Pathophysiological aspects of severe falciparum malaria in Thailand and Ghana

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Abstract

The mechanisms of parasite clearance in falciparum malaria are still unclear but the spleen is considered to be the major site of parasite removal. The process of removing intraerythrocytic parasites leaving the host erythrocyte intact is termed "pitting". We have investigated several possible mechanisms for parasite clearance. Using monoclonal antibodies to Ring Erythrocyte Surface antigen, (RESA or Pf 155) an early antigen expressed on the erythrocyte surface and staining for intraerythrocytic plasmodial DNA we have demonstrated that removal of intracellular parasites by host phagocytes leaving the erythrocyte intact, occurs *in vivo*. During acute falciparum malaria infection, red blood cells (RBC) containing abundant RESA, but no intracellular parasites, are present in the circulation. These RESA-positive parasite-negative RBC are not seen in parasite cultures *in vitro*. This indicates that in acute falciparum malaria there is active removal of intra-erythrocytic parasites by a host mechanism *in vivo* (probably the spleen) without destruction of the parasitized RBC.

The ability of parasites to form rosettes has been considered a marker of pathogenicity in malaria and those parasites with a greater potential for this phenomenon were considered inherently more pathogenic. We have reported rosetting in a Thai patient with *Plasmodium ovale* infection suggesting that rosetting is in fact a common property of all plasmodia.

Using a laser diffraction technique, we measured red blood cell (RBC) deformability over a range of shear stresses and related this to the severity of anemia in 36 adults with severe falciparum malaria. The RBC deformability at a high shear stress of 30 Pa, similar to that encountered in the splenic sinusoids, showed a significant positive correlation with the nadir hemoglobin concentration during

hospitalization ($r = 0.49$, $P < 0.002$). Reduction in RBC deformability resulted mainly from changes in non-parasitised erythrocytes.

Since reduced red blood cell deformability (RBC-D) can also contribute to impaired microcirculatory flow, RBC-D was measured and compared in 23 patients with severe falciparum malaria (seven of whom subsequently died), 30 patients with uncomplicated malaria, and 17 healthy controls. The RBC-D was significantly reduced in severe malaria and was particularly low in all fatal cases. At a low shear stress of 1.7 Pascal (Pa), a red blood cell elongation index less than 0.21 on admission to the hospital predicted fatal outcome with a sensitivity of 100% (confidence interval [Cl] 59 - 100%) and a specificity of 88% (CI = 61 - 98%). The reduction in the RBC-D appeared to result mainly from changes in unparasitized erythrocytes. This finding may provide a rationale for the use of exchange transfusion in severe malaria since this process will replace rigid cells with more deformable cells.

It has been suggested that nitric oxide (NO) plays an important but still rather unclear role in the pathogenesis of severe falciparum malaria. We measured plasma nitrate and nitrite concentrations in 70 Ghanaian children with malaria (54 with severe malaria) and 48 control subjects (33 with medical conditions and 15 surgical patients). We also studied 24 adults with severe malaria on the Thai-Burmese border and compared those who survived with those who died. After correction for renal function in the adults, there was no correlation between plasma or CSF NOx levels, or the total amount of NOx excreted in the urine, and disease severity. In the children mean nitrogen oxide concentrations were not significantly different in the 2 outcome categories and were not related to depth of coma. In children whose cerebrospinal fluid (CSF) was examined, lactate concentrations but not CSF NOx levels were elevated in fatal cases compared with survivors (P=0·032). Plasma nitrogen oxide

concentrations were negatively correlated with admission parasitaemia (r=–0·41, n=70; P<0·0001). In these two populations, elevations of plasma lactate, but not nitrite or nitrate, reflected disease severity.

Lactic acidosis and hypoglycaemia frequently complicate severe malaria in African children, and lactate is a strong independent predictor of mortality. To study these metabolic derangements we sequentially allocated 21 children with severe falciparum malaria and capillary lactate concentrations > 5mmol/l to receive quinine or artesunate, as antimalarial therapy, and dichloroacetate (DCA), an activator of pyruvate dehydrogenase or saline placebo for lactic acidosis. We then administered a primed infusion (90 min) of L - [3-$^{13}C_1$] sodium lactate and D-[6,6-D_2] glucose to determine the kinetics of these substrates. Nineteen surviving children were comparable at entry to the study. The mean (SD) glucose disposal rate in all patients was 56(16) μmol/kg/min and geometric mean (range) lactate disposal rate was 100(66-177) μmol/kg/min. Glucose and lactate disposal rates were positively correlated (r=0.62, p=0.005). Artesunate was associated with faster parasite clearance, lower insulin/glucose ratios and higher glucose disposal rates than quinine. Lactate disposal was positively correlated with plasma lactate concentrations (r=0.66; p=0.002) and time to recovery from coma (r=0.82, p<0.001; n=15). We have shown that elevated glucose turnover in severe malaria results mainly from enhanced anaerobic glycolysis and that increased lactate production is the most important determinant of lactic acidosis. Quinine differs from artesunate in its effects on glucose kinetics and basal lactate disposal rates are increased with dichloroacetate.

We also tested the hypothesis that DCA rapidly reduces hyperlactataemia in this patient population. Eighteen children with severe malaria and capillary plasma lactate > 5 mM were randomised to receive either intramuscular quinine plus a single 50

mg/kg intravenous infusion of DCA in saline, or quinine plus intravenous saline alone. Thirty minutes after treatment, the mean plasma lactate was 28% below pre-treatment baseline values in the DCA group, but was unchanged in the placebo group. Throughout the first 4 h after treatment, mean plasma lactate in the DCA-treated patients was significantly less than that in controls ($p= 0.003$). Thereafter, mean plasma lactate declined in both groups and was <2 mM 10 h after treatment. DCA was well tolerated and did not alter quinine pharmacokinetics. A single intravenous dose of DCA rapidly improved lactic acidosis in African children with severe malaria, suggesting that DCA may be a useful adjunct in the initial treatment of these patients, and may increase their chance of survival by improving a major complication of their illness.

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1.1 Introduction

"The best blood will at some time get into a fool or a mosquito." Austin O'Malley

Malaria is a common tropical disease. It is a protozoal infection caused by one or more of four species of protozoa: *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* and is most commonly transmitted through the bite of an infected female anopheline mosquito. Only *P. malariae* has an animal reservoir (Bruce-Chwatt 1985). Of approximately 400 species of *Anopheles* throughout the world, only about 60 are vectors of malaria under natural conditions, 30 of which are of major importance. Malaria can also be transmitted by blood transfusion (Williamson et al. 1999) by contaminated needles and syringes {as unfortunately reported recently in a nosocomial outbreak in Nottingham} (Evans 1997) and from mother to child before and/or during birth (Fischer 1997).

1.2 Epidemiology

Malaria is a public health problem in 90 countries worldwide, inhabited by 36% of the world population, i.e. over 2 billion people are at risk. It has been estimated that the incidence of malaria in the world may be in the order of 300-500 million clinical cases each year. Estimates of malaria mortality vary from 1.5 to 2.7 million deaths worldwide per year. Snow et al estimate that eighty per cent of the cases occur in tropical Africa, malaria accounts for 10% to 30% of all hospital admissions and is responsible for 15% to 25% of all deaths of children under the age of five especially

in remote rural areas with poor access to health services. Around 800,000 children under the age of five die from malaria every year and it is one of the major causes of infant and juvenile mortality (Snow et al. 1999). Other high-risk groups include women during pregnancy, and non-immune travellers, refugees, displaced persons, or labour forces entering endemic areas.

Malaria thus has disastrous social consequences and is a heavy burden on economic development. It is estimated that a single bout of malaria costs a sum equivalent to over 10 working days in Africa. The cost of treatment is between $US 0.08 and $US 5.3 according to the type of drugs prescribed as determined by local drug resistance. In 1987, the total "cost" of malaria - health care, treatment, lost production, *etc.* was estimated to be $US 800 million for tropical Africa and to be more than $US 1,800 million by 1995 (World Health Organisation 1998). In other parts of the world, the distribution of malaria varies greatly from country to country and within the countries themselves. In 1990, 75% of all recorded cases outside of Africa were concentrated in nine countries: India and Brazil (50%), Afghanistan, Sri Lanka, China, Indonesia, Viet Nam, Cambodia and Thailand. Within countries there are also marked differences in the distribution of the disease from one area to another. For example in India, the majority of reported cases occur in nine states and in Brazil, 80% of all cases occur in three states (World Health Organisation 1993). In Thailand it is mainly found on the western Thai-Burmese border particularly amongst the Burmese refugees (Luxemburger et al. 1996).

1.3 Historical perspective

"*Prehistoric man in the Old World was subject to malaria. It is probable that the disease originated in Africa, which is believed to be the cradle of the human race. Fossil mosquitoes were found in geological strata 30 million years old and there is no doubt that they have spread the infection through the warmer regions of the globe, long before the dawn of history.*" (Bruce-Chwatt, 1985)

Hippocrates is credited with early clinical descriptions of malaria and the symptoms of malaria have been known since time immemorial, although it was a long time before the actual causes were well understood. It was previously thought that "*miasma*" (bad air or "*mal aria*") emanating from swamps caused the disease. There is evidence that some ancient treatments were remarkably effective. The prescription of an infusion of qinghao (*Artemisia annua*) has been used in China for at least the last 2000 years, its active ingredient qinghaosu (artemisinin) having only recently been identified as a potent anti-malarial (Klayman 1985). Development of derivatives of these compounds in particular artemether and artesunate have shown them to be the most potent and well tolerated anti-malarial drugs ever discovered.

In Peru, the antifebrile properties of the bitter bark of the cinchona tree (*Cinchvona ledgeriana*) were known by local populations well before the 15th century as the "*Jesuit's bark*". Quinine was isolated from this in 1820 by the pharmacists, Pelletier and Caventou.

Although in the past people were unaware of the origin of the recurring fevers and their mode of transmission, protective measures against mosquito bites have been used for many hundreds of years. Herodotus (485-425 BC) noted that in the swampy

region of Egypt, some people slept in tower-like structures out of the reach of the mosquitoes, whereas others slept under nets. Recent studies in the Gambia have rediscovered the importance of bed nets particularly when impregnated with an insecticide (D'Alessandro et al. 1995) although they are not applicable to all regions including for example, Thailand due to differences in the biting habits of local vectors (Dolan et al. 1993).

Systematic malaria control started after the discovery of the malaria parasite by Laveran, a French army surgeon in Algeria in 1880 (for which he was awarded the Nobel Prize for medicine in 1907), and the demonstration by Ronald Ross, a Scottish army doctor in India in 1897 that the mosquito was the vector of malaria (Bruce-Chwatt, 1985; Desowitz, 1991). These findings quickly led to control strategies and, with the discovery during the World War II of the powerful insecticidal effect of DDT, the notion of eradication. Effective and inexpensive antimalarial drugs of the 4-aminoquinoline chloroquine group were also synthesised during this period. However resistance to chloroquine was first identified in Cambodia in the 1960's and spread rapidly throughout South East Asia.

Between 1955 and 1969, the WHO Global Malaria Eradication Programme launched a series of campaigns aimed at spraying the inside of homes with insecticide. The initiative proved successful in large areas of North America, Southern Europe, the former Soviet Union and some territories of Asia and South America where transmission was halted and malaria was eradicated.

In Latin America and most Asian countries however, results varied and the disease persisted especially with the increase of drug resistance. The problems and logistics associated with malaria control were considered beyond the scope of the vast

majority of African countries, and large-scale eradication was never attempted. Epidemics frequently broke out in Central America and South East Asia - culminating in a massive epidemic in 1968 in Sri Lanka, where malaria was thought to have been eradicated. They also occurred in some parts of Africa, as in 1988 in Madagascar when 25,000 people died from malaria (Desowitz, 1991).

Mefloquine was developed by the US army in the early 1970's and again resistance has been observed since the early 1980s particularly on the Thai/Cambodia and Thai/Burmese borders (Desowitz, 1991). Halofantrine was also developed by the US army and marketed by SmithKline Beecham in the 1990s but cross-resistance with mefloquine and side-effects (particularly cardiac dysrhythmias) (Nosten et al 1993) have been observed. It is also too expensive for use in countries where the average health expenditure is $10 per annum. The Chinese have developed the latest class of antimalarial the artemisinin derivatives artemether and artesunate (Klayman 1985). These are potent rapidly acting and relatively cheap. Some Western drug companies have now begun marketing these drugs for use in multi-drug resistant falciparum malaria.

The hope of global eradication of malaria was finally abandoned in 1969. Previous efforts had at least demonstrated that it was utopian to envisage eradication of the disease by campaigns based on a single strategy. It is now clear that to be effective, control programmes need to be adapted to local conditions and involve the community at large, the general health services and those involved in development. Malaria is a "Disease under Surveillance" by W.H.O., as it is considered an essential element of the world strategy of primary health care. National health administrations are expected to notify W.H.O. twice a year of those areas originally malarious with no

present risk of infection, those malaria cases imported into areas in the maintenance phase of eradication, those areas with chloroquine resistant strains of parasites, and those international ports and airports free of malaria. The new "Roll back Malaria" program, "a global partnership for halving the burden of malaria by 2010" and the "Medicines for Malaria Venture" a private-public co-operation to develop new drugs are key plans of the new director of WHO Dr Gro Harlem Brundtland. The outlook for new drugs in malaria, however, is rather bleak and the costs involved in developing them are huge with little prospect of a return for drug companies for their investment (White et al, 1999).

