Complement receptor one polymorphisms and susceptibility to severe malaria

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Abstract

Severe malaria as a result of infection with *Plasmodium falciparum* is one of the leading causes of mortality and morbidity in the world. It is responsible for the deaths of over one million children per year in tropical areas. However the number of children who develop severe disease is only a small fraction of the number that are infected, hence the need to study virulence factors associated with severe malaria. Rosetting - the binding of parasitised red blood cells to uninfected red cells to form "rosettes" has been associated with severe disease in many studies in Africa. However a study in Papua New Guinea found no association between rosetting and severe disease. It remains unclear whether rosetting is a cause or correlate of parasite virulence.

Rosetting is mediated by the interaction of at least one parasite protein: *Plasmodium falciparum* erythrocyte membrane protein one (PfEMP1) on the infected erythrocyte surface and a variety of host red cell surface receptors including complement receptor one (CR1). We reasoned that if rosetting were a cause of severe disease we would be able to identify protective polymorphisms in the CR1 gene. Here we show that CR1 deficiency is extremely common among individuals from malaria endemic populations in Papua New Guinea. This deficiency is associated with previously reported polymorphisms in the CR1 gene and, unexpectedly with alpha-thalassaemia, a red cell disorder that occurs in up to 90% of Melanesians. We tested the hypothesis that CR1
polymorphisms protect against severe malaria by genotyping samples from a case control study for polymorphisms in the CR1. We found that both CR1 deficiency alleles and alpha-thalassaemia protect against severe malaria.

We wished to test the hypothesis that rosetting was not associated with severe disease in Papua New Guinea because of widespread CR1 deficiency preventing the formation of rosettes strong enough to withstand sheer forces in the circulation. Studies on field isolates from Papua New Guinea showed that rosetting was unusual in this population, rarely mediated by binding to CR1 and not associated with disease severity. This suggests that CR1 is essential for physiologically significant rosetting associated with severe malaria. We have therefore identified a new malaria resistance gene and provided compelling evidence that CR1 mediated rosetting is an important virulence phenotype and a potential target for drug and vaccine development.
Declaration

This text of this thesis is wholly my own work. The work presented is substantially my own with the following exceptions:

1. Knops Blood Groups typings presented in section 4.3.6 were carried out by Joann Moulds (Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA)

2. Case-control study samples used in Chapter 5 were originally collected over a period of 3 years by Stephen Allen and Angela O'Donnell (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK). The DNA in the samples was amplified by whole genome PCR prior to use in this study in their laboratory.

3. Statistical analyses in Chapters 4 and 5 were checked and rechecked independently by Margaret MacKinnon, (University of Edinburgh, UK).

4. The field study and sample collection described in Chapter 6 was set up jointly with Mirjam Kaestli, (Swiss Topical Institute, Basel, Switzerland), however all the rosetting experiments and analyses were done by myself.

This work has not been submitted for any other degree or professional qualification.

Ian Cockburn - January 2004
Abbreviations

ABS - AB Serum
BSA - bovine serum albumin
CA - cofactor activity
CCP - complement control protein
c-FDA - 5-carboxyfluorescein diacetate
CR1 - complement receptor 1
CSA - chondroitin sulphate A
DAA - decay accelerating activity
DAPI - 4',6'-diamidino-2-phenylindole
DMSO - dimethyl sulphoxide
DNA - deoxyribonucleic acid
EDTA - ethylenediaminetetraacetic acid
ELISA - enzyme-linked immunosorbant assay
FITC - fluorescein isothiocyanate
FL - fluorescence
G6PD - glucose-6-phosphate dehydrogenase
Hb - haemoglobin
HRP - horseradish peroxidase
ICAM-1 intercellular adhesion molecule 1
Ig - immunoglobulin
IFA - immunofluorescence assay
iRBC - infected red blood cell
LDS - lithium dodecyl sulphate
LHR - long homologous repeat
mAb - monoclonal antibody
NADP - nicotinamide adenine phosphate
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PBMC - peripheral blood mononuclear cell
PCR - polymerase chain reaction
PEP - primer extension preamplification
PfEMP1 - *Plasmodium falciparum* erythrocyte membrane protein 1
PMS - phenazine methosulphate
PMSF - phenylmethylsulfonyl fluoride
PNG - Papua New Guinea
RFLP - restriction fragment length polymorphism
RBC - red blood cell
SCR - short consensus repeat
SDS - sodium dodecyl sulphate
SLE - systemic lupus erythematosus
SNP - single nucleotide polymorphism
VCAM-1 - vascular adhesion molecule 1
Acknowledgements

Research carried out over a period of over three years involves the input and support of many people. I hope I have not left anyone out. Firstly thanks to my supervisor Alex for the idea, the technical knowledge but above all for being around virtually all the time for support and advice. Also thanks go to my second supervisor David Walliker for his interest in my progress and moral support and to Marg for her huge input into this work in ideas and in teaching me some stats. The rest of the laboratory, Ahmed, Anne-Marie and Jean-Phillipe deserve a mention for being great. It has been a very stimulating and fun environment in which to work for the last few years. Edinburgh was not the only laboratory I worked in during my PhD. Two months were spent in Woods Hole at the Biology of Parasitism course (BoP) which was enormously influential in where I see my future work going. Thanks to the course organisers and to my fellow BoPsters, especially Markus, Janine, SooHee and Katelyn. More importantly to the work presented here some 5 months was spent at the Papua New Guinea Institute of Medical Research where I worked with Alfred Cortes who ran the lab so ably in Madang and Mirjam Kaestli of the Swiss Tropical Institute with whom the study in Chapter 6 was set up. In addition to being the most organised and efficient co-worker I know, Mirjam also taught me a thing or two about domestic hygiene. Also in PNG I must thank, Tibby MacKinnon for collection of samples in New Ireland, John Reeder and Moses Bockarie for permission to
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None of this could have been done without support from family and friends. So thanks go to my mother, and Duncan and Catherine: my brother and sister. Also thanks to friends above all (leaving out the above-mentioned and in alphabetical order): Axel, Ben, Bob (aka the other Lucy), Catherine, Clare, Gemma, Helen, Jaap, Lisa, Natasha, Simon, Titch (and Rie), and wee Janette. Finally thanks to Lucy for her love and support and frank criticism of my "terrible" abstracts.
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Appendix
Chapter 1: Introduction

In this introduction I firstly attempt to describe the problem of severe malaria both globally and by looking locally at a particular population around Madang, Papua New Guinea, that is studied in this thesis. In the remaining sections of the introduction I
outline our understanding of disease process in malaria infections such as cytoadhesion and rosetting. As one of the major themes of this thesis is malaria resistance genes I also look at how our understanding of natural resistance can inform our understanding of pathogenesis. Finally I introduce a candidate malaria resistance gene: complement receptor 1 (CR1) which is the focus of study for this thesis.

1.1 The global burden of malaria

Malaria due to *Plasmodium falciparum* is the most important tropical parasitic disease in the world and is responsible for between 1-2 million deaths per year, 90% of them in sub-Saharan Africa, and 400 - 900 million clinical episodes each year worldwide (Breman *et al.* 2001). Some 90 countries in the world are malarious, half of them in Africa, the remainder in South Asia, Central and South America and island Oceania. In these areas the mortality from malaria is less, however, morbidity due to the disease is high and the economic and social costs considerable. The problem has worsened over the last 30 years as the parasite is increasingly resistant to older and cheaper drugs such as chloroquine and pyrimethimine leading to the use of more expensive alternatives to which the parasites are already rapidly becoming resistant (Wernsdorfer 1994). Moreover political instability in many of the worst affected countries has led to the breakdown of control measures, drug distribution and research. The solution to this problem is often sought in
the development of an appropriate vaccine, which may either prevent transmission of the parasite or aim to limit the lethal effects of infection or both (Hoffman et al. 1998).

Figure 1.1

Figure 1.1: The global burden of malaria: shaded areas have regions where malaria is endemic while unshaded areas indicate regions that never had malaria or countries where malaria has been eradicated (image from the Roll Back Malaria website: http://www.rbm.who.int/)
1.1.1 Lifecycle and biology of *P. falciparum*

Malaria in humans is caused by one of four protozoan parasites of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of these, most severe disease and mortality is caused by *P. falciparum* though *P. vivax* is a major contributor to morbidity in much of the world. The other two species are rarer and while disease due to them may be unpleasant it is rarely life threatening. The life cycle is complex (reviewed in Barnwell *et al.* 1998; Beier *et al.* 1998; Frevert *et al.* 1998; Sinden 1998)) and only a brief overview is given here (Figure 1.2). The parasites are carried between human hosts by a female mosquito vector of the *Anopheles* genus that takes up haploid gametocytes. Within the mosquito midgut the gametocytes become gametes which combine to form diploid zygotes. The zygotes undergo meiosis to form ookinetes which penetrate the midgut wall, where further asexual divisions occur to form sporozoites. These invade the salivary glands of the vector and are injected into the human host where they invade the liver. From the liver emerge the blood stage forms (merozoites) that invade red blood cells (RBC) reproduce asexually and burst out (schizogony) to reinvoke fresh RBC. In *falciparum* malaria the extent of blood infection can increase exponentially even as high as 50% of peripheral RBC. From this population of circulating merozoites a number of gametocytes develop. The cycle is completed when the gametocytes are taken up by feeding *Anopheles* and a new round of sexual reproduction begins.
Figure 1.2 The malaria life cycle (image from www.niaid.nih.gov/publications/malaria/life/htm)
1.1.2 Who dies of malaria?

While the number of children who die from malaria each year is large, it represents a small fraction of the total number of clinical malaria infections. The number of clinical infections itself is small compared with parasite prevalence across the whole population. The graph in figure 1.3 shows two manifestations of malaria: parasitisation and severe disease plotted against age, this data is from Kilifi in Kenya (Marsh et al. 1999), though the pattern is repeated throughout malaria endemic areas (Molineaux et al. 1980; Cattani et al. 1986; Allen et al. 1996). Severe malaria is largely a childhood disease in areas endemic for malaria. While parasite prevalence is still rising as severe disease is falling away, suggesting that children develop immunity to severe disease while still susceptible to parasitism (Marsh et al. 1999). Some evidence for this comes from showing that children become immune to severe malaria syndromes (other than cerebral malaria) after only 1 or 2 bouts of clinical illness (Gupta et al. 1999). A molecular explanation was forwarded by Bull et al. who showed that surprisingly severe malaria seemed to be caused by relatively few common parasite variants while less severe disease was the result of many rarer variants to which immunity took a long time to acquire (Bull et al. 1998; Bull et al. 1999).
Figure 1.3 Indices of malaria in endemic areas (after Marsh et al. 1999).

Surprisingly in highly endemic areas parasite prevalence does not increase with increased transmission - i.e. increased numbers of infective bites (Marsh et al. 1999). Furthermore some studies show that cerebral malaria can be rare in areas of intense malaria transmission, perhaps because of the rapid acquisition of immunity (Marsh et al. 1999). In areas of much lower transmission (such as Southeast Asia or the south coast of Papua New Guinea) the patterns are different with transmission intensity corresponding to the burden of disease. Moreover full immunity may never be developed and so adults as well as children can be susceptible to severe disease. Often in adults the pattern of severe...
disease is unlike the classical cerebral malaria and severe anaemia observed in children in endemic areas but instead is characterised by multi-organ failure and metabolic disturbances (White 1987).

1.1.3 Syndromes of severe malaria

Traditionally malaria mortality has been attributed to two major syndromes, cerebral malaria and severe malarial anaemia. Cerebral malaria has been the focus of most research into disease pathogenesis. Strikingly the median age for cerebral malaria patients is typically much older than that for severe anaemia patients: 40 months vs. 22 months in Kenya (Marsh et al. 1997) and 45 months vs. 28 months in the Gambia (Greenwood et al. 1991), suggesting that partial exposure to malaria maybe a risk factor for severe disease. Anaemia accounts for a larger proportion of "severe" malaria requiring hospitalisation, but has a lower case fatality rate than cerebral malaria. Studies where a whole population has been subject to successful malaria control with concomitant reduction in anaemia demonstrate that malaria is a cause of anaemia and not a correlate of poor health indices in general (Bland et al. 1993). The causes of anaemia are perhaps even less well understood than the causes of cerebral malaria. The proportion of infected red blood cells can reach 50% which obviously precipitates major haemolytic anaemia. Nonetheless there remains an important group of patients with relatively low parasitaemias (<2%) who nonetheless have extremely low haemoglobin (<5g/dl - the arbitrary definition of severe anaemia) (Abdalla et al. 1980). Dyserythropoiesis has been associated with this pattern of disease in the Gambia (Abdalla et al. 1980), the cause of this may be the
stimulation of cytokine production by malaria: TNF-α and IFN-γ have been shown to suppress haemopoiesis (Young et al. 1994). Other groups have shown that parasites can have an effect on the expansion of erythroid progenitor cells (David Roberts, personal communication).

Recently the importance of metabolic disturbances in malaria has been realised, especially in cases of respiratory distress (Marsh et al. 1995). This syndrome is rarely found on its own. In about 80% of cases it is associated with severe anaemia or cerebral malaria and is a major determinant of mortality. Cerebral malaria cases with respiratory distress were nearly 4 times more likely to die than those with coma alone. The prognosis for anaemia and respiratory distress was almost as poor, with children being over 12 times more likely to die than their counterparts with just anaemia (Marsh et al. 1995).

1.1.4 Malaria in Papua New Guinea

Papua New Guinea (PNG) occupies the eastern half of the island of New Guinea and several smaller Island groups around including New Britain, New Ireland, Manus Island and Bougainville Island. PNG is an interesting place to study malaria as this relatively small country (461,691 square km - roughly 2 times the size of the island of Great Britain, with a population of 4.7 million people) contains a variety of different conditions for malaria transmission (Figure 1.4). In the highlands there is epidemic malaria with
little natural or acquired resistance to disease (Mueller et al. 2002; Mueller et al. 2002).

The south coast has intermediate malaria transmission (Flint et al. 1986) with a pattern of disease similar to that in Southeast Asia (Lalloo et al. 1996). While the north coast and is a good example of a high transmission area where severe disease is prevalent and individuals develop some degree of protective immunity. The age-dependence of parasite prevalence and severe disease is similar in this area to the situation described in figure 1.3 (Cattani et al. 1986). Thus it is similar in many respects to Africa where most malaria mortality occurs. Though there are crucial differences, notably the relatively low mortality rates and the prevalence of malaria due to *P. vivax*.

The climate is tropical and characterised by year-round rainfall. In lowland areas two seasons are defined: a wet season from October to May and a dry season from June to September. Malaria transmission is a year round occurrence with only a slight decrease in the dry season. Over the last decades much work has been done by the Papua New Guinea Institute of Medical Research to describe malarialometric indices in defined areas in different parts of the country. Outside these study areas information is more patchy. The work in this thesis was largely on individuals and populations around Madang, one such well-studied area.
Figure 1.4: Malaria endemicities in Papua New Guinea, dark shading represents areas of intense malaria transmission with >70% spleen rates, medium shading represents area where there is intermediate malaria transmission 50-69 % spleen rates, light shading represents areas where malaria is sporadic (spleen rates 10-49%) while the white areas have at worst epidemics of malaria with a spleen rate <10 %) (after Flint et al. 1986)

1.1.5 Epidemiology of malaria in Papua New Guinea

Madang is a town of around 20,000 people on the North Coast of Papua New Guinea (Figure 1.5). The town is circled by peri-urban settlements where around 13,000 people live, many of them migrants from other parts of the country. The area around the town is secondary rainforest, in which most people live by subsistence agriculture in villages of
100-700 people. Around Madang, the parasite prevalence for the population as a whole was reported as 25.7% in the dry season and 33.4% in the wet season. Among children 5-9 years old this rises to 44% and 49% respectively. Spleen rates (the enlargement of the spleen is a determinant of long term exposure to malaria) for the area around Madang town defined three different areas. In the town and surrounding areas spleen rates were less than 70% while in the foothills of the Adelbert ranges spleen rates were >75%. In other more low lying areas such as the Gogol river basin area an intermediate spleen rate was measured (Figure 1.5)(Cattani et al. 1983). Measurement of the mosquito biting rates showed that the entomological inoculation rate varied from 68 to 526 infectious bites per year with villages in the high risk area receiving more bites than those in the lower epidemiological zones (range 353-526 vs. 68-150 infected bites per year)(Burkot et al. 1988). Transmission is by members of the Anopheles punctulatus complex including An. koliensis, An. funestus and An. punctulatus. While villages varied in which species was most prevalent the vectoral capacity of the three species was similar for P. falciparum. There was also no difference in the prevalence of the species in the different epidemiological zones(Burkot et al. 1988).

The parasite prevalences in humans and the sporozoite inoculation rates are similar to those seen in many African situations. However the situation in Papua New Guinea differs in other ways. Firstly there is a high prevalence of P. vivax in the population making up around 25% of infections(Cattani et al. 1986; Bruce et al. 2000; Bruce et al. 2000; Bruce et al. 2000). A polymorphism in the only invasion receptor for this parasite
the Duffy blood group antigen has rendered much of the West African population refractory to *P. vivax* infection and therefore it has almost been eradicated in much of Africa (Miller *et al.* 1976). Secondly the mortality due to malaria is much lower in Papua New Guinea. A survey of 16,500 individuals in 75 villages measured the percentage of deaths due to malaria as 1.3 % for the population as a whole and 12.5 % for children aged from 1 to 4 years (there were no deaths from malaria among children < 1 year). Far from being a leading cause of death malaria rates only as the 6th most common cause of death among young children and 9th overall (Moir *et al.* 1989). A comparable study in The
Gambia showed that malaria was a cause of 4% of deaths in children < 1 year and 25% of deaths in children aged 1-4 (Greenwood et al. 1987). They also report that the death rate due to malaria is 6.3 - 10.7 per 1000 per year in children aged 0-4. The comparable figures for Madang would be of the order of < 1 per 1000 per year (Moir et al. 1989). In Kenya Snow et al. report a death rate due to malaria of 1.1 per 1000 per year in children aged 1-4 years however they only look at hospital deaths and estimate that only a third of deaths occur in a hospital setting. Co-infection with *P. vivax* has been suggested to be responsible for the lower levels of severe malaria compared to Africa (Williams et al. 1996). Some authors have suggested an immunological mechanism for this though clinical data where *Plasmodium* infections were used to control syphilis suggests that exposure to *P. vivax* does not alter susceptibility to *P. falciparum* infections (Maitland et al. 1997). Chloroquine use is high in this population with one survey showing that up to 14% of children being chloroquine positive in urine tests (Moir et al. 1989). However as the in vivo drug resistance rate is 85% in the most recent surveys this may have little effect other than to increase the problem of drug resistance (al-Yaman et al. 1996) and G.J Casey unpublished. Finally the role of human polymorphisms could be very important. Coastal Papua New Guineans probably have the greatest range and highest proportion of red cell disorders in the world, which means that many of them may never be at risk from severe malaria in the first place (see section 1.3).
1.1.6 Severe disease in Papua New Guinea

There is an extensive literature on severe malaria in Papua New Guinea. Allen et al. report cases of severe malaria at Madang general hospital over 3 wet seasons (October 1993- February 1996). Out of 311 children with severe malaria there were 103 cases of severe anaemia (33%) and 75 cases of cerebral malaria (24%)(Allen et al. 1996). The small proportion of children with the classical syndromes of severe malaria compared to African situations is interesting(Marsh et al. 1995). Instead many children suffer from metabolic disturbances such as hyperlactaemia (28%), acidosis (19%) and hypoglycaemia (5%). As in Africa this study found that the median ages of cerebral malaria patients was older than that of severe anaemia cases (43 months vs. 30 months). Genton et al. present a study of 134 "comatose" Papua New Guinean children in a study also based in Madang. A closer analysis reveals that in this instance "comatose" actually means impaired consciousness and only 52 of the children in this study meet the criteria of cerebral malaria, these data were collected over 2 wet seasons (April 1991 to October 1993). Moreover the case fatality for cerebral malaria in Madang has been reported as 8.0%(Allen et al. 1996) or 11.9%(Genton et al. 1997) which are much lower than in African settings where typical values are around 16-25%(Molyneux 1990; Marsh et al. 1995). It is often commented anecdotally that some severe malaria is rarer around Madang than in African regions with comparable transmission(Muller et al. 2003). While this is supported by some data that suggests a lower overall mortality due to malaria(Moir et al. 1989), and low case fatality rates(Allen et al. 1996; Genton et al. 1997), the
The proportion of hospital cases being due to severe malaria is the approximately the same in Madang as in Kilifi (cf. [Marsh et al. 1995; Allen et al. 1996]) and the proportion of deaths due to malaria among hospitalised children is the same in PNG as in various African situations (cf. [Greenwood et al. 1991; Allen et al. 1996]).
1.2 Malaria - a neglected disease

How then should we tackle malaria? Despite malaria being one of the major killers worldwide, it has been described as a "neglected disease" (Marsh 1992). Traditionally malaria control measures have been based on eradication either of the mosquito vector by insecticide spraying, or on eradication of the parasite by mass treatment with antimalarials. For as long as eradication was the ultimate aim, the disease itself (as distinct from asymptomatic infection) was not considered so important. As these measures failed and emphasis has switched to control and prevention of mortality, an understanding of the pathogenesis of disease has become a more important priority. In an article from over a decade ago, Kevin Marsh identified the key question for the study of malaria as a disease as "what factors put some children at risk of developing severe malaria?" (Marsh 1992). He gives a list of these factors and identifies them as areas for study by basic scientists and clinicians alike. Twelve years on perhaps it is interesting to review this list and see what progress has been made (Table 1.1):
Table 1.1 Factors which may play a role in determining the clinical outcome of malaria infection (after Marsh 1992)

<table>
<thead>
<tr>
<th>1. Environmental</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Vectors - multiple factors summing to give variations in number of sporozoites inoculated over time</td>
</tr>
<tr>
<td>ii. Socio-economic - e.g. availability of health care</td>
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<table>
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<tr>
<th>2. Host</th>
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<tr>
<td>iii. Acquired immunity</td>
</tr>
<tr>
<td>iv. Behavioural factors - usage of anti-malarials, farming practices</td>
</tr>
<tr>
<td>v. Nutritional Status - iron, riboflavin, p-aminobenzoic acid, others?</td>
</tr>
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<td>vi. Genetic factors</td>
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<td></td>
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<tr>
<td>Red cell polymorphisms</td>
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<td>Immune response genes</td>
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<td>Others such as T cell receptors, polymorphisms in the acute phase response and endothelial receptors.</td>
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<th>3. Parasite</th>
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<td>vii. Growth</td>
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<td>viii. Cytoadherence</td>
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<td>ix. Immune evasion</td>
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<td>x. Drug resistance</td>
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Environmental factors, and behavioural and social factors are beyond the scope of this thesis. However we are concerned with both host and parasite biology. It is fair to say that despite the efforts of a small number of excellent researchers our knowledge of host immunity to malaria is inadequate. In the scramble to find a vaccine antigen that provides effective immunity little thought has been given to what kind of immunity that vaccine should be generating. An understanding of the T cell phenotypes involved in malaria pathogenesis and protection is only just emerging (Good et al. 1999; Plebanski et al. 2000; Carvalho et al. 2002; Hisaeda et al. 2004). The potential importance of antibody responses was demonstrated initially by passive transfer of immune IgG into individuals suffering from clinical malaria, resulting in almost complete clearance of parasitaemia (though some residual parasites remained) (Cohen et al. 1961; Sabchareon et al. 1991). The targets of these protective antibodies are poorly understood, though subsequent studies have suggested roles for red cell surface antigens (Bull et al. 1998) and merozoite surface protein 1 (MSP-1) (Conway et al. 2000). One area of interest is that of dendritic cell biology, in which *P. falciparum* infected erythrocytes have been found to prevent dendritic cell maturation and turn off their antigen presentation functions through CD36 binding by parasites (Urban et al. 1999; Urban et al. 2001). In addition to having implications for the outcome of disease, any vaccination strategy will have to overcome this defect in antigen presentation in the malaria exposed immune system, as it has been demonstrated that downregulated dendritic cells can prevent proper T cell activation in malaria infections (Ocana-Morgner et al. 2003).
Overall our understanding of the molecular basis of drug resistance, cytoadherence, and growth and invasion is probably more complete. The genes for chloroquine and sulphadoxine resistance have been mapped (Wellems et al. 1991; Wu et al. 1996; Fidock et al. 1997; Fidock et al. 2000). Invasion is a process that is increasingly well understood largely due to the availability of gene knockouts of parasite proteins thought to be involved (Waterkeyn et al. 1999). Nonetheless the relative importance of different pathways and disease severity is an unknown area. Likewise our understanding of parasite adhesion and disease severity has increased enormously over the last decade, with the identification of the \textit{var} genes encoding a family of \textit{Plasmodium falciparum} erythrocyte membrane protein 1 (PfEMP1) proteins as the major determinants of adhesion (Baruch et al. 1995; Smith et al. 1995; Su et al. 1995). Likewise many of the host proteins this molecule binds to have been identified (discussed below). One thing that has become clear though, is that a simple model of cytoadhesion correlated to disease severity is insufficient (Rogerson et al. 1999). A picture is emerging (discussed below) of adhesion to different receptors being "good" or "bad" though which receptors may depend on the population and parasites in question.

The study of host polymorphisms (such as in this study) has been ongoing; many of the important genes and their significance are discussed in the next section. One development is the development of genome-wide scans for susceptibility genes (see section 1.3.4), which may allow not only for the identification of novel malaria resistance genes, but
also to compare the basis of resistance and pathogenesis in different populations (Kwiatkowski 2000).
1.3 Malaria resistance genes

Long before the first incursions of molecular biology into the study of malaria J. B. S. Haldane first suggested the idea of "malaria resistance genes" (Haldane 1949). He argued that the distribution of the red cell disorders of sickle cell anaemia and thalassaemia could be explained if these deleterious, yet common, disorders protected against malaria. For many years direct evidence for the protective effects of these disorders was not found as most of these polymorphisms have little or no effect on parasitaemias or prevalences; their effect is through protection against death. Thus it was only when researchers examined patients with severe malaria that they were able to definitively link red cell disorders with malaria (Hill et al. 1991). This makes malaria resistance genes more than merely a good example of the action of disease selection on the human genome; it means they could be used to pinpoint processes of severe disease and mechanisms to protect against them.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Allelic Variant (Disorder)</th>
<th>Association</th>
<th>Region</th>
<th>Reference</th>
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<tr>
<td>Red Cell Polymorphisms</td>
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<td>α-globin (α-thalassaemia)</td>
<td>α+-thalassaemia</td>
<td>Protection (SM*)</td>
<td>Papua New Guinea</td>
<td>(Allen et al. 1997)</td>
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<td>β-globin</td>
<td>β-thalassaemia</td>
<td>Protection (CIM)</td>
<td>Vanuatu</td>
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<td></td>
<td></td>
<td>Susceptibility (CIM)</td>
<td>Liberia</td>
<td>(Willcox et al. 1983)</td>
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<tr>
<td>Haemoglobin S</td>
<td></td>
<td>Protection (SM)</td>
<td>West Africa</td>
<td>(Hill et al. 1991)</td>
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<td>(Sickle cell anaemia)</td>
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<tr>
<td>Haemoglobin C</td>
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<td>Protection (CIM)</td>
<td>West Africa</td>
<td>(Modiano et al. 2001)</td>
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<tr>
<td>Haemoglobin E</td>
<td></td>
<td>Protection (SM)</td>
<td>Thailand</td>
<td>(Hutagalung et al. 1999)</td>
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<td>Glucose-6-phosphate</td>
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<td>Kenya</td>
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<td>dehydrogenase (G6PD)</td>
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<tr>
<td>Band 3</td>
<td>South east Asian Ovalocytosis</td>
<td>Protection (CM)</td>
<td>Papua New Guinea</td>
<td>(Genton et al. 1995; Allen et al. 1999)</td>
</tr>
<tr>
<td>Glycophorin C</td>
<td>Gerbich blood group (ΔExon3</td>
<td>Decreases invasion?</td>
<td>Papua New Guinea</td>
<td>(Mayer et al. 2002; Maier et al. 2003)</td>
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<tr>
<td>CR1</td>
<td>Knops Types</td>
<td>Decreased rosetting</td>
<td>West Africa</td>
<td>(Rowe et al. 1997)</td>
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<td>The Gambia</td>
<td>(Zimmerman et al. 2003)</td>
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* Severe Malaria
† Clinical Malaria
‡ Cerebral Malaria
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<th></th>
<th>Susceptibility (SM)</th>
<th>Protection (SM)</th>
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<tr>
<td><strong>Endothelial Receptors</strong></td>
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<tr>
<td>ICAM-1</td>
<td>ICAM-1 (Kilifi)</td>
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<td>CD36</td>
<td>Deficiency Allele</td>
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<td></td>
<td>Thailand</td>
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| Immunity Genes | | |
|----------------|------------------|
| Class I        | HLA-Bw53         | Protection (SM) |
|                | DQB1*0501        | Protection (Various) |
|                | DRB1*1302        | Protection (CM) |
| MBP            | Deficiency Allele| Susceptibility (SM) |
| Fcγlla         | Arg/Arg131       | Protection (SM) |
| iNOS-2 -969 promoter | Susceptibility (CM) |
| NOS2-69546     | Protection (SM) |
| TNF-α -308 promoter | Susceptibility (CM) |
|                | Susceptibility (SM) |
|                | Gambia, Kenya    |

References:
- Nagayasu et al. 2001
- Fernandez-Reyes et al. 1997
- Kun et al. 1999
- Pain et al. 2001
- Aitman et al. 2000
- Hill et al. 1991
- May et al. 2001
- Hill et al. 1991
- Luty et al. 1998
- Shi et al. 2001
- Burgner et al. 1998
- Kun et al. 2001
- McGuire et al. 1994
- Wattavanage et al. 1999
- McGuire et al. 1999
- Hill 1996
Sickle cell anaemia is the most well known example of malaria resistance gene. Homozygotes for the mutation suffer a severe anaemia and rarely live beyond the age of 20 but it is selected for because heterozygotes have only a 1/10 risk of suffering from severe malaria compared to individuals with normal haemoglobin genes (Hill et al. 1991). This explains why the gene can be maintained at frequencies of 20% in some populations in West Africa despite its lethality in the homozygous state (Flint et al. 1998). However despite arising on at least 5 separate occasions in Africa, India and the Middle East it is absent from Papua New Guinea. Instead the relatively small population of Melanesians has perhaps the greatest diversity of other protective genes (Flint et al. 1986; Flint et al. 1998). Papua New Guinea is an interesting place to study these genes because of the varied degrees of malaria transmission across the country. The involvement of a gene in malaria can be inferred from the relative frequencies of the genes in one area (say the holoendemic north coast) relative to those in another (for example the highlands) (Flint et al. 1986; Williams et al. 1996). Here I examine some of the most common malaria resistance genes in Papua New Guinea to give an idea of the genetic background on which malaria infections are occurring.

1.3.1 The thalassaemias

Thalassaemia is a worldwide disorder but one that reaches its highest frequencies of around Madang. Thalassaemia is caused by a defect in one of the alpha-globin or beta-
globin genes (leading to \( \alpha \)- or \( \beta \)-thalassaemia respectively), which results in an imbalance of the globin chains, defective haemoglobin production and therefore tends to result in some degree of anaemia. Around 150 polymorphisms can cause \( \beta \)-thalassaemia, while around 50 have been found that cause \( \alpha \)-thalassaemia (Flint et al. 1993; Flint et al. 1998). \( \beta \)-thalassaemia is the more severe as the gene is present in only a single copy. Polymorphisms can be extremely harmful as in \( \beta^0 \)-thalassaemia where the gene is deleted altogether, or less harmful for example due to polymorphism that causes a defect in the level of beta-globin production (\( \beta^+ \)-thalassaemia). At least one \( \beta^+ \)-thalassaemia variant has been characterised in Papua New Guinea and pockets of high frequency identified (Hill et al. 1988) though in general this polymorphism is relatively rare in Papua New Guinea compared with some African situations (Willcox 1983; Willcox et al. 1983).

\( \alpha \)-thalassaemia on the other hand is extremely common, perhaps the commonest single gene disorder in the world (Flint et al. 1998). As there are two tandem functional copies of this gene on chromosome 16 (figure 1.6). Deletions of one gene (known as \( \alpha^+ \)-thalassaemia) often have no pathology and even homozygotes for this mutation only suffer a mild anaemia as they still have 2 functional gene copies left. Deletions occur as a result of cross over events at meiosis between homologous regions in the region of the gene. There are two major types of deletion -\( \alpha^3.7 \) and -\( \alpha^4.2 \), with the latter being most common in coastal Papua New Guineans, though the rarer of the two in other parts of the world (Flint et al. 1993). \( \alpha^0 \)-thalassaemia where both copies of the gene are lost from a
single chromosome is more severe (and much rarer). Homozygotes live short lives, and even this is only possible because their $\alpha$-globin gene is substituted for by foetal haemoglobin. In Papua New Guinea the gene frequency of $\alpha^+$-thalassaemia ranges from 0 to 0.70 with a strong correlation existing between the frequency and malaria endemicity (Flint et al. 1986). Around Madang 95% of people carry at least one $\alpha$-globin deletion and this has been found to protect against severe malaria in a case-control study (Allen et al. 1997). The mechanism of protection has remained a mystery. Parasite invasion is normal in thalassaemic cells (Luzzi et al. 1991), so some investigators have suggested that reduced endothelial cytoadherence (Udomsangpetch et al. 1993) or rosetting may be the mechanism (Carlson et al. 1994). Other investigators have suggested immunological mechanisms such as increased antibody binding (Luzzi et al. 1991) or an increased susceptibility to $P. \text{vivax}$ in early life that accord subsequent protection against $P. \text{falciparum}$ (Williams et al. 1996). This latter explanation does not however explain why there is $\alpha$-thalassaemia in areas of Africa where there is no $P. \text{vivax}$ due to fixation of the Duffy negative polymorphism which forms an absolute block to parasite invasion (Miller et al. 1976; Horuk et al. 1993). None of the explanations is wholly compelling, though an understanding of the mechanism of protection could tell us about the mechanism of severe disease.
Figure 1.6 The α-globin gene structure (after Flint et al. 1998). There are two copies of the α-globin gene tandemly repeated (α1 and α2). In the gene are regions of homology labelled X, Y and Z. Cross over events between these regions at meiosis lead to deletions such as the -α3.7 and -α4.2, the regions deleted are shown by the lines below.

