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FULL PAPER

A complement receptor-1 polymorphism with high frequency in malaria endemic regions of Asia but not Africa

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Complement receptor-1 (CR1) is a ligand for rosette formation, a phenomenon associated with cerebral malaria (CM). Binding is dependent on erythrocyte CR1 copy number. In Caucasians, low CR1 expressors have two linked mutations. We determined the Q981H and HindIII RFLP distribution in differing population groups to ascertain a possible role in adaptive evolution. We examined 194 Caucasians, 180 Choctaw Indians, 93 Chinese-Taiwanese, 304 Cambodians, 89 Papua New Guineans (PNG) and 366 Africans. PCR/RFLP used HindIII for CR1 expression and BstNI for the Q981H mutation. DNA sequencing and pyrosequencing were performed to resolve inconclusive results. Gene frequencies for the L allele were 0.15 in Africans, 0.16 in Choctaws, 0.18 in Caucasians, 0.29 in Chinese-Taiwanese, 0.47 in Cambodians and 0.58 in PNG. Allelic frequency for 981H were 0.07 in Africans, 0.15 in Caucasians, 0.18 in Choctaws, 0.29 in Chinese-Taiwanese, 0.47 in Cambodians and 0.54 in PNG. The Q981H polymorphism correlates with the HindIII RFLP in most groups except West Africans and appears to be part of a low CR1 expression haplotype. The gene frequency for the haplotype is highest in the malaria-endemic areas of Asia, suggesting that this haplotype may have evolved because it protects from rosetting and CM.
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Introduction

Malaria has been a major pathogenic infection for several centuries and remains one now, with its distribution mainly localized in sub-Saharan Africa, Asia and South America.[1,2] Several species of the parasite are known but only four cause human infection, with *Plasmodium falciparum* (Pf) the most deadly. There are currently very few antimalarials serving as both prophylaxis and treatment and compouding this drug paucity is the global increase in Pf strains resistant to the available drugs.[3] Vaccine development is being carried forward as a means to abate the medico-social burden of this disease on endemic countries. However, these efforts are limited by the complex nature of the parasite life-cycle in both mosquito and human hosts and the antigenic distinctiveness of each developmental stage.[4–5] Currently, there are vaccines being developed to target against different developmental stages for the *Pf* parasites and its ligands with complement receptor 1 (CR1) proposed as a possible vaccine candidate.

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A review of the literature shows that the human CR1 binds to a major malarial adhesin, the *P. falciparum* erythrocyte membrane protein-one (PfEMP-1).[6–8] PfEMP-1 is responsible for the vast array of binding activities of parasitized erythrocytes (E), including the phenomenon called rosetting in which malaria parasitized E bind to nonparasitized ones.[9–12] The PfEMP-1 protein can interact with several types of surface molecules, including intercellular adhesion molecule 1 (ICAM-1), type A and B blood groups, thrombospondin, E-selectin, chondroitin sulfate, CD36 and CR1, as well as with soluble ligands. Moreover, the identification of CR1 polymorphisms in association with malaria in Africa,[13] raises concerns regarding the actual role of CR1 or that of the binding fragments (C3b and C4b) in immune complex clearance and malaria pathogenesis.

Human CR1 is a ~200 kDa, single-chain glycoprotein composed of tandemly arranged modules (Figure 1). The extra-membranous portion of its most common size allotype is composed of 30 short consensus repeats (SCRs). The 28 N-terminal SCRs are organized, based on a degree of homology, into four long homologous repeats (LHRs) A–D, each composed of seven SCRs.[14–15] There are two distinct sites on CR1 that interact with C3b and C4b. Site 1, which spans SCRs 1–3 in LHR A, binds C4b and, weakly C3b and carries decay-accelerating activity for the C3 convertases. Site 2 spans SCRs 8–10 in LHR B

