1.60–3.64). Because we found that members of the Kas-
sem ethnic group were under-represented in the severe cases
compared with the two control groups, it was possible that the
HLA-DRB1*04 association was confounded by ethnicity.
different from the pooled, frequency-weighted estimate, 2.20 (95% CI = 1.47, 3.31), or the OR adjusted for ethnicity, 2.22 (95% CI = 1.44, 3.38). It therefore seems that the association between DRB1*04 and severe malaria is not the result of confounding by ethnicity.

Table 4 shows calculated ORs based on the frequency of DRB1*04 in severe malaria cases, mild malaria controls, and asymptomatic controls. DRB1*04 is over-represented in severe cases relative to mild malaria controls (OR = 1.50, 95% CI = 1.05–2.13) and in mild malaria controls relative to asymptomatic controls (OR = 1.62, 95% CI = 1.13–2.33). For each of these comparisons, stratified analysis showed no significant difference in the ORs across strata, and the crude ORs did not differ from the combined, frequency-weighted ORs (data not shown). These findings suggest that DRB1*04 may be associated both with an increased risk of symptomatic malaria in general and an increased risk of severe malaria. It is likely that the case-control design leads to an underestimate of the magnitude of the effect of DRB1*04. Although subjects enrolled as severe malaria cases are unambiguously susceptible to severe malaria, mild malaria controls or asymptomatic controls may develop severe malaria in the future and may be as susceptible as subjects enrolled as severe cases. Similarly, the finding that a control is asymptomatic at the time of enrollment is only a relative indication of resistance to symptomatic malaria. The resulting misclassification of malaria susceptible subjects as malaria resistant will tend to reduce the magnitude of observed associations. The overall estimate of the effect of DRB1*04 on the risk of severe malaria (OR = 2.42, 95% CI = 1.64, 3.58) may therefore be an underestimate. Subjects who were enrolled as both cases and controls at different times were included in the analyses as both cases and controls. Counting these individuals, who accounted for 12% of the subjects, only as cases would have slightly increased both the estimate of the effect size and the corresponding confidence interval (OR = 2.57, 95% CI = 1.65–4.04).

In the KND, ~10% of children die of severe malaria before the age of 5 years. Similarly intense selective pressures presumably exist in much of sub-Saharan Africa. An HLA allele that substantially increased the risk of severe malaria would be expected to undergo strong negative selection. Table 5 shows data from the International HLA Working Group (IHWG) on the worldwide distribution of DRB1*04. The allele frequency of DRB1*04 is 4- to 8-fold lower in populations from sub-Saharan Africa than in populations from the rest of the world. This observation is consistent with a strong selective pressure against HLA-DRB1*04 in areas of intense malaria transmission.

The findings of this study support a real association between the presence of HLA-DRB1*04 and severe malaria and suggest that HLA-DRB1*04 may substantially increase susceptibility to severe malaria. The association was found in three sequential analyses of different sets of cases and controls from a large severe malaria case control study; in the third analysis, the only hypothesis to be tested was the HLA-DRB1*04 association. It is unlikely that the association is an artifact of multiple hypothesis testing. The DRB1*04 association was independent of ethnicity and consistent across all strata in this frequency-matched study. There was a graded increase in the frequency of DRB1*04 in the mild malaria controls compared with the asymptomatic controls and in the severe malaria cases compared with the mild malaria controls. HLA-DRB1*04 is less common in human populations from sub-Saharan Africa than in populations from the rest of the world, consistent with a strong negative selection by endemic *P. falciparum* malaria. Finally, previous studies in Gabon have suggested an association between DRB1*04 and susceptibility to malaria. This association may thus be the first HLA disease association for malaria that has been replicated in more than one population at different sites. Although the DRB1*04 association seems robust, the causal connection between this antigen and susceptibility to malaria is not clear. It is possible that this allele is in linkage disequilibrium with another gene that is itself responsible for malaria susceptibility. It is possible that the HLA-DRB1*04 effectively presents a key *P. falciparum* epitope, which either elicits a pre-dominant but non-protective immune response or elicits an excessive pro-inflammatory response that is pathogenic. This explanation would require that a single peptide epitope, or a small number of epitopes, from the many proteins expressed by *P. falciparum* in the mammalian host is a predominant immune target. It may be helpful to identify the HLA-DRB1*04 allele present in this population with more precision.

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