1.3.2 Southeast Asian Ovalocytosis

Southeast Asian ovalocytosis (SAO) is potentially the most informative polymorphism for gaining an insight into cerebral malaria. Two studies have shown that it has no effect on susceptibility to most syndromes of severe disease, with the exception of cerebral malaria from which it appears to confer absolute protection (Genton et al. 1995; Allen et al. 1999). In the two studies combined there were 105 cerebral malaria patients none of whom had SAO while the prevalence among 410 controls was 8.0%. Southeast Asian ovalocytosis is characterised by elliptocytic cells with very rigid membranes due to a
mutation in Band 3, which is a chloride channel and the most common protein on the erythrocyte surface (Solomon et al. 1983). The mechanism of inheritance was a mystery until the mutation was identified. Individuals with SAO were found to be heterozygous for a 27 bp deletion in the Band-3 gene (Jarolim et al. 1991). No homozygotes for the deletion have been found, though thousands of individuals have been screened (Genton et al. 1995) so it is assumed to be lethal in utero.

Some work has suggested that the rigid ovalocytic membrane might be a barrier to invasion ((Kidson et al. 1981) and Alfred Cortes, personal communication), however as individuals with ovalocytosis are just as susceptible to other severe malaria syndromes this may not be the mechanism. Moreover some work suggests that ovalocytic cells "decay" more rapidly on storage than normal erythrocytes (Dluzewski et al. 1992) and this may cause them to be refractory to invasion. The specific protection against cerebral malaria suggests a more specific mechanism. Recent work has shown that ovalocytic cells show normal adherence to endothelial receptors in static condition, but in flow conditions binding to CD36 is increased (Alfred Cortes, personal communication). CD36 is rarely expressed in the brain (Turner et al. 1998), suggesting that this may be a benign, even protective form of cytoadhesion.
1.3.3 The Gerbich blood group type

The Gerbich blood group type is caused by the deletion of exon 3 in the glycophorin C gene (Chang et al. 1991). Glycophorin molecules (such as Glycophorin A and B) are very abundant red blood cell surface proteins and have been implicated as receptors for invasion (Pasvol et al. 1983; Hadley et al. 1987; Orlandi et al. 1992; Dolan et al. 1994; Maier et al. 2003). Their functions remain unknown though there involvement in a variety of disease process has shown them to be under strong selective pressure (Baum et al. 2003). The Glycophorin C molecule is attached to the cytoskeleton and so its various blood group polymorphisms are often associated with elliptocytosis (Telen et al. 1991).

The blood group is probably only present at low frequencies in much of PNG but in one region of the Sepik river basin, the Wosera, it reaches a frequency of 0.47 (Patel et al. 2001). Strikingly the same population has an almost complete absence of Southeast Asian ovalocytosis. While no epidemiological studies have formally shown a protective effect of this polymorphism Glycophorin C has recently been identified as a receptor for the merozoite red blood cell adhesion molecule BAEBL, and is therefore important in the process of parasite invasion (Maier et al. 2003). However as there is great degeneracy in pathways of *P. falciparum* invasion it is unclear whether the loss of one pathway of invasion could exert the necessary selective pressure to account for the high frequencies of the Gerbich blood group seen. Interestingly the BAEBL gene also exhibits some functional polymorphism, with some BAEBL proteins binding receptors other that
Glycophorin C. Some authors have suggested that this is a result of selective pressure on the parasite by the presence of mutations in the Glycophorin C gene (Mayer et al. 2001).

1.3.4 The future of association studies

Whereas the basis for protection due to the thalassaemias, ovalocytosis and G6PD deficiency is incompletely known, some polymorphisms have been identified through more "rational" means (Kwiatkowski 2000). The search for genetic resistance factors is now pursued along two lines. Attempts have been made to trace genetic susceptibility to disease through segregation analysis on family studies. Studies in Cameroon and Burkina Faso have identified a locus involved in control of blood parasitaemia which contains a variety of genes for cytokines and other immune factors (Abel et al. 1992; Garcia et al. 1998; Rihet et al. 1998) while another study in Papua New Guinea showed that antibody responses to segregated between family members (Stimadel et al. 1999). In the study of severe malaria such family studies are impossible (as they would require information on both the parents and children's susceptibility to severe malaria) however genome-wide gene association studies for severe malaria are being attempted currently. Additionally a candidate gene approach has been taken where polymorphisms are searched for in proteins involved in the pathogenesis of severe disease. One classic example of this has been the identification of MHC class 1 allele HLA-Bw53 as protective in malaria, presumably because it is most effective at displaying epitopes derived from P. falciparum sporozoites that have invaded the liver (Hill et al. 1991). Common African
polymorphisms have been identified in the cytoadhesion ligands ICAM-1 (Fernandez-Reyes et al. 1997; Bellamy et al. 1998) and CD36 (Aitman et al. 2000) which are discussed below. Surprisingly case-control studies have shown that often the "African" variants cause susceptibility rather than protection, perhaps because the parasite has already had a chance to evolve to the new genetic background. We may be looking at evolutionary fossils rather than currently protective genotypes. Nonetheless this is evidence for the involvement of cytoadhesion in the pathogenesis of severe malaria.
1.4 Cytoadhesion: ligands and receptors

1.4.1 Cytoadhesion and Antigenic Variation

In the late nineteenth century Bignami and Bastianelli observed that mature trophozoite and schizont stages of *P. falciparum* are rarely found in the peripheral circulation (Bignami et al. 1889). The mature parasites have been found to sequester in the microvasculature of the brain and other tissues (MacPherson et al. 1985), perhaps to avoid the lethal immune activity of the spleen, which removes "aberrant" red blood cells including those significantly distorted by *Plasmodium* infection (Barnwell et al. 1983; Barnwell et al. 1983). An interest in cytoadherence stemmed from the role it was believed to play in the pathology of cerebral malaria - it was hypothesised early on that the binding of parasites to the endothelium of the brain would obstruct oxygen uptake, damage the blood brain barrier and lead to cerebral malaria (MacPherson et al. 1985).

Finding the parasite antigens responsible for this process was and remains an important focus of research, as a vaccine against such a molecule could prevent malaria associated pathology if not infection. Early on, however, it was noted that cytoadherence phenotypes varied (Udeinya et al. 1983) with immune pressure and so adhesion became linked with the phenomenon of antigenic variation. Many parasites undergo antigenic variation (Borst et al. 1995) generally in order to evade the immune response. Most famously, Trypanosomes which live freely in the serum cover their surfaces with one of about 1000
variant surface glycoprotein (VSG) genes. Thy can generate further diversity by splicing these to create further variation (Borst 1991). The parasite needs these genes to be able to evade the immune response in the serum. Antigenic variation was described as early as 1965 in malaria parasites (Brown et al. 1965). The linking of cytoadhesion and escape from clearance by the spleen to antigenic variation also solved the theoretical problem of why the malaria parasite which was capable of surviving inside the red blood cell would express antigens on the erythrocyte surface. At the molecular level these paradoxes were resolved with the discovery of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), the protein that mediates antigenic variation and all-known cytoadhesion phenotypes.

### 1.4.2 The parasite ligand: PfEMP1

PfEMP1 was originally discovered by Leech et al. who identified novel antigens on the infected red blood cell (iRBC) surface (Leech et al. 1984). They showed that the antigens were strain specific and bound to by strain specific serum. Moreover they showed that both the antigen and cytoadhesion disappeared upon treatment with trypsin and subsequently that the novel antigens were associated with "Knobs" on the infected erythrocyte surface which appeared to be associated with cytoadherence (Aley et al. 1984). Evidence that these novel antigens were of parasite rather than host origin came from metabolic labelling experiments and the proteins were named PfEMP1 (Howard et al. 1988). The same experiments also showed that Knobs were made up of other parasite
derived proteins under the host cell membrane. The derivation of a variety of different antigenic types from a single cloned parasite showed that each genetically distinct parasite probably carried a range of PfEMP1 molecules (Biggs et al. 1991). Roberts et al. then showed that antigenic switching in a cloned line could be correlated with switching in the adhesion phenotype (Roberts et al. 1992). The major breakthrough came in the cloning of the genes responsible for antigenic variation by a variety of groups in 1995. Su et al. discovered the gene family encoding PfEMP1 molecules fortuitously while sequencing a region of the genome to which chloroquine resistance had been mapped by classical techniques. They named the gene family var\(^1\). Probes to var genes showed that there were from 50-150 different var genes present in the genomes of particular parasite isolates (Su et al. 1995). The work of collaborators showed that expression of different antigenic determinants was correlated with the expression of different members of the multigene family var (Smith et al. 1995). At the same time Baruch et al. identified the var genes by screening an expression library of the malaria parasite with serum raised against PfEMP1 (Baruch et al. 1995). The proteins are formed from common domains and consist of a variety of "Duffy binding-like" (DBL) domains with cysteine rich interdomain regions (CIDRs) on the extracellular surface, as well as a small transmembrane region and intercellular domain called the acidic terminal sequence (ATS) (Su et al. 1995; Smith

\(^1\) var was not the original name of these genes, due to the problems Su et al. had in sequencing them they were originally called gfhl genes an abbreviation of gene from hell (K. Deitsch personal communication)
et al. 2000)(Figure 1.6). The genes are made up of two exons, one for the extracellular and one for the intracellular regions of the genes. var genes were found to be largely sub-telomeric with some internal var genes too(Hernandez-Rivas et al. 1997; Gardner et al. 1998; Bowman et al. 1999). The malaria genome project enabled the sequencing of the entire range of var genes from a single parasite line (3D7), which had 59 var genes(Gardner et al. 2002). The var genes had 16 different arrangements of their constituent domains though 38 of them were of a single type. All var genes except 1 began with a DBL-α domain with certain preferred pairings of domains existing. Nonetheless 3D7 may have an unusual var gene repertoire as it can neither be selected for rosetting (J. A. Rowe, unpublished) or adhesion to ICAM-1 (Alistair Craig, personal communication), alternatively the repertoire of var genes may be normal and instead there may be a defect in the transport of proteins to the cell surface in this strain (C. Newbold, personal communication). Given that there is very little overlap between the var gene repertoires of different parasites there are a huge number of var genes in nature. Ward et al. sequenced a variety of DBL-1 sequencies from various isolates and showed that much of the diversity in this family was generated by recombination between conserved sequences(Ward et al. 1999). In addition to classical recombination this process may have been accelerated by ectopic recombination(Freitas-Junior et al. 2000): i.e. recombination between non-homologous chromosomes occurring at telomeric ends due to similarity between these sequences and the presence of elements that tether together telomeric ends(O'Donnell et al. 2002).
Though parasites have a large repertoire of var genes they only express one at a time (Chen et al. 1998; Scherf et al. 1998). They must also do this in a co-ordinated fashion to avoid expressing all epitopes at once and allowing the host to develop total protective immunity. Thus much work has gone into attempting to determine the elements controlling var gene expression. Voss et al. showed that different 5' flanking regions exist in upstream of var genes in subtelomeric and internal locations and that these are bound to by different transcription factors (Voss et al. 2000; Gardner et al. 2002; Voss et al. 2003). Most interestingly Deitsch et al. identified a role for the var gene intron in transcriptional silencing in cooperation with the promoter, which occurred in a cell cycle dependant manner (Deitsch et al. 2001). Some groups have suggested that many var gene transcripts are expressed but only one is translated (Chen et al. 1998), however closer analysis shows that only one var gene transcript is generally made to full length (Kyes et al. 2000). Nonetheless how the parasite expresses one var gene while repressing the remainder (especially given similarity between promoters) remains unclear.

1.4.3 Receptors and disease severity

*P. falciparum* binds to a range of host endothelial molecules. The current list is:

- Thrombospondin (Roberts et al. 1985),
- CD36 (Oquendo et al. 1989),
- Intercellular adhesion molecule-1 (ICAM1) (Berendt et al. 1989),
- Chondroitin sulphate A (CSA) (Rogerson et al. 1995),
- Vascular cell adhesion molecule-1 (VCAM-1), E-
selectin (Ockenhouse et al. 1992; Treutiger et al. 1997), CD31 (Treutiger et al. 1997) and heparan sulphate (Vogt et al. 2003). Of these the most important quantitatively (and well studied) are CD36 and ICAM1: All isolates from children bind to CD36 usually at high frequency (Newbold et al. 1997). Second in both prevalence and binding strength is binding to ICAM-1 (Newbold et al. 1997; Rogerson et al. 1999). In addition to endothelial binding, *P. falciparum* infected red blood cells can bind to other red blood cells (rosettes) through CR1 (Rowe et al. 1997), platelets through CD36 (Pain et al. 2001) and to the syncytiotrophoblast cells of the placenta through CSA (Fried et al. 1996; Achur et al. 2000) to cause placental malaria (see section 1.4.4).
Figure 1.7 Adhesion receptors and PfEMP1 binding specificity. A theoretical PfEMP1 structure is shown. The molecule is made up of discrete Duffy binding like (DBL) domains numbered according to their position on the molecule. Greek letters refer to the type of DBL domain according to the classification of Smith et al. In between DBL domains are cysteine-rich interdomain regions (CIDRs). At the end of the molecule is a transmembrane region followed by the acidic terminal sequence (ATS). The location of various receptor-binding sites has been determined which is shown, though it is unlikely that a single molecule would possess all these binding specificities.

Which if any of these cytoadhesion processes are involved in disease pathology? Rosetting is the only cytoadhesion phenotype to be consistently associated with disease severity (discussed below - section 1.5). Much interest recently has been focused on the involvement of platelet adhesion in severe disease. Infected erythrocytes can bind to CD36 on platelets, which cause the parasitised cells to auto-agglutinate (Cooke et al. 1995; Pain et al. 2001). This process has been associated with disease severity (Roberts et
al. 2000; Pain et al. 2001). Other work has shown that platelets adhere to the microvasculature in cerebral malaria (Wassmer et al. 2003). In mouse models inhibition of this process by knock-out of some of the main receptors for platelet adhesion (P-selectin and ICAM-1) has led to a decrease in the pathogenesis of murine cerebral malaria (Chang et al. 2003; Li et al. 2003; Sun et al. 2003) though this is not necessarily relevant to human malaria. Endothelial binding has been less consistently associated with disease severity. Though as sequestration is a universal feature of *P. falciparum* infections (present in asymptomatic, mild and severe infections) this is, perhaps not surprising. Endothelial cytoadherence has been hypothesised to cause severe disease by microvascular obstruction, restricting blood and oxygen flow. Association studies have shown that adhesion to the endothelium *per-se* is not associated with severe malaria under static (Newbold et al. 1997; Rogerson et al. 1999) or flow conditions (Cooke et al. 1993; Cooke et al. 1995). Other studies have focussed on looking at adhesion to particular receptors (Newbold et al. 1997; Rogerson et al. 1999; Heddini et al. 2001). Rogerson et al. report no association between adhesion and disease severity, surprisingly severe disease was associated with reduced binding to CD36 and ICAM-1. Anaemia in particular was associated with low levels of adhesion, with high haematocrit being associated with increased binding. Newbold et al. find no difference in adhesion prevalence to different receptors between cerebral malaria, severe anaemia, mild malaria and asymptomatic infections. They do however report that there is an increase in the strength of binding to ICAM-1 in cerebral malaria patients. These *ex-vivo* studies are hampered by only looking at one side of the adhesion process. In severe disease there
may not be more parasites capable of binding to a given receptor, but that certain receptors are up-regulated in severe disease. Autopsy experiments have been use to demonstrate the role of adhesion in cerebral malaria (MacPherson et al. 1985) and particularly adhesion to ICAM-1 (Turner et al. 1994). Heddini et al. argue that multiple adhesion phenotypes are associated with disease severity, however if rosetting and immunoglobulin binding (which correlates with rosetting) were removed from the analysis the results would not be significant. Human genetics gives little support for a role for adhesion in causing severe malaria either. A polymorphism in CD36 coding for a stop codon and thereby truncating the protein has been implicated as a risk factor in The Gambia and Kenya (Aitman et al. 2000) though in Kenya this is contradicted (Pain et al. 2001). Similarly an ICAM-1 polymorphism associated with low binding is associated with malaria susceptibility (Fernandez-Reyes et al. 1997) in one study, protection in another (Kun et al. 1999) has no effect in another (Bellamy et al. 1998).

Perhaps it is time to reassess the paradigms of adhesion and disease severity. A simple equation of cytoadhesion = severe disease is clearly insufficient. Instead certain binding phenotypes are associated with severe disease. Binding to CD36 is probably a benign event, though it is probably quantitatively the most important receptor for adhesion, it is not expressed in the brain so is unlikely to be involved in cerebral malaria. Moreover if all CD36 binding parasites were lethal, then P. falciparum would be far too virulent a parasite to be evolutionarily successful. Some evidence from Papua New Guinea supports this hypothesis. Around 10% of individuals in Papua New Guinea have ovalocytes in
their blood due to Southeast Asian ovalocytosis; these individuals are completely protected from cerebral malaria but not anaemia (Genton et al. 1995; Allen et al. 1999).

Infected ovalocytes bind 2 times better to CD36 under flow conditions than normal infected erythrocytes (Alfred Cortes personal communication). "Bad" forms of adhesion may include rosetting, ICAM-1 binding (Newbold et al. 1997) and binding to CD36 on platelets. There is a large element of host involvement in this: the inflammatory response to disease can result in the upregulation of parasite receptors, for example TNF-α can cause the upregulation of ICAM-1 in the brain (Turner et al. 1994).

1.4.4 Placental malaria

Placental malaria represents a special case of Plasmodium falciparum adhesion. It is also some of the strongest evidence for the role of cytoadhesion in malaria pathogenesis. During pregnancy women are at risk of maternal malaria. Maternal malaria is a distinct clinical syndrome from childhood malaria and is associated with premature delivery, interuterine growth retardation and prenatal mortality in the child (especially in endemic areas), and anaemia and death in the mother (more common in non-immune women) (McGregor 1987; Beeson et al. 2001). The risk is greatest in the first and second pregnancies, but less so in multigravid women (McGregor 1984). This suggests (i) that global immunosuppression associated with pregnancy is not the mechanism and (ii) that
immunity is acquired with exposure to a distinct parasite type that causes placental malaria.

In pregnant women with malaria, the placenta is often a site of heavy sequestration of parasites, though the periphery may be free even of young, ring-stage parasites (Walter et al. 1982; Brabin 1983). It was shown that parasites from the placenta bind preferentially to CSA rather than any other matrix proteins or endothelial receptors (Fried et al. 1996). The site of binding to CSA was found to be the syncitial trophoblasts (Achur et al. 2000). The ligand for CSA binding was subsequently found to be the duffy-γ domain of PfEMP1 (Buffet et al. 1999; Reeder et al. 1999) which was hard to reconcile with observations that sera from multigravid women in one region of the world were able to prevent binding to CSA of placental isolates from other parts of the world suggesting a high degree of conservation in the CSA ligand (Fried et al. 1998). Rowe et al. identified two conserved var genes that bound to CSA, at least one of which was found expressed in placental isolates (Rowe et al. 2002). They suggest that certain var genes may be conserved as a result of selection acting on a particular gene with an important function or that some var genes have a different orientation relative to the telomere and are therefore unlikely to undergo normal or ectopic recombination.

Some observations are challenging the emerging orthodoxy on CSA binding and placental malaria (Beeson et al. 2002). One group has reported adhesion of ring stages to a limited range of endothelial cells (Pouvelle et al. 2000). This adhesion was only
observed in isolates that matured to express CSA binding \textit{var} genes. It suggests a mechanism for the "cryptic" circulation of placental parasites which have distinct phenotypes from the parasites found in the periphery of women with maternal malaria. It is unclear what the ligands or receptors for this adhesion phenomenon are. Binding of immunoglobulins may also be important in placental malaria, IgG was found to be bound to many parasites from placental isolates which failed to bind CSA (Flick \textit{et al.} 2001). This was not entirely matched by work with a laboratory strain, TM284S2 a rosetting parasite, which apparently also bound non-immune IgG. It is unclear though whether the IgG bound to the placental isolates was non-immune or parasite specific, moreover the work with TM284S2 has been impossible to reproduce (Creasey \textit{et al.} 2003). Instead it appears that CSA binding parasites bind non-immune IgM (Creasey \textit{et al.} 2003). An unresolved paradox is that IgM binding is also important in rosette formation and yet placental malaria and rosetting are almost mutually exclusive phenomena (Rogerson \textit{et al.} 2000). It was the hope of many researchers that an understanding of the apparently simpler syndrome of maternal malaria (one ligand, one receptor) would represent "trial run" in trying to fight childhood malaria. That "simplicity" may turn out to be an illusion based on not enough data rather than any biological effect.
1.5 Rosetting

1.5.1 Rosetting and the pathology of severe disease

While disease associations between adhesion and syndromes of severe malaria have been inconsistent and suggest a more complex pattern of disease than originally hypothesised, one adhesion phenotype that has been consistently associated with severe disease is that of rosetting. Early studies in the Gambia (Carlson et al. 1990; Treutiger et al. 1992) and Kenya (Rowe et al. 1995) reporting this association have been confirmed in a various other African sites (Ringwald et al. 1993; Newbold et al. 1997; Kun et al. 1998; Heddini et al. 2001; Pain et al. 2001; Rowe et al. 2002). Out of the African studies only one study in Malawi has found no association between rosetting and disease severity (Rogerson et al. 1999). In Southeast Asia and Melanesia the picture appears to be different with two large (>100 isolates studied) showing no association between severe malaria (al-Yaman et al. 1995; Angkasekwinai et al. 1998) and a couple of smaller studies giving conflicting results (Ho et al. 1991; Udomsangpetch et al. 1996). In their discussion to the study in Malawi, Rogerson et al. suggest that the lack of correlation between rosetting and disease severity could be because of host factors, such as receptor polymorphisms, a hypothesis that we test here. Associations have been made with both cerebral malaria and severe anaemia which suggests at least two potential mechanisms by which malaria may cause severe disease, firstly rosettes may be implicated in microvascular obstruction, blocking
blood flow and oxygen to the brain (Kaul et al. 1991) or they may potentiate invasion and enhance parasite multiplication rates though in vitro experiments have found no evidence of this (Clough et al. 1998). However this work was carried out in the absence of immune pressure and so may not be a good model of rosetting in semi-immune children. Nonetheless it remains unclear if rosetting is a cause or merely a correlate of severe disease. Some work argues that rosetting could perhaps be a marker for some other adhesion process (Fernandez et al. 1998), while other work hypothesises that rosetting isolates could enhance TNF-α release from macrophages (O'Dea et al. 2003).
Table 1.3: Rosetting and severe malaria worldwide

<table>
<thead>
<tr>
<th>Reference</th>
<th>Site</th>
<th>Mean* or median†</th>
<th>P value</th>
<th>n (severe)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rosetting</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe</td>
<td>Mild</td>
<td></td>
</tr>
<tr>
<td><strong>African studies:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carlson <em>et al.</em> 1990</td>
<td>The Gambia</td>
<td>35%*</td>
<td>17%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treutiger <em>et al.</em> 1992</td>
<td>The Gambia</td>
<td>28.3%*</td>
<td>8.5%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ringwald <em>et al.</em> 1993</td>
<td>Madagascar</td>
<td>30.5%†</td>
<td>5%</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Rowe <em>et al.</em> 1995</td>
<td>Kenya</td>
<td>7%†</td>
<td>1%</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Newbold <em>et al.</em> 1997</td>
<td>Kenya</td>
<td>9.5%*</td>
<td>5.4%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Kun <em>et al.</em> 1998</td>
<td>Gabon</td>
<td>16%*</td>
<td>8%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rogerson <em>et al.</em> 1999</td>
<td>Malawi</td>
<td>15%*</td>
<td>14.6%</td>
<td>NS</td>
</tr>
<tr>
<td>Pain <em>et al.</em> 2001</td>
<td>Kenya</td>
<td>6.5%†</td>
<td>4.1%</td>
<td>=0.02</td>
</tr>
<tr>
<td>Heddini <em>et al.</em> 2001</td>
<td>Kenya</td>
<td>21.4%*</td>
<td>12.9%</td>
<td>=0.01</td>
</tr>
<tr>
<td>Rowe <em>et al.</em> 2002</td>
<td>Kenya</td>
<td>14%†</td>
<td>3.5%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rowe <em>et al.</em> (unpublished)</td>
<td>Mali</td>
<td>12%†</td>
<td>1%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Southeast Asian and Melanesian studies‡</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ho <em>et al.</em> 1991</td>
<td>Thailand</td>
<td>18.3%† (9%)</td>
<td>10.7%</td>
<td>NS</td>
</tr>
<tr>
<td>al-Yaman <em>et al.</em> 1995</td>
<td>PNG</td>
<td>9%*</td>
<td>8.6%</td>
<td>NS</td>
</tr>
<tr>
<td>Udonmsangpetch <em>et al.</em></td>
<td>Thailand</td>
<td>20%† (8%)</td>
<td>5%</td>
<td>=0.02</td>
</tr>
<tr>
<td>1996</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angkasekwinai <em>et al.</em> 1998</td>
<td>Thailand</td>
<td>11.1%* (5.1%)</td>
<td>4.1%</td>
<td>NS</td>
</tr>
</tbody>
</table>

† For studies in Southeast Asia values for severe malaria studies are given mean or median for cerebral malaria with the mean or median for other severe malaria in brackets.
1.5.2 Physiology of rosettes

By standard methods rosettes are counted in the laboratory as the proportion of mature stage infected red blood cells binding to two or more other red blood cells. When parasites are cultivated from infected individuals it is necessary to grow the parasites to maturity as the vast majority of parasites in the peripheral circulation will be immature, ring-stage trophozoites, the later forms having sequestered in deep microvascular beds (see section 1.4). One important question this raises is whether the rosettes observed in static culture are physiologically significant and capable of obstructing blood flow in the microvasculature. Kaul et al. (Kaul et al. 1991) tested this hypothesis directly using an ex-vivo preparation of rat mesocceum vasculature through which they perfused rosetting and non-rosetting Malayan Camp parasites. Perfusion through tissues did not lead to the disruption of rosettes. They were also able to show rosette formation in the venular circulation but not on the arterial side, moreover there was higher peripheral resistance (ie. a slowing of blood flow) when tissues were perfused with the rosetting rather than the non-rosetting line. Other experiments have looked measured the forces required to directly detach red cells from rosettes. The direct detachment forces required to pull rosettes are apart are around $4.4 \times 10^{-10}$ N - around 5 times the force required to detach iRBC from the surface of endothelium (Nash et al. 1992). The types of forces generated in the circulation tend to be of high sheer caused by friction between the blood vessel walls and the blood flow. Therefore a variety of experiments have been performed which show that not only can rosettes withstand high sheer stresses associated with the arterial
circulation (around 1.4 to 1.6 Pa) (Nash et al. 1992; Chotivanich et al. 2000), but also that they can form in the conditions found in the circulation (Chu et al. 1997; Chotivanich et al. 2000).

1.5.3 Ligands and receptors for rosetting

Rosetting like other parasite adhesion phenotypes is mediated by PfEMP1 (Rowe et al. 1997; Chen et al. 1998), and a variety of uninfected RBC surface receptors including complement receptor 1 (Rowe et al. 1997), CD36 (Handunnetti et al. 1992), ABO blood group sugars (Carlson et al. 1992; Barragan et al. 2000), heparan sulphate-like molecules (Barragan et al. 2000) and serum factors (Scholander et al. 1996; Somner et al. 2000).

Early on it was identified that rosettes were sensitive to disruption by heparin, along with many other cell-cell interactions (Carlson et al. 1990). Subsequent work showed that a family of sulphated glycoconjugate molecules shared this ability (Rowe et al. 1994). However there remains little evidence that these glycoconjugates are present on the red cell surface, Immunofluorescence assays (IFAs) and western blots of the red blood cell surface have failed to reveal these molecules (J. A. Rowe unpublished). The only direct evidence we have for the involvement of GAGs in rosetting is that enzyme digestion of red cells with heparinases reduces their ability to rosette (Barragan et al. 2000), though we cannot rule out the possibility that heparinase is cross reacting on other molecules with
similar sugar units which are the true receptor. It has also been shown that PfEMP involved in rosetting is bound to by GAGs explaining the basis of their ability to disrupt rosettes (Chen et al. 1998). Strain specific preferences among rosetting isolates for particular blood group types was also noted early on with A and B blood group erythrocytes forming larger rosettes than O erythrocytes (Carlson et al. 1992). The role of blood group A was confirmed by the demonstration that A sugars bind to infected red blood cells and can disrupt rosettes albeit at high concentrations (Barragan et al. 2000). A number of field studies have shown that the A blood group may be a risk factor for severe disease which could be explained by this effect on rosetting (Hill et al. 1991; Fischer et al. 1998; Lell et al. 1999).

CD36 was also suggested early on as a rosetting receptor after it had been identified as an endothelial receptor (Oquendo et al. 1989). Adherent lines of PfEMP1 were found to rosette in a manner that could be reversed with anti-CD36 antibodies (Handunnetti et al. 1992). However CD36 is only present in low amounts on the surface of red blood cells (van Schravendijk et al. 1992) while anti CD36 antibodies do not reverse rosetting in field isolates (Rowe et al. 2000).

Electron micrographs from autopsy samples from cerebral malaria patients showed rosettes held together by dense fibrillar strands (Scholander et al. 1996). A similar observation had been seen in binding to endothelial cells previously (MacPherson et al. 1985). Two rosetting laboratory strains TM284+ and PAR+ were subsequently shown to
also have these fibrils. On TM284+ these were associated with knobs while on PAR+, which is a knobless parasite the fibrils are less dense. Antibodies to human immunoglobulins were found by immunogold transmission electron microscopy to bind to these strands. The involvement of immunoglobulin M (IgM) was confirmed by some painstaking work in which whole serum fractionated by a variety of means and the fractions capable of supporting rosetting were identified. In addition to IgM at least two other, unidentified, components were found to be involved in rosette formation (Somner et al. 2000). IgM binding has since been shown to be involved in rosetting in field isolates and associated with disease severity (Rowe et al. 2002). IgM binding has been associated with the DBL2β (Flick et al. 2001) and CIDR1α (Chen et al. 2000) domains of PfEMP1, however this domain specificity has been impossible to reproduce in our laboratory. However IgM does bind to PfEMP1 on western blots of native proteins (J.A. Rowe and J-P Semblat unpublished data) supporting the notion that this too is a PfEMP1 mediated adhesion phenotype.

CR1 may be the most important rosetting receptor. The DBL domains of PfEMP1 from a strongly rosetting line: R29+ were cloned and expressed in COS cells. The DBL-1 domain was found to bind red cells. A subsequent screen of red cell types showed that red cells deficient in CR1 showed reduced rosetting (Rowe et al. 1997). Subsequent work mapped the PfEMP-1 binding site to C3b and C4b binding domains on CR1 (see section 1.6.2) and showed that specific antibodies could reverse rosetting in laboratory strains and most field isolates (Rowe et al. 2000). Moreover the existence of polymorphisms in
the CR1 gene that are prevalent in African (Rowe et al. 1997; Zimmerman et al. 2003) and Papua New Guinean populations (this study) suggests that while rosetting may be a degenerate process, prevention of CR1-mediated rosetting may protect against severe disease.

Is PfEMP1 the only rosetting ligand? PfEMP1 is highly trypsin sensitive yet a subset of rosettes show a degree of resistance to disruption at high trypsin concentrations (Kyes et al. 1999). Early on a screen of antibodies to then-known parasite membrane associated proteins showed that monoclonal antibody (mAb) specific for histidine rich protein (PfHRP) disrupted rosettes (Carlson et al. 1990). However these experiments were performed in PAR+ parasites, a strain that does not express PfHRP (which means that it is Knob-), instead the mAb appeared to be cross-reacting with a 28kDa parasite derived protein. This protein in turn was found to be related to a 22kDa protein which could also apparently mediate rosetting as antibodies raised against this protein could disrupt rosttes (Helmby et al. 1993). Members of this family of small proteins were named "rosettins" though little subsequent work was done on this until Kyes et al. described the rifin family of clonally variant parasite antigens which match "rosettins" in their trypsin resistance, approximate size and correlation with "trypsin resistant" rosetting (Kyes et al. 1999).
1.6 Complement receptor 1

Complement receptor 1 (CR1, also known as CD35) has been implicated as receptor for rosetting. Nonetheless, as stated above it remains unclear if rosetting is a cause or correlate of parasite virulence. The presence of a variety of polymorphisms in this gene (described below) suggests that it has been subject to selective pressure. We hypothesise that some of these polymorphisms may have been caused by immune selection as a result of a malaria. If this were true it would be strong evidence that rosetting is a parasite virulence factor and therefore a target for anti-disease intervention. Here I describe the normal and malaria related functions of CR1 and the polymorphisms present in the gene.