1.4 Life cycle of Plasmodium falciparum

Falciparum malaria is transmitted by the bite of a female Anopheles mosquito. While taking a blood meal the mosquito injects saliva, which contains analgesic as well as anti-coagulant substances into the wound through her proboscis. The malaria sporozoites are injected in the saliva. They travel through the bloodstream to the liver within 45 minutes where they may mature and multiply asexually for 5 up to 28 days (Bruce-Chwatt, 1985) until they are released by hepatocyte rupture with as many as 30,000 uninucleate merozoites. These invade mature red blood corpuscles directly. There is a specific receptor/ligand interaction for invasion. This induces a calcium influx that activates calpain, a cytoplasmic protease that remodels the cytoskeleton. This alters the red cell deformability (Cranston et al. 1983). We have studied the clinical consequences of this altered red cell deformability in both the risk of severe disease and the development of anaemia (Chapter 3). The parasite probably enters the cell by attachment, re-orientation and active invasion using an actin myosin complex

(Pinder et al 2000). Once inside the merozoite rapidly expresses proteins that hijack the cellular metabolism of the erythrocyte. There is no intracellular defence mechanism within erythrocytes. The process of maturation then begins over a period of 48 hours with a large metabolic energy requirement. The merozoite forms tiny rings within a few hours. These are the first visible signs of infection under the microscope. These enlarge to become small and then large rings. The appearance of malaria pigment (haemozoin) marks histologically the trophozoite stage at around 26 hours. Haemozoin is the result of parasite polymerisation of haem since the parasite metabolises the globin part of haemoglobin in the host cell. Intracellular haem is toxic to the parasite and is polymerised by a still unknown mechanism into inert β-pleated sheets (Taramelli et al 1999). This process is the target of most anti-malarial drugs probably by binding to cysteine or aspartate proteases although for most the mechanism of action is still unclear (Krishna and White 1996). These proteases, falcipain and plasmepsin have been characterized and explored recently as drug targets (Singh and Rosenthal 2001).

The trophozoite matures through mid-trophozoite to late trophozoite. Then the cells divide into > 20 daughter cells producing a schizont. The schizont ruptures after 48 hours and releases daughter merozoites to start direct red cell invasion again.

Some of the parasites undergo meiosis to become gametocytes. Sexual development takes 10-12 days. Normally a variable number of cycles of asexual erythrocytic schizogony occur before any gametocytes are produced. The trigger for the switch to sexual reproduction is unknown but may relate to parasite density. The gametocytes form male and female gametes. Once drawn into the mosquito during a blood meal, the gametocytes increase in volume and escape the erythrocyte.

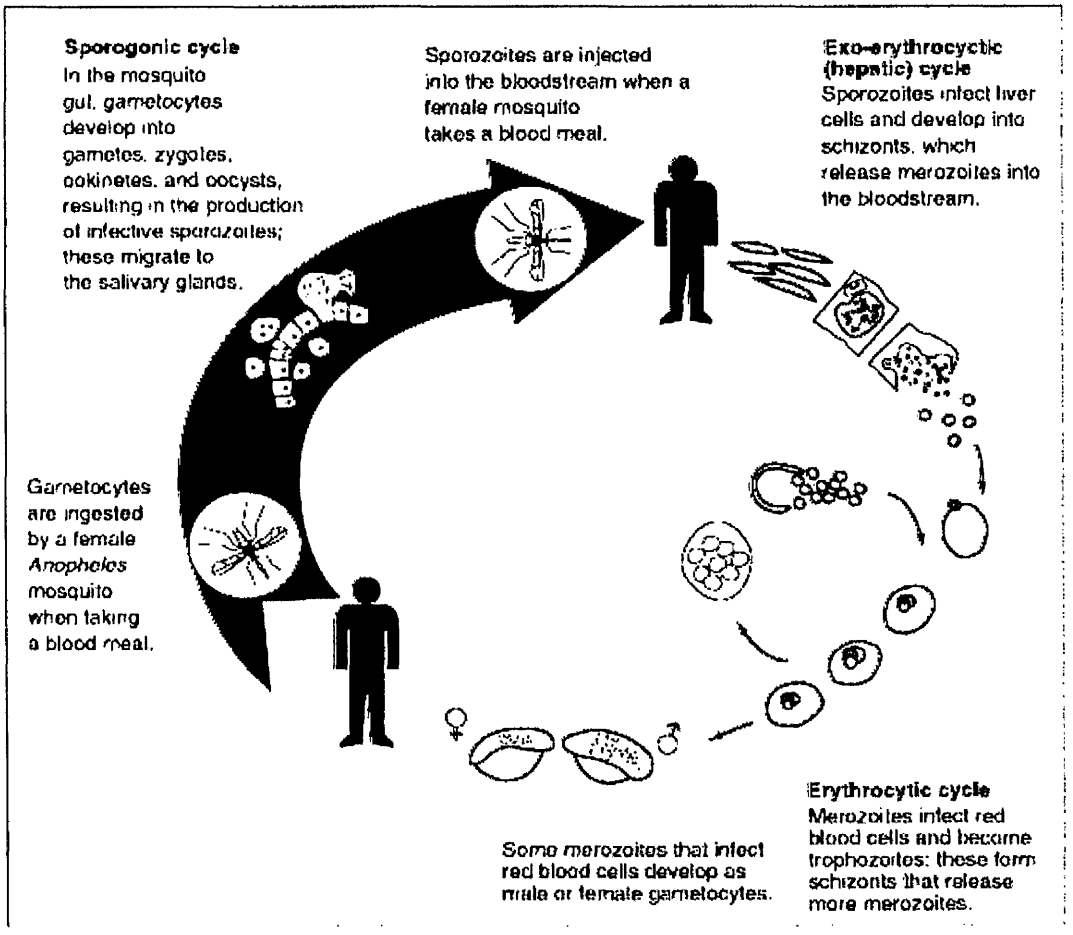
Microgametes are formed by 3 mitotic divisions within the microgamete, and are expelled explosively (Day et al, 1998). No further changes affect the female macrogamete until fertilisation where the plasmalemmas of male and female gametes fuse and the nucleus of the microgamete enters the female cytoplasm. After fertilisation, the zygote is a motionless globular cell, but after 18 to 24 hours it becomes elongated and motile, containing micronemes and a pellicle. The cell invades the microvillus border, passes through the midgut cells, and lies beneath the basement membrane. The ookinete then becomes a static oocyst, between the basal lamina and the basement cell membrane, and bounded by a thick plasmalemma. The chief source of nutrients is the haemolymph in which the oocyst develops. Sporoblasts form, and sporozoites bud off (Shahabuddin 1998).

After the cyst ruptures, the sporozoites escape into the haemocoele and migrate to and penetrate salivary gland cells where they lie in vacuoles for up to 59 days. These sporozoites develop and become up to 1000 times more infective than when in the oocyst. They are more antigenic, and bear circumsporozoite polypeptide (CSP) on their plasmalemma. Sporozoite motility is involved in their invasion of salivary gland cells and escape from the salivary gland (Day et al 1998). The sporozoites are about 12μm long and 1μm across, with a single nucleus, anterior to which lie micronemes, and posterior to which lies ER and mitochondria. They possess a complex pellicle, which is responsible for motility, and contains circumsporozoite protein. The apical penetrating region contains extensions of the microneme ducts that release an agent that interacts with host cell plasma membrane during penetration. A biting mosquito transfers about 10% of its sporozoite load into the human host's capillaries or perivascular tissue.

1.5 Pathogenesis and Host Responses

When the parasite reaches 18 hours of age the trophozoite also changes the cell surface with the appearance of electron dense "knob proteins". These are the ligands that allow for the main pathogenic characteristic of falciparum that is termed "sequestration." Sequestration describes the ability of the parasitized erythrocytes to become "sticky" and adhere to endothelial cells, letting them settle in deep tissues such as the brain, the liver, the kidneys and skeletal muscle. They adhere to the endothelium in the post-capillary venules causing slowing of the microcirculation. This allows the parasitized erythrocyte to avoid travelling to the spleen where the mechanism normally used to remove "old" uninfected rigid red cells would non-specifically remove the parasitized cell (Lewis and Swirsky, 1996). The parasitised red cell is more rigid than unparasitized red cells and can be recognised as it tries to squeeze through the splenic sinusoids. This will be discussed further in studies that we have performed relating to the rheological properties of the parasitized red blood cell (Chapter 3).

Figure 1.



The malaria transmission cycle.

The appearance of malarial parasite proteins on the surface causes another problem for the parasite. These foreign antigens can be recognised by the host. In order to escape immune detection the parasite varies these proteins, called variable surface antigens (VSA) or *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). In order to do this it uses a gene rearrangement of the variable, *var* genes on chromosomes 2 and 3 near to the teleomere. This switching on and off of the genes allows PfEMP-1 variation to occur in up to 2% of the parasite population per life cycle (Newbold 1999). Different VSAs probably differ in their pathogenic properties (Newbold 1999), for example high binding to intercellular adhesion molecule-1

(ICAM-1) on brain endothelium may lead to cerebral malaria (Turner et al. 1994) whereas adhesion to chondroitin sulphate A is important in malaria in pregnancy (Fried et al. 1998). The loss of acquired immunity to malaria that occurs amongst primigravida was shown to correlate with an absence of antibodies that block the binding of parasites to chondroitin sulphate A on the placenta which appear in subsequent pregnancies (Fried et al. 1998). VSAs may also be important in the property of "rosetting" when parasitised red cells stick to other unparasitised red cells. The role of this property of rosetting in pathogenesis will be discussed further in the text (Chapter 3).

Immunity to malaria in man is complex and not properly understood. Immunity to pre-erythrocytic stage antigens is largely mediated via CD8+ T cells and involves IFN-, nitric oxide, IL-12 and natural killer cells (Lalvani and Hill 1998). Immunity to erythrocytic stage antigens varies (in different hosts and with different parasites) but is largely mediated by antibody, helper CD4+ T cells, nitric oxide, cytokines and T cells. However the situation in man is not as well understood as in animals (Troye-Blomberg et al. 1999).

The role of nitric oxide is also controversial even in animal models since although it has an antiparasite effect which is reversible with NO synthetase inhibitors in *P. chabaudi* infection (Balmer et al. 2000), inhibitors of the inducible nitric oxide synthase do not reduce the antiparasite effect of adoptively transferred *P. chabaudi*-specific CD4+ T cells (Taylor-Robinson et al. 1993). We have performed clinical studies to measure the association if any with total nitrite excretion and severity of disease (Chapter 4). Killing of parasites probably occurs primarily in the spleen and in experiments where immune spleens were transplanted into naive recipients in the rat

model of *P. berghei* it was confirmed that the bulk of killing did occur there (Favila-Castillo et al. 1996). Asplenic patients suffer from more severe disease (Looareesuwan et al. 1987a). We have described red cells expressing malaria antigens and therefore infected but without plasmodia nucleic acid (Chapter 3). These parasites are thought to have been removed or "pitted" in the spleen.

Host factors also play a role with variation in susceptibility to severe malaria associated with tumour-necrosis factor α promoter gene polymorphisms (McGuire et al. 1994) and HLA type (Hill 1999).

1.6 Pathophysiology of severe disease

Severe falciparum malaria is defined by a combination of clinical and laboratory criteria as set out by the World Health Organisation. (Figure 2) It is associated with a much higher mortality than uncomplicated malaria (20% versus < 3%). The main defining features are as shown in Figure 2. My studies have concentrated on three main aspects of severe disease; lactic acidosis, cerebral malaria and anaemia.

