 Human Molecular Genetics, 1997, Vol. 6, No. 8 **1357–1360**

A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya

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Received April 7, 1997; Revised and Accepted May 15, 1997

The malarial parasite *Plasmodium falciparum* has acted as a potent selective force on the human genome. The particular virulence of this organism is thought to be due to the adherence of parasitised red blood cells to small vessel endothelium through several receptors, including CD36, thrombospondin and intercellular adhesion molecule 1 (ICAM-1, CD54), and parasite isolates differ in their ability to bind to each. Immunohistochemical studies have implicated ICAM-1 as of potential importance in the pathogenesis of cerebral malaria, leading us to reason that if any single receptor were involved in the development of cerebral malaria, then in view of the high mortality of that complication, natural selection should have produced variants with reduced binding capacity. We therefore sequenced the N-terminal domain of ICAM-1 from a number of Africans and discovered a single mutation present at high frequency. Genotypes at this locus from samples from a case–control study indicated an association of the polymorphism with the severity of clinical malaria such that individuals homozygous for the mutation have increased susceptibility to cerebral malaria with a relative risk of two. These counter-intuitive results have implications for the mechanism of malaria pathogenesis, resistance to other infectious agents and transplantation immunology.

INTRODUCTION

Malaria remains a major health problem in many countries and, with the development of resistance to drugs by the parasites, the mortality and morbidity inflicted by this disease is set to rise significantly. The particular pathogenicity of *Plasmodium falciparum* has been ascribed to the ability of the infected red blood cells to adhere to capillary endothelium (1). A number of endothelial molecules have been identified as receptors for parasitised cells (2), and various studies have attempted to correlate disease severity (usually recorded as cerebral malaria) with host receptor specificity. In a histopathological study, we showed that the presence of parasitised erythrocytes in cerebral vessels co-localized with endothelial expression of intercellular cell adhesion molecule 1 (ICAM-1) [RR = 4.08, $P<0.0001$ (3)]. This association is supported by results from a large case–control study in Kilifi, Kenya in which the adhesion of infected erythrocytes to ICAM-1 was highest in the cerebral malaria category compared with asymptomatic control (4). These data are consistent with a role for ICAM-1 as an endothelial receptor for infected erythrocytes in cerebral malaria.

Our hypothesis was that given the potential importance of ICAM-1 in the pathophysiology of *P.falciparum* malaria, natural variants would have been selected in endemic populations which would be protective against severe disease. We have shown previously that the binding site on ICAM-1 for infected erythrocytes maps to a region in the N-terminal immunoglobulin-like domain that overlaps, but is distinct from, the binding site for the leukocyte integrin leukocyte function antigen 1 (LFA-1) (5). This finding increases the likelihood that natural variants of ICAM-1 could exist with normal immunological function but reduced affinity for infected erythrocytes. We therefore carried out a sequence analysis of the N-terminal domain of ICAM-1 from people living in a malaria-endemic region.

RESULTS

Identification of a polymorphism in the N-terminal domain of ICAM-1

We amplified and sequenced the N-terminal immunoglobulin-like domain of ICAM-1 from the genomic DNA of 24 asymptomatic children from Kilifi, Kenya, an area of endemic malaria transmission, predicting that potentially protective mutations would be more highly represented in this group. Compared with the published 'reference' sequences of ICAM-1 (6,7), which we shall hereafter designate ICAM-1^{ref}, the only mutation found was

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an A to T transversion at position 179. This causes a lysine to methionine substitution at position 29 in the coding sequence, for which we propose the designation ICAM-1^{Kilifi}.

Table 1. Results of the ICAM-1^{ref} and ICAM-1^{Kilifi} genotyping from the case–control study

	K29/K29 (ICAM-1^{ref})	K29/M29	M29/M29 (ICAM-1^{Kilifi})	Total
Cerebral malaria	56	65	36	157
Severe anaemia	43	45	15	103
Control	135	113	39	287
Total	234	223	90	547

Cerebral malaria versus control: (3×2) $\chi^2 = 8.4$; $P = 0.015$.
Severe anaemia versus control: (3×2) $\chi^2 = 0.87$; $P = 0.65$.
Cerebral malaria versus control: (χ^2 for trend) = 8.2; $P = 0.0042$ (OR = 2.23 for homozygotes).