1.6.1 Functions of CR1

CR1 is an immune regulatory protein expressed on the surface of erythrocytes, leukocytes, macrophages, glomerular podocytes and follicular dendritic cells (Ahearn et al. 1989). Complement is an effector system of the humoral immune response (reviewed in Janeway et al. 1999); CR1 can bind to certain complement components and modify their activation or be involved in their uptake by phagocytic cells. The classical complement pathway and the role of CR1 are outlined in figure 1.8. Complement components exist in inert forms in the bloodstream until they are altered by proteolytic cleavage by various proteases that are stimulated by the binding of antibody to antigen.
and its subsequent conformational change (Figure 1.8 a). The end products of the complement pathway may act in a variety of ways. Firstly a foreign cell surface may become opsonised or coated in C3b fragments, it may then be taken up by complement receptors that can bind C3b such as CR1 (Figure 1.8 b). Secondly small fragments (C3a, C4a) formed as products of the cleavage of major early complement components (C3 and C4) may stimulate inflammation (Figure 1.8 a and b). Thirdly a membrane attack complex (MAC) consisting of the terminal complement components (C5b, C6, C7, C8 and C9) may be precipitated lysing the cell it forms on (Figure 1.8 c). An alternative pathway of complement exists in which the same cascade is initiated by spontaneous C3b binding to pathogen surfaces. The C3b can then bind a protein B (analogous to C2) which is cleaved by a protease D to form Bb. C3b-Bb complexes are active C3 convertases. This C3 convertase acts in same way as the C4b-C2b-C3b complex and from then the same downstream events as in the classical pathway can proceed (as in figure 18 b,c and e). CR1 is involved in this process in a variety of ways. As the complement system can potentially damage host cells as well as those of non-self components, it is important that host cells are protected from damage. An important role of CR1 is to control the activation of complement. CR1 can bind to C4b and displace C2b from the C3 convertase (decay accelerating activity, DAA). Once bound to C4b CR1 can act as a cofactor for factor I — a protease that breaks down some of the enzymes of complement activation — and enhance the breakdown of C4b (CA- cofactor activity) blocking the complement pathway (Figure 1.8 d)(Iida et al. 1981). In an analogous process CR1 may displace C3b from the C5 convertase and act as a cofactor for it's decay by factor I (Figure 1.8 e). On
red blood cells this complement control activity may not be the principal function of CR1 as decay accelerating factor (DAF) is present in much larger numbers and can perform a similar role (Figure 1.8 d). Instead the main role of CR1 on erythrocyte may be the clearance of immune complexes. Immune complexes are formed when antibody bound to antigen is directly opsonised by C3b fragments (Schifferli et al. 1989). In this case the complexes may bind to CR1 on the surface of RBC and be carried in the circulation to the liver where the immune complex will be cleared from the blood stream (Cornacoff et al. 1983; Ng et al. 1988; Schifferli et al. 1988). CR1 on B cells has also been implicated in giving a downregulatory signal to these cells (Jozsi et al. 2002). CR1 may be involved in malaria pathogenesis through a mechanism other than by acting as a receptor for rosetting. Erythrocyte CR1 levels are reduced in severe malarial anaemia along with other complement control proteins such as decay accelerating factor (DAF; CD55). The authors suggest that this could lead to red cell lysis by complement as part of the pathogenesis of severe malaria (Waitumbi et al. 2000; Stoute et al. 2003).
Chapter 1: Introduction

Figure 1.8

a. C4b-C2b (C3 convertase)

b. C3, C3b, C3a

c. C5, C5b

C4b-C2b-C3b (C5 convertase)

formation of membrane attack complex (cell lysis)

opsonisation (leading to uptake by phagocytosis)

C5a

C1, C2, C2a, C4a, C4b, C3b

antibody

antigen

(C3 convertase)

C3b formation of (C5 convertase)

membrane attack complex (cell lysis)

D55 factor I

CR1

factor I

DAF (CD55)

C4b-C2b

C4d, C4c, C3d, C3c
Figure 1.8: The classical complement pathway and its control by CR1: a the classical pathway is initiated when antigen is bound to antibody which fixes the large molecule C1. C1 has a protease domain C1s, which cleaves inert C4 and C2 to 4b and C2b respectively. The other products of cleavage are C2a and C4a, which are soluble inflammatory mediators. b C4b-C2b has C3 convertase activity - it can convert inert C3 to C3b which is either remains bound to the C3 convertase or is deposited on the cell's surface (opsonisation). C3b on the cell surface may be bound to by complement receptor 1 and lead to the uptake of the opsonised cell. c Alternatively the C3b-C4b-C2b complex formed may act as a C5 convertase changing inert soluble C5 to C5b which can fix the terminal components of complement (C5-C9) to form a membrane attack complex which forms pores in the surface of the cell. d CR1 is involved in this process in a number of ways, either it can displace C2b from the C3 convertase and act as a cofactor for factor I which may breakdown C4b (the role of CR1-factor I may also be performed by decay accelerating factor (DAF) or e CR1 with factor I may displace C4b and C2b from the C5 convertase and act with the protease I to breakdown C3b.
1.6.2 Structure of CR1

The predominant form of CR1 (figure 1.9), CR1*1 is a 190kDa protein consisting of 30 complement control protein (CCP) repeats, arranged into four blocks of seven CCPs, called long homologous repeats (LHRs) designated A to D and each homologous to each other (Klickstein et al. 1987) (Figure 1.9). There are three C4b binding sites in the first three CCPs of each LHR (Krych-Goldberg et al. 2001). The site on LHR-A is also the location of the decay accelerating activity of CR1. The C4b binding sites on LHRs B and C show >90% homology to each other and also bind to C3b (Vik et al. 1993) and have the cofactor activity for factor I. Recently Smith et al. determined the solution structure of CCPs 15-17 (residues 901-1095) which corresponds to the C3b/C4b binding domain. They showed that the repeats were in an extended head to tail arrangement with flexibility at the module 16-17 junction. They also showed a positively charged region on CCP 15 which is critical for C4b binding (Smith et al. 2002). The CR1 gene is linked to a variety of proteins such as decay accelerating factor (DAF; CD55), complement receptor 2 (CR2; C21) membrane cofactor protein (MCP; CD46) and C4b binding protein (C4BP) at a locus called the regulators of complement activation locus (RCA) at chromosome 1 band q32.

Western blot analyses revealed over time that there were 4 size variants of the CR1 gene. Subsequently it was determined that the structural polymorphisms (which varied in 30kDa size increments) had additions or deletions of LHR units (Table 1.4) (Dykman et
al. 1983; Dykman et al. 1984; Dykman et al. 1985). The size polymorphisms appear to have arisen as a result of unequal crossing over events between homologous regions of the gene at meiosis (Wong et al. 1989; Wong et al. 1991).

Table 1.4: Size variants of the CR1 molecule (after (Krych-Goldberg et al. 2001))

<table>
<thead>
<tr>
<th>Variant</th>
<th>CR1 size (kDa)*</th>
<th>CR1 size (kDa)†</th>
<th>No. of LHRs</th>
<th>Frequency (Caucasians)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1*1</td>
<td>190</td>
<td>220</td>
<td>4</td>
<td>0.83</td>
</tr>
<tr>
<td>CR1*2</td>
<td>220</td>
<td>250</td>
<td>5</td>
<td>0.15</td>
</tr>
<tr>
<td>CR1*3</td>
<td>160</td>
<td>190</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>CR1*4</td>
<td>250</td>
<td>280</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Under non-reducing conditions
† Under reducing conditions

The frequency of different size allotypes varies between population. Of interest is the fractionally increased frequency of the CR1*3 allele in some African populations (Table 1.5) which lack a C3b and PfEMP1 binding site.
Table 1.5 Global distribution of CR1 size polymorphisms (from Cohen et al. 1999)

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>n</th>
<th>CR1*1 (190)</th>
<th>CR1*2 (220)</th>
<th>CR1*3 (160)</th>
<th>CR1*4 (250)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black - USA</td>
<td>715</td>
<td>0.83</td>
<td>0.13</td>
<td>0.03</td>
<td>0.003</td>
</tr>
<tr>
<td>Black - Mali</td>
<td>87</td>
<td>0.79</td>
<td>0.14</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Caucasian - USA</td>
<td>426</td>
<td>0.86</td>
<td>0.11</td>
<td>0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>Chinese / Taiwanese</td>
<td>112</td>
<td>0.95</td>
<td>0.04</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Choctaw Indian</td>
<td>220</td>
<td>0.97</td>
<td>0.01</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>French</td>
<td>106</td>
<td>0.87</td>
<td>0.13</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Italian</td>
<td>71</td>
<td>0.84</td>
<td>0.15</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>Mexican</td>
<td>203</td>
<td>0.88</td>
<td>0.1</td>
<td>0.01</td>
<td>0.005</td>
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<tr>
<td>Peruvian</td>
<td>169</td>
<td>0.94</td>
<td>0.05</td>
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<td>0.003</td>
</tr>
<tr>
<td>Spaniard</td>
<td>22</td>
<td>0.8</td>
<td>0.18</td>
<td>0.02</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 1.9

- LHR-A
- LHR-B
- LHR-C
- LHR-D

Alternative deletions for CR1*3 allele

Single nucleotide polymorphisms

Ex22  Int27  Sla/Kn  Ex33

Location of epitopes recognised by antibodies used for flow cytometry and Western blotting and rosette disruption

J3B11
E11
J3D3

CCP involved in binding of C4b and decay accelerating activity

CCP involved in C3b binding and with cofactor activity for protease I
1.6.3 Expression level polymorphisms

The level of CR1 on erythrocytes is determined by a number of genetic and environmental factors (Wilson et al. 1982; Ross et al. 1985; Wilson et al. 1986). In Caucasians CR1 expression level varies between 100 and 1000 per RBC. Expression on RBC (but not the other cells expressing CR1) is controlled by two alleles associated with high (H) and low (L) expression of CR1, which are co-dominant and linked to a HindIII RFLP in intron 27 of the CR1 gene (Wilson et al. 1982; Wilson et al. 1986). Subsequent research has shown that the HindIII RFLP is linked to around nine other polymorphisms which form two distinct haplotypes again associated with high and low levels of CR1 in Caucasians (Herrera et al. 1998; Xiang et al. 1999). In the L haplotype two of the polymorphisms replace other amino acids with arginine which may make the protein more susceptible to proteolytic cleavage and explain why this haplotype is associated with low copy number of CR1 (Herrera et al. 1998; Xiang et al. 1999), accordingly we studied these polymorphisms as well. The particular polymorphisms under investigation in this study are described in table 1.6. The same work also showed that these haplotypes exist in African Americans too, however in African Americans CR1 copy number is not related to these haplotypes. This is important as it has been reported in the literature that the L allele is not protective against severe malaria based on samples from a case-control study in the Gambia (Bellamy et al. 1998) which were typed for this polymorphism. However the lack of an association between CR1 expression and H and L haplotypes in African populations suggests that this study has no functional significance (Herrera et al...
Conversely a study on Thai adults showed that in this population the L allele is present at a prevalence of 0.52 and is associated with CR1 expression (Nagayasu et al. 2001). They report in a case-control study that the LL genotype is a risk factor for severe malaria. They do not report, though, that their data also shows that the heterozygous state is associated with protection from severe disease. Two caveats of this study are its sample size (55 severe cases) and the fact that rosetting is not associated with disease severity in this population suggesting different mechanisms for disease severity in this population, consistent with the different pattern of disease in Southeast Asia compared to Africa.

**Table 1.6 SNPs in the CR1 gene linked to red cell CR1 expression level**

<table>
<thead>
<tr>
<th>Red cell phenotype*</th>
<th>exon 22</th>
<th>intron 27</th>
<th>exon 33</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nucleotide</td>
<td>amino acid</td>
<td>Size of genomic DNA RFLP</td>
</tr>
<tr>
<td>High (H) CR1 expression</td>
<td>A3650</td>
<td>His1208</td>
<td>7.4 kb</td>
</tr>
<tr>
<td>Low (L) CR1 expression</td>
<td>G3650</td>
<td>Arg1208</td>
<td>6.9 kb</td>
</tr>
</tbody>
</table>

* Typical in all populations studied to date except those of African origin (Herrera et al. 1998; Rowe et al. 2002)
1.6.4 Knops blood groups types

The Knops blood group types were mapped to CR1 in 1991 (Moulds et al. 1991). The Knops blood group antigens are not important for transfusion medicine and were not formerly associated with any disease. However they have been a focus of interest since it was discovered that certain Knops phenotypes were associated with reduced rosetting in vitro (Rowe et al. 1997). There are at least 3 allelic pairs in the blood group system Sl 1 and Sl 2, McCo and Mccb, and Kna and Knb corresponding to the blood types Sl:1 and Sl:2, McC(a+) and McC(b+) and Kn(a+) and Kn(b+). The situation is further complicated by another phenotype caused by low expression of CR1 such that none of the antigens can be detected by serotyping techniques; this is known as "Helgeson" phenotype.

Rowe et al. initially identified CR1 as a potential rosetting receptor because Helgeson cells were found to show reduced rosetting with the R29+ parasite clone. Even before it was discovered that there was a link between Knops types and rosetting existed it had already been suggested that these blood group types might have a link to malaria pathogenesis on account of their difference in prevalence between Caucasian and African populations (Table 1.7)(Miller 1994). Two of the antigens Sl:2 and McC(b+) are common only in African populations and at least one of these Sl:2 has been shown to have reduced ability to bind to COS cells expressing the DBL-1 domain that mediates rosetting in R29+ parasites(Rowe et al. 1997). These two important polymorphic sites
have recently been attributed to non-synonymous mutations in the region coding for the
in the LHR-D region (Table 1.8) (Moulds et al. 2001).

Table 1.7 Allele frequencies of Knops group types in Malians and West
Africans (Moulds et al. 2000; Zimmerman et al. 2003)

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Allele</th>
<th>n</th>
<th>Sl2</th>
<th>McC^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mali</td>
<td></td>
<td>99</td>
<td>0.75</td>
<td>0.30</td>
</tr>
<tr>
<td>West Africa*</td>
<td></td>
<td>182</td>
<td>0.79</td>
<td>0.31</td>
</tr>
<tr>
<td>The Gambia</td>
<td></td>
<td>853</td>
<td>0.80</td>
<td>0.39</td>
</tr>
<tr>
<td>Caucasian American</td>
<td></td>
<td>100</td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>Asian American</td>
<td></td>
<td>99</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Hispanic American</td>
<td></td>
<td>100</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*West Africa includes Senegal, Guinea, Sierra Leone, Ivory Coast and Ghana

Genotyping typing of samples from a case-control study in the Gambia showed no
association between the frequency of the different Knops group alleles and severe
malaria (Zimmerman et al. 2003) however a separate study in Mali has found a significant
genotype-phenotype mismatch which makes these results difficult to interpret (J.A.
Rowe, personal communication). A prospective case-control study is ongoing to examine
rosetting and disease associations with Knops phenotypes in Mali (J.A. Rowe and J.M. Mould unpublished). There is very little data on the Knops blood group antigens in Papua New Guinea though an early report in the literature stated that though Helgeson phenotype was rare among Caucasians and Africans 28 out of 67 Melanesians were nulls (Molthan 1983). It was this suggestion that lead us to examine CR1 polymorphisms in Papua New Guinea more closely.

Table 1.8 The molecular basis of the knops blood group antigens (Moulds et al. 2001)

<table>
<thead>
<tr>
<th>Blood Group</th>
<th>Antigen</th>
<th>Allele</th>
<th>Amino Acid</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>McC(a+)</td>
<td>McCa</td>
<td>McCa</td>
<td>K1590</td>
<td>A4795</td>
</tr>
<tr>
<td>McC(b+)</td>
<td>McCb</td>
<td>McCb</td>
<td>E1590</td>
<td>G4795</td>
</tr>
<tr>
<td>Sl:1</td>
<td>Sl1</td>
<td>Sl1</td>
<td>R1601</td>
<td>A4828</td>
</tr>
<tr>
<td>Sl:2</td>
<td>Sl2</td>
<td>Sl2</td>
<td>G1601</td>
<td>G4828</td>
</tr>
</tbody>
</table>
1.7 Aims of this thesis

It is unclear whether rosetting is a correlate or cause of parasite virulence. As rosetting is mediated by CR1, we would expect malaria to select beneficial forms of this gene but only if rosetting is a direct cause of severe disease. We are interested in particular in Papua New Guinea as this is a malaria endemic area where an early report suggested that CR1 deficiency might be common as many individuals were of the Helgeson (CR1 deficient) phenotype (Molthan 1983). Preliminary studies showed that 8/15 samples from neighbouring Vanuatu were also CR1 deficient. Otherwise little is known about CR1 polymorphisms in Melanesia. The aims of this study are therefore to:

1. To optimise an assay for CR1 expression that can be used in the field, or on cells collected in the field and analysed later

2. To determine the prevalence of CR1 polymorphisms and CR1 deficiency in Papua New Guinea

3. To determine the genetic basis of CR1 deficiency in Papua New Guinea

4. To determine whether common CR1 polymorphisms protect against severe malaria in Papua New Guinea

5. To investigate the effect that CR1 polymorphisms have on rosetting in Papua New Guinea.
Chapter 2: Materials and Methods

Materials and Methods

Here are described the laboratory techniques used in this study. Details of designs for epidemiological studies are given in the appropriate results chapter. Methods are ordered approximately according to their appearance in the subsequent text. All reagents were from Sigma, Poole, U.K unless otherwise stated.
2.1 Determination of CR1 expression by flow cytometry (Cohen et al. 1987; Cockburn et al. 2002)

The mean erythrocyte CR1 expression level on freshly drawn blood samples was determined using a modification of a previously published method (Cohen et al. 1987). 50 µl of whole blood was washed three times in phosphate buffered saline (PBS - 0.01M phosphate buffer, 2.7 mM potassium chloride and 0.137M sodium chloride, PH7.4), supplemented with 4 % RPMI 1640 (Invitrogen, Paisley, UK) and 1 % non-immune AB serum (ABS) (Scottish Blood Transfusion Service, Edinburgh, UK) (PBS-RPMI-ABS). The cells were resuspended in 1 ml of PBS-RPMI-ABS and 100 µl of this suspension (i.e. approximately 2.5 µl of cells) was placed in a 96 well plate, spun at 1000 g for 1 minute and the supernatant removed. The cells were then incubated at 4 °C with 40 µl of 0.5 µg/ml of the CR1 monoclonal antibody (mAb) J3D3 (Immunotech, Marseille, France) in PBS-RPMI-ABS for one hour with occasional agitation. The cells were washed three times in PBS-RPMI-ABS and resuspended in 10 µg/ml of Alexa Fluor™ 488-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, the Netherlands) followed by incubation at 4 °C for one hour with occasional agitation. Alexa Fluor™ 488 is equivalent to fluorescein isothiocyanate (FITC) but is more stable and sensitive and less prone to photo-bleaching. Negative control samples were treated as above but without the primary antibody. After the secondary incubation, cells were washed 3 times in PBS-RPMI-ABS and resuspended in PBS-RPMI-ABS supplemented with 0.37 %
formaldehyde (BDH, Poole, UK), and analysed on a FacSCAN flow cytometer (Becton Dickinson, San Jose, CA). The mean fluorescence intensity of each sample, minus the mean fluorescence intensity of the negative control was determined. A standard curve was obtained by plotting the fluorescence intensities of blood samples from donors of previously determined CR1 number (initially determined by Scatchard analysis using $^{125}$I labelled mAb, (Cohen et al. 1987)), ranging from 200 to 1000 molecules per cell (Figure 2.1). The standard curve was used to read off the CR1 expression level of unknown samples as shown in Figure 2.1. Typically each sample would have its CR1 expression tested in triplicate, with new standard curves generated for each determination.
Figure 2.1: A standard curve used to determine the mean erythrocyte CR1 expression level of unknown samples. The standard curve was plotted with data from five standards of previously determined CR1 expression levels (O), with the point at the origin representing the negative control sample with no primary antibody. The mean fluorescence intensity of six unknown samples is read off the standard curve to determine the number of CR1 molecules per cell (dotted lines). The mean CR1 expression levels of the six unknown samples were therefore determined as 448, 727, 796, 814, 894 and 967 molecules per cell.
2.2 Preservation of erythrocytes for flow cytometry and parasite culture

2.2.1 Preservation of erythrocytes or *Plasmodium falciparum* cultures by freezing in glycerol

To prepare uninfected erythrocytes or erythrocytes infected with early ring-stage trophozoites the following protocol was used. 5 volumes of glycerolyte (42.25 % w/v glycerol, 0.1 M sodium lactate, 4 mM potassium chloride, 0.1 M sodium dihydrogen phosphate adjusted to pH 6.8) were added drop-wise to 3 volumes of erythrocytes. After the first volume had been added the cells were allowed to osmotically equilibrate for 5 minutes before the remainder of the solution was added. The samples were frozen overnight at −70 °C before cryopreservation in liquid nitrogen. To use the cells for subsequent experiments or parasite culture cryopreserved cells were thawed at 37 °C and 200 μl of 12 % NaCl solution was added slowly and drop-wise to each sample. The cells were allowed to osmotically equilibrate for 5 minutes before a further drop-wise addition of 10 ml of 1.8 % NaCl followed by 10ml of 0.9 % NaCl, 0.2 % glucose solution. The cells were washed in twice incomplete RPMI1640 (iRPMI; RPMI 1640 medium with sodium bicarbonate (Invitrogen, Paisley, UK), supplemented with 2 mM L-glutamine, 25 mM HEPES, 20 mM D-glucose and 25 μg/ml gentamicin).
2.2.2 Preservation in 5 % formaldehyde(Bianco et al. 1986)

Erythrocytes were first washed three times in iRPMI. 50 µl of whole blood was resuspended at 4 % haematocrit in complete RPMI-ABS (as incomplete RPMI but with the addition of 10 % ABS) and an equal volume of fixative solution (10 % w/v formaldehyde, 4 % w/v glucose in Tris-saline (10 mM Tris, 150 mM NaCl, 10 mM sodium azide adjusted to pH 7.3)) was added. Cells could then be left at 4 °C until needed. In order to be used for flow cytometry the cells were spun down and washed three times in incomplete RPMI prior to the primary antibody incubation.

2.2.3 Preservation in 0.25 % glutaraldehyde

Erythrocytes washed thrice in iRPMI were resuspended in 0.025 % (v/v) glutaraldehyde in PBS at 2 % haematocrit for 20 minutes before being washed in PBS and resuspended at 50 % haematocrit in PBS supplemented with 1% BSA. In order to be used for flow cytometry the cells were spun down and washed three times in incomplete RPMI prior to the primary antibody incubation.
2.3 Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) genotyping of samples

2.3.1 Basic polymerase chain reaction (PCR) procedure

Polymorphic sites in the CR1 gene were detected by amplifying the surrounding region by polymerase chain reactions (PCR) of genomic DNA and cleaving the product with a restriction enzyme, which is specific for a site altered by the polymorphism. α⁺-thalassaemia, Southeast Asian ovalocytosis and Gerbich blood grouping which are caused by deletions in the α-globin, band-3 and glycophorin C genes respectively were detected by PCR alone, with primers amplifying in or around the area of the deletion. All PCRs were performed using genomic DNA extracted from whole blood or white cells on the day of receipt (usually 1 day after drawing) using the BACC-2 nucleon test kit according to the manufacturers instructions (Amersham Pharmacia, Uppsala, Sweden). PCR reactions were performed on 100 ng to 1μg DNA in 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1 % Triton X-100), 1.5 - 2.5 mM MgCl₂ (depending on reaction see relevant section), 0.2 mM of each dNTP (Roche, Lewes, UK), 0.5 pmol/μl of each primer and either 0.1 U/μl Taq polymerase (Promega, Southampton, UK) for the Intron 27 PCR reaction or 0.04 U/μl Taq polymerase for the other PCR reactions. Total reaction volumes were 50 μl or 20 μl.
Table 2.1: PCR primers and conditions used; restriction endonucleases and fragment sizes for genotyping

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primers</th>
<th>$T_A$ (°C)</th>
<th>Restriction enzyme</th>
<th>Allele</th>
<th>Fragment Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon22</td>
<td>F: 5'-TTTACATTGGATAGGCCAGAGC-3' R: 5'-CCAGAGGTAAATCTCCCTCGGA-3'</td>
<td>63</td>
<td>Rsal</td>
<td>H</td>
<td>520,162</td>
</tr>
<tr>
<td>Intron27</td>
<td>F: 5'-CAGCAGGAGGCCCAACTTCCTGACC-3' R: 5'-CCCTTGTAAAGGGAAGTCTG-3'</td>
<td>54</td>
<td>HindIII</td>
<td>H</td>
<td>1600,84</td>
</tr>
<tr>
<td>Exon33</td>
<td>F: 5'-AAGCGACAGTTCAGGACGT-3' R: 5'-GAACAGAAAAGTTCACAGCGAGG-3'</td>
<td>59</td>
<td>Mnl</td>
<td>H</td>
<td>111,80,70,33,11</td>
</tr>
<tr>
<td>Knops blood groups</td>
<td>F: 5'-TGAGCCACCTCCAACCATAT-3' R: 5'-TGAGCCBGTGTTACAGCTTG-3'</td>
<td>55</td>
<td>Mfl</td>
<td>SI 1</td>
<td>386</td>
</tr>
<tr>
<td>a-thalassemia $\alpha$2/3.7: F: 5'-CCCCTCGCAAGTCCACCC-3' R: 5'-AAAGCACTCTAGGGTCCAGG-3'</td>
<td>60</td>
<td>N/A</td>
<td>normal</td>
<td>1800</td>
<td></td>
</tr>
<tr>
<td>3.7/20.5R: F: 5'-AAAGCACTCTAGGGTCCAGG-3' R: 5'-AGACAGGAAGGCGCTG-3'</td>
<td></td>
<td></td>
<td>-$\alpha_2$ mutation</td>
<td>1628</td>
<td></td>
</tr>
<tr>
<td>a2-R: 5'-AGACAGGAAGGCGCTG-3'</td>
<td></td>
<td></td>
<td>-$\alpha_3$ mutation</td>
<td>2022/2029</td>
<td></td>
</tr>
<tr>
<td>4.2F: 5'-GGTTTACCATGTGTGCTCTC-3'</td>
<td></td>
<td></td>
<td>SI 2</td>
<td>239, 147</td>
<td></td>
</tr>
<tr>
<td>4.2-R: 5'-CGGTGTGGATCTTCTCATTTCCC-3'</td>
<td></td>
<td></td>
<td>Bsm I</td>
<td>386</td>
<td></td>
</tr>
<tr>
<td>McC²</td>
<td></td>
<td></td>
<td>MCC²</td>
<td>210, 170</td>
<td></td>
</tr>
<tr>
<td>ovulocytosis F: 5'-GGGCCCAGATGACCCTGTCG-3' R: 5'-GCCGAAGGGGTAGGCGGCTG-3'</td>
<td>70</td>
<td>N/A</td>
<td>normal</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Gerich blood GPCup 5'-CAGATCTTGTGCTCTGCAAGC-3' GPCdn 5'-TCATAACCCACCTTGAGGAGGAGG-3'</td>
<td>60</td>
<td>N/A</td>
<td>normal</td>
<td>240,264</td>
<td></td>
</tr>
</tbody>
</table>

Cycling conditions were 2 minutes at 95 °C for the hot start at which point the polymerase would be added having been left out of the original reaction mixture, followed by 35 cycles of 15 seconds at 95 °C (melting phase), 15 sec at the annealing
temperature (see table 2.1) and an extension phase of 72 °C for 1 minute per kb of expected product. Details of specific primers, cycling conditions and subsequent restriction digests are given above (Table 2.1) and in the relevant section.

2.3.2 CR1 expression associated polymorphisms

Exon 22, intron 27 and exon 33 polymorphisms (see section 1.6 for an explanation of polymorphisms in the CR1 gene) were determined by PCR followed by restriction digest according to the method of Xiang et al. (Xiang et al. 1999). For the population survey (Chapter 4) PCR was carried out using the basic PCR reaction outlined in section 2.3.1. in a volume of 50 µl. Primers and cycling conditions are outlined in Table 2.1. For samples from the case-control study (Chapter 5) a modification of this procedure was used. The template used was a primer extension preamplification (PEP) PCR product. The PEP procedure is a "whole genome" PCR reaction that allows large amount of PCR template to be produced from small amounts of genomic DNA (Zhang et al. 1992). The reactions were performed in 20 µl volumes on 96 well PCR plates. A thermostable Taq polymerase was used (Platinum Taq, Invitrogen, Paisley, UK) with its associated buffer. An initial incubation at 95 °C for 2 minutes was added at the beginning of the usual cycling to activate the polymerase.
Restriction digests were performed to determine the genotype of the samples at three linked polymorphisms in the CR1 molecule. For exon 22 RsaI (Promega Southampton, UK), was used, for intron 27 HindIII (Promega, Southampton, UK) was used and for exon 33 MnlI (New England Biolabs, Beverly, MA). In each case 5 U of enzyme was added to 1 µl of 10× the appropriate restriction enzyme buffer (as supplied by the manufacturer with the enzyme) and 0.1 µl bovine serum albumin (BSA), total reaction volume was made up to 10 µl with the PCR product (typically 10-100 ng) and deionised water. Samples were incubated at 37 °C for 5 hours before the reactions were stopped by cooling to 4 °C. Products were run on 2% agarose gel to determine fragment sizes, the expected fragment sizes for each polymorphism are given in table 2.1.

2.3.3 Knops blood group genotyping

The genotypes that determine the Knops blood groups in CR1 were determined by PCR of a region of SCR24 and SCR25 (both in one exon) that contains both the polymorphisms associated with both the Sl 2 and McCb mutations. Primers for the reaction are given in table 2.1. The basic reaction given in 2.3.1 was used with an MgCl2 concentration of 1.5 mM. To distinguish the Sl 2 genotype from Sl 1 digestion with MfeI (New England Biolabs, Beverly, MA) was performed as follows 5 U of enzyme were added to 10 µl PCR product, 2 µl 10× buffer, made up to 20 µl with dH2O, and incubated as in section 2.3.2. To distinguish the McCa and McCb alleles 5 U of BsmI (New England
Biolabs, Beverly, MA) was used instead of MfeI. Expected fragment sizes for both
typings are given in table 2.1.

2.3.4 Ovalocytosis genotyping

Genotyping for the deletion in the band-3 gene associated with Southeast Asian
ovalocytosis was carried out according to the method of Jarolim et al. (Jarolim et al.
1991). This PCR amplifies a region of genomic DNA around the 27bp deletion that
does the ovalocytosis phenotype therefore heterozygotes for this gene will have an
additional small 148 bp fragment in addition to the normal 175 bp fragment. The
homozygous state for this condition has never been observed and is presumably lethal in-
utero. The basic PCR reaction was used (Section 2.3.1) with 2.5 mM MgCl₂; primers and
conditions are given in table 2.1.

2.3.5 α⁺-thalassaemia genotyping

α⁺-thalassaemia genotyping was carried out according to the method of Chong et
al. (Chong et al. 2000). Only the -α³⁷ and -α⁴² deletions were typed as these are the most
common in coastal Papua New Guinea (Flint et al. 1998). This uses a multiplex PCR to
amplify different PCR products depending on the chromosomal structure around the α-
globin locus. A different reaction mixture was used from the basic mixture given in
section 2.3.1. The MgCl$_2$ concentration was 1.5mM, in addition dimethyl sulfoxide (DMSO) was added to the reaction mixture at a final concentration of 5% while betaine was added to a final concentration of 1M. DMSO prevents non specific amplification in multiplex PCR reactions, while betaine stabilises primer annealing for GC rich sequences (Chakrabarti et al. 2002). Each of the primers was used at a final concentration of 0.4 pmol/µl; primer sequences and cycling conditions are given in table 2.1.

2.3.6 Gerbich blood group genotyping

The Gerbich blood group phenotype is caused by the deletion of exon 3 from the glycophorin C gene. To determine if an individual is homozygous for this deletion we used a PCR strategy using genomic DNA as described by Patel et al (Patel et al. 2001). They use a primer pair that recognises sequences in both exon 2 and exon 3 amplifying different sized products from each (264 bp and 240 bp respectively) absence of an exon 3 (240bp) product signifies homozygosity for the exon 3 deletion and Gerbich phenotype. This protocol gives no way of distinguishing glycophorin C $\Delta$ exon 3 heterozygotes from normal individuals.
2.4 Determination of CR1 allotypes

2.4.1 Preparation of solubilized red blood cell membranes

Red blood cell membranes were prepared by adding 1ml of hypotonic lysis solution (5 mM Na₂HPO₄ with the following protease inhibitors: 0.348 mg/ml phenylmethanesulfonyl fluoride (PMSF), 1.16 μg/ml ethylene diaminetetracetic acid (EDTA), 0.686 μg/ml pepstatin A and 3.7 μg/ml iodoacetamide) to 0.4 ml RBC. This was centrifuged at 13000 g for 10 minutes at 4 °C and the supernatant removed. The process was repeated approximately 4 times until all of the red colour due to haemoglobin had been removed.