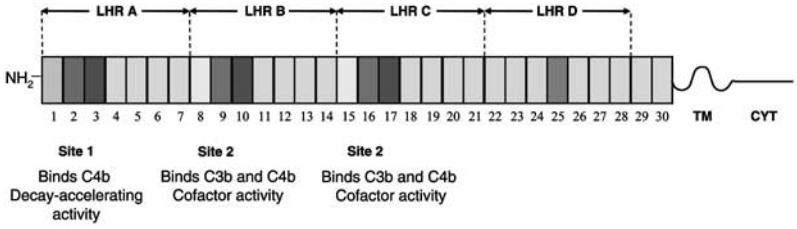

Figure 1 Diagrammatic representation of the CR1 gene. Each SCR folds independently into a globular domain with the hydrophobic core being formed by the conserved residues. This structure is held together by two disulfide bonds, one near the N-terminus, between the first and the third cysteine residue, and one near the C-terminus, between the second and the fourth cysteine residue (from Krych-Goldberg *et al*, 2002; reprinted with permission).

Table 1 Phenotypic distribution of Q981H amino-acid polymorphism among several populations

Ethnic groups	*N*	*Q/Q*[a]	*Freq (%)*	*Q/H*	*Freq (%)*	*H/H*	*Freq (%)*
Black Africans (Mali)	366	319	92	45	7	2	1
Caucasians (USA)	194	143	74	44	22	7	4
Choctaw (USA)	180	127	71	40	22	13	7
Asians (Taiwan-Chinese)	93	45	48	42	45	6	7
Asians (Cambodia)	304	85	28	150	49	69	23
[b]Pacific Asians (PNG)	89	22	25	38	42	29	33

[a]Single letter amino-acid sequence nomenclature is used to identify SCR 16 alleles (Q = glutamine, H = histidine).
[b]Papua New Guinea.

and the nearly identical SCRs 15–17 of LHR C, and binds both C3b and C4b efficiently, although it has a higher affinity for C3b. It is also the major site of CR1 cofactor activity and is indispensable for the decay accelerating activity of the C5 convertases. The two other CR1 ligands, C1q and mannose-binding lectin, bind to LHR D but localization to particular SCRs is not known.[16–17]

Genomic studies of the CR1 gene reveals three types of genetic polymorphisms: firstly, a size variation created by LHR duplications and deletions; secondly, a *Hind*III restriction fragment length polymorphism,[18] which, in Caucasians but not in Africans, correlates with E-CR1 copy number and the third represented by the Knops blood group system. Recently, a number of other CR1 polymorphisms have been identified in Caucasians linked to constitutive E-CR1 expression levels.[19–21] Two of these newly identified single-nucleotide polymorphisms (SNPs) occur in regions of known ligand-binding domains, at amino acid I643T (T2078C) at the 3′ end of SCR 10, and at Q981H (G3093T) in SCR 16. The finding of these polymorphisms resulted in the hypothesis that they constituted a low expression (L) SNP haplotype that could have ligand-binding activity different from CR1 encoded by the high expression allele (H).[21]

The Q981H mutation may provide a better understanding of malaria pathogenesis and host genetics, as it is located in the proposed binding region for PfEMP-1 protein of the malaria parasite. Questions of scientific importance that might arise from this observation are (1) what will be the distribution of this SNP in natural populations from differing malaria endemic regions, and (2) could this give an insight into clinical and *in vitro* observations relating to malaria pathogenesis in these population groups? Our study was designed to determine the distribution and genetic frequency of the Q981H mutation in natural populations from malaria-endemic and nonendemic regions and to ascertain its association or otherwise with the *Hind*III polymorphism, in order to better understand its role in disease pathogenesis and host genetics.

Results

Phenotypic frequencies for the Q981H mutation are shown in Table 1. In Cambodia, the highest frequency of the Q981H mutant allele (H) (0.53) was found in the plateau minority (Hmong from the Mondulkiri district) followed by 0.50 in the urban population of Phnom Penh, and 0.46 and 0.42 in two rural populations of Sam Peov Loum and Ratanakiri, respectively (Table 2). There is a gradual upward shift in the frequency of the mutant allele (HH) as one moves from Africa to Asia (Figure 2), affirming the presence of this mutant allele in the study populations in that order, with its probable implication for malaria pathogenesis.