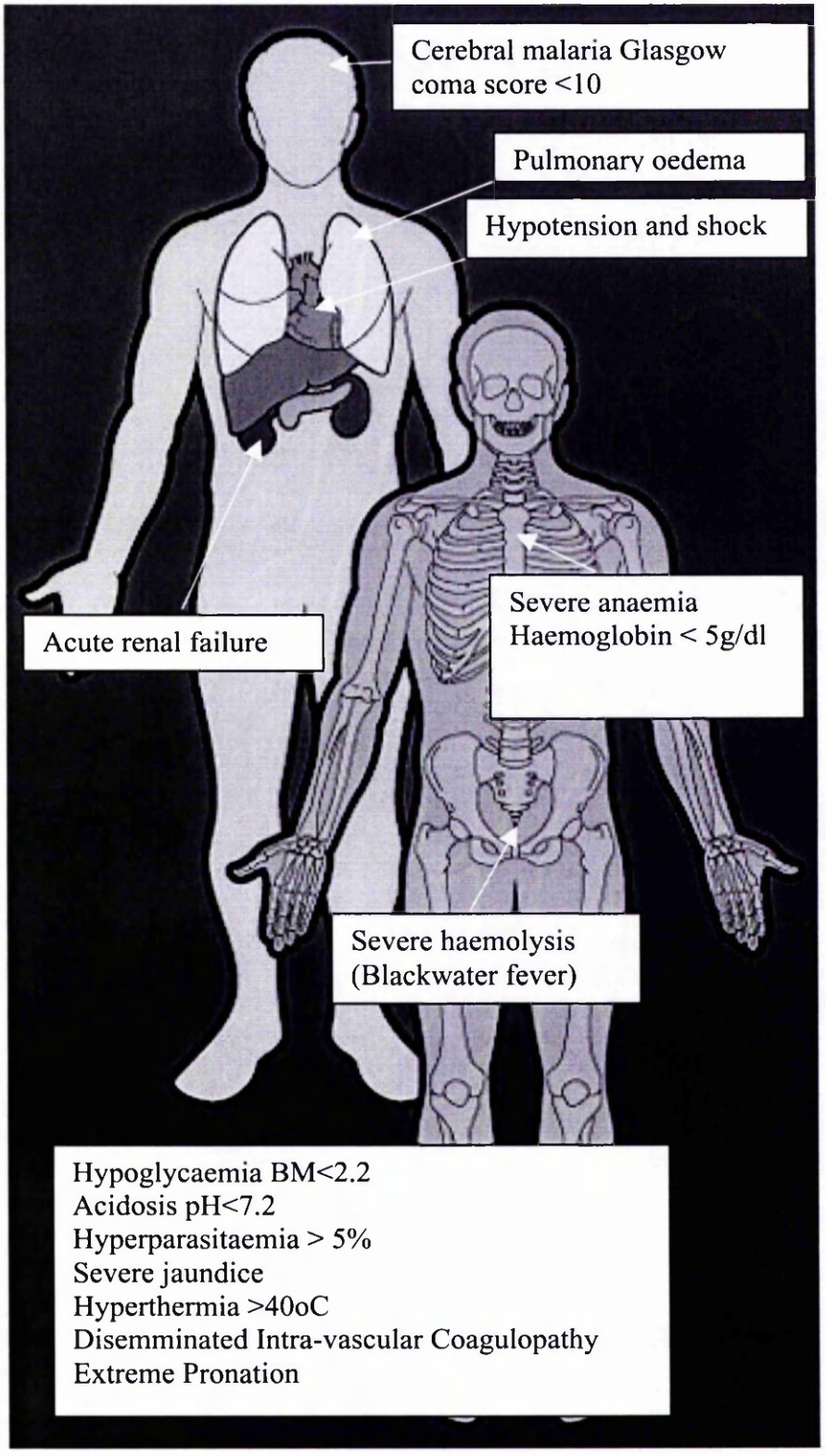
Acidosis Metabolic acidosis is a common cause of death in severe malaria. Lactate concentration is proportionally related to disease severity and the risk of death (Krishna et al. 1994b; White and Ho 1992), is multi-factorial and exacerbated by hypoxia, anaemia and malnutrition (Newton and Krishna 1998). It is associated with other metabolic disturbances such as hypoglycaemia. Lactate is generated by host and parasite anaerobic glycolysis (Zolg et al. 1984) with the majority from the host in the form of L (+) lactate and approximately 7% in the form of the laevorotatory enatomer D (-) lactate from the parasite (Jagt et al. 1990). In normal subjects the liver and

kidneys rapidly clear lactate. However in severe malaria both hepatic (Pukrittayakamee et al. 1991) and renal (van Velthuysen and Florquin 2000) function are impaired. In general terms accumulation in plasma *L*-lactate may be secondary to tissue hypoxia (type A) through circulatory insufficiency, severe anaemia, mitochondrial enzyme defects and inhibitors (carbon monoxide, cyanide) - or in which overproduction and/or decreased hepatic removal of lactate can occur (type B) through hypoglycaemia, glycogen storage diseases, seizures, diabetes mellitus, ethanol, hepatic failure, malignancy, and salicylate overdose (DuBose 1999). In severe malaria there is obviously a combination of many of these factors and lactic acidosis can be further exacerbated by thiamine deficiency (Krishna et al. 1999). Hypoglycaemia in malaria results from a failure of hepatic gluconeogenesis and an increase in the consumption of glucose by both host and parasite. This is worsened by the hyperinsulinaemic effect of quinine.

In our studies we used a stable isotope technique to measure both glucose and lactate turnover in children with severe malaria. By using artesunate, an artemisinin derivative to treat our patients we avoided the potentially confounding effects of quinine induced hyperinsulinaemic hypoglycaemia that was present in previous studies.

Dichloracetate (DCA) is a simple chemical that activates pyruvate dehydrogenase and so reduces lactic acidosis (Stacpoole, 1989). It has been used in intensive care patients with lactic acidosis due to a variety of causes and also in patients with mitochondrial myopathies (Stacpoole, 1992; Stacpoole 1993). We performed pilot studies to establish the safety and efficacy of DCA in children with severe malaria and measured the pharmacokinetics and dynamics.

Figure 2. Criteria for the diagnosis of severe falciparum malaria. Adapted from (World Health Organization 1990)



Cerebral malaria Neurological impairment in malaria can be permanent, especially if associated with prolonged hypoglycaemia (Nguyen et al. 1996). Altered consciousness and coma in malaria can also be the result of seizures that are commoner in children than adults (White et al. 1988). Opthalmological changes of haemorrhage and oedema indicating the pathology deeper in the brain can be seen in the retina in around 15% of patients (Lewallen et al. 1999). Although the main pathogenic process is sequestration and microvascular obstruction, the underlying cause of the coma in cerebral malaria is still unknown (White and Ho 1992). The main mechanisms proposed previously were hypoxia although diffuse white matter multiple small haemorrhages with microglial reaction (Durck's granulomas) are seen at post-mortem rather than infarcts or increased capillary permeability causing cerebral oedema and raised intracranial pressure (White and Ho 1992). These theories have not been validated and in fact the use of steroids in cerebral malaria to reduce supposed oedema was deleterious (Warrell et al. 1982). More recently various neurotransmitters have been implicated such as quinolinic acid and tryptophan metabolites (Dobbie et al 2000). In particular the effect of nitric oxide has been proposed by Clark (Clark, Rockett, and Cowden 1991; Clark, Rockett, and Cowden 1992) to be the major component of coma through a mixture of hypoxia and its direct neuromodulatory effects (Clark and Cowden 1999). This is claimed to be important in the generation and maintenance of induction in general anaesthesia (Johns et al 1992). Although there has been *in vitro* evidence for the protective and deleterious effects of nitric oxide there have been few clinical studies performed. As part of our studies we measured total nitrites in age/sex matched control patients and patients with severe

and uncomplicated malaria. These studies were performed both in children and in adults. We also for the first time measured nitrite in the cerebro-spinal fluid.

Anaemia As discussed previously the invasion of red blood cells by parasites inevitably results in their destruction. Potential causes of haemolysis include loss of infected cells by rupture (before or at schizogony) or phagocytosis, removal of uninfected cells due to antibody sensitization or other membrane changes, and increased reticuloendothelial activity, particularly in organs such as the spleen. Decreased production results from marrow hypoplasia seen in acute infections, and dyserythropoiesis (Weatherall and Abdalah 1982; Phillips and Pasvol 1992) possibly induced by cytokines. Ninety-four per cent of 169 patients with cerebral malaria in one study developed anaemia (haematocrit less than 35 per cent) and 30 per cent required blood transfusion to maintain the haematocrit at more than 21 per cent (Phillips et al. 1986). The haemolytic destruction of erythrocytes leads to jaundice, exacerbated by reductions in hepatic blood flow (Pukrittayakamee et al. 1991), haemoglobinuria (Blackwater fever) and subsequent renal tubular damage (van Velthuysen and Florquin 2000).

Chapter 2. Study Sites and Statement of Personal Contribution

2.1 Mae Sot District Hospital, Tak Province, Thailand. This is a 300-bedded hospital located on the Thai Burmese border. It has 5 medical wards; an 8 bedded intensive care unit with facilities for mechanical ventilation, peritoneal dialysis and care of central venous access. It also has two surgical theatres and an obstetric unit. It has basic X-ray capabilities, basic biochemistry and haematology including a Coulter counter and access to blood transfusion from Tak Provincial Hospital. The average life expectancy in Thailand is 66 years for men, the infant mortality rate is 29 per 1,000 live births, the maternal mortality rate is 200 per 1,000 live births and the expectation of dying before your fifth birthday is 3.7%. This is compared to the United Kingdom where the average life expectancy is 75 years, the infant mortality rate is 7 per 1,000 live births, the maternal mortality rate is 9 per 1,000 live births and the expectation of dying before your fifth birthday is 0.9% (World Health Organisation 1998).

As well as the local population, the hospital serves a large refugee population of Burmese people, particularly the Karen who live on either side of the border. Malaria transmission is low in this area with a seasonal peak during the rainy season that starts in late spring until late summer (Luxemburger et al. 1996). Severe disease occurs at all ages. Multiple drug resistance is an increasing problem in this area (Nosten et al. 1991) and the main vectors are *Anopheles minimus, A. maculatus* and few *A. darus* (S.W. Lindsay, personal communication 2000). I worked together with Dr. Ronatrai Ruangveerayuth, the medical division director. This site had not previously been involved in studies run by the Wellcome Unit and so I was involved in setting up the studies from an early stage. The Wellcome Trust sponsored Shoklo Malaria Research

Unit (SMRU) has been running large community based studies into new anti-malaria treatments in the area since 1986 under the supervision of Dr Francois Nosten. However there were no hospital-based studies in severe disease before my arrival.

My work involved planning studies, applying for ethical approval with the appropriate Thai authorities, ordering equipment and supplies and establishing a clinical laboratory in Mae Sot Hospital. As far as clinical work was concerned, I saw all patients admitted with a possible diagnosis of malaria, confirmed the diagnosis and resuscitated the patients, I then made arrangements for admission to one of the wards or the intensive care unit and was responsible for their continuing medical care. I randomised the patients where appropriate into treatment studies and took the relevant samples. I made thin blood films, counted and staged parasites, measured haematocrit, lactate and glucose using the YSI analysers and measured red cell deformability using the LORCA analyser. I entered data onto computer, analysed the results and was helped to write the final papers. The work on red cell deformability was done in conjunction with Dr. Arjen Dondorp, University of Amsterdam, Netherlands and clinical assistance came from Dr Yupin Supputamongkol, and Dr. Kenechanh Chanthapadith. More detailed laboratory work on culture of malaria parasites was done by Dr. Kesinee Chontavanich and Khun Kamolrat Silamut. Overall supervision was by Professors Nick White in Bangkok and Piet Kager in Amsterdam.

2.2 Komfe Anokye Teaching Hospital, Kumasi, Ghana. This is a 1,500-bedded teaching hospital in Kumasi, capital of the Ashanti area in North Western Ghana with a population of 700,000. The average life expectancy is Ghana is 58 years, the infant mortality rate is 74 per 1,000 live births, the maternal mortality rate

is 740 per 1,000 live births and the expectation of dying before your fifth birthday is 10.7% (World Health Organisation 1998). It is linked to the Institute of Science and Technology (IST) in Kumasi and has its own medical school. There were no reliable laboratory services in the hospital. I worked in the Department of Paediatrics with Dr Tsiri Agbenyega and Dr. Baffoe-Bonnie. Again there had been no previous studies at this site and I was involved from an early stage in its development. This was part of the St. George's Tropical Medicine Research Programme under the supervision of Professor George Griffin and Dr. Sanjeev Krishna from the Division of Communicable Diseases, St. George's Hospital, London.

We arranged for a special care unit to be built between two wards. I was responsible for ordering and transportation of equipment and drugs into Ghana. We used some equipment from the unit in Thailand. Since the peak malaria seasons are at different times of the year in Thailand and Ghana I was, rather uniquely, able to study severe malaria in both children and adults throughout the year. As far as clinical work was concerned I saw patients on the ward with malaria and confirmed the diagnosis of severe malaria. I measured haematocrit, glucose and lactate, resuscitated and then randomised patients into the trials. We had no access to mechanical ventilation. I collected samples from the patients, froze and then transported them back to the United Kingdom for analysis. The chromatography was done elsewhere and the mathematical and statistical modelling was done in Florida. Help with clinical work was by Dr. Krishna, Dr Agbenyega, Dr. Beddo-Addo in Ghana and Professor White in Bangkok, Prof. Griffin in London and Prof. Stacpoole in Florida were overall supervisors.