2.4.2 SDS-PAGE; Western Blotting

2 μl of ghosts per sample were taken and added to 4× loading buffer (4.36 M Glycerol, 564 mM Tris base, 424 mM Tris HCl, 292 mM lithium dodecyl sulfate (LDS), 2.04 mM EDTA, 0.88 mM Serva Blue, 0.7 mM Phenol Red), adjusted to 1× with distilled water and heated to 70 °C for 10 mins before being loaded onto a prepoured 3-8 % NuPAGE tris acetate gel (Invitrogen, Paisley, UK) and run at 150 V in running buffer (0.05 M Tricine, 0.05 M Tris Base, 3.5 mM sodium dodecyl sulfate (SDS)). The gel was then blotted onto a nitrocellulose membrane according to the manufacturers instructions.
Briefly the gel and membrane were sandwiched between blotting paper and sponge pads held in a blotting module consisting of cathode and anode plates and saturated with transfer buffer (10 % methanol, 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA), the blot was maintained at 30V for an hour whereupon the blot was retained for probing and the gel discarded. Blots were blocked with 5 % powdered milk solution in PBS-Tween (0.1 % Tween20 diluted in PBS) for an hour, they were then totally immersed in a petri dish with 0.5 μg/ml E11 anti-CR1 mAb in PBS Tween for an hour. The blot was then washed for 15 minutes in PBS-Tween, 5 further minutes in fresh PBS-Tween, 5 minutes in 5 % blocking solution prepared as above, and 5 more minutes in PBS-Tween. The blot was then immersed in 5 μg/ml horseradish peroxidase (HRP)-conjugated Goat anti Mouse Ig antibody (Dako, Kidlington, UK) in PBS-Tween, for 1 hour and then washed as above before being exposed using an enhanced chemiluminescence system (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. Both short and long exposures were developed.
Figure 2.2: CR1 allotype determination. Western blot erythrocyte ghosts from New Ireland probed with the E11 antibody to identify CR1 allotypes. All samples are homozygous for the CR1*1 allotype (190 kDa) except for samples 17 and 20 which are heterozygotes for the CR1*1 and CR1*3 allotype (160 kDa). Numbers on the left are the sizes of protein markers (in kDa).
2.5 Glucose-6-phosphate dehydrogenase activity assay

To assay for glucose-6-phosphate dehydrogenase (G6PD) activity we used a commercial test (Sigma procedure no. 400, Sigma Diagnostics, Poole, UK). The natural enzyme activity of G6PD catalyses the conversion of glucose-6-phosphate to 6-phosphogluconate with the reduction of nicotinamide adenine phosphate (NADP) to NADPH. NADPH can reduce a blue dye (dichlorophenol indophenol) in the presence of phenazine methosulphate (PMS), to form NADP and a colourless dye complex.

To prepare a red cell haemolysate 50 μl of blood is added to water. The amount of water depends on the haemoglobin (Hb) concentration of the sample. For PNG samples from healthy individuals we assumed an Hb concentration of 12 g/dl so blood was lysed in 2 ml of distilled water, whereas for Europeans we assumed an Hb concentration of 15 g/dl and blood was lysed in 2.5 ml distilled water. The mixture was allowed to stand for minutes at room temperature. The substrate solution in a "single determination vial" was reconstituted with 1 ml of Trizma buffer solution and 1 ml of lysate was then added to the vial and 2 ml of Mineral Oil layered over the top of the reaction mixture. The vial was then placed in a 37 °C water bath and observed at 10 minute intervals to determine the reaction endpoint (the colour should have changed to reddish brown). If the reaction time was greater than 60 minutes the sample was considered G6PD deficient. Control samples from Caucasians were used as positive controls (with normal G6PD activity) while a
commercial standard deficient preparation was used as a negative control, this was treated in the same way as a normal blood sample.
2.6 Direct anti-globulin test for ABO typing

ABO typing was performed by the method of slide agglutination. Briefly two drops of blood of ~20 μl each were dropped on either end of a normal microscope slide. To one drop was added a drop of anti-A serotyping reagent (Diagnostics Scotland, Edinburgh, UK) and to the other a drop of anti-B serotyping reagent (Diagnostics Scotland, Edinburgh, UK). Agglutination of the blood was detected by eye and could be seen as clumping of the red cells. If the blood was agglutinated by the anti-A reagent alone the sample was A, likewise if the blood was agglutinated with the anti-B reagent. If both reagents gave positive tests the sample was AB, and if neither agglutinated the samples the sample was O.
2.7 Parasite culture methods

2.7.1 Medium and Red cells

Parasites were grown in complete RPMI (cRPMI), while incomplete RPMI (iRPMI - see section 2.2.1) was used for washing parasites. To make cRPMI 10% normal pooled human serum, (all ABO types permissible - Scottish Blood Transfusion Service, Edinburgh, UK) was added to iRPMI.

Parasites were cultivated in O blood group red blood cells, which were either provided by the local blood transfusion service (Scottish Blood Transfusion Service, Edinburgh, UK) or from local donors. In either case the blood was treated the same. 10 ml of blood was layered over lymphocyte separation medium (Bio-Whittaker, Walkersville, MD) and spun at 1800 g on a bench top centrifuge. Lymphocyte separation medium separates the red blood cells, which spin to the bottom of the tube from the peripheral blood mononuclear cells (PBMCs), which are suspended in the layer of lymphocyte separation medium and the plasma, which forms the top of the supernatant. The blood was then washed twice in iRPMI (by resuspending in iRPMI and spinning down for 5 mins at 1800 g). Before being resuspended at 50 % haematocrit and stored at 4 °C until use.
2.7.2 Parasites

Parasites in this study are of three varieties: i. "strains" or "lines" are named and defined parasite cultures maintained in vitro, ii. "clones" are populations derived from a single cell and iii. "isolates" are samples taken from patients infected with *P. falciparum*.

Parasite cultures used in this work are:

1. R29 a rosetting clone derived from the ITOR strain (originally from Brazil) (Roberts *et al.* 1992)
2. TM284 a rosetting line derived from a Thai patient with severe malaria. (Carlson *et al.* 1992)
3. Palo Alto R+ (PAR+) is a rosetting clone isolated from a Ugandan strain: Palo Alto (Helmby *et al.* 1993). PAR+ is also known as FCR3S in some publications (Chen *et al.* 1998).

It should be noted that despite these presumed different origins of these parasites extensive RFLP analysis of these strains especially around the chloroquine resistance locus has shown that both PAR+ and R29+ are descended from the FCR3 line originally isolated by Trager in 1976 (Trager *et al.* 1976). The misattribution of these lines is probably a result of laboratory contamination (D. Walliker, personal communication). TM284+ is however genetically distinct.
2.7.3 Culture procedures

Parasites were grown under low oxygen tension using modifications of the method of Trager and Jensen (Trager et al. 1976). All procedures were carried out in laminar flow hood using aseptic techniques and the plastics used in the handling of parasites or human blood products were decontaminated by rinsing in 1% virkon (Alpha Labs, Eastleigh, UK).

Cultures were maintained at 2-5% parasitaemia in human group O erythrocytes in cRPMI. Flasks were incubated at 37 °C under an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen. Fresh red cells were added approximately every 48 hours, depending on parasitaemia. The packed cell volume of red cells was approximately 2% haematocrit; i.e. 200 μl of red cells would be maintained in 10 ml of media. Typically laboratory parasite lines such as those used in this study would give 5-fold reinvasion in a 48 cycle, thus cultures would typically have to be diluted 1 in 5 with fresh blood every 2 days to maintain constant parasitaemia.

Maturity of cultures was assessed using Giemsa stained thin films. A small aliquot (typically 200 μl) of culture would be and spun down by brief spinning in a microfuge. The supernatant would be removed, leaving enough media to resuspend the cells at 40% haematocrit. 5-10 μl would be used to make the smear and the slide would be dried with a hair dryer. The smear would be fixed by brief emersion in methanol and then stained.
with freshly prepared Giemsa. Giemsa is prepared with 10% Giemsa staining solution (BDH, Poole, UK) in Giemsa buffer (BDH, Poole, UK)

2.7.4 Freezing and thawing parasites

To allow for the long term storage and transport of parasites, parasites are stored frozen in liquid nitrogen. The same techniques are used as for the storage of red blood cell samples (see Section 2.2.1).

2.7.5 Synchronising parasites (Lambros et al. 1979)

Synchronous cultures of parasites are required for experimental work. Only parasites older than 20 hours post-invasion are permeable to sorbitol. They can therefore be lysed (by osmotic lysis), leaving younger ring forms and uninfected erythrocytes.

Parasite cultures are spun down (2 mins 1000 g) and the medium (supernatant) removed. The culture is then incubated in 5 % D-sorbitol (made up in distilled water and autoclaved) and incubated at 37 °C for 15 mins, before being washed twice in 10 ml iRPMI. Once the cells are washed the culture may be resuspended in cRPMI and gassed and incubated as usual.
2.8 Rosetting protocols

2.8.1 Measuring the rosetting frequency

An aliquot of parasite culture adjusted to 2% haematocrit was stained by adding ethidium bromide to a final concentration of 20 µg/ml (1 µl of a 1 mg/ml solution in 50 µl of culture suspension). 10 µl of this suspension was placed on clean glass slide and covered with a 22 mm × 22 mm coverslip that had previously had the edges sealed with Vaseline. Slides were blinded before counting in experiments where several slides were analysed, and then viewed on an epifluorescence microscope using a combination of fluorescence and bright field. The illumination was adjusted so that ethidium bromide stained infected erythrocytes and unstained uninfected erythrocytes could be viewed simultaneously. At least 200 infected cells were counted and assessed for rosetting, with the binding of two or more uninfected erythrocytes constituting a rosette. The rosetting frequency is the percentage of mature (pigmented trophozoite) infected erythrocytes forming rosettes.
2.8.2 Enrichment of cultures for rosetting parasites

Enrichment for rosetting was performed by two methods, one for knob+ parasites such as TM284+ and R29+ and the other for knob- parasites such as PAR+.

For knob+ parasite the parasites in culture medium were spun down and the supernatant removed, the parasites were then resuspended in 1 volume of iRPMI and then mixed with 2 volumes of gelofusine (Millpledge, Clarborough, UK). The tube would be incubated for 15 minutes at 37 °C in which time the contents would separate into two layers. The top layer contained non-rosetting knob+ parasites, while the bottom layer would contain rosetting trophozoites, ring stage parasites, and uninfected erythrocytes. To enrich for rosetting parasites the bottom layer would be retained and washed twice in iRPMI before being returned to culture in cRPMI.

For PAR+, which is knob-, a different technique was used. Parasite cultures would be spun down and resuspended in 5ml of fresh cRPMI. This parasite suspension would be layered over a layer of 60% percoll. Percoll solutions of a given concentration would be made by diluting 90% percoll stock (90 ml percoll, 10 ml 10× RPMI made up from powdered RPMI with L-Glutamine according to the manufacturers instructions (Invitrogen, Paisley, UK)) with the appropriate amount of iRPMI. Having layered the parasites over the percoll the tube would be spun at 1800 g in a bench-top centrifuge for
10 minutes. Rosetting trophozoites, and uninfected erythrocytes would be spun down to a pellet at the bottom of the tube while a non-rosetting trophozoite layer would be suspended above this. To select for rosetting parasites the pellet would be retained washed in iRPMI and returned to culture in cRPMI.

2.8.3 Labelling erythrocytes with 5-carboxyfluorescein diacetate (c-FDA)

To label 10 μl of packed erythrocytes with c-FDA, a stock solution of 15 mg/ml c-FDA in PBS should be diluted 50× in cRPMI-ABS to make the labelling solution (300 μg/ml). 1 ml c-FDA labelling solution was incubated with 10 μl of packed cells for 30-45 minutes at 37 °C. The cells were then washed four times in iRPMI and resuspended in 200 μl cRPMI-ABS.

2.8.4 Assessment of rosette formation and competition with c-FDA-labelled RBC(Carlson et al. 1992)

To assess the capacity of red cells from a given donor to form rosettes we used a competition assay in which "test" cells labelled with c-FDA competed to form rosettes against control cells in which the culture was grown. For our experiments the control
cells were O+ erythrocytes from a single donor with a CR1 expression of 996 molecules per cell.

Cultures of rosetting parasites grown in control cell by normal means would be spun down and resuspended at 5 % haematocrit in cRPMI-ABS. Existing rosettes were disrupted by addition of heparin. The amount of heparin depended upon the strain used from 100 μg/ml for PAR+ and R29 to 1mg/ml for TM284+ as the sensitivity of rosettes to heparin varies (Rowe et al. 1994). The cultures were checked as above (Section 2.8.1) that the disruption was complete. 10 μl of packed cells were added to 10 μl of test cells labelled as in section 2.8.2. The mixture was washed three times to remove heparin and resuspended in binding medium with 10 % AB serum. Binding medium is similar to iRPMI but made with powdered RPMI (Invitrogen, Paisley, UK) without the addition of sodium bicarbonate, it is less sensitive changes in the oxygen tension than normal iRPMI so can be used for short periods where the cultures are not gassed. The mixture of test cells is left under agitation for 1 hour to allow rosettes to reform. The samples then had ethidium bromide added and slides were prepared as above. The slides were viewed under UV light with a green filter. 100 cells in rosettes were counted and scored as green or unstained. This proportion was divided by the ratio of stained to unstained cells in the mixture as a whole (from at least 200 counted cells) to give a "rosetting quotient".
2.8.5 **Rosette reversal by mAbs and polyclonal Abs in laboratory strains and field isolates (Rowe *et al.* 2000)**

To try an qualitatively dissect the contributions of different rosetting receptors in a robust assay that could be used in a field setting we used a panel of antibodies that interact with different previously identified rosetting receptors to disrupt rosettes.

20 μl aliquots of parasite culture were spun down and resuspended in binding medium with AB serum and reagents were added to the correct final concentration. J3B11 (a gift of J. Cohen (University of Reims, Reims, France) was added to a final concentration of 1 μg/ml. J3B11 an antibody specific for the C3b binding sites in CR1 has been previously shown to disrupt rosettes in lab strains and field isolates (Rowe *et al.* 2000). Disruption was compared to results with an IgG1 isotype control at the same final concentration (Serotec, Oxford, UK). A rabbit polyclonal to human immunoglobulins A, G and M (Dako, Kidlington, UK) has been shown to disrupt rosettes in strains such as PAR+(Rowe *et al.* 2000) in which immunoglobulins play a large role in rosetting adhesion interactions (Somner *et al.* 2000). This antibody was used diluted 1/50 in binding medium, rabbit serum at the same final immunoglobulin concentration (1/100 dilution) was used as a negative control. Heparin has also been shown to disrupt rosettes (Rowe *et al.* 1994), though it is unclear whether it is a rosetting receptor (Barragan *et al.* 2000). This was added to a final concentration of 100 μg/ml. The samples were
incubated in the reagents for 1 hour at 37 °C before the rosetting frequency was counted by the usual means (section 2.8.1).
2.9 Immunofluorescence assay (IFA) for the detection of human Iggs on erythrocytes (Rowe et al. 2002)

The immunofluorescence assay (IFA) to detect human immunoglobulin on the surface of iRBCs was carried out with unfixed parasite cultures at the mature pigmented trophozoite stage. Only monoclonal antibodies and immunoglobulin free BSA were used to prevent cross-reactivity of reagents. Aliquots of culture suspension at 2% haematocrit were washed once in 750 µl cold PBS then in 750 µl of cold PBS-1% Immunoglobulin free-BSA (PBS-1% BSA). Mouse mAbs to human IgM (Serotec, Oxford, UK) and an isotype control (Mouse IgG1 (Serotec, Oxford, UK) were then incubated at a final concentration of 1 µg/ml in 50ml of PBS-BSA for 1 hour on ice with regular agitation. An anti CR1 mAb (7G8 (a gift from R. Taylor, University of Virginia School of Medicine, Charlottesville, VA) at the same concentration was used as a positive control for the detection of molecule associated with the red cell surface.

Following the incubation in primary antibody the cells were washed twice in 750 ml cold PBS and incubated for 45 minutes on ice in 50 ml of PBS-1% BSA with the highly cross absorbed Alexa Flour 488-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) at a 1:200 dilution and 1 µg/ml 4',6'-diamidino-2-phenylindole (DAPI). After washing firstly in 750 ml cold PBS and then in 750 ml PBS-1% BSA the cells were resuspended at 30% haematocrit in PBS-1% BSA and then used to make a smear on a
clean microscope slide. After air drying the smear was fixed briefly with methanol and then dried. The smear was then overlaid with 15 ml of vectashield mountant (Vector Laboratories, Peterborough, UK) and a coverslip (22 mm × 22 mm). The edges of the coverslip were sealed with nail varnish. Slides were viewed with an Olympus BX-50 microscope and images recorded with a digital camera and OpenLab software. The percentage of iRBCs binding IgM was determined by counting at least 200 infected cells.

Figure 2.3

Figure 2.3 IgM binding of rosetting parasites: the figure shows three multinucleate parasites (schizonts) detected with DAPI staining, the top parasite is binding IgM as detected by mouse mAb to IgM and Alexa Fluor 488™ conjugated goat anti mouse IgG.
2.10 Statistical analysis

Details of statistical procedures are given in each results chapter. Unless otherwise stated statistics were calculated using StatView (SAS Institute, Cary, NC).
Chapter 3:

A simple method for accurate quantification of complement receptor 1 on erythrocytes preserved by fixing or freezing\(^2\)

3.1 Introduction

Before we can study erythrocyte complement receptor 1 (CR1) in populations in Papua New Guinea we need a suitable assay for determining the mean CR1 expression per cell.

\(^2\) These results are published as Cockburn et al. 2002 (see Appendix)
When drawing blood from children only small blood volumes are available, on which a variety of assays must be performed. Additionally the loss of CR1 on storage of blood (Pascual et al. 1993) means that the assay must either be performed immediately or the cells must be preserved such that the assay can be performed later. Several methods currently exist to quantify the mean number of CR1 molecules per erythrocyte including an ELISA method and a flow cytometry method (Cohen et al. 1999). Both methods give equivalent results however we prefer to use the flow cytometry technique as only small quantities of blood are required. Nonetheless the technique has previously only been validated for fresh blood samples (Cohen et al. 1987). Erythrocyte CR1 loss during sample storage, though, would be a problem when working in field areas for malaria where facilities for flow cytometry are rarely available. We therefore tested a variety of methods for erythrocyte preservation in order to accurately determine the CR1 expression level on erythrocytes after storage.
3.2 Results

3.2.1 Erythrocytes from healthy donors fixed in 5% formaldehyde and frozen in glycerol can be used for CR1 determinations.

To determine if we could quantify CR1 expression using healthy preserved erythrocytes we performed the following experiment. We drew blood into Vacutainers containing 0.12ml of 15% EDTA (BD Vacutainer systems, Plymouth, UK) from five individuals with known CR1 expression. These individuals are used as standards in our laboratory and have had their CR1 levels determined by flow cytometry compared to a standard curve based on individuals who have had their CR1 expression level determined by Scatchard analysis using $^{125}$I labelled mAb (Cohen et al. 1987). We also drew blood from six healthy individuals of unknown CR1 expression and determined their CR1 expression by flow cytometry when fresh (see section 2.1). Aliquots of all 11 samples (standards and test samples) were preserved in four different ways (described in 2.2). The first was simply left in the EDTA vacutainer (EDTA), the second was fixed in 0.25% glutaraldehyde (Pattanapanyasat et al. 1993), the third was fixed in 5% formaldehyde as outlined by Bianco et al. (Bianco et al. 1986) and the final aliquot was frozen in glycerolyte (Freezing). After 1, 2, 4, 6 and 8 weeks the EDTA preserved, fixed and
Figure 3.1: CR1 expression levels determined after preservation of healthy erythrocytes compared to CR1 levels determined on fresh erythrocytes. 

(a) Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks.

(b) Erythrocytes stored in EDTA at 4 °C for 6 weeks.

(c) Cryopreservation
erythrocytes cryopreserved in liquid nitrogen for 8 weeks. The CR1 expression levels of
the six test samples determined by comparison to freshly drawn (absolute) standards
are shown as black squares (■), whereas the CR1 expression levels of the six test
samples determined by comparison to preserved (relative) standards are shown as
white squares (□). The relationship between the CR1 levels measured on preserved
cells compared to fresh cells was analysed by linear regression. The regression line for
relative standards is given and a dashed line while a solid line is the regression line for
the data derived with absolute standards. Equations are given at the top of each graph.
frozen samples underwent flow cytometry to assess erythrocyte CR1 expression. To determine the optimum method of measuring CR1 levels on stored cells we assessed them in two ways. Firstly we compared them to a standard curve derived from freshly drawn standards at each time point (absolute standards) and secondly, we compared them to a standard curve derived from the standards at each time point that had been drawn and preserved in an identical way to the test samples at the start of the experiment (relative standards).

For each of the six healthy test samples, the CR1 level measured on the preserved cells using both absolute and relative standards was compared to the CR1 level of the samples when freshly drawn, and the data were analysed by linear regression. The best results were obtained with the samples fixed in 5% formaldehyde. Comparison to relative standards gave accurate results throughout the 8 weeks of the experiment (Table 3.1 and Figure 3.1a white squares), whereas comparison to absolute standards was accurate up to 4 weeks after the blood was drawn, but thereafter gave an underestimate of true CR1 numbers (Table 3.1 and Figure 3.1a, black squares). Preservation in EDTA also gave good results providing the test samples were compared to relative standards (Figure 3.1b, white squares). This was true up to 6 weeks from the date of drawing (Figure 3.1b), after which time, the extent of haemolysis of the samples became too great for further use. When absolute standards were used to assess the CR1 level of EDTA-preserved cells, this was accurate up to two weeks after the blood was drawn, and thereafter resulted in an underestimation of the number of CR1 molecules per cell (Figure 3.1b, black squares).
Fixation of erythrocytes in 0.25% glutaraldehyde resulted in high background fluorescence of all samples and made the forming of a standard curve impossible (data not shown), therefore this method was not suitable for CR1 quantification. Preservation of erythrocytes by freezing in glycerol gave accurate results when relative standards were used (Figure 3.1c, white squares), and this was true throughout the course of the experiment. The use of absolute standards gave good results at some time points (eg week 8, Figure 3.1c, black squares), however, at earlier time points the use of absolute standards gave widely varying estimates of CR1 numbers, therefore we would not recommend the use of absolute standards with frozen cells.

### Table 3.1 Assessment of CR1 expression level over the course of 8 weeks on erythrocytes fixed with 5% formaldehyde

<table>
<thead>
<tr>
<th>Week</th>
<th>CR1 measured using absolute standards</th>
<th>CR1 measured using relative standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of CR1 molecules per cell when fresh</td>
<td>448</td>
</tr>
<tr>
<td></td>
<td>Week 1</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>Week 6</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td>Week 8</td>
<td>350</td>
</tr>
</tbody>
</table>
3.2.2  Erythrocytes from systemic lupus erythematosus (SLE) patients can also be preserved for flow cytometry

Having determined the efficacy of freezing and fixing in 5% formaldehyde for preserving healthy erythrocytes we set out to verify these techniques with erythrocytes from patients with a disease known to affect CR1 level on erythrocytes such as systemic lupus erythematosus (SLE)(Ross et al. 1985; Jouvin et al. 1986; Wilson et al. 1987).

Accordingly we repeated the experiment using the same CR1 standard donors and unknown test samples from 7 French SLE outpatients. On this occasion we preserved the cells only by fixing in 5% formaldehyde and freezing and assessed the CR1 expression with relative and absolute standards at 4 and 8 weeks only.

The repeated experiment using samples from donors with a disease in which CR1 levels are affected (SLE) confirmed that fixing in 5% formaldehyde and freezing give good results with relative standards are used (Figure 3.2 a and b, white squares). Systematic underestimation of CR1 expression is observed when fixed samples are used with absolute standards (Figure 3.2a, black squares). However fair determinations of CR1 expression are also possible using absolute standards and frozen cells (Figure 3.2b, black squares; data from week 4 not shown) though relative standards should be used if
possible. Crucially both techniques worked well with samples showing low (<100 molecules per cell) CR1 expression.

Figure 3.2

Figure 3.2: CR1 expression levels determined after preservation of erythrocytes from SLE outpatients compared to CR1 levels determined on fresh SLE erythrocytes. 

- **a**: Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks.
- **b**: Erythrocytes cryopreserved in liquid nitrogen for 8 weeks. Legend and analysis as in Figure 3.1.
3.3 Discussion

We have therefore shown that accurate CR1 quantification may be performed after preservation of either diseased or healthy erythrocytes using either a freezing technique or fixation in 5% formaldehyde, providing that the CR1 expression level standards are collected and fixed in an identical fashion. Cryopreservation of erythrocytes in glycerol is reliable and allows for potentially long-term storage of samples, but does require liquid nitrogen for freezing and transportation of cells, which may not always be available during field studies. Fixation of erythrocytes in 5% formaldehyde is quick and economical and gives an accurate determination of CR1 level for at least 2 months after sample collection. A summary of the advantages of each method is given in Table 3.2. These methods will facilitate further studies of CR1 expression level and disease susceptibility in areas lacking facilities for flow cytometry.
**Table 3.2 Comparison of methods for the preservation of samples for CR1 level determination**

<table>
<thead>
<tr>
<th>Preservation Method*</th>
<th>Maximum time of storage for accurate CR1 determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Using absolute standards</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>Up to 2 weeks after blood drawn</td>
</tr>
<tr>
<td>5% formaldehyde</td>
<td>Up to 4 weeks after blood drawn</td>
</tr>
<tr>
<td>Freezing in glycerol</td>
<td>Inconsistent results - not recommended</td>
</tr>
<tr>
<td>Using relative standards</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>Up to 6 weeks after blood drawn</td>
</tr>
<tr>
<td>5% formaldehyde</td>
<td>Up to 8 weeks after blood drawn†</td>
</tr>
<tr>
<td>Freezing in glycerol</td>
<td>Up to 8 weeks after blood drawn†</td>
</tr>
</tbody>
</table>

* Fixation in 0.25% glutaraldehyde was also tested but was not acceptable at any timepoint due to high background fluorescence

† Longer periods of storage may be acceptable but this would require validation.
Samples were tested only up to 8 weeks in these experiments.
Chapter 4: CR1 polymorphisms in Papua New Guinea

High frequency of erythrocyte complement receptor 1 (CR1) deficiency in Papua New Guinea due to independent effects of CR1 mutations and $\alpha^+$-thalassaemia$^3$

4.1 Introduction

In 1949 J B S Haldane proposed that the distribution and prevalence of a variety of red blood cell disorders could be explained if they were to provide protection against

$^3$ These results are previously published as Cockburn et al. 2004
malaria (Haldane 1949). In coastal regions of PNG there is a high prevalence of α+-thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency and ovalocytosis, all of which have previously been associated with malarial protection (Yenchitsomanus et al. 1986; Genton et al. 1995; Allen et al. 1997; Allen et al. 1999). Recently a variety of new potential disease associations with blood group types have been reported. Many individuals in the Wosera region in North West Papua New Guinea are Gerbich negative. Erythrocytes of this type lack glycophorin C - an abundant red cell protein of unknown function. Gene disruption studies have shown that glycophorin C is the receptor for the invasion ligand erythrocyte-binding antigen 140 (EBA-140 also known as BAEBL) (Maier et al. 2003). Other studies have identified an emerging Duffy negative polymorphism that makes erythrocytes refractory to invasion by P. vivax (Zimmerman et al. 1999).

Red cell polymorphisms are not merely biological curiosities. They can be used to determine important disease processes. While we have extensive evidence that rosetting is associated with severe disease ((Carlson et al. 1990; Rowe et al. 1995) and see introduction) we do not know if it is an a priori cause of severe malaria, or merely a marker for some other underlying disease process. Given that CR1 is an important rosetting receptor (Rowe et al. 1997; Rowe et al. 2000), we reasoned that if rosetting is directly involved in malaria pathology then CR1 polymorphisms should protect against severe disease by reduced rosette-mediated microvascular obstruction. The Knops blood group antigens S12 and McCb are common in West Africa but rare in Caucasians, a
distribution that suggests malarial selection, however direct evidence for a protective effect is lacking (Zimmerman et al. 2003). CR1 deficiency is thought to be rare in most populations, however, a review article (Molthan 1983) stated that 28/67 Melanesians had CR1 deficient red cells (Helegeson phenotype). No experimental details were given, and no further information was published to validate this claim. A pilot study in our laboratory found that 9/15 samples from Vanuatu were also Helgeson phenotype (J.A. Rowe and J.M. Moulds unpublished data). In Caucasians red blood cell CR1 expression varies between 50 and 1200 molecules per cell (Moldenhauer et al. 1987; Cohen et al. 1989), with levels of less than around 200 molecules per cell being associated with Helgeson phenotype (Moulds et al. 1991). CR1 expression is genetically determined and is associated with at least three linked single nucleotide polymorphisms (SNPs) in the CR1 gene (in exon 22, intron 27 and exon 33 - see section 1.6) (Wilson et al. 1986). These SNPs comprise high (H) and low (L) CR1 expression haplotypes associated with CR1 level on erythrocytes alone but not other cell types such as B cells and macrophages (Wilson et al. 1986; Xiang et al. 1999). Given the prevalence of individuals with Helgeson blood and the burden of malaria in Melanesia we wanted to examine the erythrocyte CR1 expression level of individuals from Papua New Guinea and determine if this was under the same genetic control as in Caucasians. We also wanted to examine the prevalence of Knops group types and size polymorphisms.
We obtained blood samples from three sites within PNG and also from a control sample in Edinburgh. In all cases informed verbal consent was given by all donors following an explanation of the aims of the project. The PNG Medical Research Advisory Committee approved all protocols. Blood samples came from New Ireland and Madang, both of which have holoendemic malaria with a spleen enlargement rate >70% (Cattani et al. 1986; Flint et al. 1986). Buffy coats from blood samples collected in East Highlands Province in 1985 were kindly made available to us by the PNG Institute of Medical Research. These samples enabled us to genotype a highlands population to compare with the coastal and island ones. The highlands samples came from Andakombi, a village 2000m above sea level, where transmission is rare as the parasite cannot complete its lifecycle at the lower temperatures within the lifespan of the mosquito vector. Malaria is, at worst, epidemic in this population Samples from each study site were collected and processed in slightly different ways. In Edinburgh blood was drawn from healthy volunteers and processed fresh. In New Ireland 5ml of blood was drawn from 49 adult volunteers into EDTA and shipped to the UK, which took 2 weeks, the blood was then processed as fresh. In Madang blood was collected, the buffy coat was used for DNA extraction and the red cells from each samples were both frozen in glycerolyte and transported to Edinburgh by dry shipper, or fixed in 5% formaldehyde according to the method of Bianco et al. (Bianco et al. 1986) and kept at 4°C until used for flow cytometric
analysis. The Buffy coats from East Highlands Province had been stored at -60 °C since collection and were shipped to the UK by dry shipper where DNA was extracted from them.