The *Hind*III polymorphism was also examined in the study population and a similar pattern of gene frequencies was found (Table 3). Significantly, the mutant expressing H allele of the Q981H polymorphism correlates very strongly with the *Hin*dIII L allele in Cambodia, southern China and Taiwan, strongly in Papua New Guinea (PNG), less strongly in the Caucasians and Choctaws and weakly in Mali. These observations appear to be part of a low expression SNP haplotype. The gene frequencies for the two mutant 981H and *Hind*III L polymorphisms reach their highest frequencies

in the malaria endemic areas of Asia and the Pacific (Figure 3).

Pyrosequencing was carried out for confirmation, particularly in cases with inconclusive findings. This involved interpreting pyrogram peaks and interpreting amino-acid changes-pyrosequencing annotation as depicted in Figure 4.

Discussion

The low expression allele for CR1 was initially described as a *Hin*dIII RFLP that correlated with E-CR1 density in Caucasians.[18] Later reports identified SNPs in the coding regions of the CR1 gene that appear linked to the low constitutive expression CR1 allele.[18–21] According to a recent report,[21] the low expression allele encodes a CR1 protein that has greater binding activity for C4b than the high expression allele, and this difference in binding is due to a histidine (*vs* a glutamine) at amino-acid residue 981 in SCR 16. That the amino-acid change from glutamine to histidine at residue 981 in SCR 16 could affect ligand binding is not surprising as the change from a Q to an H increases the net positive charge at this site. This polymorphism, thus, seems to be part of an SNP haplotype that may have important implications for malaria pathogenesis.

Erythrocytes having low CR1 expression have been shown to form reduced number of rosettes with Pf-infected cells.[7] In Africa, rosetting has been shown to correlate with disease severity.[22] Since E having low CR1 copy numbers form fewer rosettes, it has been postulated that low E-CR1 might protect from severe malaria. In a study by Cockburn *et al*,[23] a high incidence of individuals having low E-CR1 was found in PNG. When they studied a cohort of malaria patients, they further found that low CR1 expression protected from severe malaria.

Table 2 Genotypic frequencies of Q981H polymorphism among malaria-endemic and nonendemic populations

Malaria endemicity	*Gene frequencies*	
	Q allele	*H allele*
Nonendemic		
Caucasian (USA)	0.85	0.15
Choctaw (USA)	0.82	0.18
Endemic		
Black Africans (Mali)	0.93	0.07
S. Chinese-Taiwanese	0.71	0.29
Pacific Asians (PNG)	0.46	0.54
Cambodians	0.53	0.47

Table 3 Genotypic frequencies of *Hin*dIII polymorphism in malaria-endemic and nonendemic population groups

Malaria endemicity	*Gene frequencies*	
	H allele	*L allele*
Non-endemic		
Caucasian (USA)	0.82	0.18
Choctaw (USA)	0.84	0.16
Endemic		
Black Africans (Mali)	0.85	0.15
S. Chinese-Taiwanese	0.71	0.29
Pacific Asians (PNG)	0.42	0.58
Cambodians	0.53	0.47