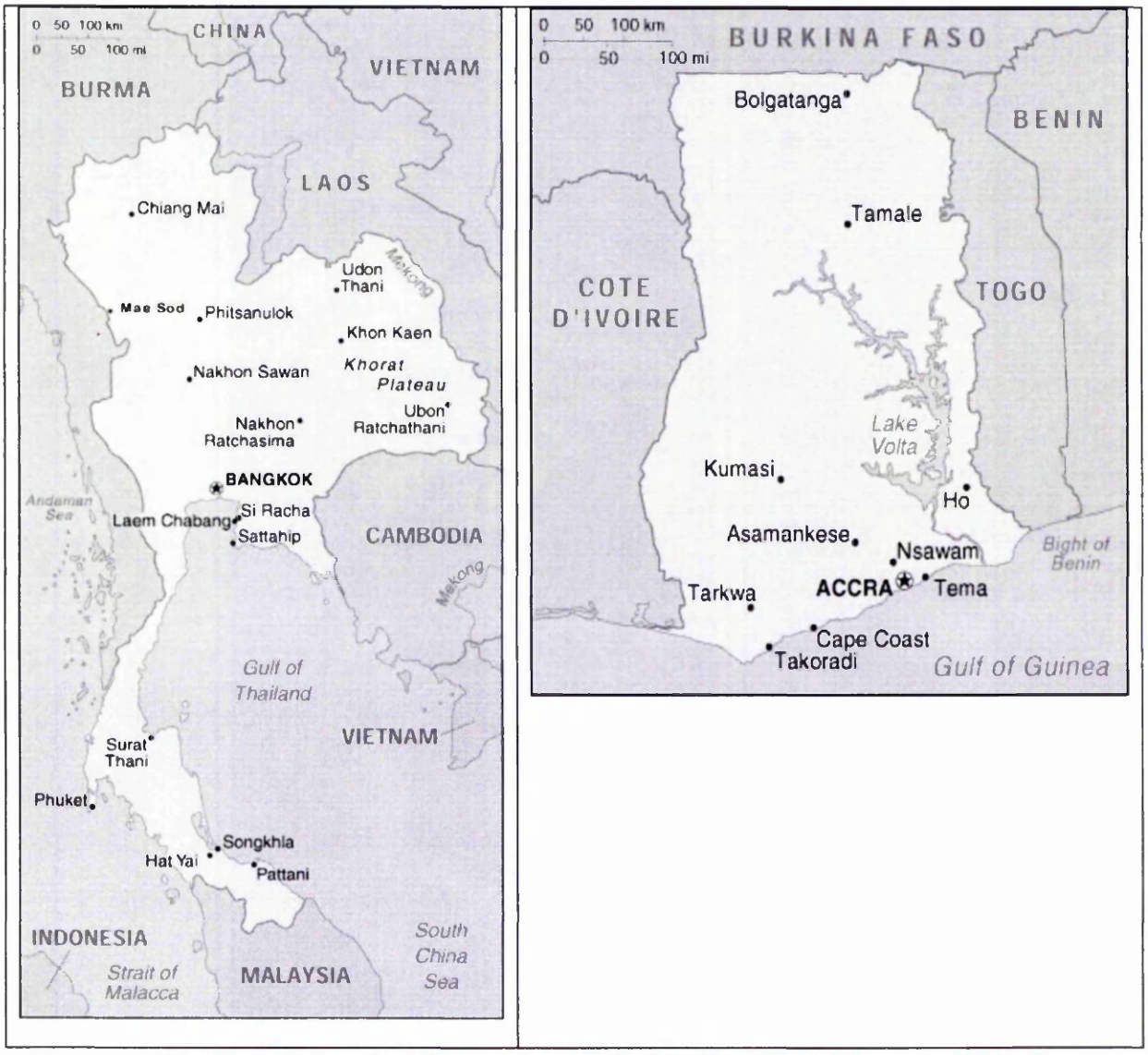


Figure 3. Maps showing study sites in Thailand and Ghana.

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In vivo Removal of Malaria Parasites From Red Blood Cells Without Their Destruction in Acute Falciparum Malaria

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Abstract

During acute falciparum malaria infection, red blood cells (RBC) containing abundant ring-infected erythrocyte surface antigen (Pf 155 or RESA), but no intracellular parasites, are present in the circulation. These RESA-positive parasite negative RBC are not seen in parasite cultures *in vitro*. This indicates that in acute falciparum malaria there is active removal of intraerythrocytic parasites by a host mechanism *in vivo* (probably the spleen) without destruction of the parasitized RBC. This may explain the observed disparity between the drop in haematocrit and decrease in parasite count in some hyperparasitemic patients. The fate of these "once-parasitized" RBC *in vivo* is not known.

Introduction

The mechanisms of parasite clearance in human malaria *in vivo* are unclear. The spleen is considered the major site of parasite removal (World Health Organization 1990) and splenic function is increased in acute malaria; the clearance thresholds for both mechanical filtration and Fe receptor-mediated parasitized erythrocyte clearance are lowered (Ho et al. 1990a; Looareesuwan et al, 1987). Parasite clearance is delayed considerably in splenectomized patients. Despite evidence from animal studies in the 1960s that the spleen could remove intraerythrocytic parasites leaving the host erythrocyte intact, a process known as pitting, parasite clearance in human malaria has been considered generally to result in obligatory destruction of the parasitized RBC (Davis et al. 1990). Anemia is an inevitable consequence of malaria infection (World Health Organization 1990), but we have observed that the haematocrit in some patients with heavy parasitaemias does not decrease as much as would be expected from destruction of the parasitized RBC (unpublished observations, 1992).

This is particularly evident with the rapid parasite clearance that follows treatment with artemisinin or one of its derivatives. To establish whether pitting occurs in human malaria, we have used detection of the Pf 155 or Ring Erythrocyte Surface antigen (RESA), which is expressed immediately following merozoite invasion, as a measure of parasitization and correlated this with the presence of intraerythrocytic parasites.

Materials and Methods

Preparation of cells. *Plasmodium falciparum*-infected RBC were obtained from admission blood samples from patients with uncomplicated (n = 22) and severe (n = 26) falciparum malaria admitted to Mae Sot Hospital, Tak Province, western Thailand or the Hospital for Tropical Diseases. Bangkok. The World Health Organization criteria were used to define severe malaria. Patients requiring hospitalization, but without one of these criteria, were considered to have uncomplicated falciparum malaria. These patients had no previous antimalarial treatment and were enrolled in prospective studies of malaria treatment, which will be described in detail elsewhere. Blood samples were also taken for full blood count, routine biochemistry, glucose and lactate measurements. A laboratory strain of *P. falciparum,* TM267R, cultured in O^+ RBC (RBC) for longer than 3 years was used as a standard comparator (Trager and Jensen 1976). Blood samples containing ring-stage parasites were washed three times in isotonic saline solution, the Buffy coat was removed, and the packed RBCs were resuspended to 50% haematocrit in heat-inactivated fetal calf serum. Thin blood films were made from the RBC suspension, air-dried, and fixed with methanol. In some assays, the thin films were fixed with 1% glutaraldehyde in saline solution, washed, and air-dried. All fixed thin film slides were stored at -20^0C until use. Admission

samples from patients with *P. vivax* malaria infection and also samples from healthy donors were processed and stored as described above and used as negative controls.

In vitro culture. During the continuous *in vitro* culture, the patients' RBCs were replaced progressively with normal erythrocytes from healthy 0^+ donors, and ring-stage parasites in these normal RBCs were maintained *in vitro* using continuous culture conditions (Trager and Jensen 1976a). Ring-infected RBCs from these cultures were then used to prepare samples as described above. The following antibodies were tested: 1) pooled sera from *P. falciparum*- immune Thai donors (kindly provided by S. Thaithong, Chulalongkorn University, Bangkok. Thailand); (2) a human monoclonal antibody (MoAb) 3362 recognizing the *P. falciparum* RESA antigen (RU 1986) (Udomsangpetch et al. 1986); (3) serum from a *P. vivax*-infected Thai patient: and (4) serum from a healthy Thai donor with no previous malaria exposure. All sera were heat inactivated and absorbed twice against group AW RBCs to deplete antiRBC antibodies that might interfere in the assay.

Antibody staining. The antisera were used at 1:50 dilution. Each antibody (5 μL) was placed on a demarcated area on the thin blood films, incubated in a humidified chamber for 30 minutes at room temperature, and washed twice in isotonic saline solution. Rabbit antihuman immunoglobulin - G (5 μl) conjugated with fluorescein isothiocyanate (FITC) was then added to the previously stained area on the thin films and incubated for 30 minutes under the same conditions. After washing, all thin films were mounted with 50% glycerol in saline solution containing 10 μg/ml ethidium bromide to visualize the intraerythrocytic parasite DNA. The stained thin films were then examined with an ultraviolet (UV) light microscope at 1,000x magnification. The FITC-stained ring-infected RBCs and the number of FITC-stained

uninfected RBCs (RESA-RBC) were determined and expressed as numbers/1,000 RBCs.

In the patients whose RBCs were used for cell preparation, serum was also tested at a single dilution (1:50) for the presence of antibodies to RESA using the staining procedures as described above.

Statistical analysis. Data were analyzed using Statview 4.1 (Abacus Concepts Inc. Palo Alto, CA. 1994). Normally distributed data were compared by the Student's t-test and non-normally distributed parameters were compared using Mann-Whitney U test. Correlations were assessed by Spearman's method. The X^2 test with Yates' correction was used for comparison of groups.

Results

Laboratory isolate. Glutaraldehyde-fixed or methanol-fixed thin blood films of ring-infected RBCs from a laboratory parasite strain TN4267R were tested with pooled sera of *P. falciparum* immune donors, and the human MoAb 3362 to RESA. Both antibodies reacted with only the membrane of ring-infected RBCs and gave similar intensity and pattern of staining. This indicated that fixation of blood films with either glutaraldehyde or methanol provided equal antibody access to the RESA and that immune sera could substitute for the MoAb in routine staining.

All sera preabsorbed with AB^+ ± RBCs showed no reactivity with normal RBCs in the indirect immunofluorescence assay. Serum from a *P. vivax*—infected patient and a healthy donor did not stain the ring form-infected RBCs. None of the antibodies stained uninfected RBCs in the TN4267R parasite culture.

Acute malaria. Of the 48 patients studied, 26 had severe falciparum malaria (12 cerebral malaria, 3 acute renal failure, 1 severe anemia, 2 jaundice, 8

hyperparasitaemia) and the other 22 had uncomplicated malaria. Four patients with severe malaria (15%) died. The geometric mean (range) parasite counts were higher in the severe malaria group; 1,174,830/μL (3.010 to 1,210,920) compared with the uncomplicated group; 26,540/μL (2.070 to 221.560); $P < .05$. The mean (standard deviation [SD]) haematocrit was similar in the severe and uncomplicated malaria groups; 32 (9)% and 35 (8)%, respectively. The clinical and laboratory values for the patients with severe malaria are shown in Table 1. Thin blood films from malaria patients on admission that contained bright ethidium bromide-stained parasite nucleic acid were then stained with the pooled immune sera or MoAb 3362 and showed the typical RESA pattern of staining on the infected RBCs. This pattern was also seen on some uninfected RBCs (RESA-RBC) containing no ethidium bromide (Fig 4). The absolute number of RESA-RBC was higher in patients with severe malaria (geometric mean [range] 21,720/μL [5,650 to 166.5501 compared with 7,200/μL [2,390 to 39,190] in patients with uncomplicated malaria. The median (range) ratio of parasitized RBCs to RESA-RBCs was 8.63 (0.133 to 50) for severe patients and 2.917 (0,278 to 20) for uncomplicated patients ($P < .05$). There was a significant positive correlation between absolute parasitaemia and absolute RESA-RBC numbers ($r^2 = .52$. $P = .0004$), *i.e.* RESA-RBC accounted for 50% of the variation of parasitaemia (Fig 5). There were no significant correlations for any other markers of disease severity including spleen size, haematocrit, lactate, or glucose (Krishna et al. 1994b; World Health Organization 1990). There was no significant difference for duration of previous illness before presentation or for outcome.

Continuous in vitro culture. When all of the patients' RBCs had been replaced by RBCs from a healthy donor in continuous culture, the infected RBCs stained as

before with pooled immune sera or human MoAb 3362, but either no RESA-RBC was detected or, in eight patients' cultures, very low numbers were seen (median, 100 cells/mL. range. 100 to 500). There was no significant difference in the numbers of RESA-RBC between mild and severe patients *in vitro*.

Anti-RESA antibodies. Sera from *P. falciparum*-infected patients were tested for antibodies to RESA by indirect immunofluorescence assay. Thirteen sera showed reactivity with RESA at 1 in 25 dilution. Ten patients had uncomplicated disease and three had severe disease ($P = .011$) There was no significant difference in the number of RESA-RBC or measures of diseases severity between the antibody positive and antibody negative patients, although there was a significantly lower parasitaemia in the antibody positive patients ($P = .009$).