Table 2. Allele and genotype frequencies for ICAM-1^{Kilifi} in Kilifi (Kenya)

Polymorphism	Allele	Frequency
K29/M	K29	0.668
	M29	0.332
Genotype	Expected frequency	Observed frequency
K29/ K29	0.45	0.47
K29/ M29	0.44	0.39
M29/ M29	0.11	0.14

Table 3. The mean fluorescence intensity (MFI) of ICA-1^{ref} and ICAM-1^{Kilifi}

mAb	Location	ICAM-1^{Kilifi}/ICAM-1^{ref}
1G12	Domain 1 (D26)	0.848 ± 0.009
2D5	Domain 1 (D26)	1.097 ± 0.231
CBR-IC1/4	Domain1 (D26)	0.933 ± 0.320
7.5C2	Domain 1 (D26)	2.017 ± 0.513 ($n = 3$)
7F7	Domain 1 (L43)	0.898 ± 0.124
15.2	Domain 1 (K40) (L43)	0.958 ± 0.336 ($n = 6$)
BBA4	Domain 1	0.016 ± 0.009
RR1.1	Domain 1 (P70)	0.983 ± 0.058
6E6	Domain 2 (reporter mAb)	1
8.4A6	Domain 2	1.008 ± 0.461

The MFI of ICAM-1^{ref}, ICAM-1^{Kilifi} and pLTM-1 per 5000 transfected cos cells was measured using a panel of mAbs mapping to domains 1 and 2 ($n = 2$). The MFI was corrected for background using the value obtained for each mAb on pLTM-1-transfected COS cells. The MFI ratio ICAM-1^{Kilifi} /ICAM-1^{ref} was calculated for each mAb using mAb 6E6 as a reporter.

Association of the K29/M polymorphism with cerebral malaria

To determine the relationship of this mutation to clinical disease, we obtained DNA samples from the large group of Kenyan children recruited in our case–control study of risk factors for severe malaria (8). We designed specific oligonucleotides to the ICAM-1^{ref} and ICAM-1^{Kilifi} sequences, together with a control oligonucleotide to use as probes on PCR-amplified DNA encoding domain 1 from a blinded panel of 547 samples (287 community control; 157 cerebral malaria; 103 severe anaemia) (Table 1). To our surprise, on unblinding the study, we found that the homozygous ICAM-1^{Kilifi} genotype was associated with susceptibility to cerebral malaria with a relative risk of 2.23 and heterozygotes with a relative risk of 1.39 (χ^2 for trend = 8.2; $P = 0.0042$). No significant differences were seen comparing the community control and severe anaemia groups. The allele and genotype frequencies are shown in Table 2.

Analysis of *P.falciparum*-infected erythrocytes binding to ICAM-1^{Kilifi}

One explanation for increased susceptibility to cerebral malaria is that ICAM-1^{Kilifi} is expressed on cerebral endothelium where it binds infected erythrocytes with increased avidity compared with ICAM-1^{ref}. We and others previously have demonstrated important roles in infected erythrocyte adhesion for amino acid sequence residues 15–20 and 43–47 (5,9). Molecular modelling suggests that the K29/M mutation is at the edge of one of these two regions, and we recently have found that some monoclonal antibodies (mAbs) that block infected erythrocyte adherence have epitopes sensitive to mutations in the region from residues 26 to 30 (10). It was possible, therefore, that the mutation could have effects on the adhesion of infected erythrocytes. To test this, we used PCR-directed mutagenesis to construct an ICAM-1 cDNA containing the K29/M mutation and expressed this transiently in COS cells. No effect on the overall conformation of the domain was seen using a panel of anti-ICAM-1 (mAbs) previously mapped to different parts of the ICAM-1 structure, including three mapping to the D26QPKL loop (10,11) where the ICAM-1^{Kilifi} mutation resides (Table 3). However, the epitope for mAb BBA4 is destroyed and mAb 7.5C2, which maps to the D26 loop, shows increased binding to ICAM-1^{Kilifi}, indicating that the structures of the two ICAM-1 variants are different. Binding of infected red cells to ICAM-1^{ref} and ICAM-1^{Kilifi} was compared using a quantitative static adhesion assay (5). The ratio of adherence (ICAM-1^{ref}: ICAM-1^{Kilifi}) was 1.43 ± 0.22:1 (95% ci 1–1.86:1) showing that there was no significant difference in binding to the two alleles. We cannot yet exclude a difference in adhesion between ICAM-1^{ref} and ICAM-1^{Kilifi} under shear flow conditions, where it is known that ICAM-1 mediates rolling adhesion (12).