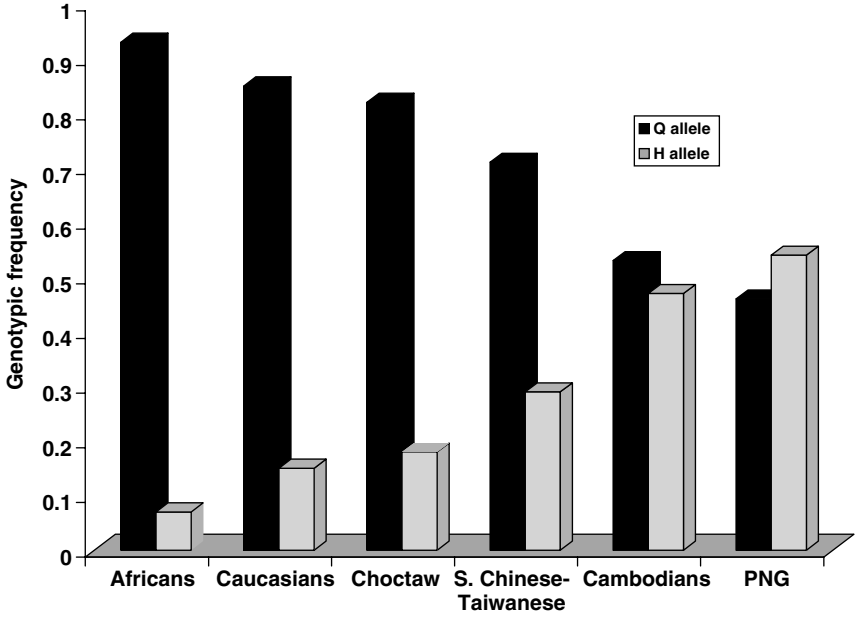

Figure 2 Graphical representation of the 981 allelic distribution of the wild-type (QQ) and mutant expression (HH) genes in various populations. The figure shows an increase in the frequency of the mutant H allele from one population to the other and a decrease in the wild-type allele (Q) clearly depicting the evolution of this gene, probably attributable to human migration from Africa to Asia and then to other parts of the world.

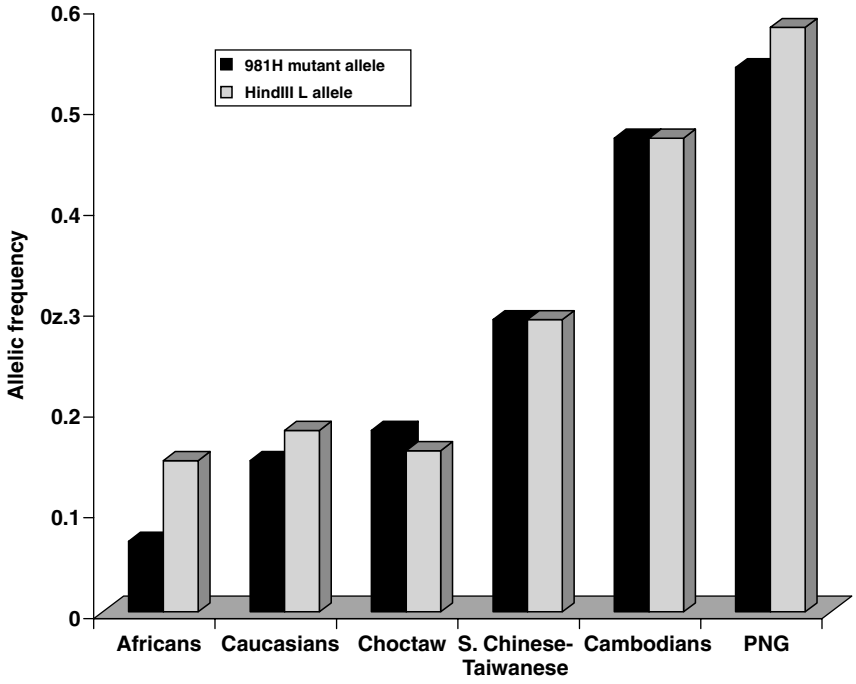

Figure 3 Comparisons of the gene frequencies for the mutant 981H and *Hin*dIII low expression allele (LL) polymorphisms in malaria-endemic and nonendemic populations. The mutant H allele of the Q981H polymorphism correlated very strongly with the *Hin*dIII L allele in Cambodia, southern China and Taiwan, strongly in PNG, less strongly in the Caucasians and Choctaws and weakly in Mali, and appears to be part of a low expression SNP haplotype. The gene frequencies for the mutant alleles reach their highest frequencies in the malaria endemic areas of Asia and the Pacific.