Figure 4.1

Figure 4.1: Map of Papua New Guinea (excluding Bougainville Island) showing locations of study sites for this study ( ■) and principal towns ( □).
4.3 Results

4.3.1 CR1 expression in Papua New Guinea

We measured the RBC CR1 levels of healthy adult volunteers from two highly malarious regions of PNG (Madang and New Ireland) and from a control Caucasian population from Edinburgh (UK). CR1 levels in Edinburgh varied between individuals in the range 253-1181 molecules per cell, with a mean of 786 (Figure 4.2). These results are similar to CR1 levels described in other Caucasian populations (Table 4.1), showing that our assay for CR1 (Cockburn et al. 2002) is comparable to those used previously. In PNG, CR1 levels were significantly lower than in Edinburgh (Figure 4.2, \( P < 0.001 \)), with 79% of individuals in Madang and 55% of individuals in New Ireland having fewer than 200 CR1 molecules per cell, this is the approximate maximum expression level of Helgeson phenotype erythrocytes associated with reduced rosetting (Moulds et al. 1992; Rowe et al. 1997). The mean RBC CR1 levels in Madang and New Ireland are the lowest in the world (Table 4.1). These results indicate that in malarious regions of PNG, CR1 deficiency is extremely common and could therefore play a major role in influencing susceptibility to severe malaria.
Figure 4.2

Figure 4.2: Low CR1 expression is common in Papua New Guinea. Graph shows CR1 expression of individuals from two Papua New Guinea populations and a control sample from Edinburgh. CR1 expression is in molecules per cell; each data point represents a single individual. To the right are shown the means and standard deviations for each population.
### Table 4.1: Red cell CR1 levels and frequency of the CR1 low expression allele in different populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean red cell CR1 (range)</th>
<th>Gene frequency (L allele)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>613 (161-712)</td>
<td>0.21</td>
<td>(Cohen <em>et al.</em> 1989)</td>
</tr>
<tr>
<td>UK</td>
<td>624 (119-1220)</td>
<td>0.27</td>
<td>(Moldenhauer <em>et al.</em> 1987)</td>
</tr>
<tr>
<td>USA (Blacks)</td>
<td>635 (158-1023)</td>
<td>0.25</td>
<td>(Herrera <em>et al.</em> 1998)</td>
</tr>
<tr>
<td>USA (Whites)</td>
<td>547 (89-1166)</td>
<td>0.25</td>
<td>(Herrera <em>et al.</em> 1998)</td>
</tr>
<tr>
<td>India</td>
<td>648 (140-1294)</td>
<td>0.23</td>
<td>(Kumar <em>et al.</em> 1995)</td>
</tr>
<tr>
<td>Mali</td>
<td>415 (58-1032)</td>
<td>0.14</td>
<td>(Rowe <em>et al.</em> 2002)</td>
</tr>
<tr>
<td>China</td>
<td>446 (27-1039)</td>
<td>0.28</td>
<td>(Moulds <em>et al.</em> 1998)</td>
</tr>
<tr>
<td>Edinburgh (UK)</td>
<td>786 (253-1181)</td>
<td>0.23</td>
<td>This Study</td>
</tr>
<tr>
<td>New Ireland (PNG)</td>
<td>253 (40-839)</td>
<td>0.73</td>
<td>This Study</td>
</tr>
<tr>
<td>Madang (PNG)</td>
<td>124 (0-439)</td>
<td>0.60</td>
<td>This Study</td>
</tr>
<tr>
<td>Eastern Highlands</td>
<td>ND</td>
<td>0.41</td>
<td>This Study</td>
</tr>
<tr>
<td>(PNG)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined
4.3.2 CR1 expression in Papua New Guinea is associated with polymorphisms in the CR1 gene

We wanted to see if CR1 deficiency in Papua New Guinea was due to the L allele that causes low CR1 in Caucasians and all populations studied to date outside Africa (Moldenhauer et al. 1987; Kumar et al. 1995; Herrera et al. 1998; Nagayasu et al. 2001; Rowe et al. 2002). We genotyped 25 samples from Edinburgh, 45 samples from New Ireland and 36 samples from Madang at the three CR1 SNPs in exon 22, intron 27 and exon 33. In 3 samples from New Ireland and 11 from Madang there was discordance between at least one of the SNPs and the expected haplotypes. In all populations there was a significant association between CR1 expression and all three SNPs \(P < 0.05\). In Madang where there was a higher degree of discordance the \(P\) values for association by one way ANOVA were \(P < 0.001\) for exon 22, \(P = 0.020\) for intron 27 and \(P = 0.040\) for exon 33. As the strongest association with CR1 expression was shown by the exon 22 allele we typed the remaining samples for this polymorphism alone. At all three study sites the exon 22 genotype was associated with a highly significant effect on RBC CR1 level, with carriers of the G3650 low (L) expression allele having significantly lower CR1 levels than HH individuals (Figure 4.3, \(F_{2,96} = 50, P < 0.001\)). The frequency of the CR1 L allele in the malarious regions of PNG is the highest described in the world to date (Table 4.1), and significantly different to Edinburgh, \(\chi^2 > 6, P < 0.01\). DNA samples were also studied from the non-malarious Eastern Highlands Province of PNG, and the frequency of the L allele was found to be significantly lower than in the malarious conditions.
regions (Table 4.1, \( \chi^2 = 8.1, P < 0.01 \)). This may indicate selection for the L allele in areas with high malaria mortality, although other explanations cannot be excluded such as founder effects and inbreeding (which are likely to occur in isolated populations such as those found in PNG (Flint et al. 1993; Flint et al. 1998)) or some other, as yet unknown, disease association. Another test was made to determine whether the HL heterozygotes had a CR1 expression level that was different to the weighted mean of the two homozygotes (using the CONTRAST option in SAS): no significant difference was found confirming that the alleles were acting in a co-dominant fashion \( (P > 0.05, F_{1,96} = 0.2) \). The above data indicate that RBC CR1 levels in Melanesians are associated with polymorphisms in the CR1 gene. However, even when matched for exon 22 genotype, CR1 expression levels in Edinburgh, Madang and New Ireland remained significantly different from each other \( (F_{2,96} = 66.0, P < 0.001) \). For example, the CR1 levels of HH individuals from PNG are lower than those of HH individuals from Edinburgh (Figure 4.3), suggesting that additional factors influence CR1 levels in Melanesians.
Figure 4.3: CR1 expression is associated with SNPs in the CR1 gene. Each point represents the CR1 expression of a single individual in molecules per cell. Results are displayed by population of origin and CR1 genotype. CR1 genotype is based on typing at the n3650 polymorphism in exon 22.
4.3.3 α'-thalassaemia is associated with low CR1 expression

We investigated whether other RBC polymorphisms that occur commonly in PNG such as ovalocytosis (Allen et al. 1999), glucose-6-phosphate dehydrogenase (G6PD) deficiency (Yenchitsomanus et al. 1986) and alpha-thalassaemia (Yenchitsomanus et al. 1986) could affect RBC CR1 levels. Ovalocytosis is known to reduce the expression of many RBC surface antigens (Booth et al. 1977), therefore it seemed a likely candidate for an additional modifying influence on CR1 levels. Ovalocytosis and G6PD deficiency were both relatively uncommon, occurring in up to 15% of individuals, and were not associated with RBC CR1 level (ovalocytosis, F1,115 = 0.2, P > 0.10 and G6PD deficiency, F1,115 = 1.8, P > 0.10). Alpha-thalassaemia occurred in 89% of individuals in Madang, and 23% of individuals in New Ireland. In both populations only -α4.2 mutations were observed except for one individual in the New Ireland population who was homozygous for the -α3.7 mutation, this individual was subsequently excluded from the analyses. At both sites, individuals with one or more alpha-thalassaemia mutations had RBC CR1 levels significantly lower than those of non-thalassaemic individuals (Figure 4.4 a, F1,111 = 12.4, P < 0.001). The effect of alpha-thalassaemia genotype on RBC CR1 level was independent of the CR1 exon 22 polymorphism, as both genotypes were statistically significant when simultaneously included in the analysis (F2,110 = 10, P < 0.001 for alpha-thalassaemia genotype; F2,110 = 44, P < 0.001 for exon 22 genotype). Thus, an individual’s RBC CR1 level is dependent upon both their CR1 exon 22 genotype and their alpha globin genotype (Figure 4.4 b).
Figure 4.4: CR1 expression is related to α*-thalassaemia as well as SNPs in the CR1 gene. a. CR1 deficiency in Papua New Guinea is associated with α*-thalassaemia. CR1
expression in New Ireland and Madang is shown in molecules per cell. Data is subdivided by population and α⁺-thalassaemia genotype. The effects of α⁺-thalassaemia and CR1 polymorphisms are independent of each other. Mean CR1 expression for the Madang population subdivided by CR1 genotype (n3650 polymorphism) and α⁺-thalassaemia (-α⁺² deletion) is given, error bars are standard errors. Thalassaemia genotypes are -α/αα - heterozygotes for the -α⁺² deletion, -α/-α homozygotes for the same deletion and αα/αα normal α-globin gene structure. Dark bars represent the HH genotype, grey bars the HL genotype and white bars the LL genotype. * = no representative of this genotype.
4.3.4 **Individuals with low CR1 expression are more susceptible to asymptomatic infection**

Even after accounting for differences in the frequency of the CR1 L allele and α-thalassemia mutations, there were still significant differences between the populations in red cell CR1 expression level (see Figure 4.4 b). Waitumbi et al. have shown that Kenyan children with malaria have reduced CR1 expression that is restored upon recovery of the patient (Waitumbi et al. 2000). We therefore considered whether malaria infection could be influencing the level of red cell CR1 in Melanesians by assessing the blood samples collected in Madang for the presence of malaria parasites. All samples came from healthy adult donors: therefore clinical malaria was not seen, though low level asymptomatic parasitaemia was common, with 31/96 individuals having detectable parasites (14 *P. falciparum* infections, parasitaemia range 2.60 - 4.07 log(parasites/μl); 17 *P. vivax* infections, parasitaemia range 2.90 - 4.62 log(parasites/μl), 3 *P. malariae* infections, parasitaemia range 3.08 - 3.20 log(parasites/μl), and zero *P. ovale* infections). The CR1 levels of individuals infected with *P. malariae* or *P. vivax* were not significantly different to individuals not infected with these species (*P*>0.05). However, the mean red cell CR1 expression level of *P. falciparum*-infected individuals was 70 molecules per cell compared to 133 in individuals not infected with *P. falciparum* which was statistically different (*P* < 0.01, *F*1,73=8.0). When the exon 22 CR1 genotype was included in the statistical model, the difference in CR1 expression level between *P. falciparum*-infected
and uninfected individuals was no longer significant (Table 4.2; \( P > 0.05, F_{1,71}=1.3 \)). This could be explained if the lower level of CR1 expression observed in infected individuals is due to preferential infection of individuals with low CR1 expression rather parasite infection causing low CR1 levels.

**Table 4.2: The relationship between CR1 expression, CR1 genotypes and asymptomatic malaria infection in adults**

<table>
<thead>
<tr>
<th>Infection</th>
<th>All genotypes</th>
<th>LL  (n)</th>
<th>HL  (n)</th>
<th>HH  (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em> +</td>
<td>70* (14)</td>
<td>61 (12)</td>
<td>105 (1)</td>
<td>143 (1)</td>
</tr>
<tr>
<td><em>P. falciparum</em> -</td>
<td>133 * (82)</td>
<td>67 (26)</td>
<td>155 (38)</td>
<td>180 (18)</td>
</tr>
<tr>
<td><em>P. vivax</em> +</td>
<td>125 (17)</td>
<td>75 (8)</td>
<td>78 (4)</td>
<td>93 (5)</td>
</tr>
<tr>
<td><em>P. vivax</em> -</td>
<td>123 (79)</td>
<td>63 (30)</td>
<td>90 (35)</td>
<td>89 (14)</td>
</tr>
<tr>
<td><em>P. malariae</em> +</td>
<td>49 (3)</td>
<td>21 (2)</td>
<td>105 (1)</td>
<td>N/A (0)</td>
</tr>
<tr>
<td><em>P. malariae</em> -</td>
<td>126 (93)</td>
<td>68 (36)</td>
<td>155 (38)</td>
<td>178 (19)</td>
</tr>
</tbody>
</table>

*significant difference \( (P < 0.01 \) by one-way ANOVA)

We therefore examined the distribution of *P. falciparum*-infected and uninfected individuals across the exon 22 CR1 genotypes (Table 4.3). We found that 12 out of the 14 (86%) of the infected individuals were homozygous for the L allele compared with 26 out of 82 (32%) of non-*P. falciparum* infected individuals: this was a significant departure from expected values \( (P < 0.001 \) by \( \chi^2 \) test). These results suggest that *P. falciparum* may
preferentially infect people with low CR1 expression or low CR1 individuals being less effective at clearing parasites, rather than the presence of the parasite being the cause of reduced CR1 levels. No association was found between \(\alpha^{+}\)-thalassemia genotype and \(P. falciparum\) infection (\(P>0.10\) by \(\chi^2\) test).

### Table 4.3 Prevalence of infection among asymptomatic controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HH</th>
<th>HL</th>
<th>LL</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. falciparum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate (%)</td>
<td>1/19 (5)</td>
<td>1/39 (3)</td>
<td>12/38 (32)</td>
<td>0.0007*</td>
</tr>
<tr>
<td>median density†</td>
<td>3.32</td>
<td>2.94</td>
<td>3.11</td>
<td>0.99‡</td>
</tr>
<tr>
<td>(range)</td>
<td>(-)</td>
<td>(-)</td>
<td>(2.60-4.08)</td>
<td></td>
</tr>
<tr>
<td><strong>P. vivax</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate (%)</td>
<td>5/19 (26)</td>
<td>4/39 (10)</td>
<td>8/38 (21)</td>
<td>0.21*</td>
</tr>
<tr>
<td>median density</td>
<td>2.90</td>
<td>3.08</td>
<td>3.20</td>
<td>0.15‡</td>
</tr>
<tr>
<td>(range)</td>
<td>(2.90-3.08)</td>
<td>(3.08-4.62)</td>
<td>(2.90-3.55)</td>
<td></td>
</tr>
</tbody>
</table>

* \(\chi^2\) test

†parasite density given in log (parasites/\(\mu\)l)

‡ Kruskal Wallis test
4.3.5 CR1 size variant frequencies in Papua New Guinea

Samples from New Ireland and Madang were typed for the CR1 size variants. There were 7 CR1*3 chromosomes out of 192 in Madang (gene frequency = 0.04) with the remainder being CR1*1. In New Ireland, 4 out of 94 genes were CR1*3 (gene frequency = 0.04), 1/94 was CR1*2 (gene frequency = 0.01) and the remainder were CR1*1 (gene frequency = 0.95). These frequencies were not significantly different by Fisher’s exact test ($P > 0.10$). After grouping all individuals with the CR1*3 genotype across both populations this polymorphism had no significant effect on CR1 expression level ($P > 0.10$, $F_{1,115} = 0.3$).

4.3.6 Knops Blood Group antigens in Papua New Guinea

The 47 samples from New Ireland were typed by direct agglutination assays with antisera to Sl:1, McC(a+), McC(b+) and Kn(a+). 30/47 (64%) were of the Helgeson phenotype (i.e. negative for all antigens, usually due to low expression with less than 100-200 molecules CR1 per cell (Moulds et al. 1992)). Of the Helgeson phenotype individuals, 10 had fewer than 100 CR1 molecules per cell, 13 had 100-200 CR1

---

4 These serotyping assays were carried out by Dr Joann Moulds (Drexel University College of Medicine, Philadelphia, PA)
molecules per cell, and 7 had more than 200 CR1 molecules per cell. Of the samples that were positive for at least one Knops antigen, there were no examples of the McC(b) phenotype, suggesting that this antigen may not occur in Melanesian populations. In addition, ten samples from each of the three PNG sample sites were typed by PCR for the Knops allelic pairs, $Sl\, 1$ and $Sl\, 2$, and $McC^a$ and $McC^b$. Consistent with the typing data, all samples were homozygous for $Sl\, 1$ and $McC^a$, and the $Sl\, 2$ and $McC^b$ alleles that have been reported at high frequencies in malarious regions of Africa were not detected.
4.4 Discussion

Here we show that erythrocyte CR1 deficiency is extremely common in Papua New Guinea. Individuals from lowland and island populations have the lowest recorded levels of CR1 in the world. The low level of CR1 expression is associated with an extremely high frequency of the L allele of CR1 in this population and, surprisingly, with alpha-thalassaemia which is likewise extremely common in the northern coastal regions of Papua New Guinea with 89% of individuals in Madang having at least one alpha-thalassaemia mutation. Moreover we show that there is a relatively high frequency of the small CR1*3 size allele (0.04 overall) which is rare in non-malarious populations (Cohen et al. 1999).

Given the involvement of CR1 in *P. falciparum* rosetting we hypothesise that CR1 deficiency has been selected for by malaria. We have tested this hypothesis directly by analysing samples from a severe malaria case control study (see chapter 5). Some circumstantial evidence in this study for the involvement of CR1 in protection from malaria comes from the genotyping of some blood samples from the PNG highlands, an area that has at worst, epidemic malaria. Here the gene frequency of the L allele is 0.41, significantly lower than in Madang (0.61) or New Ireland (0.73) \( (P < 0.001 \) for both populations, \( \chi^2 \) test). Though we cannot be sure that in the highlands this allele is linked to CR1 expression it seems probable given the common ancestry of these
Chapter 4: CR1 polymorphisms in Papua New Guinea

populations(Kirk 1992; Redd et al. 1999). Despite a possible malaria based hypothesis to explain the low levels of CR1 in PNG populations, the only study so far done(Nagayasu et al. 2001) suggests that low CR1 levels are actually a risk factor for malaria in Thailand. Nagayasu et al. found firstly that the intron 27 RFLP in CR1 is linked to expression level in this population with the low expression allele having a frequency of 0.52. The high frequency of the L allele suggests malarial selection, however a small study of severe and mild malaria patients compared to healthy individuals found that homozygotes carrying the low expression allele are more susceptible to severe disease, a fact they suggest may be attributed to a failure to control complement induced damage and the deposition of immune complexes(Adam et al. 1981; Gupta et al. 1988). This kind of pathology is typical of South East Asian patterns of disease characterised by multi-organ failure including renal failure(White 1998). The data they present shows some evidence that heterozygotes are protected from severe disease a fact concordant with the gene frequency of the low expression allele seen in this study. In addition to the previous study in Thailand, our own data shows that low levels of CR1 predispose to the presence of infection, especially with Plasmodium falciparum. Most protective polymorphisms result in lower or unaltered parasite prevalence (ovalocytosis(Cattani et al. 1987; Allen et al. 1999), β-thalassemia(Willcox et al. 1983)sickle cell anaemia(Ntoumi et al. 1997), and G6PD(Ruwende et al. 1995)), so this would suggest that the G3650 allele is a risk factor for malaria in PNG. One study has however shown increased parasite prevalence among α⁺-thalassemic heterozygotes in Vanuatu(Williams et al. 1996). The observation
that individuals with low CR1 are more prone to infection could in part explain this observation.

Case control studies on the ICAM-1^{kiifi} mutation, another polymorphism believed to affect adhesion characteristics of malaria parasites, have shown that a given polymorphism can be protective in some populations but a susceptibility factor in others (Craig et al. 2001). ICAM-1^{kiifi} is associated with susceptibility to cerebral malaria in Kenya (Fernandez-Reyes et al. 1997), has no effect in The Gambia (Bellamy et al. 1998) and is protective in Gabon (Kun et al. 1999). The reasons for these differences are unknown but probably lie in the patterns of disease and population genetic factors. In PNG the pattern of disease is different from Southeast Asia and the host genetics are different, notably the much higher prevalence of α^+^-thalassemia mutations in this population which also affects CR1 expression (Flint et al. 1993; Flint et al. 1998).

The reduction CR1 expression among α^+^-thalassemic individuals, is one of the most striking results from this study. α^+^-thalassemia has previously been shown to confer protection from severe disease in the Madang region with heterozygotes having a relative risk of 0.6 while homozygotes have a relative risk of 0.4 (Allen et al. 1997). But while the protective effect of this disorder is well known, the mechanism is debated. Parasite growth is unimpaired in thalassaemic cells (Luzzi et al. 1991) so investigators have sought explanations based on reduced cytoadherence or immune recognition (Udomsangpetch et al. 1993). Carlson et al. (Carlson et al. 1994) showed a
large reduction in rosetting with thalassaemic RBC. They argued that this could be a result of microcytosis - thalassaemic cells have around 15% less surface area than normal cells (Luzzi et al. 1991). However the thalassaemic cells still rosetted less well than microcytic controls with normal haemoglobin. Microcytosis is also insufficient to explain CR1 reduction, which is around 50% in thalassaemic individuals (Figure 4.4). As CR1 is preferentially lost in vesicles from the cell surface as RBC age or become ATP-depleted (Pascual et al. 1993) it is possible that biochemical abnormalities in thalassaemic RBC promote a similar process that would reduce their CR1 expression. We might speculate that low CR1 levels on thalassemic red cells could enhance immune recognition of parasite antigens as it could theoretically increase the deposition of complement components like C3b on infected red cells and hence facilitate phagocytosis of infected cells. Another notable result from the both Madang and New Ireland group is a gene frequency of 0.04 for the small CR1*3 size allele. This is higher than in other populations studied other than populations in Mali and Black Americans (Cohen et al. 1999). The CR1*3 allele could confer protection as these alleles lack one of the potential binding sites for PfEMP1 and may allow only weak rosetting (Rowe et al. 2000). This hypothesis, though, has yet to be tested, even in in-vitro experiments.

I have argued that low CR1 has been selected for as it reduces parasite rosetting and therefore severe disease. However a previous study showed no correlation between rosetting and severe disease in PNG (al-Yaman et al. 1995), how can these observations be reconciled? This study was based at the town hospital in Madang and the patients and
controls came from region from which encompasses the village from which our Madang samples came. The study showed rosettes forming in every isolate studied at rates higher than those seen in African studies where many isolates do not rosette at all (Rowe et al. 1995). These otherwise anomalous results could be explained if the rosettes observed were formed in the effective absence of CR1 and were therefore not physiologically significant. If the rosettes were bound only by weak interactions they may not have been sustainable in the conditions of shear stress that would be encountered in the capillary systems of the human body and could not contribute to severe disease pathology. In the remaining chapters I have tried to resolve these paradoxes and questions. Firstly by testing the hypothesis that CR1 deficiency is a result of malarial selection and secondly by studying rosetting in this population, the extent to which it occurs, its disease associations and the receptors involved.
Chapter 5:

The CR1 L allele protects individuals against severe malaria

5.1 Introduction

We have demonstrated the high frequency of CR1 polymorphisms in Papua New Guineans that are rare in other populations. High frequencies of an otherwise rare allele

5 These results are published as Cockburn et al. 2004 (see Appendix)
may be the result either of chance - acting through the processes of genetic drift and founder effects, or they may occur as a result of natural selection in a particular environment (Flint et al. 1998). Given the high level of malaria transmission in coastal populations of PNG (Flint et al. 1986; Burkot et al. 1988) and the involvement of CR1 binding in *Plasmodium falciparum* pathology (Rowe et al. 1997) we decided to test the hypothesis that the high frequency of CR1 deficiency in this population was due to protection it provides from severe malaria. If CR1 deficiency did protect against severe malaria it would also provide strong indirect evidence that rosetting is a cause and not a correlate of disease severity. In order to do this we were fortunate to have access to DNA samples from a large case-control study carried out previously at the Madang general hospital (Allen et al. 1997; Allen et al. 1999). These samples had been previously typed for α-thalassaemia and ovalocytosis, and full clinical information was available for each patient. We were able to type these samples at the exon 22 polymorphism to determine if the CR1 L allele does indeed protect against severe malaria.

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6 The painstaking work of carrying out a case-control study over a period two years was done by Steve Allen and Angela O'Donnell. The genotyping of samples and statistical analysis is my work.
5.2 Study design

5.2.1 Case-control study

The case-control study design has been described extensively previously (Allen et al. 1997; Allen et al. 1999) and only an outline is given here. Samples were collected from children admitted to the paediatric ward of the Madang general hospital between October 1993 and February 1996. The hospital is the main referral hospital for the province including the village of Udisis from which our community blood samples described in Chapter 4 came. Only individuals who had lived in Madang province for more than a year were included in the study. In this case-control study individual severe malaria cases were paired with a community control matched for age, sex, ethnicity and residence. Severe malaria was defined according to World Health Organisation criteria (Warrell et al. 1990). Coma was defined as a Blantyre coma score of \( \leq 2 \) with asexual stages of \( P. falciparum \) in the peripheral blood and no alternative diagnosis. The Blantyre coma score is a modification of the more commonly used Glasgow coma score adapted for use with pre-verbal children (Molyneux 1990). Severe anaemia was defined as a haemoglobin < 5 g/dl and a parasitaemia > 10 000 parasites /\( \mu l \) though lower parasite densities were admissible if the child had taken anti-malarial drugs prior to admission. Acidosis was defined as plasma bicarbonate < 15 mmol/l, hypoglycaemia as plasma glucose < 2.2 mmol/l and hyperlactaemia as \( \geq 5 \) mmol/l. Clinical features of the cohort of severe
malaria patients in this study have also been described extensively previously (Allen et al. 1996).

One matched community control child was recruited for each index case. A child was found from a family neighbouring the house of the index case. The child had to be of the same age (±1 year), the same ethnicity (defined according to the languages spoken by the parents) and the same sex as the index child. Controls were collected within one month of admission of the index case.

5.2.2 Laboratory analysis

Blood was drawn into EDTA tubes for white cell counts and into heparin and fluoride oxalate for biochemical analysis. White and red cell counts were done on a coulter counter (Coulter Electronics, Luton, UK) Biochemical analyses on samples were carried out by dry slide chemistry (Kodak-Ektachem, Rochester, NY). Samples had been previously typed for ovalocytosis according to the method of Jarolim et al. (Jarolim et al. 1991), for α⁺-thalassaemia by southern blotting and haemoglobin electrophoresis (Old et al. 1983), and for glucose-6-phosphate dehydrogenase deficiency using Sigma procedure no. 400 (Sigma, Poole, UK) as described in Section 2.5. The samples were typed for the exon 22 polymorphism in the CR1 gene as described in section 2.3. Exon 22 PCRs were performed on DNA samples that had previously been amplified by primer extension.
preamplification (PEP or whole genome) PCR as the remaining genomic DNA from the case-control study was small. This technique amplifies genomic DNA non-specifically to increase the amount of template available (Zhang et al. 1992).
Chapter 5: Low CR1 protects against severe malaria

5.3 Results

5.3.1 The CR1 HH genotype is a risk factor for severe malaria

Out of 311 case-control study pairs we were able to genotype 186 index cases and 186 controls for the CR1 polymorphism. The other samples could not be genotyped either due to the unavailability of DNA samples or PCR insensitivity. We had complete genotypic information (ovalocytosis, α^+thalassaemia and CR1 exon 22) for 180 cases and 178 controls. We performed logistic regression using case or control status as the outcome variable and incorporating thalassaemia and ovalocytosis genotypes into the model. This form of regression analysis takes no account of the pairing samples and simply compares the two groups as whole. We found that CR1 HL heterozygotes were strongly protected against severe malaria relative to the HH genotype (OR [95% confidence interval]: 0.33 [0.14-0.78], P = 0.01; Table 5.1) while LL homozygotes had reduced odds ratios but this was not statistically significant (OR [95% confidence interval: 0.55 [0.24-1.28], P = 0.16; Table 5.1]). HH individuals were found to have a odds ratio of severe malaria of 2.26 (95% confidence interval: 1.00 - 5.12; P = 0.05) compared to the other two genotypes combined. The LL genotype is apparently less protective than the heterozygous state (though this difference in itself is not statistically significant (Table 5.1)), which implies that there may be a malaria-associated cost to having a level of CR1 expression that is extremely low. We also carried out conditional
logistic regression analysis. While this is a more sensitive statistical test as it compares matched pairs directly, many case-control pairs without complete data for both individuals had to be removed from the analysis. The results from this analysis were not materially different from those with ordinary logistic regression analysis.

5.3.2 Different disease types

Some malaria-associated polymorphisms protect against one severe syndrome but not another, for example ovalocytosis has been shown in two studies to be protective against cerebral malaria but not any other complication of severe disease (Genton et al. 1995; Allen et al. 1999), while α⁺-thalassaemia protects against severe malarial anaemia, acidosis and hyperlactaemia (Allen et al. 1997). We therefore subdivided the cases and controls into overlapping groups of the following syndromes (defined as in section 5.2.1): severe anaemia, cerebral malaria, acidosis, hyperlactaemia and hypoglycaemia. Due to the relatively small numbers in the last three groups we also pooled these samples as "metabolic complications".

The HL genotype confers significant protection against severe anaemia (Table 5.1), while the LL genotype has a trend towards protection. The HL and LL genotypes also have low odds ratios relative to the HH genotype for susceptibility to cerebral malaria, however the P values for this are large reflecting the small sample size. Conversely there is a trend
towards the LL genotype being a risk factor for the three metabolic determinants of
disease severity both when they are pooled or treated separately but this does not reach
statistical significance.
## Table 5.1: Case-control study: summary of results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Adjusted odds ratio* (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All severe cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=180</td>
<td>n=178</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>21 (12%)</td>
<td>9 (5%)</td>
<td>0.33 (0.14-0.77)</td>
<td>0.01</td>
</tr>
<tr>
<td>HL</td>
<td>57 (37%)</td>
<td>81 (46%)</td>
<td>0.55 (0.24-1.28)</td>
<td>0.16</td>
</tr>
<tr>
<td>LL</td>
<td>102 (56%)</td>
<td>88 (49%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe anaemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=113</td>
<td>n=109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>15 (13%)</td>
<td>5 (5%)</td>
<td>0.30 (0.10-0.93)</td>
<td>0.017</td>
</tr>
<tr>
<td>HL</td>
<td>34 (30%)</td>
<td>45 (41%)</td>
<td>0.46 (0.15-1.40)</td>
<td>0.15</td>
</tr>
<tr>
<td>LL</td>
<td>64 (57%)</td>
<td>59 (54%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=45</td>
<td>n=47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>4 (9%)</td>
<td>2 (4%)</td>
<td>0.40 (0.06-2.50)</td>
<td>0.33</td>
</tr>
<tr>
<td>HL</td>
<td>15 (33%)</td>
<td>21 (46%)</td>
<td>0.60 (0.10-3.69)</td>
<td>0.55</td>
</tr>
<tr>
<td>LL</td>
<td>26 (58%)</td>
<td>23 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperlactaemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=44</td>
<td>n=42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>4 (9%)</td>
<td>4 (10%)</td>
<td>1.07 (0.20-5.54)</td>
<td>0.93</td>
</tr>
<tr>
<td>HL</td>
<td>14 (32%)</td>
<td>18 (43%)</td>
<td>1.99 (0.38-10.37)</td>
<td>0.41</td>
</tr>
<tr>
<td>LL</td>
<td>26 (59%)</td>
<td>20 (47%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidosis</td>
<td>n=29</td>
<td>n=32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>2 (7%)</td>
<td>2 (6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>12 (41%)</td>
<td>15 (47%)</td>
<td>1.41 (0.13-14.93)</td>
<td>0.78</td>
</tr>
<tr>
<td>LL</td>
<td>15 (52%)</td>
<td>15 (47%)</td>
<td>1.80 (0.17-18.84)</td>
<td>0.62</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>n=13</td>
<td>n=12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>1 (8%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>6 (46%)</td>
<td>4 (32%)</td>
<td>∞ (0-∞)</td>
<td>NA</td>
</tr>
<tr>
<td>LL</td>
<td>6 (46%)</td>
<td>8 (64%)</td>
<td>∞ (0-∞)</td>
<td>NA</td>
</tr>
<tr>
<td>Metabolic syndromes†</td>
<td>n=58</td>
<td>n=61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Odds ratios for the HL and LL genotypes are calculated in relation to the HH genotype after adjusting for the potential confounding factors ovalocytosis and alpha-thalassaemia.

† Hypoglycaemia, acidosis and hyperlactaemia.
5.3.3 Interaction between α*-thalassaemia and CR1 genotype

Since both α*-thalassaemia and the CR1 L allele are associated with both low CR1 and protection against severe malaria one hypothesis is that the protection these mutations afford is through their effect on CR1 expression. A second hypothesis is that α*-thalassaemia has an effect on severe malaria which is independent of CR1 expression - this might be the only protective effect of thalassaemia or could act side by side with effects on CR1 expression. It is also possible that CR1 genotypes are protective through an effect independently of CR1 expression, i.e. if the two alleles were functionally distinct for which there may be some evidence. These models are summarised in Figure 5.1.

As no information exists for the actual erythrocyte CR1 expression of samples in this study, to try and differentiate between these models we determined the odds ratio of developing severe malaria for each combination of CR1 and thalassaemia genotype. If both genotypes were acting through CR1 expression we would expect the odds ratios to correlate with expected CR1 expression for that genotype. Nonetheless this may be confounded by the relatively small sample size, and the possibility that the LL genotype carries a malaria-associated cost, probably independent of its effect on rosetting.
Figure 5.1 Models of the protective effects of α+-thalassaemia and CR1 polymorphisms against malaria. Either they act together through CR1 expression (light arrows) or α+-thalassaemia and CR1 may have effects on severe malaria independently of CR1 expression (dark arrows).

We calculated odds ratios by logistic regression compared to the remainder of the population, which had a case to control ratio of 1:1 (182 cases, 179 controls). Only two genotype odds ratios differ significantly from 1 (Table 5.2). The HL (CR1 genotype); α-/α- (thalassaemia genotype) combination combines the most beneficial alleles of both genotypes and provides the strongest protection relative to all other alleles (OR = 0.39; P = 0.0009), however is not associated with the lowest CR1 expression. This may be expected as very low CR1 expression could be a risk factor for malaria (section 5.3.1).
Conversely HH; αα/αα individuals are most at risk of severe malaria (P = 0.015) as none of these 7 individuals were in the control category. This tentatively suggests that the two effects are acting through CR1 expression as this genotype is associated with the highest CR1 expression.

### Table 5.2: Combined thalassaemia and CR1 genotypes

<table>
<thead>
<tr>
<th>α⁺-thalassaemia</th>
<th>CR1</th>
<th>No. cases/</th>
<th>OR* (95% confidence intervals)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no. Typed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αα/αα</td>
<td>HH</td>
<td>7/7</td>
<td>∞†</td>
<td>0.015‡</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>12/21</td>
<td>1.33 (0.54-3.26)</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>15/28</td>
<td>1.15 (0.53-2.50)</td>
<td>0.73</td>
</tr>
<tr>
<td>α⁻/αα</td>
<td>HH</td>
<td>7/14</td>
<td>0.98 (0.34-2.88)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>24/49</td>
<td>0.94 (0.51-1.72)</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>38/62</td>
<td>1.70 (0.97-2.49)</td>
<td>0.063</td>
</tr>
<tr>
<td>α⁻/α⁻</td>
<td>HH</td>
<td>7/10</td>
<td>2.35 (0.59-9.30)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>22/69</td>
<td>0.39 (0.22-0.68)</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>50/101</td>
<td>0.95 (0.60-1.51)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

* Odds ratios for each two-locus genotype are expressed relative to the remainder of the population, which had a case to control ratio of 0.50 (182 cases, 179 controls). They were calculated using logistic regression fitting two-locus genotype as a 2-level fixed effect factor (i.e. of or not of that genotype).

† Not estimable because all people in this class had severe malaria.

‡ Tested for deviation in frequency from the remainder of the population using Fisher's exact test.
Another way of attempting to distinguish between the two models would be to look for evidence of linkage disequilibrium between thalassaemia and CR1 genotypes. If there is linkage disequilibrium and particular combinations of genotypes are favoured that may be evidence for both genotypes acting through a common mechanism, perhaps CR1 expression. The control samples alone (which should represent an unbiased population sample with respect to individuals' genotypes) were analysed for linkage disequilibrium using the a number of permutations method in the program Genetix (Universite de Montpellier, Montpellier, France). This method gives the probability of linkage disequilibrium for two loci where there is information on the genotype at two loci but does not require family information. Also being a permutations (exact) test it makes no assumptions about the distribution of the genotypes - in other words it can be used with alleles not in Hardy-Weinberg equilibrium. Nonetheless there is no statistically significant evidence for linkage disequilibrium between these two loci ($P = 0.08$).