DISCUSSION

Recent data estimate malaria-specific mortality in Africa in children under 5 as 10 000 per year; i.e. 5% of all live births (13). This figure is in line with that calculated from the current gene frequencies of haemoglobin S (14). Since death from malaria in endemic areas always occurs before reproductive age, it is clear that this disease remains as one of the major selective forces on the human genome. This is evidenced by the increasing list of high frequency polymorphisms that have apparently been selected because of the protection they confer against death from malaria (15). Here we have identified a mutation in ICAM-1 with a gene frequency of >30% which is associated with susceptibility to life-threatening malaria. While this association strengthens the link between ICAM-1 and cerebral malaria, a mutation which confers susceptibility to this syndrome is unlikely to have arisen at such high frequency in the absence of some counteractive selective advantage. ICAM-1^{Kilifi} is not the only known susceptibility allele; a mutation in the TNF2 promotor has also been associated with disease severity in the Gambia (16), but this allele is only present at very low frequency. To investigate whether this phenomenon

was specific to the study area, we typed 99 unrelated reference Caucasian samples, but no individuals with the ICAM-1^{Kilifi} allele were identified. This was in agreement with other data which showed no polymorphisms in domain 1 of ICAM-1 in DNA samples from 40 multigenerational families from the CEPH collection (17). Screening of 20 Gambian samples produced a similar frequency of the ICAM-1^{Kilifi} allele to that seen in Kenya, indicating that ICAM-1^{Kilifi} is a high frequency, African mutation (data not shown). The relationship of genotype to disease severity in West Africans remains to be determined.

We speculate that other biological selective forces at least as powerful as malaria must result in a compensatory selective advantage for individuals bearing the ICAM-1^{Kilifi} gene, and the relatively low increased risk of cerebral malaria seen in this study would allow for this. ICAM-1 plays a central role in the immune response, mediating leukocyte–leukocyte and leukocyte–endothelial interactions. There is direct and indirect evidence that the loop containing the ICAM-1^{Kilifi} mutation makes a contribution to the binding sites for the physiological ligands LFA-1 and fibrinogen, both of which influence leukocyte adhesion. The loop is also implicated in the binding site for the major serogroup of rhinovirus, for which ICAM-1 is the cellular receptor (18–20). Hence, the mutation may exert a protective effect against rhinovirus infection, which although unlikely to constitute a significant selection pressure *per se* may predispose individuals to acute bacterial respiratory infections, an important cause of childhood mortality in Africa (21). Alternatively, the mutation may result in an altered affinity for LFA-1 (20) or for fibrinogen, which can act as a bridging molecule in some leukocyte–endothelial interactions (10), with beneficial immunomodulatory effects. These might involve either enhanced protective immune responses to pathogens or decreased ICAM-1-dependent, leukocyte-mediated tissue damage from exposure to infectious agents. As an example, colonic ulceration due to *Shigella dysenteriae* has been shown, *in vitro*, to be largely neutrophil-mediated (22). Alternatively, the long-term outcome of childhood infection with chronic agents such as hepatitis B may be shaped by the magnitude of ICAM-1-dependent cellular responses and the tissue destruction caused by such chronic inflammation. Finally, the presence of an expressed structural isoform of this ubiquitous adhesion molecule has important theoretical implications for transplantation immunology and transfusion medicine, the significance of which remains to be determined.

Children in East Africa face a large number of diseases which compromise their survival to reproductive age. Under these circumstances, it is inevitable that genes controlling the outcome of such infections will be subject to selective pressures which result in a complex balance of both resistance and susceptibility alleles. This will be of particular relevance when the genes concerned have central roles in the immune response. The identification of the ICAM-1^{Kilifi} mutation therefore offers an opportunity to investigate the important interplay between susceptibility and resistance to infectious agents.

MATERIALS AND METHODS

Case–control study design

Samples were chosen from a much larger case–control study. Index cases of severe malaria including cerebral malaria and severe anaemia, in the age range of 6 months to 7 years, were recruited in Kilifi hospital according to strict clinical definitions (23). For each index case, an age- and time-matched non-severe malaria control that did not require hospitalization was recruited. These two cases were each used to recruit an age-, time- and location-matched control chosen from the community at random.

PCR amplification of ICAM-1

A 262 bp fragment encompassing the entire coding sequence of ICAM-1^{ref} domain 1 was amplified by PCR using a 5′-biotinylated primer ICG1-Bio (5′-BiotinGACCTGGCAATGCCCAGACATCTGTGTCC-3′) and a 3′ primer ICG2 (5′-ACGAATTCGTACACGGTGAGGAAGGTTTTAGCTGTTG-3′). Human DNA was extracted from venous blood and ~100 ng amplified under the following conditions: 100 µl reaction mixture containing 0.2 µM of each primer, 200 µM of each dNTP, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% tissue culture gelatin, 0.5 U *Taq* DNA polymerase. The amplification conditions were: one cycle at 95°C for 3 min, 60°C for 1 min, 74°C for 1 min; 38 cycles at 95°C for 1 min, 60°C for 1 min, 74°C for 1 min and one final cycle at 95°C for 1 min, 60°C for 1 min, 70°C for 10 min. Template-free controls were included in each amplification and each product was verified by electrophoresis in a 1.5% agarose gel.