Discussion

The objective of these studies was to seek evidence of previous parasitization in unparasitized RBCs in acute falciparum malaria. The parasite-derived antigen Pf 155 or RESA (Perlmann et al. 1984) was chosen because it is associated with dense granules in the apical part of the merozoite and is deposited in the erythrocyte membrane during invasion (Brown et al. 1985) (Aikawa et al. 1991a). RESA is, therefore, present in the RBC membrane from a very early stage of infection and acts as a "footprint" of RBC parasitization by a *P. falciparum* parasite. RESA is a well-characterized polypeptide antigen that is highly conserved and is immunogenic in man (Cowman 1984). Immune sera and MoAb 3362 gave a distinctive pattern of rim fluorescence over the glutaraldehyde-fixed erythrocyte membrane, but not on unfixed cells (Udomsangpetch et al. 1986). RESA is first synthesized within mature trophozoites and accumulates within merozoites. When RBCs from acute malaria

patients were stained with pooled immune serum or MoAb3362, the pattern of antibody binding on some of the unparasitized RBCs was typical of RESA staining. Pooled immune serum is likely to have recognized a number of different parasite antigens, but the relative immunodominance of RESA was such that the two patterns of staining appeared identical.

There are three possible explanations for these observations. First, that in some cases, the merozoites invade the RBCs, then die spontaneously and are degraded rapidly within the cytoplasm. Second, that the young parasites are extruded actively by the RBC (either alive or dead), and third, that the parasites are somehow extracted from the RBC leaving it intact. If the parasites had simply died, then some DNA degradation products would still be expected to have stained by the ethidium bromide. This was not observed. The discrepancy between the numbers of RESA positive, parasite negative cells seen *in vivo* and *in vitro* suggests that active extrusion of parasites by the RBCs is unlikely to be quantitatively important. The most likely explanation is that host defense responses (probably the spleen) are involved in removal of intraerythrocytic parasites *in vivo* (Ferrante 1990; Nnalue and Freidman 1988; Abdalla and Weatherall 1982; Phillips et al 1986). The spleen normally removes residual host nuclear material from erythrocytes, but how it recognizes damaged intraerythrocytic parasites is not known. The possibility that immature or killed intraerythrocytic parasites could he removed from within RBCs either by phagocytic cells or by active extrusion without their destruction, was first raised by observations in experimental simian malaria by Conrad and Dennis (Conrad and Dennis 1968). This was further supported by ultrastuctural studies of the spleen in Rhesus monkeys infected with *P. knowlesi* (Conrad and Dennis 1968) (Schnitzer et al.

1971) (Schnitzer et al. 1972) (Schnitzer et al. 1973). Human neutrophils *in vitro* have also been shown to be capable of extracting *P. falciparum* parasites from RBCs, leaving the RBCs intact (Kumaratilake et al. 1994) and parasitized RBCs treated with antimalarial drugs have been recorded extruding dead trophozoites (Gu and lnselhurg 1989). This evidence in experimental systems supports our findings that removal of intraerythrocytic parasites occurs naturally *in vivo* when the host immune system can act, but not under *in vitro* parasite culture conditions in the absence of leukocytes and the spleen. It is likely that the parasites removed were in the first 24 hours of their 48-hour asexual life cycle because the morphology of the RESA positive cells was normal. In the second half of the asexual life cycle, cytoadherence takes place. and these adherent RBCs would have remained sequestered in the microcirculation whether or not they were still parasitized (Molyneux et al. 1989). As most of the intraerythrocytic hemoglobin is consumed by more mature parasites, the hemoglobin concentration of the once parasitized cells was probably not reduced markedly. Thus, in the short-term, these once parasitized and antigenically marked RBCs. which occur in numbers approximately similar to the parasitized RBCs, will contribute to oxygen carriage and delivery, but their ultimate fate is not known. RESA is not expressed on the cell surface (Aikawa et al. 1991b), so increased antibody binding and thus immune recognition would not be expected, but it is associated with the RBC cytoskeleton (Ruangjirachuporn et al. 1991) (through binding with spectrin) and could theoretically alter RBC deformability leading to splenic removal. Further study of the survival and the effects of different antimalarial drugs on the numbers of these cells in the circulation will be required.

Table 1.

Summary of Clinical and Laboratory Variables in 26 Patients With Severe Falciparum Malaria (median, range unless indicated)	
Temperature (°C)	38.8 (36.5 – 40)
Pulse (beats/min)	111 (88 – 140)
Systolic blood pressure (mm Hg)	100 (80 – 140)
Respiratory rate (breaths/min)	35 (20 – 48)
Glasgow Coma Score	15 (3 – 15)
Parasite count/mL geometric mean (range)	174,830 (3,010 – 1,210,910)
RESA-RBC count/μL geometric mean (range)	21,720 (5,650 – 166,550)
Ratio parasite count to RESA-RBC median (range)	8.63 (0.2 – 75)
Lactate (mmol/L) Mean, (SD)	6.8 (5.3)
Blood urea nitrogen (mg/dl)	27.5 (12 – 108)
Serum creatinine	1.15

(mg/dL)	(0.6 – 7.6)
Total bilirubin (mg/dL)	2.85 (0.6 – 23.3)
SGOT (U/L)	49 (18 – 335)
SGPT (U/L)	20 (5 – 91)
White cell count (cells x 10^9/L)	7.6 (4.7 – 8.8)
Hemoglobin (g/dL)	9.9 (5.5 – 15.2)
Platelet count (cells x 10^9/L)	93 (20 – 191)

Figure 4. Immunofluorescence photomicrograph (original magnification x 1.000) stained with ethidium bromide and FITC-conjugated antiRESA demonstrating a parasitized RBC on the left and a RESA positive, parasite negative RBC on the right.

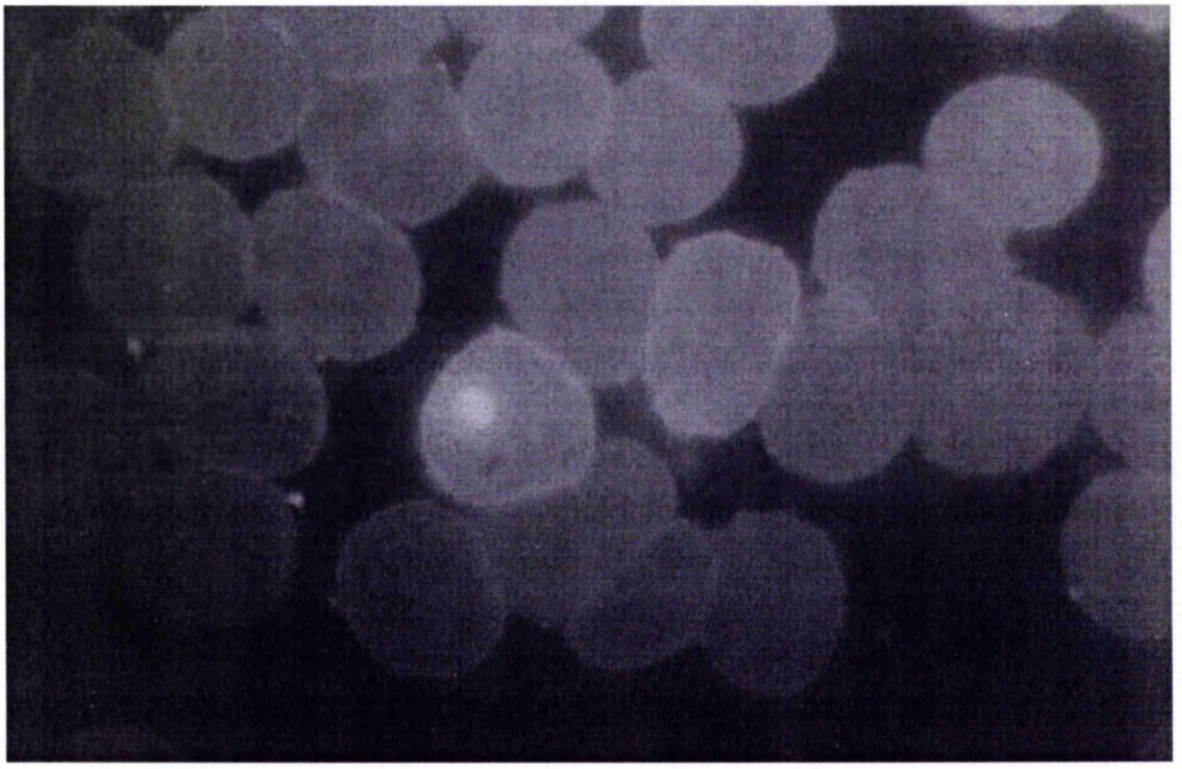
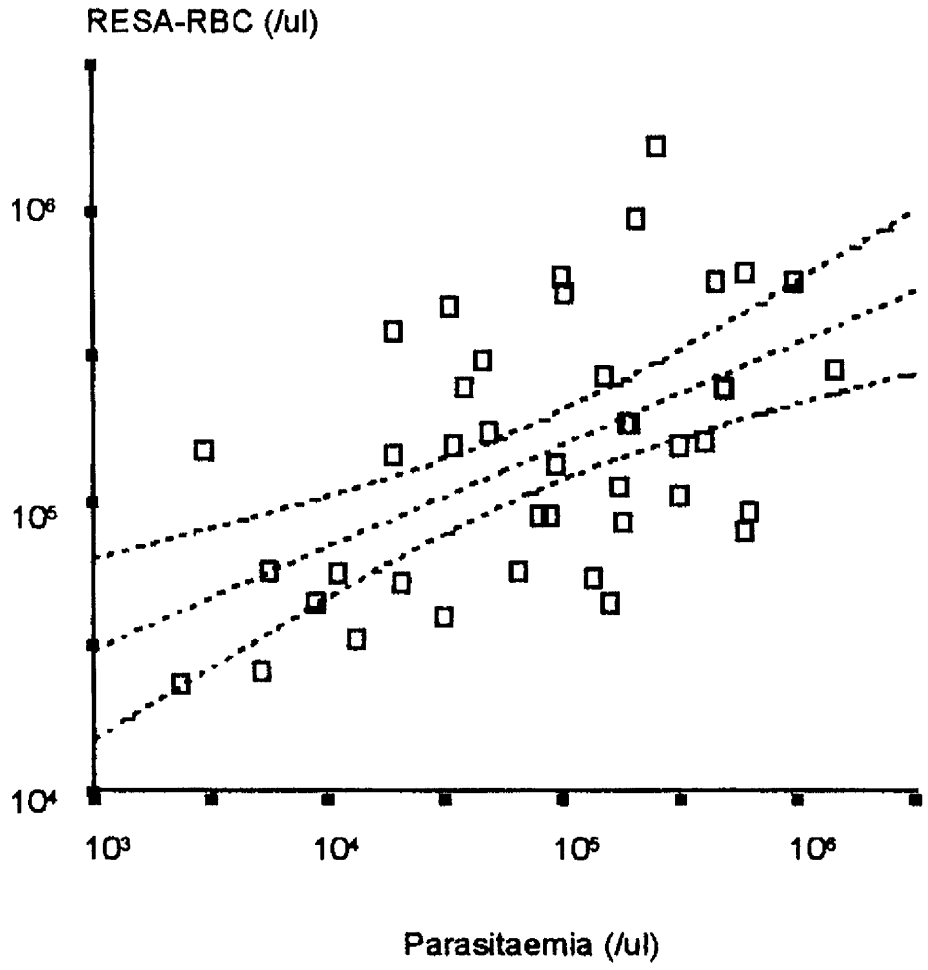


Figure 5. Linear regression analysis of parasitaemia with the number of RESA positive, parasite negative RBCs (RESA-RBC) (mean. 95% confidence interval for the slope).



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Short Report. Rosette formation in Plasmodium ovale infection

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Abstract.

Red blood cells infected by mature stages of *Plasmodium ovale* obtained from a 56-year-old Thai patient formed rosettes readily with uninfected erythrocytes. *Ex vivo* the ring stage-infected erythrocytes matured well under the *in vitro* conditions used for *P. falciparum* culture, and the infected erythrocytes formed rosettes when the parasites became mature trophozoites. These rosettes were stable and remained intact until completion of schizogony. *Plasmodium ovale* rosettes were similar to those formed by *P. falciparum* and *P. vivax* - infected erythrocytes. Rosette formation appears to be a common property of three species of human plasmodia.

Introduction

Four species of plasmodia cause malaria in humans. Of these, only *Plasmodium falciparum* infections are potentially lethal (World Health Organization 1990). The pathology of severe falciparum malaria is related to sequestration in which infected erythrocytes stick in the deep vasculature and the resultant slowing of blood flow and focusing of toxic damage causes vital organ dysfunction (White and Ho 1992). Two *in vitro* phenomena observed are thought to be related to this mechanism: cytoadherence or the adhesion of infected erythrocytes to vascular endothelial cells (Udienya et al. 1981) and rosette formation in which two or more uninfected erythrocytes adhere to an infected erythrocyte (Udomsangpetch et al. 1989). The ability of parasites to form rosettes has been considered a marker of pathogenic potential and those parasites with a greater potential for this phenomenon are considered inherently more pathogenic (Rowe et al. 1995). Rosette formation has been correlated with cerebral malaria and with other manifestations of severe falciparum malaria in

studies in Africa (Carlson et al. 1990b; Hassler et al. 1990; Ringwald et al. 1993), but not in Southeast Asia or Oceania (Al-Yaman et al. 1995; Ho et al. 1991). The human malaria parasites causing benign malaria have been considered incapable of cytoadherence or rosette formation (Handunnetti et al. 1989), although we have recently reported that *P. vivax*-infected erythrocytes readily form rosettes (Udomsangpetch et al. 1995), and now report here rosette formation by *P. ovale*-infected erythrocytes.