However, an examination of the distribution of genotype combinations (Table 5.3) shows that there is significant departure from the expected values if the genotypes were in Hardy-Weinberg equilibrium, giving further evidence for strong selection on these two loci ($\chi^2 = 18.485; \text{df} = 4; P < 0.001$).
Table 5.3: Distribution of CR1 and thalassamia genotypes among control individuals

<table>
<thead>
<tr>
<th>Thalassaemia genotype</th>
<th>αα/αα</th>
<th>α-/αα</th>
<th>α-/α-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1 genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>0 (1*)</td>
<td>7 (6)</td>
<td>3 (7)</td>
<td>10 (14)</td>
</tr>
<tr>
<td>HL</td>
<td>9 (6)</td>
<td>25 (29)</td>
<td>47 (38)</td>
<td>81 (73)</td>
</tr>
<tr>
<td>LL</td>
<td>13 (7)</td>
<td>24 (37)</td>
<td>51 (47)</td>
<td>88 (91)</td>
</tr>
<tr>
<td>Total</td>
<td>22 (14)</td>
<td>56 (72)</td>
<td>101 (93)</td>
<td>179</td>
</tr>
</tbody>
</table>

* expected value given in brackets

5.3.4 Clinical Parameters

No significant difference was observed among any of the clinical parameters measured between the different CR1 genotypes in the group of severe disease patients (Table 5.3). The median value for haemoglobin is slightly lower in the HH genotype reflecting the susceptibility to anaemia, while the lactate level in individuals with the LL genotype is higher which accords with the result that the LL genotype may predispose to hyperlactaemia. Finally there is a trend towards lower mortality in the LL group.
Table 5.3: Indices of severity of malaria and mortality subdivided by genotype for the CR1 exon 22 polymorphism

<table>
<thead>
<tr>
<th></th>
<th>HH</th>
<th>HL</th>
<th>LL</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemoglobin (g/dl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>4.45 (2.4 - 10)</td>
<td>4.85 (2.3 - 16.6)</td>
<td>4.70 (1.4 - 13.3)</td>
<td>0.66</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>60</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>2.8 (1.7 - 8.0)</td>
<td>2.85 (1.2 - 15.4)</td>
<td>3.40 (1.2 - 15.6)</td>
<td>0.55</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>58</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Bicarbonate (mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>13.9 (8.8 - 32.5)</td>
<td>14.9 (8.4 - 48.3)</td>
<td>14 (4.4 - 40.7)</td>
<td>0.36</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>47</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Pma (log parasites/µl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>3.91 (1.60-5.41)</td>
<td>4.09 (1.60-5.60)</td>
<td>4.24 (1.60-5.63)</td>
<td>0.65</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>55</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Coma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/total for genotyp (%)</td>
<td>4/21 (19)</td>
<td>15/59 (25)</td>
<td>28/103 (27)</td>
<td>0.74</td>
</tr>
<tr>
<td>Death</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/total for genotype (%)</td>
<td>1/22 (4.5)</td>
<td>5/60 (8.3)</td>
<td>3/104 (2.8)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Analyses for haemoglobin, lactate, bicarbonate and parasitaemia are by Kruskal-Wallis test. For coma and death analysis is by χ² test.

### 5.3.5 Infection in control individuals

In section 4.3.4 we found that in a population survey of healthy adults infection with slide-detectable *P. falciparum* was significantly associated with the LL genotype. The control sample from the case-control study gave us an opportunity to determine if this
observation was also true in children. However we found that for both *P. falciparum* and
*P. vivax* there was no association between genotype and infection prevalence (Table 5.4).

### Table 5.4: Prevalence of infection among asymptomatic controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>P. falciparum</th>
<th>P. vivax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate (%)</td>
<td>Median density (range)</td>
</tr>
<tr>
<td>HH</td>
<td>3/12 (25)</td>
<td>3.32 (2.20-3.93)</td>
</tr>
<tr>
<td>HL</td>
<td>33/79 (42)</td>
<td>2.94 (1.60-4.76)</td>
</tr>
<tr>
<td>LL</td>
<td>32/87 (37)</td>
<td>3.11 (1.60-5.09)</td>
</tr>
<tr>
<td></td>
<td>Rate (%)</td>
<td>Median density (range)</td>
</tr>
<tr>
<td>HH</td>
<td>1/12 (8)</td>
<td>2.68 (-)</td>
</tr>
<tr>
<td>HL</td>
<td>7/79 (9)</td>
<td>2.90 (1.90-3.94)</td>
</tr>
<tr>
<td>LL</td>
<td>16/87 (18)</td>
<td>1.99 (1.60-3.47)</td>
</tr>
</tbody>
</table>

* *χ²* test
† parasite density given in log (parasites/μl)
‡ Krucksal Wallis test

The reasons for the difference between these two samples are unclear. It may be that the small sample size of the survey in chapter 4 gave an artefactual result. However this survey is not directly comparable to the population survey described in chapter 4. In this case the samples were from children who would have yet to develop functional immunity to malaria, whereas the individuals in the other survey were adults. As these individuals were controls they would have been collected largely in the wet season, whereas the samples from the adults were collected in June: the dry season. A difference in infection
rate or clearance of parasites among CR1 genotypes depending on season and age could be interesting, though to confirm that this difference was real a variety of cross sectional studies in different seasons would have to be analysed.

5.3.6 Gene frequencies of the L allele vary significantly between populations

As the children in this study came from a variety of genetic backgrounds we could also use the control sample to study the gene frequency of the L allele in population from different parts of Papua New Guinea with different malaria endemicities (Table 5.5). The 186 controls were grouped according to their parents' language into groups of Madang, Sepik, Highlands, Other Coastal and Mixed. The Sepik has some of the most intense malarial transmission in the country (Genton et al. 1995; Genton et al. 1995). While the other coastal regions include the southern coastal areas that have less intense transmission than the north coast (Brown 1995).
### Table 5.5 Frequency of the L allele among different ethnic groups

<table>
<thead>
<tr>
<th></th>
<th>Madang</th>
<th>Sepik</th>
<th>Highlands</th>
<th>Other Coastal</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>HL</td>
<td>61</td>
<td>3</td>
<td>1</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>LL</td>
<td>60</td>
<td>14</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>19</td>
<td>2</td>
<td>35</td>
<td>3</td>
</tr>
</tbody>
</table>

| Gene frequency (L allele) | 0.71 | 0.82 | 0.25 | 0.64 | 0.5 |
| P*                   | -    | 0.027 | 0.015 | 0.14 | 0.21 |

*P = difference between population from the Madang population ($\chi^2$ test)

The data show that there are significant differences in the gene frequency of the L allele between different populations in Papua New Guinea. The Sepik individuals have the highest L allele frequency in the world, which is significantly higher than in Madang. The Highlanders have a much lower gene frequency though as the sample number is very small we should not infer anything from this data. Better evidence for low frequencies of the L allele in this population comes from data presented in 4.3.2. Individuals from the southern coastal regions had a lower overall frequency of the L allele but this did not reach statistical significance. These data support the notion of a geographical distribution of the L allele that correlates with the transmission intensity of malaria and therefore a role for CR1 in malaria pathogenesis.
5.4 Discussion

This study shows that alleles associated with CR1 deficiency, found commonly in Papua New Guinea, protects against severe malaria. Genotypes associated with low CR1 in both the α-globin gene (Allen et al. 1997) and the CR1 gene itself have now been associated with protection against severe malaria. There is also evidence for variation in the prevalence of the L allele depending on malaria endemicity. As parasites invade and grow normally in both CR1 deficient (Soubes et al. 1999) and thalassaemic red blood cells (Luzzi et al. 1991) the simplest interpretation is that these polymorphisms are protecting against severe disease through their known effects on rosetting (Carlson et al. 1994; Rowe et al. 1997). Positive selection of rosette-reducing polymorphisms is strong evidence that CR1-mediated rosetting plays a causal role in the pathogenesis of severe malaria.

CR1 deficiency appears to be rare in African populations studied to date (Herrera et al. 1998; Rowe et al. 2002) despite its protective effect in Melanesians. The H and L haplotypes do exist in Africa but are not associated with CR1 expression (Herrera et al. 1998; Rowe et al. 2002). This suggests (though other explanations are plausible) that the SNPs themselves are non-functional and that the lesion causing CR1 deficiency arose on an L chromosome only after humans left Africa. We would have a better understanding of these issues if the functional basis of CR1 deficiency is definitively identified. Instead African populations possess other CR1 polymorphisms - particular Knops blood group
antigens (SI 2 and McCb) localised to the CR1 protein reach high frequencies in West Africa. SI 2 has been associated with low rosetting (Rowe et al. 1997) but not through CR1 deficiency (Moulds et al. 1991). We might also speculate that in order for CR1 expression to be sufficiently low to prevent rosette formation both CR1 and α-thalassaemia mutations have to be present in a population i.e. there must be linkage disequilibrium between the genes. While there is no direct evidence for that presented here, we cannot rule it out as our sample size is small. This might account for the exceptional frequency of both alleles in coastal Papua New Guineans, but their lower prevalence elsewhere. Clearly the selective pressure of malaria has acted in different ways on the CR1 gene in diverse populations.

The rarity of CR1 deficiency in much of the rest of the world suggests that without malaria the L allele carries a cost. The high prevalence among our healthy adult donors demonstrates that it is clearly not responsible for a severe syndrome like sickle cell anaemia. One study has linked the L allele to systemic lupus erythematosus (SLE) in which immune complexes accumulate (Wilson et al. 1987), but most find no association (Moldenhauer et al. 1987; Cohen et al. 1989; Yen et al. 1989; Satoh et al. 1991; Kumar et al. 1995; Kiss et al. 1996). In this study we find that HL individuals are more protected than LL individuals, raising the possibility that very low CR1 expression prevents proper parasite clearance or contributes to pathology. In the previous chapter we referred to the a small study of CR1 polymorphisms in Thailand, this also showed a high frequency of the L allele (f = 0.52) showing that it had undergone selection, though they
Chapter 5: Low CR1 protects against severe malaria

report that LL homozygotes are at risk of severe malaria (Nagayasu et al. 2001). Our analysis of their data indicates that HL heterozygotes in Thailand, as in PNG, may be protected from severe disease. However, perhaps due to the small number of cases, this effect was not statistically significant (severe malaria cases versus healthy controls, HL versus HH, OR 0.395, 95% CI 0.126-1.23, P=0.11). Our findings and those of Nagayasu et al. support some older work that argued that impaired handling of immune complexes contributes to some severe malaria syndromes such as renal failure and pulmonary oedema, which are common in South East Asia (Adam et al. 1981; White 1987).

The case-fatality rates for severe malaria are high, even in areas with intensive care facilities, so there is an urgent need for new approaches to treatment (Warrell 1999). Our results suggest that therapies aimed at inhibiting CR1-mediated rosetting (Rowe et al. 1997), such as soluble recombinant CR1 (Weisman et al. 1990), could be of benefit in severe malaria. Alternatively, development of a vaccine against the parasite rosette-mediating ligand PfEMP1 may prevent some cases of severe disease. The selective pressure of malaria on a major rosetting receptor indicated by our results strongly supports a direct role for CR1-mediated rosetting in the pathogenesis of severe disease, and emphasises that further research to develop rosette-inhibiting interventions should be a high priority.
Chapter 6:

Rosetting is infrequent in Papua New Guinea, not associated with disease severity and rarely mediated by CR1

6.1 Introduction

Our previous work has shown that CR1 deficiency is extremely common in Papua New Guinea and that genotypes associated with low CR1 on erythrocytes are protective against severe malaria. We have hypothesised that the mechanism of protection is
through reduced rosetting. Here I set out to test that hypothesis firstly by testing the ability of a range of CR1 deficient erythrocytes to adhere to rosetting laboratory isolates, and secondly by studying rosetting in isolates from severe and uncomplicated malaria patients from Papua New Guinea. A previous study found that rosetting in Papua New Guinea was not associated with disease severity (al-Yaman et al. 1995) in contrast to most studies in Africa (Carlson et al. 1990; Ringwald et al. 1993; Rowe et al. 1995). This challenged the idea of rosetting as a cause of severe disease. However the data presented in the previous chapter suggests an alternative idea: that due to polymorphisms in one of the main receptors for rosetting in Papua New Guinea the nature and disease associations of rosetting may be significantly altered. The aim of this study is to understand the epidemiological difference between African and Papua New Guinean rosetting studies in molecular terms and so gain a better understanding of the molecular basis of severe disease.

Combining two pieces of information – the importance of CR1 for rosetting in Africa and the low level of CR1 on the red blood cells of most Melanesians, we set out to test the hypothesis that the rosetting previously observed in malaria patients in PNG is mediated through receptors other than CR1. We hypothesise that the rosettes seen in CR1 deficient Melanesians are not as strong as those mediated via CR1 and may be unable to withstand physiological shear forces, therefore such rosettes are not implicated in the pathogenesis of severe disease. Furthermore, a minority of Melanesian individuals have sufficient CR1 expression for this to be used by the parasite for rosetting, and we predict that in
these patients, stronger rosettes could be formed that may be associated with severe disease. We decided to test this hypothesis by comparing the host receptors for rosetting and CR1 expression in severe and uncomplicated malaria cases. The study also gave us an opportunity to observe \textit{ex-vivo} if rosette formation was impaired as a result of any of the common red cell polymorphisms that exist in Papua New Guinea. In addition to $\alpha^+$-thalassaemia we also typed samples for the deletion of exon 3 in glycophorin C responsible for the Gerbich blood group type and for Southeast Asian ovalocytosis.
6.2 Methods and Study Design

6.2.1 In-vitro studies with laboratory rosetting clones

We first of all set out to determine the ability of uninfected erythrocytes from a variety of donors from Papua New Guinea to form rosettes with three laboratory isolates: Palo Alto R+, TM284+ and R29+. These isolates vary in their use of different host receptors, R29+ forms rosettes through CR1, while PAR+ and TM284+ are known to bind immunoglobulins (Scholander et al. 1996; Somner et al. 2000). PAR+ seems to bind the A blood group antigen while TM284+ may preferentially bind the B blood group antigen (Carlson et al. 1992). The erythrocytes used for these experiments were frozen red blood cells from the Madang donor group described in section 4.2. To measure the rosetting ability of an erythrocyte sample we performed a competition assay in which fluorescently labelled "test" erythrocytes competed with control erythrocytes (in which the parasites were grown) to form rosettes. The relative proportion of cells in rosettes that were labelled gave a relative rosetting score for that isolate. The full method is described in section 2.8.4.
6.2.2 Field study design

We wished to compare rosetting frequency and receptor usage between parasite isolates from severe and uncomplicated malaria patients. We also compared CR1 expression, CR1 genotypes and thalassaemia between severe and uncomplicated malaria patients, and asymptomatic controls. However as the number of severe cases collected was small we collected asymptomatic controls matched to uncomplicated malaria patients as well as to severe malaria patients. Severe malaria was defined according to World Health Organisation criteria described in (Warrell et al. 1990), in section 5.2.1. The various criteria are summarised in table 6.1. Uncomplicated malaria was defined as an acute febrile illness (axillary temperature ≥ 37 °C) with detectable parasitaemia and no manifestation of severe malaria. As rosetting may be affected by drug treatment (Udomsangpetch et al. 1996; Chotivanich et al. 1998) we excluded patients who had taken anti-malarials in the previous 2 weeks. All protocols were approved by the Papua New Guinea Medical Research Advisory Committee (Study Number 03.05).
### Table 6.1: Severe malaria criteria (Warrell et al. 1990)

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defining criteria of severe malaria</td>
<td></td>
</tr>
<tr>
<td>Cerebral Malaria</td>
<td>Blantyre coma score ≤ 2</td>
</tr>
<tr>
<td>Severe anaemia</td>
<td>Haemoglobin &lt; 5g/dl</td>
</tr>
<tr>
<td>Pulmonary oedema*</td>
<td>diagnosed by X ray</td>
</tr>
<tr>
<td>Acidosis*</td>
<td>plasma bicarbonate &lt; 15 mmol/L</td>
</tr>
<tr>
<td>Hyperlactaemia</td>
<td>&gt; 5 mmol/L blood lactate</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>glucose &lt; 2.2 mmol/L or 40 mg/dl</td>
</tr>
<tr>
<td>Renal Failure*</td>
<td>creatinine &gt; 265 μmol/L (3mg/dl)</td>
</tr>
<tr>
<td>Convulsions</td>
<td>&gt; 1 / 24 hours</td>
</tr>
<tr>
<td>Respiratory distress</td>
<td>Chest indrawing, grunting, flaring nostrils, irregular breathing</td>
</tr>
<tr>
<td>Circulatory collapse</td>
<td>systolic blood pressure &lt; 60</td>
</tr>
<tr>
<td>Haemoglobinuria</td>
<td></td>
</tr>
<tr>
<td>Abnormal bleeding</td>
<td></td>
</tr>
<tr>
<td>Other manifestations of severe malaria</td>
<td></td>
</tr>
<tr>
<td>Hyperparasitaemia</td>
<td>≥ 20%</td>
</tr>
<tr>
<td>Prostration</td>
<td>Inability to sit without support, or for infants inability to breastfeed</td>
</tr>
<tr>
<td>Impaired consciousness</td>
<td>Blantyre coma score 3 - 4</td>
</tr>
<tr>
<td>Jaundice</td>
<td>detected clinically or defined by serum bilirubin &gt; 50 μmol/L</td>
</tr>
</tbody>
</table>

* Indicates criteria that we were unable to test for
6.2.3 **Collection of patient samples**

Samples were collected at the paediatric outpatients department of Madang general hospital, which is the main referral centre for the region. This hospital was also the base for the case-control study described in chapter 5. Children were assessed by nursing staff and either treated as outpatients or admitted to the paediatric ward. No intensive care facilities exist, so treatment consists of oral/intramuscular drug treatment with quinine and artemether, and palliative care. Individuals were enrolled in the study that had a provisional diagnosis of malaria. A local nurse employed by the project took up to 2ml of blood into EDTA tubes from each patient having obtained consent from the parents following an explanation of the aims of the project. The parents of the child would be asked questions about the patient history and a clinical assessment would be made to assess fever, coma score (Molyneux 1990), respiratory status and extent of prostration (if any). Diagnostic tests would be made on the spot for hypoglycaemia using Glucotrend test strips (Roche, Lewes, UK), hyperlactaemia using the Accutrend, Lactate system (Roche, Rotkrenz, Switzerland) and haemoglobin using the Hemocue B-Hemoglobin system (Hemocue, Lake Forest, CA) according to the manufacturer's instructions. Thick and thin smears for slide diagnosis of malaria were prepared. If the patient showed sign of impaired consciousness the nursing staff would take a CSF sample by lumber puncture and the hospital laboratories would check for meningococcal bacteria. As sample collection was slow and many patients had only been referred after receiving drugs at
local clinics, we also collected samples from the town clinic in Madang using identical protocols.

### 6.2.4 Laboratory analysis of patient samples in Madang

Samples were taken to the Papua New Guinea Institute of Medical Research campus at Yagaum about 10 miles south of Madang town. Here thick and thin smears were stained with giemsa and the parasitaemia counted to confirm or discount the provisional diagnosis of malaria. Slide positive samples with no alternative diagnosis were considered malaria cases and then used for experiments in Madang and prepared for analysis in the UK. Direct antiglobulin testing was used to determine the ABO blood group type of the patent (Section 2.6). Blood samples were spun through lymphocyte separation medium (Bio-Whittaker, Walkersville, MD), the white blood cells were then removed and used for DNA extraction using the Nucleon BACC2 DNA extraction kit according to the manufacturers instructions (Amersham, Little Chalfont, UK). The remaining red cells were washed twice in 10 ml iRPMI and up to 200 μl of packed cells were put into culture according to standard methods (Section 2.7.3). 50 μl of blood was fixed for subsequent flow cytometry (Section 2.2.2) any remaining blood cells were frozen in glycerolyte (Section 2.2.1). The cultures were checked for growth approximately every 12 hours by giemsa staining (Section 2.7.3). Once most parasites reached the late trophozoite/early schizont stage the rosetting frequency was measured as
in Section 2.8.1. If the rosetting frequency was found to be >10% then antibody disruption assays were performed with a panel of antibodies and reagents specific for various rosetting receptors to try and determine which of these may be used in rosetting in these isolates (Section 2.8.5).

6.2.5 Laboratory analysis of patient samples in Edinburgh

CR1 expression level was determined by flow cytometry on the fixed red blood cells compared to fixed standards as described (Section 2.1)(Cockburn et al. 2002). Genotypes for the n3650 polymorphism in CR1, -α\(^{3.7}\) and -α\(^{4.2}\) thalassaemia, South-East Asian ovalocytosis and ΔExon3 in Glycophorin C were carried out as described (Section 2.3 and references therein).

6.2.6 Collection of control samples

Asymptomatic control samples were collected for 36/43 clinical malaria samples. Controls were matched by age (patient age ± 20%), ethnicity (determined by parents home province), location, and were collected in the same season as the clinical malaria samples. Location matching of samples was as follows: patients were considered to come from one of four areas, Madang town and periurban settlements; the North Coast, a narrow strip of plantations, villages and small offshore islands at sea level; the foothills
of the Adelbert ranges, and the south coast and trans-Gogol, the large basin and flood
plain of the Gogol river which empties into the Pacific Ocean about 20 miles south of
Madang (Figure 6.1). To collect samples we would inform the village leaders the day
before of our intention to come and find children for the study. Once an appropriate child
was found and informed consent obtained from the parents, 2ml of blood would be
drawn, a slide prepared for parasite counts and the child's haemoglobin would be
measured. In the lab the blood would be processed as for clinical samples but without
parasite culture.
Figure 6.1: Madang town and the surrounding area showing the regions from which samples were collected and the villages in which controls were collected.
6.3 Results 1: Rosetting in Papua New Guinea

6.3.1 Erythrocytes from Papua New Guineans rosette poorly with laboratory strains

We tested a total of 46 different erythrocyte samples from the Madang population described in section 4.2 for their rosetting ability. Thirty samples were tested with R29+, 10 with TM284 and 10 with PAR+ (4 samples were tested with more than two isolates). The control individual for these experiment was a Caucasian O+ donor with a CR1 expression determined as 996 per erythrocyte, the individual was HH for their CR1 genotype. In addition to the Papua New Guinean samples we also tested 2 other Caucasian control samples with R29+ to ensure that our control donor did not have unusually highly rosetting red blood cells (Figure 6.1 a). One of the other Caucasians was O+, their CR1 expression = 491 per erythrocyte and their CR1 genotype was HL, while the other Caucasian was O-, their CR1 expression = 928 per erythrocyte and their CR1 genotype was HH. Neither individual showed significantly different rosetting from the control donor. This result is supported by previous studies which have shown very little variation in the rosetting of red blood cells from Caucasians once blood group is accounted for (Carlson et al. 1992; Carlson et al. 1994).
Figure 6.2: Rosetting of RBC from donors from PNG with rosetting laboratory clones. Clones are R29+ (a), PAR+ (b) and TM284+ (c). Bars are the relative rosetting abilities.
of individual donor RBC each tested at least in duplicate. Error bars are standard errors. White bars represent O samples. In dark bars represent A and AB samples and pale bars represent B blood group samples. In dark bars A and B blood groups are represented collectively by dark bars. Numbers on the X axis represent the erythrocyte CR1 expression level of the blood cells.
Rosetting was almost abolished in 13 O RBC from 13 donors from Papua New Guinea tested with R29+ (Figure 6.2 a) with their mean (sd) relative rosetting ability being 0.154 (0.121). Some rosetting was seen with A and B erythrocytes from Papua New Guinea though 10 out of 17 samples still showed significantly reduced rosetting relative to the control. R29+ is known to bind to CR1 but does not appear to bind to other rosette mediators such as IgM (Rowe et al. 2002). We therefore decided to look at some samples with two other strains: PAR+ and TM284+ which probably require IgM for optimal rosette formation (Scholander et al. 1996; Somner et al. 2000). Rosette formation was also severely impaired with Papua New Guinea erythrocytes in these isolates (Figure 6.2 b+c), particularly TM284, suggesting that CR1 is involved in the rosetting of these parasites in addition to other binding interactions. We compared the ability of these parasites to bind O erythrocytes from Papua New Guinea (to avoid the compounding factors of A and B blood groups for which parasites vary their affinity) and found significant differences between the ability of all the laboratory clones to form rosettes with melanesian erythrocytes ($P < 0.01$ all pairwise differences, one-way ANOVA), probably reflecting the fact that TM284+ and PAR+ use IgM in addition CR1 as a rosetting receptor (Figure 6.3).
Figure 6.3: Laboratory strains vary in their ability to rosette with CR1 deficient cells. Y
axis is the mean of the relative rosetting of O blood group samples tested with the
respective strains.

6.3.2 Rosetting is dependent upon blood group and thalassaemia in vitro

We used the large number of samples we had tested with strain R29+ to try and
determine the main factors affecting the ability of a given sample to rosette. We therefore
analysed the data by ANOVA initially incorporating blood group, thalassaemia, and CR1
expression into the statistical model. In this analysis the relative contributions of these
variables to the final rosetting ability can be analysed. Unsurprisingly blood group was
the major determinant of the final rosetting ability \( (P = 0.0032; \text{Figure 6.4} \ a) \). Strikingly there was a significant difference in rosetting between cells from non-thalassaemic individuals and homozygous thalassaemic individuals \( (P = 0.05) \) (Figure 6.4 \ b) with heterozygotes being similar to normal individuals. This was true when CR1 expression was included in the model. Thus it appears that the mechanism by which thalassaemic cells show reduced rosetting is both through reduced CR1 expression and a second effect - probably microcytosis\( (\text{Carlson et al. 1994}) \), but potentially altered binding of immunoglobulins\( (\text{Luzzi et al. 1991}) \). Within the PNG sample increased CR1 expression did not significantly increase rosetting whether or not blood group was included in the model \( (P > 0.2) \). However all samples tested had low CR1 expression and the range tested was small \( (\text{minimum CR1 expression} = 0, \text{maximum} \ = 258) \) so perhaps this not surprising \( (\text{Figure 6.4} \ c) \).
Figure 6.4: Factors potentially affecting R29+ rosetting in vitro. 

a. blood group, b. thalassaemia and c. CR1 expression. 

Thalassaemia genotypes are
- \( \alpha^+/\alpha^+ \): normal \( \alpha^- \)-globin gene structure,
- \( \alpha^+/-\alpha^- \): \( \alpha^- \)-thalassaemia trait,
- \( \alpha^-/-\alpha^- \) for \( \alpha^+ \)-thalassaemia. In a
and $b$ the y axis is the mean of the relative rosetting abilities of samples with the given trait. In $c$ individual observations are plotted against CR1 expression. O erythrocytes are denoted by squares (trendline is dotted) while other blood groups are denoted by circles with a solid trendline. Neither trendline denotes a significant relationship between Cr1 expression and relative rosetting of the cells tested ($P>0.6$).
6.3.3 Rosetting is not associated with severe malaria in Papua New Guinea

Having demonstrated that red cells from Papua New Guinea do not support rosetting well with laboratory strains we wanted to see if rosetting occurred naturally in Papua New Guinea. Accordingly we carried out a study in Madang to measure rosetting and to try to determine the receptors used for rosetting. It was possible to assess the rosetting frequency in 13 severe malaria cases and 25 cases of symptomatic non-severe malaria. The median rosetting frequencies were 2% and 3.5% respectively (Figure 6.5). Therefore rosetting was not associated with disease severity in PNG (P=0.74, Mann Whitney U test). In non-severe malaria cases the median rosetting frequency is comparable to figures from the rest of the world (Table 1.3 and references therein). The median frequency of rosetting for severe malaria cases is lower than any other severe malaria cohort in the world (Table 1.3). This data confirms the previous result in PNG that rosetting is not associated with severe disease in this population (al-Yaman et al. 1995). There are however a number of differences between the studies. The previous study is much larger and uses age-matched controls, it also only includes cerebral malaria cases. In our study there were only a few examples of each disease type so the results were not separated, however taken together it appears that rosetting is not a major determinant of disease severity in PNG. Our median frequencies for rosetting were much lower than the other study in PNG suggesting a systematic difference in counting rosettes or preparing parasites.
Figure 6.5: Rosetting is not associated with severe disease in Papua New Guinea. Each point represents mean rosetting frequency of a single isolate. Rosetting frequency is the percentage of mature stage parasites forming rosettes, disease severity is according to WHO criteria (Warrell et al. 1990).

Having confirmed the previous result that rosetting is not associated with severe disease in Papua New Guinea we wished to try and determine what is "different" about rosetting in Papua New Guinea compared to African situations. We hypothesised that rosettes in Africa and perhaps Papua New Guineans with severe disease might be stronger and held together by qualitatively and quantitively different interaction than rosetting in PNG patients with uncomplicated malaria.
6.3.4 CR1 is rarely a receptor for rosetting in Papua New Guinea

To try and develop a qualitative picture of the receptors used for rosetting in Papua New Guinea we used the rosettes disruption assays described in Section 2.8.5. We used the antibody J3B11 to disrupt rosettes which are formed via CR1. J3B11 is an anti-CR1 monoclonal that binds to the region of CR1 which PfEMP1 uses as a receptor and is capable of disrupting rosettes as well as preventing de-novo rosette formation (Rowe et al. 2000). As positive control we tested the ability of this antibody to disrupt rosettes from an R29+ culture (Figure 6.6 a), it was able to reduce rosetting to ≈ 50% of the original culture and an isotype control. A similar result was also found for PAR+ though not for TM284+ (Figure 6.6 a). We were concerned that the presence of other receptors (such as the A blood group antigen) would prevent the J3B11 from disrupting rosettes so we tested its ability to disrupt R29+ rosettes formed in A blood. A lower but still significant reduction was observed in this experiment (Figure 6.6 a). To test for the involvement of immunoglobulins in rosette formation we attempted to disrupt rosettes with a rabbit polyclonal serum raised against human immunoglobulins as described previously (Rowe et al. 2000). However this was not possible even with strains such as PAR+ and TM284+ which are known to bind immunoglobulins by other means (Somner et al. 2000) (Figure 6.6 b).
Figure 6.6: Rosetting through CR1 is rare and may be associated with higher CR1 expression. 

\(a\) Disruption of laboratory strains with the J3B11 mAb (dark bars) compared to isotype control (light bars), results are expressed as % of the rosetting frequency of the parent culture, results are the mean and standard error of at least three experiments. All experiments performed in O blood except R29+(A) in which experiments were
performed with R29+ parasites in A blood b. A rabbit anti-immunoglobulins serum does not disrupt rosettes (dark bars) compared to a rabbit serum immunoglobulins control (light bars). c. Rosette disruption in field isolates from Papua New Guinea following treatment with a variety of reagents relative to control treatments is shown. Light shading is 25-50% disruption, medium shading is 51-75% disruption and dark shading is 76-100% disruption. CR1 copy number is in mean molecules per cell, rosetting frequency (rf) is expressed as percentage of late stage parasites.
For analysis of receptor use in PNG we used field isolates that rosetted at a frequency of > 10% were included in the experiments. Out of 8 isolates tested only 3 showed rosette disruption with an anti-CR1 antibody, J3B11 (Figure 6.6 c) compared to 14/15 Kenyan isolates and 5/10 Malawian isolates (Rowe et al. 2000). Interestingly CR1 was a receptor for rosetting in the two isolates with the highest rosetting frequency and in two isolates that had amongst the highest CR1 expression. The anti-immunoglobulins polyclonal serum, which is recorded as disrupting rosettes in many African, isolates (Rowe et al. 2000) only disrupted one isolate weakly. The failure of the positive control means that we should reject this data (Figure 6.6.b). An alternative way of assessing immunoglobulin binding in these isolates would be to analyse them by indirect fluorescence assay (Rowe et al. 2002). To test the efficacy of this technique we prepared anti-human IgM IFAs on sample of the laboratory strains. The IgM binding ability of TM284+ and PAR+ was clearly illustrated while R29+ was found not to bind IgM in agreement with previous data (Rowe et al. 2002) (Figure 6.7). As frozen isolates of many of the PNG samples exist it should be possible to test these for IgM binding in due course. No staining was observed in negative controls with an isotype control for the anti-IgM mAb. Heparin, a highly sulphated sugar molecule disrupted most of the rosettes efficiently, suggesting that a carbohydrate interaction might be important in the absence of CR1 mediated rosetting (Barragan et al. 2000; Barragan et al. 2000). Finally 6/8 of these isolates were of the A or B blood group type. When we examined the proportion of high rosetting isolates (> 5% rosettes) we found a significant excess of non-O blood groups among the strongly rosetting isolates ($P = 0.017$ by Fishers exact test, see also figure 6.8 a).
Figure 6.7: IgM binding of laboratory rosetting lines detected by indirect immunofluorescence assay (IFA). Results are expressed as % of infected erythrocytes binding IgM. Results are means and standard errors of at least 2 experiments.