Direct sequencing

The 262 bp PCR product was purified in 1.5% low melting point agarose gel and recovered after β-agarase digestion. Fifty µl of streptavidin magnetic beads (Dynabeads M-280^{TI}) were washed twice with 100 µl of TE/0.1 M NaCl using the magnetic particle concentrator (MPC) to recover them from suspension. After the final wash, the beads were resuspended in 100 µl of TE/2 M NaCl and 100 µl of PCR product was added. To optimize the recovery of PCR product, the beads were incubated for 30 min at room temperature, then placed in the MPC to remove the beads. The PCR product was denatured with NaOH 0.1 M for 30 min, followed by two washes in 0.1 M NaOH , one in TE/0.1 M NaCl and one in TE. The single-stranded PCR product was resuspended in water for sequencing with the PCR primer ICG2 using sequenase version 2.0 (US Biochemical) and Mn^{2+} to obtain sequences close to the primer.

Genotyping

Domain 1 of ICAM-1 was amplified in 547 genomic DNA samples. The PCR product was denatured and spotted onto Hybond N$^+$ (Amersham) according to the manufacturer's instructions in 96-well plate format using a vacuum manifold. We designed two allele-specific oligonucleotides, (A) 5′-AGCCCATGTTGTTG-3′ for the ICAM-1^{Kilifi} allele and (B) 5′-CCCAAGTTGTT-3′ for the ICAM-1^{ref} allele. An internal domain 1 ICAM-1^{ref} oligonucleotide (C) 5′-TTGGTTGGCTATCTTCTTGC-3′ was used as a control. The oligonucleotides were end-labelled with Redivue [γ-^{32}P]ATP (Amersham) and purified using a Sephadex G25 spin column. Oligonucleotides A and B were hybridized to the membranes under the following conditions: pre-hybridization at 47°C in 0.5 M sodium phosphate 7% SDS for 30 min and hybridization at 47°C in 0.5 M sodium phosphate/7% SDS overnight. For ICAMD1-WTB: pre hybridization at 25°C in 0.5 M sodium phosphate/7% NLS for 30 min followed by hybridization at 25°C in 0.5 M sodium phosphate/7% NLS overnight. Excess probe was removed by four 1 min washes in 3× SSC 0.1% SDS according to the following

temperatures for each oligonucleotide: ICG2-WT at 55°C, ICAMD1-M at 47°C and ICAMD1-WTB at 27°C. The filters were autoradiographed. The accuracy of this method was confirmed by direct sequencing of 16 samples as described above.

ICAM-1^{Kilifi} expression construct

PCR site-directed mutagenesis was used to incorporate the A179/T single point mutation into an ICAM-1^{ref} CDM8-based expression vector (5). Two PCR reactions using ICAM-1^{ref} CDM8 as a template were performed. A 778 bp fragment was amplified using a 5′ primer ICAMD1-M (5′-AGCCCATGTTGTTG) which incorporates a T at position 179 and a 3′ primer ICAM-10 (5′-ACGAATTCGTTGGGCGCCGGAAAGC-3′) from the end of domain 3. A second PCR reaction was carried out using a 5′ primer ICAM-5del (5′-ACATCGATCTCCCCCTCAAAAGTCATCCTG-3′) from the start of domain 1 and a 3′ primer ICAMD1-Mcomp1 (5′-GCCCAACAACATGGGCTGG-3′) complementary to ICAMD1-M. Both PCR products were then used as templates for a third PCR reaction using the 5′ primer ICAM-5del and the 3′ primer ICAM-10. The PCR product was then digested with *Cla*I and *Eco*RI and cloned into *Cla*I–*Eco*RI-digested pLTM-1.

P.falciparum-infected erythrocyte binding assay

Binding of erythrocytes infected with the A4 clone of *P.falciparum* to COS cells transfected with pLTM-1, ICAM-1^{ref} and ICAM-1^{Kilifi} was carried out as described previously (5). The number of infected red cells bound to 300 COS cells was counted for three experiments, including four coverslips for each condition. Counts were normalized to 2% Hct, 10% parasitaemia, and correction for transfection efficency was made by measuring the mean fluorescence intensity (MFI) with mAb 6E6.

ACKNOWLEDGEMENTS

The authors would like to thank all the members of the Molecular Parasitology Group in Oxford for their support, Robert Pinches for parasite culture, Nancy Hogg, Dario Altieri and Andy Gearing for gifts of monoclonal antibodies, Adrian Hill for providing Gambian DNA samples and John Todd for the Caucasian DNA samples. The work was supported by Kemri, the UNDP/WORLD BANK/WHO special programme for research and training in tropical diseases (TDR), The Lister Institute for Preventive Medicine and The Wellcome Trust. A.R.B. is a Lister Institute Research Fellow; K.M. is a Wellcome Trust Senior Research Fellow in Clinical Science. D.F.-R. is supported by CONICIT. This paper is published with the permission of the Director of Kemri.

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