A 56-year-old woman from the northwestern border of Thailand originally diagnosed as having *P. vivax* infection was admitted to Sangklaburi Hospital in Kanchanaburi. The patient was febrile (temperature = 38^0C) and mildly anemic (haematocrit = 28%) but was able to walk. A thin blood film made on admission showed a *P. ovale* infection. The parasitaemia was 1.4% with a predominance of ring forms (Figure 6A). The patient was blood group B. Rosette formation was then assessed by mounting one drop of a fresh EDTA-blood specimen diluted 1:10 with normal saline on a microscope slide and examined using a light microscope at 1,000X magnification. This showed no rosette formation nor aggregation of the erythrocytes. She was given a single dose of mefloquine (15 mg of base/kg) and a 14 day course of primaquine (0.15 mg of base/kg). A subsequent EDTA-blood specimen taken 15 hr later showed some pigment-containing trophozoites and schizonts on the thin blood film (Figure 6B and C) that formed rosettes readily (Figure 6D).

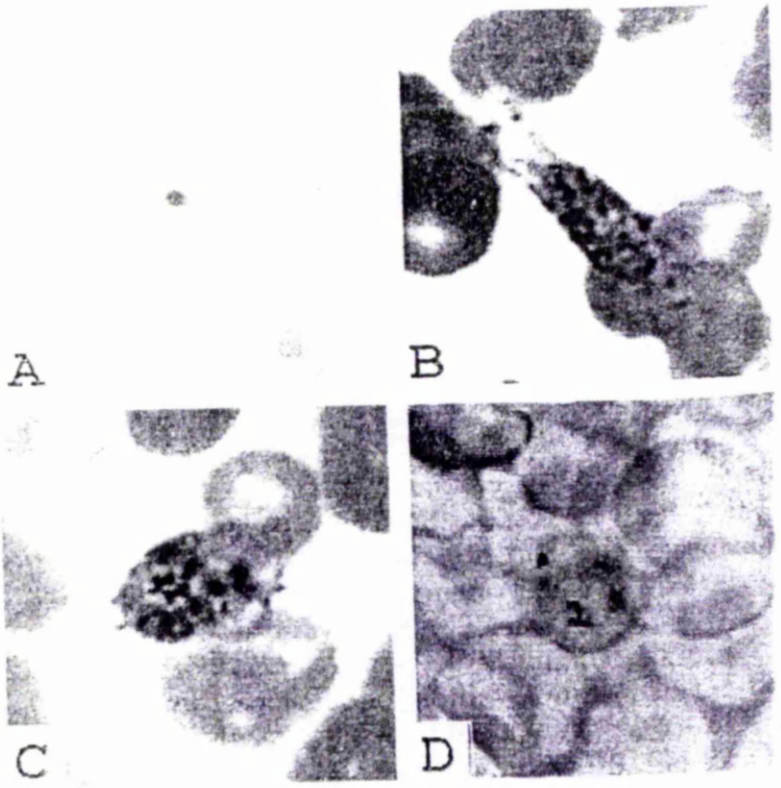
To document the development of rosette formation *in vitro*, a culture of the pretreatment parasites was prepared as described previously for *P. falciparum in vitro* (Trager and Jensen 1976b). An admission EDTA-blood specimen was washed three times with RPMI 1640 medium, the leukocytes in the Buffy layer removed, and the

erythrocytes were then diluted to haematocrit in malaria culture medium containing RPMI 1640, pH 7.4, 10% heat-inactivated AB serum, 25 mM PES, and 20 μg/ml of gentamicin. This was then cultured at 37^0C in air plus 5% CO_2 for 45 hr. A thin blood film made after *in vitro* culture for 24 hr showed that the parasites had matured to the pigment-containing trophozoite stage. Infected erythrocytes with pigment-containing parasites formed rosettes with uninfected erythrocytes. No uninfected erythrocyte aggregations were seen. By 36 hr of culture parasites had matured to the schizont stages, and again the schizont-infected erythrocytes formed rosettes. Examination of the thin blood film after 45 hr of culture showed a few ring-infected erythrocytes while most of the schizonts had become pyknotic. The remaining schizont-infected erythrocytes all still formed rosettes.

The observations that two species of plasmodia infecting humans, *P. ovale* and *P. vivax,* which do not sequester and do not cause severe clinical disease, readily form rosettes (Udomsangpetch et al. 1995) challenges the hypothesis that rosette formation alone is a marker of parasite pathogenicity. This further emphasizes the differences between the properties of rosette formation and cytoadherence. It does not exclude the possibility that rosette formation contributes significantly to microvascular obstruction and thus pathogenicity in *P. falciparum in vivo*, but suggests that it would do so only in conjunction with cytoadherence leading to sequestration, and/or high parasite burdens. Rosette formation appears to be a common property of these three species of *Plasmodium* under *in vitro* conditions.

Figure 6.

Thin blood films of the patient showing A. a ring form of *Plasmodium ovale* (on admission), B, a pigmented trophozoite, C, a schizont (15-hr after admission), and D, a red blood suspension with rosette formation by a *P. ovale* infected erythrocyte (reversed Field's staining, magnification X 1.000).



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Prognostic significance of reduced red blood cell deformability in severe falciparum malaria

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Abstract.

Severe falciparum malaria is associated with microvascular obstruction resulting from sequestration of erythrocytes containing mature stages of the parasite. Since reduced red blood cell deformability (RBC-D) can contribute to impaired microcirculatory flow, RBC-D was measured in 23 patients with severe falciparum malaria (seven of whom subsequently died), 30 patients with uncomplicated malaria, and 17 healthy controls. The RBC-D, measured by ektacytometry, was significantly reduced in severe malaria and was particularly low in all fatal cases. At a low shear stress of 1.7 Pascal (Pa), a red blood cell elongation index less than 0.21 on admission to the hospital predicted fatal outcome with a sensitivity of 100% (confidence interval [Cl] 59—100%) and a specificity of 88% (CI = 61 - 98%). The reduction in the RBC-D appeared to result mainly from changes in unparasitized erythrocytes. Reduced deformability of unparasitized red blood cells in severe malaria may contribute to impaired microcirculatory flow and a fatal outcome in severe falciparum malaria.

Introduction

Infection with *Plasmodium falciparum* remains a major cause of death in the tropics, with an annual global mortality of 1—2 million people and a mortality rate in severe malaria of 15—30% (World Health Organization 1990; World Health Organization 1993). The sequestration of red blood cells containing the mature forms of the parasite in the microcirculation of the vital organs is considered to be the essential pathologic feature of the infection, but precisely how this causes death is not known. Sequestration interferes with microcirculatory flow and tissue metabolism and may focus the release of host- or parasite-derived toxins to the vital organs (White

and Ho 1992). The cytoadherent, parasitized red blood cells impede the passage of the uninfected red blood cells, which are forced to deform more than usual in their transit through the microcirculation. Since reduced red blood cell deformability (RBC-D) has been associated with impaired tissue perfusion in other conditions (Mokken et al. 1992) we investigated the relationship between RBC-D and disease severity in falciparum malaria.

Patients and Methods

The study was carried out in May and June 1995 in the provincial hospital of Mae Sot, Tak Province, Thailand. Malaria transmission is low in this area with a seasonal peak during the rainy season that starts in late spring (Luxemburger et al. 1996). Severe disease occurs at all ages. Multiple drug resistance is an increasing problem in this area.

Patients and clinical procedures.

Consecutive adult patients, admitted to Mae Sot Hospital with acute falciparum malaria were included provided that written informed consent was obtained from the patients or their attendant relatives. Disease severity was classified according to standard criteria (World Health Organization 1990). Exclusion criteria were an age less than 14 years, pregnancy, and a history of previous antimalarial drug treatment - within 24 hr of admission. Previous quinine treatment was checked in a baseline blood sample by a rapid quinine dipstick method in all patients (Silamut et al. 1995). A full clinical examination was performed on admission and all details were recorded in a standard form. In cases of cerebral malaria a lumbar puncture was performed to exclude other causes of altered consciousness. Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride (20 mg of salt/kg infused

over a 4-hr period followed by 10 mg/kg every 8 hr) followed by oral tetracycline or intravenous artesunate (2.4 mg/kg immediately, then 1.2 mg/kg at 12 and 24 hr and then daily) followed by mefloquine in a comparative study that will be published elsewhere. Full supportive care was given as described previously (World Health Organization 1990). If necessary, patients were transferred to an intensive care unit for mechanical ventilation, peritoneal dialysis, or haemodynamic support and monitoring. This investigation was part of studies approved by the Ethical and Scientific Review Subcommittee of the Ministry of Public Health, Thailand.

Laboratory methods.

Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting (White and Silamut 1989). Baseline blood samples were taken for full blood count, glucose, lactate, and routine biochemistry. Hemoglobin (Hb) electrophoresis was assessed from stored frozen samples (—20^0C). Mean red blood cell diameter (MCD) and red blood cell morphology were evaluated in a thin smear using a calibrated light microscope. The RBC-D was measured immediately by ektacytometry using a Laser-assisted Optical Rotational Cell Analyzer (LORCA;® Mechatronics, Hoorn, The Netherlands) (Hardeman et al. 1994). Blood samples from healthy Thai adults of the same age range were used as controls. With this method a defined shear stress is applied to a red blood cell suspension in a high viscous medium (5% polyvinylpyrrolidone in phosphate-buffered saline) at a constant temperature of 37^0C, in a small gap between two concentric rotating cylinders. Because of the applied shear stress the cells elongate and align themselves in the fluid layer, thus forming a grid. A laser beam is directed through the fluid layer and forms a diffraction pattern behind it. The ellipticity of this diffraction pattern is

directly proportional to the mean ellipticity of the red blood cells (i.e., the amount that the normally discoid erythrocyte is deformed). The unit of deformability is the elongation index (El), defined by the length of the long axis minus the short axis divided by the length of the long axis plus the short axis of the deformability pattern. This is determined by computer analysis of the diffraction pattern, using iso-intensity lines for curve fitting. Red blood cell deformability was assessed at three shear stresses (1.7, 9.5, and 30 Pascal [Pa]) corresponding approximately to shear stresses encountered *in vivo* in the venules, the arterioles and capillaries, and in arteries with significant stenosis, respectively (Chien 1987). Reproducibility was a major drawback in former filtration methods for measuring RBC-D, but it is very good with ekta-cytometry (Bessis, Mohandas, and Feo 1980; Hardeman et al. 1994; Mokken et al. 1992).

Table 2.

Admission clinical and laboratory variables in 23 patients with severe malaria in Mae Sot Hospital, Tak Province, Thailand

Variable*	Survivors (n = 16)	Fatal cases (n = 7)	p
Age (years)	28 ± 8	28 ± 14	NS
Days with fever prior to admission	3.5 ± 1.8	4.3 ± 1.8	NS
Pulse rate (per mm)	114 ± 11	109 ± 15	NS
Blood pressure(mm of Hg)			
Systolic	108 ± 12	104 ± 14	NS
Diastolic	61 ± 13	63 ± 20	NS
Temperature (C)	38.9 ± 9	38.2 ± 1.4	NS
Median coma score	15	8	0.004
Packed cell volume (%)	31 ± 12	31 ± 9	NS
Parasitaemia (%)	6 ± 6	10 ± 11	NS
Creatinine (mg/dl)	1.15 ± 0.22	2.35 ± 2.36	0.029
Lactate (mmol/liter)	4.4 ± 2.4	13.1 ± 9.2	0.019
Glucose (mmol/liter)	9.5 ± 6.3	8.9 ± 3.5	NS
MCD (μm)	6.2 ± 0.5	6.2 ± 0.2	NS
RBC-D at SS = 1.7 Pa	0.252 ± 0.033	0.189 ± 0.014	0.0001

(EI)			
RBC-D at SS = *9.5* Pa (El)	0.507 ± 0.035	0.450 ± 0.034	0.0014
RBC-D at SS = 30 Pa (El)	0.588 ± 0.029	0.547 ± 0.033	0.0077

MCD mean cell diameter of red blood cells; RBC-D = red blood cell deformability; SS = shear stress; El = elongation index; Pa = Pascal.

Except for median coma score, values are the mean ± SD.

Comparisons by Mann-Whitney U test; all other comparisons by Student's t-test.

Statistical methods.

Statistical analyses were carried out using SPSS 6.1 statistical programs (SPSS Corporation, Chicago, IL). Normally distributed data were analyzed using Student's t-test and analysis of variance, with application of the least significant difference method for multiple comparisons. The Mann-Whitney test was used to compare non-normally distributed variables. Correlations were assessed by the method of Pearson for normally distributed variables, and the method of Spearman for the remainder. A multiple logistic regression model was used (Forward Logistic Regression; SPSS 6.1 statistical programs) to determine the most discriminating prognostic indicators and their relative contributions in predicting outcome (death or survival).

Results

Clinical details.