6.3.5 Associations with rosetting *ex vivo*

Our experiments with laboratory adapted rosetting *P. falciparum* clones (section 6.3.2) and others have shown that rosetting is reduced in RBC from O blood group (Carlson *et al.* 1992), low CR1 and ((Rowe *et al.* 1997) and α-thalassaemic individuals (Carlson *et al.* 1994). We wished to examine if these associations still existed in *ex vivo* samples. There was a trend towards increased rosetting in A, B and AB blood group individuals as reported in other studies (Figure 6.8 *a;* *P* = 0.19, Mann Whitney U test) (al-Yaman *et al.*).
1995; Rowe et al. 1995; Barragan et al. 2000). As in section 6.3.2 we were unable to find a correlation between CR1 expression and rosetting within our Papua New Guinean samples (Figure 6.8 b; $P = 0.26$, one-way ANOVA), however as almost all samples are deficient in CR1 it may be impossible to identify a correlation. Nor was there any evidence for HH (Figure 6.8 c; $P = 0.456$, Kruskal Wallis test) or non-thalassaemic individuals (Figure 6.8 d; $P = 0.782$, Kruskal Wallis test) having higher levels of rosetting. However the sample size here is small and the numbers of individuals with higher CR1 expression genotypes smaller still. The other difficulty with such observations is that individuals may be infected with a variety of different strains of \textit{P. falciparum} - thus a high CR1 individual is just as likely to be infected with a non-rosetting strain as a low CR1 individual. We found no correlation between rosetting and haemoglobin ($P = 0.70$), age ($P = 0.45$) or parasitaemia ($P = 0.94$). Out of the isolates tested for rosetting we identified 1 Gerbich negative individual (XB27) whose parasites rosetted at high frequency (18%) despite a low CR1 expression (36 molecules per cell). There was also one individual with Southeast Asian ovalocytosis (XB115), the isolate showed 26% rosetting (Figure 6.6 c). These results suggest anecdotally that any protection afforded by these genotypes is not due to preventing rosetting.
Figure 6.8: The effect of blood group, CR1 expression, CR1 genotype and $\alpha^*$-thalassaemia on rosetting *ex vivo*. 

a. Relationship between bloods groups A, B and AB with rosetting. 
b. No correlation between CR1 expression and rosetting, CR1 expression in molecules per cell, rosetting in all figures is in % of mature stage parasites in rosettes. 
c. Relationship between rosetting and CR1 genotypes CR1 genotypes are defined as in figure 4.3. 
d. Relationship between rosetting and $\alpha^*$-thalassaemia, thalassaemia genotypes are $\alpha\alpha/\alpha\alpha$: normal $\alpha$-globin gene structure, $-\alpha/\alpha\alpha$, $\alpha^*$-thalassaemia trait and $-\alpha/-\alpha$ for $\alpha^*$-thalassaemia.
6.4 Results 2: Case-control study data

Above we have given as full an analysis of the characteristics of rosetting in Papua New Guinea as is possible given the relatively small sample size available. The collection of community controls gave us an opportunity to expand our case-control study presented in the previous chapter and to gain information on CR1 expression itself and its effect on severe and uncomplicated malaria.

6.4.1 Severe malaria in Papua New Guinea

It is commonly asserted that severe malaria is rarer in Papua New Guinea than Africa (Moir et al. 1989) (and see section 1.1.5), though due to the difficulties of standardising studies in different locations it has not been possible to test this directly. Our study provides circumstantial evidence for this, as only 14 severe malaria cases were recorded in 4 months, though this excludes drug treated cases. Details of the cases are given in Table 6.2. Previous studies suggest that in Madang around half of all severe malaria patients have taken medication prior to admission (al-Yaman et al. 1995). Of the 14 severe malaria cases none died of the disease. This accords with rigorous population surveys that find very low levels of malaria induced mortality in this population (Moir et al. 1989) and with the low case fatality for severe malaria (Allen et al. 1996; Genton et al. 1997). Overall there were 3 children with cerebral malaria, with three others showing
signs of impaired consciousness, 2 children with severe malarial anaemia, 3 with hypoglycaemia, 1 with hyperlactaemia, 5 in respiratory distress, 5 with a history of convulsions and 3 were prostrated (who were not also in coma). 3 children had only 1 manifestation of severe malaria, 7 had 2, 3 had 3 and 1 child met 4 severe disease criteria.
Table 6.2: Severe malaria cases in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Coma</th>
<th>Prostration</th>
<th>Convulsions /24hr</th>
<th>Respiratory distress</th>
<th>Parasitaemia (%)</th>
<th>Hb g/dl</th>
<th>Lac mmol/L</th>
<th>Gic mmol/L</th>
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<tbody>
<tr>
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<td>2</td>
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<td>5.1</td>
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<td>ND</td>
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<td>3.9</td>
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<td>1</td>
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<td>9.9</td>
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<tr>
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<td>8.8</td>
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<td>7.2</td>
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<td>2</td>
<td>NO</td>
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<td>9.1</td>
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* CSF analysis negative for menigococcal bacteria (otherwise not done)

Shading indicates severe malaria criteria met
We went on to compare the laboratory and population parameters between the severe and uncomplicated malaria cases. The results are summarised in table 6.3. The mean age of uncomplicated malaria cases was 4 years 0 months which was significantly more than the mean age of severe malaria cases 2 years 7 months ($P = 0.007$ Student’s T test) perhaps reflecting the acquisition of immunity with age (Marsh 1992).

### Table 6.3 Indices of severity between severe and uncomplicated malaria cases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Severe malaria</th>
<th>Uncomplicated malaria</th>
<th>Asymptomatic controls</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin*(g/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>8.24 (2.19)</td>
<td>8.80 (1.93)</td>
<td>10.43 (1.40)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>$n$</td>
<td>14</td>
<td>28</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>4.16 (1.54)</td>
<td>2.71 (0.85)</td>
<td>ND</td>
<td>0.0082</td>
</tr>
<tr>
<td>$n$</td>
<td>7</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>4.79 (3.05)</td>
<td>6.78 (4.07)</td>
<td>ND</td>
<td>0.156</td>
</tr>
<tr>
<td>$n$</td>
<td>12</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pma‡(log$_{10}$(P/μl))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>5.04 (3.80-5.68)</td>
<td>4.87 (3.47-5.50)</td>
<td>ND</td>
<td>0.49</td>
</tr>
<tr>
<td>$n$</td>
<td>14</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Haemoglobin, lactate and glucose were found to be normally distributed. Analysis is by T test or one-way ANOVA for these variables.  
†By one-way ANOVA across all three classes, however there is no significant difference between severe and uncomplicated malaria cases ($P = 0.40$)  
‡Parasitaemia had a skewed distribution data is given as median and range with number of observations in italics, analysis is by Mann Whitney U test.
Haemoglobin was not significantly different between severe and uncomplicated malaria cases, perhaps reflecting the small number of severe anaemia patients in this study. Lactate was significantly associated with disease severity even though only one patient met the criteria for hyperlactaemia (> 5 mmol/L). This is in accordance with previous data showing that in Madang lactate levels are one of the most powerful predicates of disease severity (Allen et al. 1996). The lack of significant difference in parasitaemia between severe and uncomplicated malaria cases is not an original result but once again emphasises that parasite load is not a principal determinant of disease severity.

### 6.4.2 Erythrocyte CR1 expression level may be reduced in malaria infections

Having shown in the previous chapter and elsewhere (Allen et al. 1997) that genotypes associated with low CR1 expression such as the L allele and α'-thalassaemia protect against severe malaria, we wanted to use our data to compare the actual CR1 expression between severe and uncomplicated malaria cases and their matched controls. To our initial surprise CR1 expression was slightly lower in individuals with both severe and uncomplicated malaria than on controls (Figure 6.9 a and b) though this was not significant by one-way ANOVA with just CR1 expression in the statistical model ($P > 0.10$ for both syndromes).
Figure 6.9: CR1 expression is dependent upon malaria infection and CR1 genotype. *a.* Severe malaria and *b.* uncomplicated malaria. Dark bars are the mean CR1 expression level of control individuals, while the light bars are the mean CR1 expression levels of infected individuals all subdivided by CR1 genotype. Error bars are standard errors.

One possible explanation for this data is that malaria infection itself is directly affecting erythrocyte CR1 expression. This has been observed for severe anaemia patients in Kenya (Waitumbi *et al.* 2000; Stoute *et al.* 2003). We subdivided our malaria cases and controls by CR1 genotype. The data was then analysed by 2-way ANOVA with both CR1 expression and case or control status in the model. We found that for any given genotype the malaria cases (severe or uncomplicated) had much lower CR1 expression than genotype matched controls ($P < 0.05$ by 2-way ANOVA for both severe and uncomplicated malaria). We examined the distribution of CR1 genotypes among severe
and uncomplicated malaria cases and controls. Despite the decreased CR1 expression overall among malaria cases, in accordance with the data in chapter 5 there we find a higher gene frequency of the H allele among the severe and mild cases compared to their controls. This is not statistically significant, presumably due to the small sample sizes (gene frequency (H) for severe malaria = 0.25, controls = 0.22, \( P > 0.2 \) by \( \chi^2 \) test; gene frequency (H) for uncomplicated malaria = 0.34, controls = 0.16, \( P = 0.10 \) by \( \chi^2 \) test).

There was no difference between the mean erythrocyte CR1 expression of samples from severe and uncomplicated malaria patients (Figure 6.9 a and b, \( P > 0.2 \)) suggesting that the severity of disease does not affect CR1 expression in this population. The sample sizes were too small to compute logistic regression analyses to determine the odds ratios for risk of severe malaria among the different genotypes.

Overall CR1 expression among these samples is dependent on CR1 genotype \( (P < 0.0001) \) and infection (severe or mild) \( (P = 0.019) \) by multi-way ANOVA with thalassaemia also included in the model. There is also a trend for lower CR1 expression in thalassaemic individuals as reported in chapter 4 \( (P = 0.19) \).
6.5 Discussion

In the work presented here I aimed to determine what the effects of CR1 deficiency in Papua New Guinea were on rosetting and severe disease. We confirmed the results of a previous study in showing that rosetting was not associated with severe disease (al-Yaman et al. 1995). Although in contrast to al-Yaman et al. (al-Yaman et al. 1995) we found a very low overall frequency of rosetting in this population. We also show that CR1 is rarely a receptor for rosetting and that rosetting in this population does not correlate with CR1 expression or genotypes associated with higher CR1 expression. Our study was hampered by difficulties in finding severe malaria cases, though we hypothesise that the low level of severe disease in Papua New Guinea might be in part a direct result of the rarity of CR1-mediated rosetting.

Our *in vitro* data in which we measured the ability of erythrocytes from CR1 deficient Papua New Guineans clearly demonstrates that these cells rosette poorly with rosetting laboratory strains. This was especially true with R29+ which is well documented as rosetting via CR1 (Rowe *et al.* 1997; Rowe *et al.* 2000). Rosetting ability was found to be dependent on blood group (a result echoed in the *ex-vivo* data), and on thalassaemia. The thalassaemia effect was still present when CR1 expression was included in the model, showing reduced rosetting in these cells was not only mediated by low CR1 but by another factor too: possibly microcytosis. Though the effect of thalassaemia on rosetting
in vitro has been reported previously (Carlson et al. 1994) it has not been possible to replicate the result in other laboratories (David Roberts personal communication). Moreover in the study by Carlson et al. (Carlson et al. 1994) the origin of the cells is not given, though it may have been Papua New Guinea, and the data might have been attributable to low CR1 expression caused mostly by the L allele as well as thalassaemia. These data accounting for all known factors affecting CR1 expression show that the effect of thalassaemia is probably real and dependent on both a reduction in CR1 expression and a second factor too.

A previous study on rosetting in Papua New Guinea found that rosetting was not associated with cerebral malaria (al-Yaman et al. 1995). This was counter to all African studies then published (Carlson et al. 1990; Ringwald et al. 1993; Rowe et al. 1995) and suggested that rosetting might not, after all, be a major parasite virulence factor. However our data showing that CR1 deficiency is almost universal in Papua New Guinea suggests another hypothesis: that CR1 is essential for disease-causing rosettes. This could be because rosettes formed without CR1 are too weak to withstand the sheer forces in the circulation - and therefore that the rosettes previously observed in in vitro culture from Papua New Guineans would not be able to cause severe disease in vivo. Though we too found no association between rosetting and severe disease our results differed from those of the previous study in finding many isolates without rosettes at all. This systematic difference may be explained by differences in counting protocols, we cultured parasites at 2% haematocrit however al-Yaman et al., cultured at 5%. This does not affect rosette
formation (J. A. Rowe, DPhil Thesis) but may cause an overestimation of rosetting as many non-specific cell-cell contacts may be seen on a slide at 5% haematocrit. Nonetheless when our results from Papua New Guinea are compared to those from studies in Africa from our group (Rowe et al. 1995; Rowe et al. 2002) (which use identical methods) then it appears that rosetting frequencies in this population are lower than in Africa especially in the severe malaria group. In Kenya an association between parasitaemia and rosetting has been reported, this association is particularly strong in isolates from severe cases (Rowe et al. 2002). The authors suggest that this correlation is indicative of increased invasion or immune evasion by rosetting parasites. In Papua New Guinea we do not see such a correlation, which would be expected if there were no selective advantage to the rosette formation observed in this population.

Initially we aimed to determine if there was a difference in receptor use in rosettes formed in culture from severe and uncomplicated parasite isolates. However this study was hampered by a lack of severe cases and a lack of highly rosetting isolates. We were, however, able to examine the receptor choice across all isolates. Strikingly an anti CR1 antibody disrupted rosetting in only 3/8 isolates. The two isolates with greater than 50% rosette disruption had the highest rosette frequencies. Only 3 of the 8 isolates tested for rosette disruption had erythrocytes with CR1 expression >50 molecules per cell and two of those showed rosette disruption with an anti-CR1 antibody, these two individuals also had at least one H allele. All other high rosetting isolates had very low CR1 expression so it was not unexpected that their rosettes were not disrupted with the anti-CR1 antibody.
The rarity of CR1 adhesion probably explains why we were unable to detect any associations between erythrocyte CR1 expression level or the genotypes controlling this phenotype and rosetting frequency. Another hindrance to trying to detect differences in rosetting frequency between the different genotypes in Papua New Guinea is the fractional difference in CR1 expression between them. As almost all individuals have low CR1 expression, a large sample size would be required to detect what are likely to be marginal differences between genotypes at a significant level. We have hypothesised that CR1 mediated rosettes are stronger than others, though this has not yet been tested experimentally. To test this we intend to study difference parasite isolates under conditions of sheer stress and to measure the detachment forces required to directly disrupt rosettes (Nash et al. 1992; Chu et al. 1997; Chotivanich et al. 2000).

We must reconcile the fact that rosetting appears to have exerted selective pressure on the CR1 gene and yet is not apparently a cause of severe disease. We hypothesise that a proportion of rosettes formed in Papua New Guinea are formed via CR1 and that these are capable of causing severe disease. Rosetting studies may fail to detect an association because they do not distinguish between physiologically significant rosettes and in vitro artefacts. Moreover as rosetting is only rarely a cause of severe disease, most severe disease in this population is caused by other factors such as immunopathology and metabolic disturbances. Thus rosetting as a cause of severe disease is very difficult to detect on this background. It is as though we have arrived at the scene of the crime too late since CR1 deficiency has almost reached fixation in this population and there is now
very little CR1 binding causing pathology in this population. Only large studies such as
that presented in chapter 5 could have the power to detect the selective effect of rosetting
parasites and severe disease on CR1, whereas smaller studies such as this one have little
power to detect the rare cases where rosetting is a cause of severe disease. Finally we
might consider if CR1 deficiency has affected the evolution of the parasite. If there is no
CR1 to bind to form rosettes, CR1 binding is unlikely to confer an advantage to the
parasite. Therefore there may have been selection in the parasite population against CR1
binding variants. There is a precedent for this in Papua New Guinea. The merozoite red
cell binding ligand BAEBL has various alternative types, one of which mediates invasion
via glycophorin C, while the other variants use an alternative receptor (Mayer et al. 2002).
In Papua New Guinea most parasites express the alternative version of BAEBL are
found (Mayer et al. 2002), perhaps because glycophorin C binding parasites have been
selected against by the Gerbich mutation which is found in Papua New Guinea and
prevents invasion via glycophorin C (Mayer et al. 2002; Maier et al. 2003).
Chapter 7:

Conclusions, confusions and future work

Pedants and philosophers of science point out that there is no such thing as a scientific conclusion; a scientist's work, it seems, is never done. But inasmuch as we have to agree on something to get anywhere I would regard the following as more-or-less settled.

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7 Most philosophers of science seem to have very little idea of what researchers actually do and are rightly ignored by scientists, but one good account of the scientific method is "The rationality of science" by W.H. Newton-Smith (Newton-Smith 1981)
1. We have been able to validate a method for the determination of erythrocyte CR1 for use on cells that have been fixed or frozen. This method is therefore usable for field settings and for analysing blood long after it has been taken.

2. Individuals from Papua New Guinea have low erythrocyte CR1 expression, which is genetically determined by polymorphisms in the CR1 gene and α⁺-thalassaemia. We have not ruled out the involvement of other genetic and environmental factors such as infection having an effect on CR1 expression.

3. The CR1 L allele confers protection against severe malaria.

4. Erythrocytes from Papua New Guineans with low CR1 expression show reduced rosetting compared to high CR1 Caucasian erythrocytes with rosetting laboratory *P. falciparum* clones.

5. Rosetting is rare in Papua New Guinea, not mediated by CR1 and not associated with disease severity.

The data that leads us to these conclusions is in each of the results chapter and the reasons for accepting them are given in the corresponding discussion section. Thus far in my discussions I have tried to restrict myself to what is in the literature. But when thinking of future directions for work what is more interesting is to look at the things we do not know and to speculate on what we might want to know:

1. We have not demonstrated formally that the CR1 L allele protects as a result of reduced erythrocyte CR1 expression rather than some other functional effect.
2. We do not know if the protective effect of thalassaemia is a result of its effect on CR1 expression or whether it acts by some other means.

3. We do not know why low CR1 has been selected for in Papua New Guinea but not in African populations.

4. We do not know if the mechanism of protection by the L allele is reduced rosetting (we have only hypothesised this).

5. If reduced rosetting is the protective mechanism of this polymorphism we must reconcile that with the fact that rosetting is not apparently associated with severe disease in this population, so how can it exert the necessary selective pressure?

6. Does low CR1 expression explain the lower mortality due to malaria in Papua New Guinea compared to Africa?

I shall try and examine each of these in order though many of the issues are overlapping. It shall be up to other scientists, or their funding councils, to decide if any of these leads are worth following.

Our study shows that the L allele is protective against severe malaria, it does not show that the low CR1 expression *per se* protects against severe malaria. The L allele is associated with two things: firstly it consists of a number of polymorphisms in the coding region of the CR1 molecule at least one of which lies in a binding site for CR1 (Herrera *et al.* 1998; Xiang *et al.* 1999), secondly in populations outside Africa the L allele is associated with reduced erythrocyte CR1 expression (Table 4.1 and references therein).
The strongest evidence that the L allele is acting through CR1 expression is the fact that the coding polymorphisms in the L allele exist in African populations but are not apparently selected for in this area (Rowe et al. 2002). Therefore we believe that the effect must be on CR1 expression. Sceptics could point out that there is in fact not much difference in the mean CR1 expression between HH and LL individuals CR1 in Papua New Guinea as most individuals have low CR1. However a subset of individuals with H alleles will have high CR1 expression (the range in Madang goes up to 439 and in New Ireland to 839) and it is on individuals such as these that selection will act. So it does seem likely that the L allele is acting through CR1 expression.

We might legitimately ask how little CR1 is protective - do I simply need a bit less CR1 than my neighbour, say 300 molecules per cell to his 400 to be protected against malaria relative to him or do I need to get my CR1 levels below a particular threshold, say 100 molecules per cell before I see any benefit. If the mechanism of protection is indeed rosetting this is tied up with the amount of CR1 required for stable rosette formation. The data in section 6.3.1 suggests that all the Papua New Guinea samples (with O blood) show reduced rosetting compared with Caucasian samples even those with intermediate CR1 expression. This suggests a threshold, i.e. above around 200 molecules per cell rosetting can be sustained, below this amount and you are protected. However these are static assays, under conditions of sheer stress such as in the circulation rosettes formed in static culture with red cells with 250 molecules/cell of CR1 may come apart whereas those formed with RBC expressing 900 molecules of CR1 may hold together. These
"dose response" experiments are some of the basic experiments in the rosetting field that have not been done.

It is unclear if thalassaemia is acting through CR1 expression or independently of this. There is no clear correlation between the risk a particular genotype combination confers to malaria and mean CR1 expression associated with it. We point out that HH; αα/αα individuals are the most at risk of severe malaria, but this could be result of simply having 4 risk loci, it does not mean that the loci are acting through the same mechanism. Far better evidence of genes acting in concert comes from linkage disequilibrium studies, which show if genes are selected together. As such we would expect the L allele and α⁺-thalassaemia to be found together, even despite the fact that they are not genetically linked (on the same chromosome). Evidence from our own small population survey finds no evidence of linkage disequilibrium occurring between these genes, though much larger surveys may be necessary to reveal this. On a global scale there is linkage disequilibrium in that the highest frequencies of α-thalassaemia occur where there is the highest level of the L allele i.e. in Southeast Asia and Papua New Guinea(Flint et al. 1993; Flint et al. 1998). In Africa where there does not appear to be any genetically determined CR1 deficiency α-thalassaemia is much rarer(Flint et al. 1993; Flint et al. 1998). The discussion in the preceding paragraph about how much or little CR1 is protective suggests an explanation for this: these results could be rationalised if both a lesion in the CR1 gene and α-thalassaemia were required for CR1 levels to be low enough to be protective against severe malaria. If the threshold for rosetting were 200 molecules per
cell, having a couple of L chromosomes may not be sufficient to have low enough CR1 levels (see Figure 4.2) but having a α-thalassaemia gene can lower the CR1 sufficiently to be protective, thus the two genes may have been selected together in Papua New Guinea and perhaps parts of Southeast Asia. If this is true it seems that in our population thalassaemia is protecting through CR1 deficiency. However thalassaemia does exist in Africa, so what is it doing there? Most probably α-thalassaemia has a protective effect against malaria independent of erythrocyte CR1 deficiency. Many candidates have been put forward: impaired growth (Senok et al. 1997) (though cf. (Luzzi et al. 1990)), altered cytoadhesion (Udomsangpetch et al. 1993) and altered immune recognition (Luzzi et al. 1991). All of these are controversial and hard to test in the field, but perhaps they all have some truth in them. Of course no one has yet checked that the populations in Africa (which contains more genetic diversity than the rest of the world) that do have α-thalassaemia do not also have a CR1 deficiency allele.

So why has the L allele or erythrocyte CR1 deficiency not been selected for in Africa? CR1 deficiency seems to be as rare in Africa as in Northern Europe. One reason may lie in the preceding paragraph - without a second allele like α-thalassaemia with which to act a lesion in the CR1 gene affecting its erythrocyte expression may not confer any selective advantage. An alternative explanation is that the genetic lesion that causes CR1 deficiency (and is linked to the SNPs that constitute the L allele in non-African populations) never arose in African populations. We cannot know if this is the case until we know what that genetic lesion is. One hypothesis is that the H and L chromosome are
ancient and that the lesion arose on an L chromosome, possibly only after humans left Africa. Mapping the site of the genetic lesion would probably enable dating of the mutation by tracing the most recent common ancestor of populations that have the same mutation. Further support for the idea that the lesion has only arisen once come from the fact that it is probably seen on only one genetic background - the L allele. If the lesion had arisen many times the question of why it had not arisen in Africa would be even harder to skirt around.

The final explanation for the lack of CR1 deficiency in malaria endemic populations in Africa might be the presence of alternative CR1 polymorphisms such as the Knops bloods groups types notably Sl:2 and McCb which may have been selected instead. Can several malaria resistance genes be selected together, or is one enough and so there is less pressure for more such genes to be selected for in a population? The presence of α-thalassaemia, CR1 deficiency, ovalocytosis, Gerbich blood groups and glucose-6-phosphate dehydrogenase polymorphisms in the same populations in Papua New Guinea suggests that they can be selected together. Our own data shows that the most protected people against severe malaria are those with two most beneficial genotypes: α-/α- and HL - in other words the protective effects appear to be additive. However all these polymorphisms are in different genes from each other, what happens when several different polymorphisms arise in a single gene such as CR1. If the polymorphisms arise on different backgrounds - if the Sl 2 allele arises on an H chromosome, while CR1 deficiency arises on an L chromosome, those alleles are going to in direct competition
with each other. The usual outcome of such a head to head battle is that the least beneficial allele will disappear, or more likely fail to establish itself in the first place. Only in circumstances such as balancing selection do polymorphisms establish themselves at frequencies intermediate between 0 and 1.

In the final chapter I attempt to test the hypothesis that the mechanism by which low CR1 protects against severe malaria is reduced rosetting. To do this properly would require a large case-control study in which we were able to identify individuals with high CR1 whose parasites rosetted well in their blood via CR1 and who developed severe disease. The work presented does not do this, but it does give some evidence in favour of this hypothesis and finds none that can rule it out.

The evidence for reduced rosetting being the mechanism of protection is the following: rosetting appears to occur at a lower level in Papua New Guinea compared to what is seen in Africa. In particular comparison of African and Melanesian data shows that a pool a group of individuals are missing from the Melanesian study - severe malaria cases with high numbers of rosettes. Circumstantial evidence for reduced rosetting being the mechanism of protection by low CR1 comes from the observation that rosetting is not associated with severe disease in this population. This could be explained if the lack of CR1 in this population meant that rosetting simply was no longer an important determinant of disease severity - thus in Papua New Guinea severe disease is probably caused other parasite phenotypes such as ICAM1 adhesion(Newbold et al. 1997), TNF-α
release (Clark et al. 1991; Clark et al. 1993; Turner et al. 1998) and metabolic disturbances (Marsh et al. 1995). In 1999 trying to explain why rosetting was not associated with severe disease in a population in Malawi Rogerson et al. comment "It will be important to examine the prevalence of rosette formation receptors in different populations in relation to the frequency and intensity of rosette formation and in relation to the association between rosette formation and disease severity in these populations" (Rogerson et al. 1999). This certainly appears to be the case in Papua New Guinea.

However, if this hypothesis is true we would expect to find individuals who are homozygous for the H allele, with high CR1 expression and a high frequency of rosette forming parasites among the severe cases. No such individuals were found. However individuals with high CR1 expression are few and far between in this population. These individuals would also have to suffer the misfortune to be infected with a parasite capable of taking advantage of the high levels of CR1. Such parasites may have been selected against as we discussed in the previous chapter. Nonetheless we did find two individuals with high rosetting frequencies and at least one H allele, the rosettes from these patients could be disrupted with an anti-CR1 antibody (Figure 6.6). As stated in the discussion to chapter 6 it appears that we are looking at a selective process that is almost complete and so people unfortunate enough to have "risky" levels of CR1 expression are rare. Only a large survey such as that in chapter 5 could have the power to detect the protective effect of the L allele relative to the rare H allele.
We set out to determine if rosetting was a cause or correlate of parasite virulence. By showing the crucial importance of CR1 polymorphisms in disease susceptibility and by studying their effect on rosetting we appear to have some strong evidence that rosetting is a cause of severe malaria. However can we rule out the possibility that CR1 adhesion rather than rosetting might be the true. In other words could rosetting via CR1 be a correlate of another adhesion process: parasite binding to macrophages and B cells via CR1 which is the true cause of severe disease. There is a small amount of evidence that rosetting lines can cause enhanced TNF-α release (O'Dea et al. 2003) perhaps by some interaction with macrophages. Nonetheless there is a certain amount of evidence against this - notably the fact that the polymorphisms we have discussed: the CR1 L allele and α+-thalassaemia are red cells specific (Wilson et al. 1986).

The final and most controversial question I want to tackle is whether the low CR1 expression seen in Papua New Guinea could explain the low malaria mortality in this population. Firstly as we saw in section 1.1.6 it is still controversial that there is actually lower mortality than would be expected around Madang, though three things convince me that it is - firstly the low mortality observed in a large population census around Madang (Moir et al. 1989), secondly the relatively low case fatality rates for cerebral malaria (Allen et al. 1996), and thirdly (the least scientific but for me the most compelling) the low number of cases of severe malaria that we saw in the study in Chapter 6 over a four month period. To explain low mortality in Papua New Guinea some
authors have suggested the prevalence of *P. vivax*, other protective polymorphisms and a limited range of parasites. However while some researchers have suggested that infection with *P. vivax* might provide some immunity to *P. falciparum* (based on epidemiological studies of α*-thalassaemia* which enhances susceptibility to clinical infection with both (Williams *et al.* 1996)) there is no direct evidence of this. Moreover if *P. vivax* were beneficial then mutations in the Duffy blood group antigen should never have arisen in Papua New Guinea (Zimmerman *et al.* 1999). The presence of malaria resistance genes such as ovalocytosis and the Gerbich blood group are unlikely to have much effect on overall mortality as they rarely reach frequencies > 10% in this population (Genton *et al.* 1995; Allen *et al.* 1999) and this study. Parasite diversity does indeed appear to be limited in neighbouring Vanuatu where there is virtually no malaria mortality (Maitland *et al.* 2000), however PNG is a much larger island, and studies find no shortage of diversity at either the AMA-1 or MSP-1 loci (Felger *et al.* 1994; Cortes *et al.* 2003). The low level of CR1 conferred by α*-thalassaemia* and CR1 polymorphisms could however be responsible for reduced mortality: there is a hypothesised protective mechanism, which there is not for cross-protection with *P. vivax*. The polymorphisms are together present in virtually the entire population around Madang, but not in Africa whereas polymorphisms such as ovalocytosis, though absent from Africa are too rare in Papua New Guinea to have an overall effect on mortality. Finally low CR1 and its protective effect have been documented in this study, whereas no evidence exists for limited parasite diversity.
What, then, is the overall significance of these results? We have identified a new malaria resistance gene - the L allele of the CR1 gene. Secondly we have identified a novel mechanism by which α^+/-thalassaemia might protect against severe disease. Protective polymorphisms in erythrocyte CR1 are clear evidence that CR1 is an important rosetting ligand for many parasite strains in the wild and not merely an unusual adhesion phenotype of the R29+ parasite clone. Finally the fact that protective polymorphisms that reduce CR1 binding and rosetting have been selected for is the strongest evidence yet that rosetting is a cause, and not a correlate of, parasite virulence and mortality. That means it is an important target for the development of drugs and vaccines against severe malaria.


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Bibliography


Appendix

Publications arising from this work

At the time of writing two publications have arisen from the work presented here, which are reproduced here:


A simple method for accurate quantification of complement receptor 1 on erythrocytes preserved by fixing or freezing

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Abstract

The mean number of complement receptor 1 (CR1) molecules on erythrocytes differs between normal individuals within the range of 100–1000 molecules per cell. In some disease states such as systemic lupus erythematosus (SLE), acquired immune deficiency syndrome (AIDS), insulin-dependent diabetes mellitus and malaria, erythrocyte CR1 levels are reduced and CR1 function may be impaired. Current methods for determining erythrocyte CR1 levels by flow cytometry require the use of freshly drawn blood samples because CR1 is lost from erythrocytes during storage. In order to facilitate field studies of associations between erythrocyte CR1 levels and disease, we have developed and validated an assay to quantify CR1 on both healthy and diseased erythrocytes that have been fixed in 5% formaldehyde or frozen in glycerol. These methods enable blood samples to be collected in areas lacking the facilities for flow cytometry and stored for later accurate quantification of CR1. Such procedures will be of particular benefit for future investigations of erythrocyte CR1 expression level and malaria susceptibility.

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Keywords: Complement receptor 1; Erythrocyte; Flow cytometry; Formaldehyde; Glycerol; Malaria

1. Introduction

The level of complement receptor 1 (CR1) on erythrocytes is important in a variety of disease processes. Reduced levels of erythrocyte CR1 correlate with disease severity in acquired immune deficiency syndrome (AIDS), systemic lupus erythematosus (SLE) (Kazatchkine et al., 1987) and insulin-dependent diabetes mellitus (Ruuska et al., 1992). In the case of SLE, low CR1 levels may cause pathology because the normal function of CR1 in regulating complement activation and removing immune complexes from the circulation is impaired (Gibson and Waxman, 1994). In malaria infection, reduced levels of erythrocyte CR1...
have been implicated in the pathogenesis of severe malarial anaemia (Waitumbi et al., 2000). Conversely, we have suggested (Rowe et al., 1997) that low erythrocyte CR1 may protect against severe malaria by reducing the virulence-associated rosetting phenomenon whereby Plasmodium falciparum-infected erythrocytes bind to uninfected erythrocytes to form clumps of cells that may block microvascular blood flow and contribute to severe disease (Kaul et al., 1990). Further elucidation of the role of erythrocyte CR1 in malaria and other diseases requires the investigation of CR1 expression levels and disease associations in a variety of populations and patient sets.

Several methods currently exist to quantify the mean number of CR1 molecules per erythrocyte, including an ELISA technique and a flow cytometry method (Cohen et al., 1999). Both methods give equivalent results, although flow cytometry is preferable in many circumstances because it can be performed on very small quantities of blood. This is a particular advantage when studying young children or when multiple investigations are to be carried out on a single blood sample. One drawback, however, is that the flow cytometry method can only be used to determine CR1 numbers on fresh blood samples, because CR1 is lost from erythrocytes during storage (Pascual et al., 1993). This is a particular problem when working on malaria and AIDS in some field study areas in developing countries where facilities for flow cytometry may not always be available. We therefore set out to test a variety of methods of erythrocyte preservation in order to develop an assay to determine accurately by flow cytometry the CR1 expression level on erythrocytes after storage.