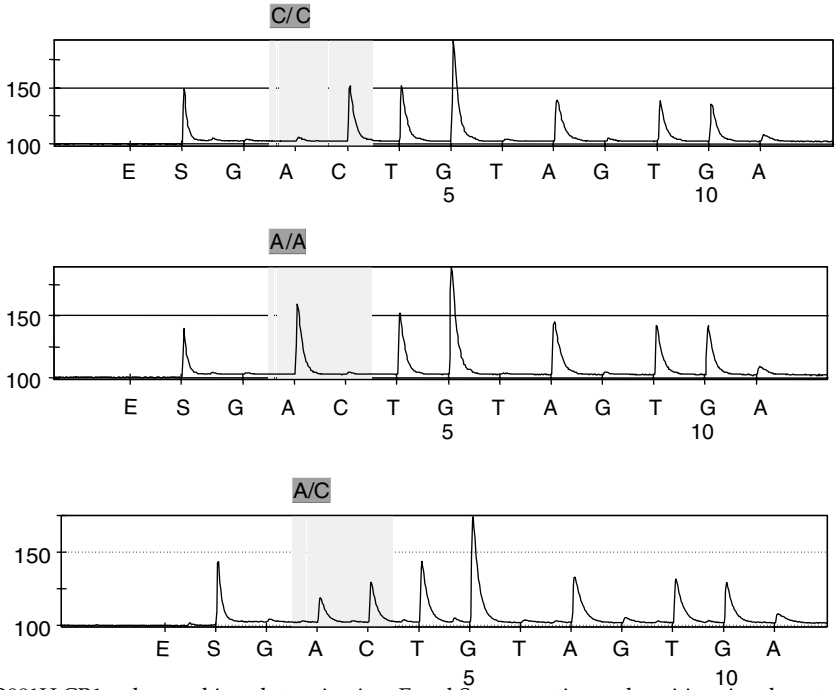

Figure 4 Pyrograms of Q981H CR1 polymorphism determination. E and S are negative and positive signal controls, respectively. An absent base G was then injected as a further base line control, followed by the subsequent ACTGTAGTGA injections, leading to peaks proportional to the number of incorporated nucleotides, either absent, heterozygous or homozygous at a given position (here the polymorphic AC position), or to the number of repetitive bases (here G for the fifth injection). The different bases ATCG are not incorporated with the same yield, leading to slightly different peak heights, which can be normalized by computer analysis. Crude primary data are presented.

Thus, low E-CR1 expression may hinder parasite binding resulting in the formation of fewer or no rosettes.

It is important to note that both the *Hin*dIII and Q981H mutations increase in frequency and correlate positively among Asians peaking in malaria endemic areas of Cambodia. We believe that this is further evidence that CR1 and rosetting play a major role in malaria pathophysiology. According to Cockburn *et al*,[23] the high

prevalence of CR1 deficiency in Southeast Asia and PNG could explain why rosetting is not associated with malaria severity in this region. Results from *in vitro* cultures of samples from these regions indicate the formation of weak rosettes, which may not withstand the pressure of peripheral circulation and therefore, are insignificant in disease pathogenesis. In Africa, however, E-CR1 deficiency seems to be less important since other CR1 polymorphisms, that is, Knops blood group antigens,[24] appear to confer a protective advantage against malaria infection in this continent (manuscript submitted). These observations support the notion that the selective pressure of malaria has acted differently on the CR1 gene in different populations. The high incidence of these CR1 mutations in Asia depicts yet another example of the evolutionary pressure exerted by *Plasmodium* on human population, with these mutations a response to adaptive evolution in these locations as a result of human migration from Africa to Asia.[25–27]

In PNG, we found a high rate of the heterozygous (Q981H) gene state indicating that this mutation may be still evolving in this location resulting in a significant shift from the wild (QQ) to the mutant types (HH). Interestingly, in PNG,[23] there is greater protection from severe malaria among individuals heterozygous for the *Hin*dIII H/L alleles. The low E-CR1 density found in LL patients may protect from cerebral complications of malaria but predispose to severe malarial anemia. This situation is reminiscent of the heterozygous advantage afforded by hemoglobin S.[28]

Accordingly, we propose that the Q981H polymorphism is an adaptive evolutionary phenomenon probably compensatory for the shortcomings of the *HindIII* polymorphism (low E-CR1). Immune complexes (ICs) containing activated complement components C4b and C3b bind to specific sites in CR1; C4b binds primarily at site 1, while C3b binds only at site 2 (Figure 1). The Q981H mutation may have evolved to potentiate the clearing of IC in patients having low E-CR1. Alternatively, since PfEMP-1 reportedly binds to the same region as IC, it is possible that the Q981H mutation adversely affects parasite binding and may be protective in Asians. Thus, the Q981H polymorphism might provide plausible explanations for the seemingly divergent disease pathogenesis observed in natural populations from the continents.