A total of 23 patients with severe malaria were included in the study. The

comparison groups comprised 30 adult patients with uncomplicated falciparum malaria and 17 healthy volunteers. In the severe malaria group 12 had cerebral malaria, four developed pulmonary edema requiring assisted ventilation, one patient became anuric and was dialyzed, and one patient developed nosocomial pneumonia. Seven patients (30%) subsequently died (of whom four received quinine and three artesunate). There were no deaths in the group with uncomplicated malaria. Clinical and laboratory details are shown in Table 3. The mean (SD) time to fever clearance in severe malaria was 68.5 (54.8) hr and the corresponding time to parasite clearance was 63.4 (24.2) hr. Within the group of patients with severe malaria MCD of the red blood cells did not differ significantly between survivors and fatal cases. There were five patients with severe malaria and severe microcytosis (MCD < 6.0) all of whom survived. Of these five patients four likely to suffer from thalassemia because of high HbA2 levels and target cells in the thin smear. The RBC-D at 1.7 Pa varied between 0.24 and 0.28 in this group. One patient who died was later shown to have had an HbE haemoglobinopathy. The MCD of the red blood cells was 6.0 μm and RBC 1.7 at Pa was 0.20 in this patient. Six patients with intracellular haemolysis were found to be negative for glucose-6- phosphate dehydrogenase deficiency.

Red blood cell deformability.

Red blood cell deformability was reduced in proportion to disease severity (Table 3). The most striking difference was between fatal cases with severe malaria and survivors (Figure 7). Severely reduced RBC-D (El <0.21) at a shear stress of 1.7 Pa predicted fatal outcome with a sensitivity of 100% (confidence interval [CI] = 59—100%) and a specificity of 88% (CI = 61—98%). Parasitaemia on admission did not correlate either with the RBC-D (correlation coefficient = —0.08, $P = 0.6$) or with

survival. In a multiple logistic regression analysis, with the parameters listed in Table 2 as variables, RBC-D at a shear stress of 1.7 Pa was the strongest predictor of mortality (Wald statistic = 4.5). The only other variable that contributed significantly to the model was the Glasgow Coma Score on admission (Wald statistic = 2.1). There was a significant correlation between admission values for plasma lactate levels and RBC-D (correlation coefficient = —0.44, $P = 0.04$). There was no relation between the MCD of the red blood cells and RBC-D at 1.7 Pa. In fatal cases, the RBC-D on admission was not significantly different from the RBC-D 2-12 hours before death (mean ± SD RBC-D at 1.7 Pa = 0.186 0.018 and 0.197 ± 0.039, respectively). There was no significant change in RBC-D during the time of admission (up to 168 hr).

Table 3.

Mean ± SD red blood cell deformability (RBC-D) on admission in 30 patients with uncomplicated falciparum malaria, 23 patients with severe falciparum malaria, and 17 healthy controls in Mae Sot Hospital, Tak Province, Thailand*

Study group			
Variable	Uncomplicated malaria	Severe malaria	Healthy controls
n	30	23	17
Age, years (mean ± SD)	27 ± 10	28 ± 10	32 ± 7
RBC-D at SS = 1.7 Pa (EI)	0.270 ± 0.027	0.232 ± 0.041	0.284 ± 0.017
RBC-D at SS = 9.5 Pa (EI)	0.520 ± 0.020	0.489 ± 0.043	0.544 ± 0.011
RBC-D at SS = 30 Pa (EI)	0.602 ± 0.016	0.576 ± 0.035	0.617 ± 0.010

Differences in RBC-D at all levels of shear are significant ($P < 0.05$) between all study groups (analysis of variance), except for RBC-D at SS = 1.7 Pa in uncomplicated malaria patients compared with healthy controls.

•SS = shear stress; El = elongation index.

Discussion

During their passage through the microcirculation, red blood cells must undergo

considerable deformation because their diameter (7.5 μm) exceeds the average midpoint diameter of the capillaries (3—7 μm). Red blood cell deformability is therefore an important determinant of microvascular blood flow (Nash 1991). Red blood cells infected with *P. falciparum* parasites become progressively less deformable as the intra-erythrocytic parasites mature (Cranston et al. 1983a; Nash et al. 1989). Early studies showed that the filterability of red blood cells in uncomplicated malaria was reduced, suggesting that uninfected red blood cells might also be less deformable, although the relationship of red blood cells filterability to the rheologic conditions encountered *in vivo* is uncertain (Areekul and Yamarat 1988; Lee et al. 1992). The present study shows that red blood cells in patients with acute falciparum malaria are less deformable than in healthy subjects and that this rigidity increases with increasing severity of the infection.

The red blood cells deformability estimate obtained by the LORCA is a summation of the RBC-D of all the red blood fractions, with contributions to the overall value that are proportional to their size (Streekstra GJ, *A Biplane Rheoscope for the Measurement of Red Cell Deformation and Orientation in a Couette Flow.* Thesis, University of Utrecht, Utrecht, The Netherlands, August 1994). Since the majority of red blood cells even in severe malaria is uninfected, this reduction in RBC-D results mainly from changes in the unparasitized erythrocytes. The relative unimportance of the parasitized red blood cells to this measurement is supported by the lack of correlation between parasitaemia and RBC-D in this study. Moreover, the most rigid cells containing the mature parasites are usually sequestered in the microcirculation and are not present in the peripheral blood samples used.

Several prognostic factors have been identified in severe malaria including

depth of coma, hyperparasitaemia, the predominance of late stages of parasite development or a high proportion of neutrophils containing malaria pigment, hypoglycemia, elevated plasma levels of tumor necrosis factor, elevated lactate levels in the blood and cerebrospinal fluid, and the severity of acidosis (Field and Niven 1937; Molyneux et al. 1989; Silamut and White 1993; White and Ho 1992; World Health Organization 1990; Phu et al, 1998; Grau et al. 1989; Krishna et al. 1994; Kwiatowski et al. 1990; Taylor, Borgstein, and Molyneux 1993; Taylor et al. 1988; White et al. 1985). Multiple logistic regression analysis showed that a markedly reduced mean RBC-D was the strongest predictor of mortality in this small series. Severely reduced RBC-D lowers microcirculatory blood flow since red blood cells have to deform in order to pass through the smaller capillaries. In blood vessels lined by cytoadherent rigid parasitized erythrocytes, there must be considerable luminal obstruction. However, some flow is often maintained, presumably by even more than usual red blood cell deformation (White and Ho 1992). Any reduction in the deformability of uninfected erythrocytes would be expected to further compound the microvascular obstruction caused by cytoadherent erythrocytes and inter-erythrocytic adhesion (rosette formation). This effect would be greatest in the tissues of vital organs such as the brain where sequestration is greatest. This mechanism could also contribute to the lactic acidosis by inducing anaerobic glycolysis. Host tissues are quantitatively the most important source of lactate in severe malaria (Krishna et al. 1994b; White and Ho 1992). The predictive value of lactic acidosis for mortality in malaria and the correlation between venous lactate concentrations and reduced mean RBC-D could be explained by this causal relationship. Reduced RBC-D is not an epiphenomenon related to direct effects of the lactate ion or acidaemia because acidification of the

suspension medium (to pH 6.9) with lactate does not reduce RBC-D significantly at any shear stress as measured with the LORCA (Hardeman MR, unpublished data).

The mechanisms underlying the reduction in RBC-D of uninfected cells in severe malaria are not known. There was no significant increase in RBC-D during the time of admission, or shortly after recovery, suggesting irreversible damage to the uninfected red blood cells. In this study, we could not follow the patients after recovery. We have observed that normalization of the mean RBC-D in nonimmune Dutch travelers with falciparum malaria took 2—4 weeks (Dondorp AM, unpublished data). Reduction in RBC-D was most significant at the lower shear stresses. Changes in the flexibility of the red blood cell membrane are likely to be an important factor, since RBC-D at low shear stresses is very susceptible to membrane changes (Mohandas et al. 1980). Nauman and others have identified a heat-labile exoantigen produced by *in vitro* cultures of *P. falciparum* that binds reversibly to normal red blood cells and reduces their deformability (Nauman et al. 1991). We have also found that soluble products of *P. falciparum* in culture reduce the RBC-D of normal erythrocytes (Unpublished data). Red blood cell morphology and mean red blood cell diameter were not important parameters of RBC-D in this study. Although RBC-D is diminished in thalassemia (Vasselon et al. 1981), in the present study the level of disturbance is smaller than found in the fatal cases. An increase in temperature up to 41^0C did not reduce RBC-D of normal erythrocytes *in vitro* as measured by LORCA, suggesting that fever was not a major contributor to this effect. The role of systemic host factors or endothelial cell malfunction in reducing RBC-D is not known.

If reduced RBC-D is a cause rather than an effect of potentially lethal organ dysfunction in severe malaria, then measures to correct this abnormality may save

lives. Exchange transfusion is widely used in the management of severe malaria, although it has never been clear how it might be of benefit (Editorial 1990). The parasitized red blood cells causing pathology are sequestered and not available for exchange. However, removal of rigid unparasitized cells and their replacement by more deformable new erythrocytes would provide a plausible explanation for the apparent benefit from this treatment. Patients with severely reduced RBC-D form a subgroup that might benefit from exchange transfusion. In conclusion, this study shows that the mean RBC-D is an important predictor of and may be a contributor to mortality in severe falciparum malaria. This reduction in mean RBC-D is mainly due to a reduction in the RBC-D of unparasitized erythrocytes.

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Red blood cell deformability as a predictor of anemia in severe falciparum malaria

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Abstract.

Decreased erythropoiesis and increased clearance of both parasitized and noninfected erythrocytes both contribute to the pathogenesis of anemia in falciparum malaria. Erythrocytes with reduced deformability are more likely to be cleared from the circulation by the spleen, a process that is augmented in acute malaria. Using a laser diffraction technique, we measured red blood cell (RBC) deformability over a range of shear stresses and related this to the severity of anemia in 36 adults with severe falciparum malaria. The RBC deformability at a high shear stress of 30 Pa, similar to that encountered in the splenic sinusoids, showed a significant positive correlation with the nadir hemoglobin concentration during hospitalization ($r = 0.49$, $P < 0.002$). Exclusion of five patients with microcytic anemia strengthened this relationship ($r = 0.64$, $P < 0.001$). Reduction in RBC deformability resulted mainly from changes in unparasitized erythrocytes. Reduced deformability of uninfected erythrocytes at high shear stresses and subsequent splenic removal of these cells may be an important contributor to the anemia of severe malaria.

Introduction

Anemia is an important cause of morbidity and mortality in falciparum malaria (Phillips et al. 1986; World Health Organization 1990). The pathogenesis of anemia in malaria is multifactorial and incompletely understood. It is thought to result from a combination of parasitized erythrocyte destruction at schizont rupture, accelerated removal of parasitized and unparasitized red blood cells, and defective erythropoiesis (Looareesuwan et al. 1987b; White and Ho 1992). Of these factors, removal of un-parasitized red blood cells is the most important, accounting for approximately 90%

of the reduction in the haematocrit in malaria (Price RN et al, Am. J. Trop. Med. in press). We have shown previously that the threshold for splenic removal of complement-damaged or antibody-coated erythrocytes in acute malaria is lowered, suggesting enhancement of both mechanical filtrative function and Fc receptor-mediated clearance (Looareesuwan, 1987b; Nash et al. 1988). Reduced red blood cell (RBC) deformability is thought to play an important role in the removal of senescent blood cells from the circulation by the spleen (Nash et al. 1988). Since reduced RBC deformability might also play a role in the clearance of both parasitized and unparasitized red blood cells in malaria, we have measured RBC deformability in relation to the development of anemia in severe falciparum.

Patients and Methods

Study site.

The study was carried out during the rainy season months from May until July in both 1995 and 1996, in the provincial hospital of Mae Sot, Tak province, in western Thailand. Malaria transmission is low in this area with a seasonal peak during the rainy season that starts in late spring (Luxemburger et al. 1996). Severe disease occurs at all ages. Multiple drug resistance is an increasing problem in this area.

Patients and clinical procedures.

Consecutive adult patients admitted to Mae Sot Hospital with severe falciparum malaria were included, providing that written informed consent for blood sampling was obtained from the patients or attendant relatives. Disease severity was classified according to standard criteria (World Health Organization 1990). Exclusion criteria were an age less than 14 years, pregnancy, and previous antimalarial drug treatment

within 24 hr of admission. Previous quinine treatment was checked in a baseline blood sample by the rapid dipstick method in all patients (Silamut et al. 1995). Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride (20 mg salt/kg infused over a 4-hr period followed by 10 mg/kg every 8 hr) followed by oral tetracycline, or intravenous artesunate (2.4 mg/kg initially, then 1.2 mg/kg at 12 and 24 hr and then daily), followed by mefloquine in a comparative study, the results of which will be published elsewhere. Full supportive care was given as described previously (World Health Organization 1990). A control group of 22 healthy age- and sex-matched Thai volunteers provided a blood sample for measurement of RBC deformability.