2. Methods and study design

2.1. Determination of CR1 expression by flow cytometry

The mean erythrocyte CR1 expression level on freshly drawn blood samples was determined using a modification of a previously published method (Cohen et al., 1987). Whole blood (50 μl) was washed three times in PBS (all reagents from Sigma, Poole, Dorset, UK unless otherwise stated) supplemented with 4% RPMI 1640 (Gibco Life Technologies, Rockville, MD) and 1% AB serum (Blood Transfusion Service, Edinburgh, UK) (PBS-RPMI-ABS). The cells were resuspended in 1 ml of PBS-RPMI-ABS and 100 μl of this suspension (i.e. approximately 2.5 μl of cells) was placed in a 96-well plate, spun at 1500 rpm for 1 min and the supernatant removed. The cells were then incubated at 4°C with 40 μl of 0.5 μg/ml of the CR1 monoclonal antibody J3D3 (Immunotech, Marseille, France) in PBS-RPMI for 1 h with occasional agitation. The cells were washed three times in PBS-RPMI-ABS and resuspended in 10 μg/ml of Alexa Fluor™ 488-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, the Netherlands) followed by incubation at 4°C for 1 h with occasional agitation. Alexa Fluor™ 488 is equivalent to fluorescein isothiocyanate but is more stable and sensitive and less prone to photo bleaching. Negative control samples were treated as above but without the primary antibody. After the secondary incubation, cells were washed three times in PBS-RPMI-ABS and resuspended in 10 μg/ml of Alexa Fluor™ 488-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, the Netherlands) followed by incubation at 4°C for 1 h with occasional agitation. Alexa Fluor™ 488 is equivalent to fluorescein isothiocyanate but is more stable and sensitive and less prone to photo bleaching. Negative control samples were treated as above but without the primary antibody. After the secondary incubation, cells were washed three times in PBS-RPMI-ABS and resuspended in 0.37% formaldehyde (BDH, Poole), and analysed on a FacSCAN flow cytometer (Becton Dickinson, San Jose, CA). The mean fluorescence intensity of each sample, minus the mean fluorescence intensity of the negative control was determined. A standard curve was obtained by plotting the fluorescence intensities of blood samples from donors of previously determined CR1 numbers (standards, initially determined by Scatchard analysis using 125I labelled mAb (Cohen et al., 1987)) ranging from 200 to 1000 molecules per cell. The standard curve was used to read off the CR1 expression level of unknown samples.

2.2. Preservation of blood samples

After obtaining informed consent, 5 ml of blood was drawn from 11 donors (five standards and six unknown samples) into Vacutainers containing 0.12 ml of 15% EDTA (BD Vacutainer systems, Plymouth, UK). We then preserved or fixed aliquots of all 11 blood samples in four different ways. The first was simply left in the EDTA Vacutainer into which the blood had been drawn. The second aliquot was fixed in 0.25% glutaraldehyde according to the method of Pattanapanyasat et al. (1993), in which washed cells are resuspended in 0.25% (v/v) glutaraldehyde in PBS at 2% haematocrit for 20 min before being washed in
PBS and resuspended at 50% haematocrit in PBS supplemented with 1% BSA. The third aliquot was fixed in formaldehyde as outlined by Bianco et al. (1986). These cells were first washed three times in incomplete RPMI (RPMI 1640 medium with sodium bicarbonate (Gibco Life Technologies), supplemented with 2 mM L-glutamine, 25 mM HEPES, 20 mM D-glucose and 25 μg/ml gentamicin). Whole blood (50 μl) was resuspended at 4% haematocrit in complete RPMI (as for incomplete RPMI but with the addition of 10% ABS) and an equal volume of fixative solution (10% w/v formaldehyde, 4% w/v glucose in Tris-saline (10 mM Tris, 150 mM NaCl, 10 mM sodium azide adjusted to pH 7.3) was added. The fixed samples and the EDTA-preserved samples were stored at 4 °C for the remainder of the experiment. The final aliquot of blood was frozen in glycerol by adding drop-wise five volumes of freezing solution (42.25% w/v glycerol, 0.1 M sodium lactate, 4 mM potassium chloride, 0.1 M sodium dihydrogen phosphate adjusted to pH 6.8) to three volumes of erythrocytes and then freezing overnight at −70 °C before cryopreservation in liquid nitrogen. At the appropriate time (see below), cryopreserved cells were thawed at 37 °C and 200 μl of 12% NaCl solution was added slowly and drop-wise to each sample, followed by a further slow drop-wise addition of 10 ml of 1.8% NaCl followed by 10 ml of 0.9% NaCl, 0.2% glucose solution. The cells were washed in incomplete RPMI 1640 and were then ready for flow cytometry.

2.3. Study design

To determine whether the flow cytometry could be used to quantify CR1 expression level using preserved erythrocytes from healthy donors we performed the following experiment. We drew blood from five individuals with known CR1 expression who were then used as standard donors for CR1 quantification as described above. We also drew blood from six test subjects who had unknown erythrocyte CR1 levels. We determined the mean number of CR1 molecules per cell from the fresh blood samples of the six unknowns according to the flow cytometry protocol. We then preserved or fixed aliquots of all 11 blood samples according to the methods outlined above. After 1, 2, 4, 6 and 8 weeks, the EDTA preserved, fixed and frozen samples underwent flow cytometry to assess erythrocyte CR1 level. In order to determine the optimum method of measuring CR1 levels on the stored cells, we assessed them in two ways. Firstly, we used a standard curve derived from freshly drawn standards at each time point (absolute standards) and, secondly, we derived a standard curve from the standards that had been drawn and preserved in an identical way to the test samples at the start of the experiment (relative standards).

Having determined the most promising techniques for the preservation of healthy erythrocytes (fixing in 5% formaldehyde and freezing—see Results and discussion) we then proceeded to determine whether these techniques were reliable with diseased erythrocytes which may have reduced CR1 levels. Accordingly, we repeated the experiment using the same CR1 standard donors and unknown samples from seven individuals with SLE whose symptoms met the American Rheumatism Association criteria for the disease. The erythrocytes were preserved by fixing in 5% formaldehyde and freezing in glycerol as described above, and the CR1 expression levels assessed with relative and absolute standards at 4 and 8 weeks.

3. Results and discussion

Mean erythrocyte CR1 levels were measured on six fresh blood samples from healthy donors (unknown samples) by flow cytometry using a standard curve derived from five donors with known CR1 levels (standards) as shown in Fig. 1. Erythrocytes from the six healthy test samples and the five standard donors were then preserved by fixing or freezing as described under Methods and study design. For each of the six test samples, the CR1 levels were then measured on the preserved cells at various time points (1, 2, 4, 6 and 8 weeks after the blood was drawn) using both absolute (freshly drawn) and relative (preserved) standards as described in Methods and study design. At each time point, the CR1 levels of the preserved cells were compared to the CR1 levels of the samples when freshly drawn, and the data were analyzed by linear regression. The best results were obtained with erythrocytes preserved by fixation in 5% formaldehyde. Comparison with the relative standards gave accurate results throughout the 8 weeks of the experiment (Table 1 and Fig. 2A, open squares), whereas...
Fig. 1. A standard curve used to determine the mean erythrocyte CR1 expression level of unknown samples. The standard curve was plotted with data from five standards with previously determined CR1 expression levels (Δ), with the point at the origin representing the negative control sample with no primary antibody. The mean fluorescence intensity of six unknown samples was read off the standard curve to determine the number of CR1 molecules per cell (dotted lines). The mean CR1 expression levels of the six unknown samples were determined as 448, 727, 796, 814, 894 and 967 molecules per cell.

comparison to absolute standards was accurate up to 4 weeks after the blood was drawn, but thereafter gave an underestimate of true CR1 numbers (Table 1 and Fig. 2A, filled squares). Preservation in EDTA also gave good results providing the test samples were compared to relative standards (Fig. 2B, open squares). This was true up to 6 weeks from the date of blood

Fig. 2. CR1 expression levels determined after the preservation of healthy erythrocytes compared to CR1 levels determined on fresh erythrocytes. (A) Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks. (B) Erythrocytes stored in EDTA at 4 °C for 6 weeks. (C) Erythrocytes cryopreserved in liquid nitrogen for 8 weeks. The CR1 expression levels of the six test samples determined by comparison to freshly drawn (absolute) standards are shown as filled squares (□), whereas the CR1 expression levels of the six test samples determined by comparison to preserved (relative) standards are shown as open squares (○). The relationship between the CR1 levels measured on preserved cells compared to fresh cells was analysed by linear regression. The regression equation for the data determined using relative standards is shown at the top of each graph, and the regression equation for the data determined using absolute standards is shown at the bottom of each graph. RBC, red blood cell.
suitable for CR1 quantification. Preservation of erythrocytes by freezing in glycerol gave accurate results at all time points when relative standards were used (Fig. 2C, open squares, data from week 8). The use of absolute standards gave good results at some time points.

withdrawal (Fig. 2B), after which time, the extent of haemolysis of the samples became too great for further use. When absolute standards were used to assess the CR1 level of EDTA-preserved cells, this was accurate up to 2 weeks after the blood was drawn, and thereafter resulted in an underestimation of the number of CR1 molecules per cell (Fig. 2B, filled squares). Fixation of erythrocytes in 0.25% glutaraldehyde resulted in high background fluorescence of all samples and it impossible to generate a standard curve (data not shown). Therefore, it was concluded that this method was not suitable for CR1 quantification. Preservation of erythrocytes by freezing in glycerol gave accurate results at all time points when relative standards were used (Fig. 2C, open squares, data from week 8). The use of absolute standards gave good results at some time points.

Table 1
Assessment of CR1 expression level over the course of 8 weeks on erythrocytesfixed with 5% formaldehyde

<table>
<thead>
<tr>
<th>No. of CR1 molecules per cell when fresh</th>
<th>448</th>
<th>727</th>
<th>796</th>
<th>814</th>
<th>894</th>
<th>967</th>
</tr>
</thead>
</table>

CR1 measured using absolute standards

| Week | 425 | 719 | 816 | 826 | 929 | 1072 |
| Week 2 | 421 | 860 | 886 | 1064 | 1308 | 1173 |
| Week 4 | 436 | 740 | 804 | 833 | 889 | 1014 |
| Week 6 | 294 | 529 | 585 | 606 | 632 | 746 |
| Week 8 | 350 | 575 | 645 | 690 | 760 | 788 |

CR1 measured using relative standards

| Week | 427 | 757 | 865 | 877 | 993 | 1152 |
| Week 2 | 355 | 732 | 754 | 907 | 1117 | 1001 |
| Week 4 | 442 | 760 | 826 | 856 | 915 | 1045 |
| Week 6 | 408 | 753 | 835 | 866 | 904 | 1071 |
| Week 8 | 406 | 682 | 769 | 824 | 909 | 944 |

Table 2
Summary of methods for the preservation of erythrocytes for CR1 level determination

<table>
<thead>
<tr>
<th>Preservation method</th>
<th>Maximum time of storage for accurate CR1 determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Using absolute standards</td>
<td>Up to 2 weeks after blood drawn</td>
</tr>
<tr>
<td>EDTA</td>
<td>Up to 4 weeks after blood drawn</td>
</tr>
<tr>
<td>5% formaldehyde</td>
<td>Inconsistent results – not recommended</td>
</tr>
<tr>
<td>Freezing in glycerol</td>
<td></td>
</tr>
</tbody>
</table>

Using relative standards

| EDTA | Up to 6 weeks after blood drawn |
| 5% formaldehyde | Up to 8 weeks after blood drawn |
| Freezing in glycerol | Up to 8 weeks after blood drawn |

Fig. 3. CR1 expression levels determined after preservation of erythrocytes from seven donors with SLE compared to CR1 levels determined on fresh SLE erythrocytes. The mean CR1 levels when fresh were 104, 218, 275, 493, 794, 867 and 896 molecules per cell. (A) Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks. (B) Erythrocytes cryopreserved in liquid nitrogen for 8 weeks. Symbols and analysis as in Fig. 2.
(e.g. week 8, Fig. 2C, filled squares), although, at two earlier time points the use of absolute standards gave widely differing estimates of CR1 numbers (data not shown), and we would not recommend the use of absolute standards with frozen cells. A summary of the results from each method of preservation is given in Table 2.

Our results indicate that CR1 levels can be accurately measured on erythrocytes from healthy donors preserved by formaldehyde fixation or freezing, as long as similarly preserved erythrocytes are used to generate the standard curve. To determine whether these methods are also reliable using erythrocytes from donors with disease states that may reduce CR1 numbers, we repeated the experiment using samples from seven donors with SLE. Once again, we found that fixing in 5% formaldehyde and freezing in glycerol both gave good results over 8 weeks when relative standards were used (Fig. 3A and B, open squares). As previously, an underestimation of CR1 expression was observed when fixed samples were used with absolute standards (Fig. 3A, filled squares), while frozen cells with absolute standards gave good results in some cases (Fig. 3B, filled squares; data from week 8) but was not reliable at all time points. Crucially, both the fixing and freezing techniques worked well with samples showing low CR1 expression (<250 molecules per cell).

We conclude that accurate CR1 quantification may be performed after preservation of either diseased or healthy erythrocytes using either a freezing technique or fixation in 5% formaldehyde, providing that the CR1 expression level standards are collected and fixed in an identical fashion. Cryopreservation of erythrocytes in glycerol is reliable and permits potentially long-term storage of samples, but does require liquid nitrogen for freezing and transportation of cells, which may not always be available during field studies. Fixation of erythrocytes in 5% formaldehyde is quick and economical and gives an accurate determination of CR1 level for at least 2 months after sample collection. These methods will facilitate further studies of CR1 expression level and disease susceptibility in areas lacking facilities for flow cytometry.

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References

A human complement receptor 1 polymorphism that reduces Plasmodium falciparum rosetting confers protection against severe malaria


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Parasitized red blood cells (RBCs) from children suffering from severe malaria often adhere to complement receptor 1 (CR1) on uninfected RBCs to form clumps of cells known as "rosettes." Despite a well-documented association between rosetting and severe malaria, it is controversial whether rosetting is a cause or a correlate of parasite virulence. CR1-deficient RBCs show greatly reduced rosetting; therefore, we hypothesized that, if rosetting is a direct cause of malaria pathology, CR1-deficient individuals should be protected against severe disease. In this study, we show that RBC CR1 deficiency occurs in up to 80% of healthy individuals from the malaria-endemic regions of Papua New Guinea. This RBC CR1 deficiency is associated with polymorphisms in the CR1 gene and, unexpectedly, with α-thalassemia, a common genetic disorder in Melanesian populations. Analysis of a case-control study demonstrated that the CR1 polymorphisms and α-thalassemia independently confer protection against severe malaria. We have therefore identified CR1 as a new malaria resistance gene and provided compelling evidence that rosetting is an important parasite virulence phenotype that should be a target for drug and vaccine development.

Severe malaria remains one of the largest causes of childhood mortality in the world. The number of deaths is high at ∼1–2 million per year; however, this number represents a small fraction of the total number of clinical malaria episodes that occur worldwide, estimated to be ∼400–900 million (1). Much work has been done to determine the factors that lead to the development of severe malaria, with parasite virulence phenotypes and host genetic factors being two major foci of research. One such Plasmodium falciparum virulence phenotype is rosetting, an adhesion property in which parasitized RBCs bind to unparasitized RBCs to form clumps of cells (2). Rosetting is thought to contribute to malaria pathology by causing microvascular obstruction and impaired tissue perfusion (3, 4). Rosetting has been associated with severe malaria in many studies in Africa (e.g., refs. 5–7), although no association with severe disease was seen in Southeast Asia (8) or Papua New Guinea (PNG) (9). This inconsistency in the association of rosetting with severe disease has led some investigators to question the role of rosetting in malaria pathogenesis (10).

Rosetting is mediated by the parasite ligand PfEMP1 on the surface of infected RBCs (11, 12), binding to a variety of uninfected RBC receptors, including complement receptor 1 (CR1) (11, 13). CR1 is an immune-regulatory protein found on RBCs and a variety of leukocytes, and its functions include control of complement activation and the clearance of immune complexes (14). On RBCs, CR1 levels vary between individuals in the range of 50–1,200 molecules per cell (15–21). In Caucasians, RBC CR1 levels are genetically determined and are associated with at least three single-nucleotide polymorphisms (SNPs) in the CR1 gene (in exon 22, intron 27, and exon 33; ref. 22). These SNPs comprise high (H) and low (L) CR1 expression haplotypes that are codominant and are associated with CR1 level on RBC, but not on other cell types, such as B cells and macrophages (15). We have shown previously that CR1-deficient RBCs from LL homozygotes with <200 CR1 molecules per cell show greatly reduced rosetting with P. falciparum-infected RBCs (ref. 11 and J.A.R., unpublished data). We reasoned that if rosetting is important in malaria pathology, then CR1 deficiency should protect against severe disease by reducing rosette-mediated microvascular obstruction. CR1 deficiency is thought to be rare in most populations; however, a review article (23) stated that 28 of 67 Melanesians had CR1-deficient RBCs. No experimental details were given, and no further information was published to validate this claim. This tantalizing suggestion that CR1 deficiency could be very common in Melanesians led us to examine the prevalence and genetic basis of CR1 deficiency in PNG and to determine whether RBC CR1 deficiency protects against severe malaria.

Methods

Study Sites and Sample Collection. We collected fresh blood samples into EDTA from healthy adult volunteers at two malaria-endemic sites within PNG (New Ireland and Madang) and from Edinburgh, United Kingdom. Informed consent was given by all donors after the aims of the project were explained, and the PNG Medical Research Advisory Committee approved all protocols. In Edinburgh and New Ireland the blood samples were analyzed fresh, whereas in Madang,uffy coats were removed for DNA extraction and the RBCs were fixed in 5% formaldehyde (24) before shipping to the United Kingdom. Buffy-coat samples from the Eastern Highlands Province (PNG) were kindly made available to us by the PNG Institute of Medical Research and came from a village 2,000 m above sea level, where no regular malaria transmission exists. New Ireland and Madang both have intense year-round malaria transmission with a spleen enlargement rate of >70% (25, 26).

Determination of RBC CR1 Level. The mean RBC CR1 level on both fresh and fixed blood samples was determined as described (24). In brief, the CR1 level was determined by flow cytometry using the anti-CR1 monoclonal antibody J3D3, with comparison to a standard curve derived from a set of reference RBCs with known

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PNG, Papua New Guinea; G6PD, glucose-6-phosphate dehydrogenase; CR1, complement receptor 1; SNP, single-nucleotide polymorphism.

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CR1 levels. The reference RBCs used to establish the assay had their CR1 expression initially determined by the use of 125I-labeled antibody and Scatchard analysis to determine the number of antigenic sites per cell, controlling for the number of antibody-binding sites per CR1 molecule (24). We have shown that fresh and fixed RBC samples are similar and that CR1 level can be assessed accurately on fixed RBCs provided that the reference RBCs used to generate the standard curve are fixed in the same manner (24).

DNA Extraction and Genotyping. DNA was extracted from buffy coats by using the Nucleon BACC I kit (Amersham Pharmacia Life Science). Samples were genotyped for three SNPs in the CR1 gene at nucleotide 3650 in exon 22, a HindIII restriction fragment length polymorphism in intron 27, and at nucleotide 5507 in exon 33 by PCR and restriction digest as described (22). α-Thalassemia genotyping for the −α7 and −α4 deletions was done by multiplex PCR according to the method of Chong et al. (27). Southeast Asian ovalocytosis genotype was determined by PCR as described (28). DNA samples for the case-control study were as described (29, 30) and were amplified by primer-extension preamplification (31) before CR1 genotyping. α-Thalassemia genotyping for the case-control study was carried out by Southern blotting (29).

Determination of Glucose-6-phosphate Dehydrogenase (G6PD) Deficiency. Whole-blood samples from Madang and New Ireland were screened for G6PD deficiency by using Sigma Procedure no. 400 (Sigma).

Statistical Analysis. Statistical tests for differences in RBC CR1 levels between populations, between CR1 genotypes, and between α-thalassemia genotypes were performed by using multivway ANOVA and F tests in the statistical package SAS (SAS Institute, Cary, NC). Further analyses were performed to test for population differences in the frequencies of the CR1 alleles and α-thalassemia genotype by using the χ2 test or Fisher’s exact test when the numbers of observations per cell were small. Odds ratios for the protective effects of genotypes in the case-control study were derived by logistic regression analysis in SAS. Data were analyzed by using both conditional and unconditional logistic regression; conditional logistic regression allows for matching of case-control pairs and maximizes power. However, because CR1 genotyping was not carried out in pairs, information was unnecessarily lost by using conditional logistic regression, and so results from unconditional analyses were used. This choice made no difference in the outcome of the analysis, nor did the fitting of ethnic groups in the model make a difference in the outcome.

Results
We measured the RBC CR1 levels of healthy adult volunteers from two highly malarious regions of PNG (Madang and New Ireland) and from a control Caucasian population from Edinburgh, United Kingdom. CR1 levels in Edinburgh varied between individuals in the range of 235–1,181 molecules per cell, with a mean of 786 (Fig. 1a). These results are similar to CR1 levels described in other Caucasian populations (Table 1), showing that our assay for CR1 (24) is similar to those used previously. In PNG, CR1 levels were significantly lower than in Edinburgh (Fig. 1a, P < 0.001), with 79% of individuals in Madang and 55% of individuals in New Ireland having fewer than 200 CR1 molecules per cell. The mean RBC CR1 levels in Madang and New Ireland are the lowest in the world (Table 1). These results indicate that, in malarious regions of PNG, CR1 deficiency is extremely common and could therefore play a major role in influencing susceptibility to severe malaria.

To ascertain whether RBC CR1 levels in Melanesians are genetically determined, we studied the three SNPs in the CR1 gene that have been associated with CR1 expression levels in Caucasians (22). The CR1 exon 22 SNP (A/G at nucleotide 3650) showed the strongest association with RBC CR1 levels in all three populations; therefore, only exon 22 data are shown. At all three study sites the exon 22 genotype had a highly significant effect on RBC CR1 level, with carriers of the G3650 low (L) expression allele having significantly lower CR1 levels than HH individuals (Fig. 1 b–d). The RBC CR1 levels of HL individuals were intermediate between those of HH and LL individuals, as expected for codominant alleles. The frequency of the CR1 L allele in the malarious regions of PNG is the highest described in the world to date (Table 1, significantly different to Edinburgh, χ2 > 6, P < 0.01). DNA samples were also studied from the nonmalarious Eastern Highlands Province of PNG, and the frequency of the L allele was found to be significantly lower than in the malarious regions (Table 1, χ2 = 8.1, P < 0.01). This may indicate selection for the L allele in areas with high malaria mortality, although other explanations cannot be excluded. The data above indicate that RBC CR1 levels in Melanesians are associated with polymorphisms in the CR1 gene. However, even when matched for exon 22 genotype, CR1 expression levels in Edinburgh, Madang, and New Ireland remained significantly different from each other (F2,96 = 66.0, P < 0.001). For example, the CR1 levels of HH individuals from PNG are lower than those of HH individuals from Edinburgh (Fig. 1 b–d), suggesting that additional factors influence CR1 levels in Melanesians.

We investigated whether other RBC polymorphisms that occur commonly in PNG such as ovalocytosis (30), G6PD deficiency (32), and α-thalassemia (32) could affect RBC CR1 levels. α-Thalassemia occurred in 89% of individuals in Madang and in 23% of individuals in New Ireland. At both sites, individuals with one or more α-thalassemia mutations had RBC CR1 levels significantly lower than those of nonthalassemics individuals (Fig. 2 a and b, F1,111 = 12.4, P < 0.001). The effect of α-thalassemia genotype on RBC CR1 level was independent of the CR1 exon 22 polymorphism, because both genotypes were statistically significant when simultaneously included in the analysis (F2,110 = 10, P < 0.001 for α-thalassemia genotype; F2,110 = 44, P < 0.001 for exon 22 genotype). Thus, an individual’s RBC CR1 level depends on both their CR1 exon 22 genotype and their α-globin genotype (Fig. 2c). Significant population differences still remained after including α-thalassemia and CR1 exon 22 genotype in the statistical model, suggesting that additional factors that influence RBC CR1 levels in Melanesians are undetected. Ovalocytosis and G6PD deficiency occurred in up to 15% of individuals in Madang and New Ireland and were not associated with RBC CR1 level (ovalocytosis, F1,115 = 0.2, P > 0.10; G6PD deficiency, F1,115 = 1.8, P > 0.10).

To determine whether the low CR1 levels in PNG influence susceptibility to severe malaria, we studied DNA samples from a case-control study carried out in Madang. These samples have been used previously to show that α-thalassemia and ovalocytosis confer protection against severe malaria (29, 30). One hundred eighty severe malaria cases and 179 community controls (matched for age, sex, ethnicity, season, and residential location) were genotyped for the CR1 exon 22 polymorphism. The data were analyzed by logistic regression, incorporating the previously identified protective factors of ovalocytosis (30) and α-thalassemia (29) into the model. We found that HL individuals for the CR1 exon 22 polymorphism were significantly protected against severe malaria (odds ratio, 0.33; P = 0.01; Table 2). The protective effect of the HL genotype was most prominent when present on a homozygous α-thalassemia background (odds ratio, 0.13; 95% confidence intervals, 0.02–0.67; P = 0.005). In contrast, HH/normal α-globin individuals, who would be expected to have the highest CR1 levels (Figs. 1 and 2), were most at risk of severe malaria, with this genotype being found in seven
Fig. 2. RBC CR1 deficiency in Melanesians is associated with α-thalassemia. (a and b) RBC CR1 levels in relation to α-thalassemia genotype. Thalassemia genotypes are the following: −α/α, heterozygotes for the −α^4.2 deletion (32); −α/−α, homozygotes for the −α^4.2 deletion; and αα/αα, normal α-globin gene structure. Only one individual carried the −α^3.5 deletion (32); therefore, this case was excluded from the analysis. (c) The effect of α-thalassemia and CR1 exon 22 genotype on RBC CR1 levels are independent of each other. The mean CR1 level for the Madang population subdivided by CR1 genotype (n3650 polymorphism) and α-thalassemia (−α^4.2 deletion) is shown, and standard errors are indicated. The HH/normal α-globin genotype was not represented.

Discussion

This study shows that RBC CR1 deficiency is extremely common in malaria-endemic regions of PNG and that polymorphisms associated with CR1 deficiency confer protection against severe malaria. As parasites invade and grow normally in both CR1-deficient normal (33) and thalassemic RBCs (34), the simplest interpretation of our findings is that these polymorphisms protect against severe malaria through CR1 deficiency bringing about reduced rosetting (11, 35). Positive selection of rosette-reducing polymorphisms in a human population with high malaria mortality is strong evidence that CR1-mediated rosetting plays a causal role in the pathogenesis of severe malaria.

The case-control study results shown here indicate that heterozygotes for the CR1 low-expression allele (HL) are significantly protected from severe malaria, whereas homozygotes (LL) have a reduced odds ratio, but this reduction does not reach statistical significance. It is unclear whether the difference between the levels of protection provided by the HL and LL genotypes is genuine, and a larger sample size would be required to resolve this issue. The only previous small study of CR1 polymorphisms and malaria suggested that the LL genotype is a risk factor for severe malaria in Thai adults (36). In Southeast Asia, severe malaria is characterized mainly by metabolic disturbances and multiorgan failure (37) rather than by severe anemia and cerebral malaria, which occur commonly in children

Table 2. The effect of the L allele on susceptibility to severe malaria

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Severe malaria cases, n (%)</th>
<th>Community controls, n (%)</th>
<th>Adjusted odds ratio (95% CI)*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH</td>
<td>21 (12)</td>
<td>9 (5)</td>
<td>1</td>
<td></td>
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<tr>
<td>HL</td>
<td>57 (32)</td>
<td>81 (46)</td>
<td>0.33 (0.14–0.77)</td>
<td>0.01</td>
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<tr>
<td>LL</td>
<td>102 (56)</td>
<td>88 (49)</td>
<td>0.55 (0.24–1.28)</td>
<td>0.16</td>
</tr>
<tr>
<td>Severe anemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>15 (13)</td>
<td>5 (5)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>34 (30)</td>
<td>45 (41)</td>
<td>0.30 (0.10–0.93)</td>
<td>0.04</td>
</tr>
<tr>
<td>LL</td>
<td>64 (57)</td>
<td>59 (54)</td>
<td>0.46 (0.15–1.40)</td>
<td>0.17</td>
</tr>
<tr>
<td>Cerebral malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>4 (9)</td>
<td>2 (4)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>15 (33)</td>
<td>21 (46)</td>
<td>0.40 (0.06–2.50)</td>
<td>0.33</td>
</tr>
<tr>
<td>LL</td>
<td>26 (58)</td>
<td>23 (50)</td>
<td>0.60 (0.10–3.69)</td>
<td>0.55</td>
</tr>
<tr>
<td>Other severe malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>5 (9)</td>
<td>6 (10)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HL</td>
<td>18 (31)</td>
<td>25 (43)</td>
<td>1.18 (0.29–4.79)</td>
<td>0.82</td>
</tr>
<tr>
<td>LL</td>
<td>35 (60)</td>
<td>27 (47)</td>
<td>2.23 (0.56–8.87)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

*Odds ratios for the HL and LL genotypes are calculated in relation to the HH genotype after adjusting for the potential confounding factors ovalocytosis and α-thalassemia. CI, confidence intervals.

†Hypoglycemia, acidosis (low plasma bicarbonate), and hyperlactatemia; the odds ratios were similar for all three syndromes when analyzed separately.
severe cases but no controls \( (P = 0.015 \text{ by Fisher's exact test}) \). When the cases were subdivided into different severe malaria syndromes, the odds ratios for the HL genotype were reduced for severe anemia and cerebral malaria but not for other forms of severe malaria (a pool of metabolic complications, Table 2). The odds ratios for the LL genotype were also reduced for all severe malaria cases, severe anemia, and cerebral malaria but did not reach statistical significance (Table 2).

**Table 1. RBC CR1 levels and frequency of the CR1 low-expression \( (L) \) allele in different populations**

<table>
<thead>
<tr>
<th>Population</th>
<th>No. tested</th>
<th>Mean RBC CR1 in molecules per cell (range)</th>
<th>Gene frequency of the CR1 L allele</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edinburgh, U.K.</td>
<td>31</td>
<td>786 (235-1181)</td>
<td>0.23</td>
<td>This study</td>
</tr>
<tr>
<td>New Ireland (PNG)(^*)</td>
<td>47</td>
<td>253 (40-839)</td>
<td>0.73</td>
<td>This study</td>
</tr>
<tr>
<td>Madang (PNG)(^*)</td>
<td>96</td>
<td>124 (0-439)</td>
<td>0.60</td>
<td>This study</td>
</tr>
<tr>
<td>Eastern Highlands Province (PNG)(^\dagger)</td>
<td>17</td>
<td>ND</td>
<td>0.41</td>
<td>This study</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>60</td>
<td>624 (119-1220)</td>
<td>0.27</td>
<td>16</td>
</tr>
<tr>
<td>France</td>
<td>84</td>
<td>613 (161-712)</td>
<td>0.21</td>
<td>17</td>
</tr>
<tr>
<td>African Americans</td>
<td>54</td>
<td>635 (158-1023)</td>
<td>0.25</td>
<td>19</td>
</tr>
<tr>
<td>Caucasian Americans</td>
<td>53</td>
<td>547 (89-1166)</td>
<td>0.25</td>
<td>19</td>
</tr>
<tr>
<td>India</td>
<td>48</td>
<td>548 (140-1294)</td>
<td>0.23</td>
<td>18</td>
</tr>
<tr>
<td>Mali</td>
<td>149</td>
<td>415 (58-1032)</td>
<td>0.14</td>
<td>21</td>
</tr>
<tr>
<td>China</td>
<td>100</td>
<td>446 (27-1039)</td>
<td>0.28</td>
<td>20</td>
</tr>
<tr>
<td>Thailand</td>
<td>30</td>
<td>ND</td>
<td>0.52</td>
<td>36</td>
</tr>
</tbody>
</table>

The L allele is associated with low RBC CR1 levels in all populations studied to date, except for Africans or African Americans \( (19, 21) \). ND, not determined.

\(^*\)Sites in PNG with intense malaria transmission.

\(^\dagger\)Site in PNG with no regular malaria transmission.
in PNG (38). Our data for the small subgroup of children with metabolic forms of severe malaria indicated a trend toward increased risk for LL individuals. This is possible that the L allele showed a large reduction in rosetting with thalassemic individuals than in those with normal g-globin (Fig. 2c). The mechanism responsible for which is ≤0.5% lower in thalassemic individuals than in those with normal g-globin (Fig. 2c). The mechanism responsible for low CR1 on thalassemic RBCs is unclear. CR1 is preferentially lost in vesicles from the cell surface as RBCs age or become ATP-depleted (43, 44). It is possible that biochemical abnormalities in thalassemic RBCs promote a similar process that would reduce their CR1 level.

The high prevalence of CR1 deficiency in Southeast Asia and PNG could explain why rosetting is not associated with malaria severity in this region (8, 9). Rosetting is greatly reduced in in vitro cultures, presumably by using other RBC receptors such as the ATP-depleted (43, 44). It is possible that biochemical abnormalities in thalassemic RBCs promote a similar process that would reduce their CR1 level.

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