Materials and methods

Subjects

The subjects recruited for the study were volunteers who signed the informed consent document. Some of the donors were recruited from previous research for which they assented to further studies and analysis of their samples. The study population consisted of 194 healthy Caucasians from New Jersey, 180 native American-Indians of the Oklahoma Choctaw nation, 366 Black Africans from Mali and 93 blood donors from Southern China and Taiwan. Additional groups included 69 individuals from the Madang area of PNG and 304 Cambodians (49 from a high plateau minority, 79 and 41 from two rural populations of Sam Peov Loum and Ratanakiri, respectively, and 135 adult female subjects from the urban population of Phnom Penh). In Cambodia, the population of Phnom Penh is very mixed from heterogeneous origins and thus reciprocal populations in the country remain very diverse, representing small isolates with both genetic and environment original features. The African population from Mali is composed of 26 ethnic groups but the predominant tribes in Bandiagara are Dogon (80%) and Peuhl (10–15%). The group from Mali is subdivided into 36 medical students from Bamako, 116 adults from Bancoumana and Bamako, 29 adults from Bandiagara and 185 adults and children from Doneqoubougou.

Sample collection

The protocols under which these samples were obtained was approved by the respective institutional review boards from the University of Texas-Houston Medical School, Drexel University College of Medicine, the University of Maryland, University of Reims France, Mackay Memorial Hospital Taiwan, Medical Research Council of PNG and the University of Bamako, Mali. Following informed consent, 3–10 ml of venous blood was drawn into EDTA tubes by venipuncture from individuals ranging in age from 1–65 years regardless of sex. In some of the Cambodian patients, DNA was obtained from cervical smears that were collected as part of a large scale screening for cervical cancer in the population group (reported elsewhere).

Extraction of genomic DNA

After centrifugation of the EDTA tubes, buffy coat was removed and genomic DNA extracted using the Puregene DNA extraction kit from Gentra Systems according to the manufacturer's instructions (Minneapolis, MN, USA). For patients recruited from Cambodia, DNA was extracted using the QIAamp tissue kit from QIAGEN (Courtaboeuf, France).

Polymerase chain reaction

PCR amplification for the Q981H SNP was carried out in a final volume of 50 μl, containing 1.0 μl genomic DNA, 5.0 μl (3.5U/μl) of Mg-free buffer, 4.0 μl of magnesium chloride, 1.0 μl of 0.2 mM PCR nucleotide mix, 0.4 μl of 2.5 U of *Taq* polymerase (Promega, USA) and 1.0 μl each of both primers. Sequences for primer pairs used for amplification are 5′-GCTACATGCACGTTGAGACCT-TAC-3′ (SCR16a-F) and 5′-AGCAAGCATACA-GATTTTCCCC-3′ (SCR16a-R). Amplification using these primers yielded a 366 bp product, containing the 5′ exon of SCR 16. The PCR cycling conditions were 94°C 1 min, 55°C 1 min and 72°C 2 min, with a further 72°C 10 min hold for a total of 40 cycles. The amplification products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide and observed by ultraviolet transillumination.

Restriction fragment length polymorphism determination

For restriction fraction length polymorphism determination, 18 μl PCR product, 2.5 μl 1 × buffer, 2.5 μl 10 × bovine serum albumin and 2.0 μl of *Bst*NI were digested at 60°C for 2 h and analyzed on a 2% ethidium bromide gel. Using this protocol, the wild-type 3093G residue yielded fragments of 54, 91 and 221 bp, while the 3093T mutant yielded fragments of 54 and 312 bp (Figure 2).

The SNP determination by *Hind*III digestion was assessed as previously described.[25]

DNA sequencing and pyrosequencing

The amplification product was prepared for sequencing with the Exonuclease I and Shrimp Alkaline Phosphatase (EXOSAP-IT™) according to the manufacturer's guidelines (USB, Cleveland, OH, USA). This degrades residual single-stranded primers, extraneous single-stranded DNA produced in the PCR and hydrolyses remaining dNTPs from the PCR mixture. DNA sequencing was performed as a confirmatory step by using fluorescence-based sequencing protocols on an ABI 377 DNA Sequencer (Perkin Elmer, Foster City, CA, USA). Alternatively, confirmation was carried out by the pyrosequencing technique to validate results obtained by RFLP and dye terminator sequencing (B Donvito, unpublished) or in a handful of cases, to resolve inconclusive findings from RFLP and DNA sequencing.

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