A second control group comprised 12 adult Dutch travellers who presented with uncomplicated falciparum malaria at the Academic Medical Centre in Amsterdam. This group was treated with either oral sulfadoxine/pyrimethamine or halofantrine. A blood sample was taken on admission and at days 3, 7, 14, 21, and 28 after start of the treatment for assessment of parasitaemia, haemoglobin level, and RBC deformability.

This investigation was part of studies approved by the Ethical and Scientific Review Sub-committee of the Ministry of Public Health, Thailand. The study in Dutch travellers was approved by the Medical Ethical Committee of the Academic Medical Centre in Amsterdam.

Laboratory methods.

Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting (White and Silamut 1989). Blood samples were taken every 12 hr for a full blood count, routine biochemistry, lactate, glucose (assessed daily), and assessment of RBC deformability. Immediately after

venepuncture, RBC deformability was measured with a laser-assisted optical rotational cell analyzer (LORCA®; Mechatronics, Hoorn, The Netherlands) (Hardeman et al. 1994). With this method a defined shear stress is applied to an RBC suspension in a high viscous medium (5% polyvinylpyrrolidone in phosphate-buffered saline buffer, viscosity = 30 mPa.sec at 37nC) at a constant temperature of 37^2C in a small gap between two concentric cylinders. Because of the applied shear stress caused by rotation of the outer cylinder, the cells elongate and align themselves in the fluid layer.

A laser beam is directed through the fluid layer and forms a diffraction pattern on a screen behind it. The ellipticity of this diffraction pattern is directly proportional to the mean ellipticity of the red blood cells. This ellipticity is described by the elongation index (El) defined by the formula El = (length of the long axis — length of the short axis)/(length of the long axis + length of the short axis). This is determined by computer analysis of the diffraction pattern. Red blood cell deformability was assessed at a range of shear stresses from 1.7 Pa to 30 Pa. Shear stresses of 1.7 Pa and above are encountered in the capillaries (Chien 1987). Higher shear stresses are also likely to occur in the sinusoids of the spleen where red blood cells have to squeeze through the small intercellular gaps. Poor reproducibility has been a major drawback in earlier methods of measuring RBC deformability by filtration, but it appears to be very good with ektacytometry (Bessis, Mohandas, and Feo 1980; Mokken et al. 1992).

In the patients that were included in 1996, the mean cell volume (MCV) was measured with a Coulter Counter (Coulter, Inc., Hialeah, FL). Because of the lack of availability of the Coulter machine in 1995, the MCV were calculated from the mean

cell diameter (MCD) during this year. The MCD was assessed by light microscopy by averaging 200 red blood cells in a thin smear. The MCV was calculated from these values by using a nomogram (England 1982). The thin smear was also checked for the appearance of target cells and other RBC abnormalities. Hemoglobin electrophoresis was performed on the admission blood samples. Admission plasma samples were also stored at —20^{0}C for subsequent measurement of bilirubin, transaminases, lactate dehydrogenase, and serum iron.

Statistical methods.

Statistical analyses were carried out using SPSS 6.1 statistical programs (SPSS Corporation, Chicago, IL). Comparisons of means were made by Student's test, with a Bonferroni correction for multiple comparisons if applicable. Non-normally distributed parameters were compared by the Mann-Whitney U test. Correlations were assessed by the method of Pearson for normally distributed variables, and the method of Spearman for the remainder. A multiple regression analysis was performed to determine the most discriminating prognostic parameters in predicting the severity of the anemia, defined as the lowest hemoglobin level that was reached during admission.

Results

A total of 42 patients with severe malaria were included in the study. Six patients were excluded from further analysis because they were given blood transfusions shortly after admission: one was thalassaemic with a severe microcytic anaemia (haemoglobin level = 3.3 g/dl), another (haemoglobin level = 7.8 g/dl) received a blood transfusion 24 hr after admission because of unstable

haemodynamics partly related to atrial fibrillation. Four patients received exchange transfusion because of severe illness. Six patients (17%) subsequently died. Clinical and laboratory details are shown in Table 4. The mean (± SD) time to fever clearance in severe malaria was 67 (± 30) hr and the corresponding time to parasite clearance was 68 (± 19) hours. The mean RBC deformability at admission at a shear stress of 30 Pa of patients, expressed as EI (SD), was 0.586 (0.030) (range 0.508—0.624), and was significantly lower than the RBC deformability in healthy controls (El [SDI = 0.608 [0.005 <0.05).

Red blood cell deformability and anemia.

Figure 7 shows the correlation between the mean RBC deformability on admission and the nadir value in the hemoglobin level during hospitalization. The admission RBC deformability at a stress of 30 Pa correlated significantly with the degree of anemia (defined as the absolute hemoglobin concentration that developed during admission (n = 36, Pearson r = 0.49, $P< 0.002$). This correlation was strongest when the RBC deformability was measured at a high level of shear. At a lower shear stress of 1.7 Pa, the correlation coefficient between RBC deformability and the degree of anemia was 0.38 ($P <0.02$) for all patients (Figure 8).

The correlation between admission RBC deformability at high shear and the severity of subsequent anemia was even stronger (r = 0.64, $P < 0.001$) when five cases with microcytic anemia (MCV < 80 fl) were excluded from the regression analysis (Figure 7). All patients with microcytic anemia had a hemoglobin concentration < 9.0 g/dl and showed relatively more deformable red blood cells at these low hemoglobin levels than did the remaining patients. The cause of the microcytic anemia was iron deficiency (serum iron level = 2.3 mmol/L, iron binding capacity = 75 mmol/L) in

one case, and thalassemia in the other four, who had normal serum iron levels, target cells in the blood smear, and (in two cases) high hemoglobin A_2 levels on electrophoresis.

There was no significant correlation between the change in hemoglobin concentration and the change in RBC deformability over the same period of time. Red blood cell deformability at the time of the nadir in the hemoglobin level was not significantly different from the RBC deformability on admission (Table 4). Six patients with macroscopic haemoglobinuria had similar RBC deformability values at a shear stress of 30 Pa as the remaining patients (mean [SD] RBC deformability = 0.588 [0.0191] compared with 0.586 [0.032]). None of these haemoglobinuric patients were deficient for glucose-6-phosphate dehydrogenase.

The group of 12 Dutch travellers that returned from the tropics with uncomplicated malaria did not have high parasitaemias (mean [SD] = 1.5% [1.3%]) and developed only mild anemia, with a mean (SD) nadir in hemoglobin concentration of 12.5 (1.3) g/dl. The RBC deformability was only slightly decreased with a mean (SD) El of 0.595 (0.009) at a shear stress of 30 Pa. Nevertheless, in this group the percentage improvement in hemoglobin concentration during the four-weeks follow-up correlated significantly with the percentage improvement in the RBC deformability over the same time period ($r = 0.67$, $P = 0.018$). Moreover, the linear regression line describing the correlation between these two parameters nearly crossed the zero point (% improvement in hemoglobin level = 1.5% + 3.5 X % improvement in RBC deformability). The RBC deformability started to normalize two weeks after the start of treatment.

Factors related to anemia.

Besides RBC deformability, none of the other clinical or laboratory variables listed in Table 4 showed a significant correlation with the degree of anemia during the course of the disease. In particular, the admission parasitaemia was not a predictor of anemia (correlation coefficient = 0.13, not significant). In a multiple regression analysis (forward stepwise regression) with the variables listed in Table I as explanatory variables, the RBC deformability at admission (at a shear stress = 30 Pa) was the only parameter that contributed significantly to the model (adjusted $R^2 = 0.39$, B = 45.1, SE(B) = 10.1, $P = 0.0001$, F = 20.0).

Discussion

Anemia in acute falciparum malaria is caused by increased destruction of both infected and noninfected erythrocytes and decreased erythropoiesis. In severe malaria, anemia develops rapidly. The decrease in hemoglobin concentration is often considerably greater than could be accounted for by destruction of parasitized cells only. Anemia results largely from accelerated RBC destruction (Davis et al. 1990; Lee et al. 1992; Looareesuwan et al. 1987b; Phillips et al. 1986; White and Ho 1992). Labeling studies have shown rapid clearance of uninfected red blood cells by the spleen (Looareesuwan et al. 1987b). The importance of the enhanced clearance of uninfected cells is illustrated by the lack of correlation between parasitaemia and the severity of the anemia evident in this and previous studies (Molyneux et al. 1989; Phillips et al. 1986). The mechanism for this enhanced clearance of uninfected red blood cells remains to be elucidated. Evidence of an immune-mediated mechanism is unconvincing, although a role for antibody-mediated clearance cannot be ruled out since the spleen in acute malaria shows a lowered threshold for clearance of

erythrocytes coated with immunoglobulins, and it therefore may be difficult to demonstrate increased antibody binding in circulating erythrocytes (Abdalla and Weatherall 1982; Ho et al. 1990b; Lee et al. 1992; Looareesuwan et al. 1987a).

In this study, the RBC deformability on admission in patients with severe falciparum malaria was significantly lower than in healthy controls. Red blood cells infected with *Plasmodium falciparum* parasites become progressively less deformable as the intra-erythrocytic parasites mature (Cranston et al. 1983b; Nash et al. 1989). However, the mean RBC deformability obtained with LORCA is a summation of the RBC deformability of all the RBC fractions in the peripheral blood, with contributions to the overall value that are proportional to their size (Streekstra GJ, 1994. *A Bi Plane Rheoscope for the Measurement of Red Cell Deformation and Orientation in a Couette Flow.* Thesis, University of Utrecht, Utrecht, The Netherlands). Since the majority of red blood cells even in severe malaria is uninfected, the reduction in RBC deformability in the patients in this study results mainly from changes in the unparasitized erythrocytes.

Reduced RBC deformability does not result from a nonspecific response to severe infections. In a group of 14 septicaemia patients in the intensive care unit of the Academic Medical Centre in Amsterdam, there was no correlation between RBC deformability and severity of anemia and the mean (SD) RBC deformability at 30 Pa, expressed as the El, was 0.594 (0.020) (range = 0.574—0.614).

This study shows a clear correlation between the RBC deformability on admission and subsequent anemia in severe falciparum malaria. Reduced RBC deformability and anemia could both be independent markers of overall disease severity, but a causal relationship seems more likely, i.e., clearance of less deformable red

blood cells from the circulation by the spleen, a mechanism that is also thought to account for the clearance of senescent erythrocytes (Nash et al. 1988). The relationship between reduced RBC deformability and anemia was most prominent at the shear stresses encountered normally in the spleen where red blood cells have to squeeze through the small intercellular gaps in the sinusoids of the spleen (width = 0.5—2m) (Chen and Weiss 1973). In a recent study, we showed that in severe falciparum malaria, RBC deformability at a lower shear stress of 1.7 Pa correlated strongly with mortality, suggesting impairment of microcirculatory flow by rigid red blood cells (Dondorp et al. 1997). This shear stress corresponds with that encountered in the capillaries (average diameter = 3—5 mm) (Chien 1987).

The mean RBC deformability did not change significantly during hospitalisation, which was generally up to seven days after the start of treatment. Longer follow-up, to study if the improvement in RBC deformability is related to recovery from anaemia, was unfortunately not possible in the Thai patients. However, follow-up in a group of 12 Dutch travellers with uncomplicated falciparum malaria showed that the improvement in haemoglobin levels over a four-week period correlated closely with the improvement in RBC deformability over the same time period. These findings further support a causal relationship between the two parameters.

The correlation between RBC-D and the severity of anaemia that developed in patients with severe malaria was even stronger when the patients with microcytic anaemia, resulting from either a haemoglobinopathy or iron deficiency, were excluded. The few patients with microcytic anaemia showed a slight but nonsignificant decrease in RBC deformability at 30 Pa compared with healthy

controls (mean [SD] = 0.600 [0.020] and 0.608 [0.005], respectively, Figure 7). The RBC deformability can be reduced in both iron deficiency and thalassemia (Schrier, Rachmilewitz, and Mohandas 1989; Yip et al. 1983). We were not able to genotype all patients but it is unlikely that thalassemia was a significant confounder in the normocytic malaria patients. Of 95 well-defined patients with various forms of α- and β- thalassaemia and haemoglobin E and haemoglobin Constant Spring (hemoglobin - CS) disease studied in Bangkok, only 10 had a normal MCV > 80 fl. Seven had haemoglobin CS (4) or haemoglobin thalassaemia (3) with a normal RBC deformability at 30 and slight or no anaemia (mean haemoglobin levels Hb 11.5 g/dl and 13.4 g/dl, respectively) (Dondorp AM, unpublished data).

The mechanisms underlying the reduction in RBC deformability of uninfected cells in severe malaria are known. Mohan and others showed damage of the uninfected erythrocyte membrane through lipid peroxidation in *P. falciparum* co cultured with blood monocytes (Mohan et al. 1995). Nauman and others have reported on a heat-labile exoantigen produced by *in vitro* cultures of *P. falciparum* that binds reversibly to normal red blood cells and reduces their deformability (Nauman et al. 1991). We also think that a soluble factor produced by the parasite the most likely cause of a reduction in RBC deformability in patients with falciparum malaria. Preliminary data shows that plasma from patients with acute falciparum malaria mixed with healthy donor red blood cells can rigidify these cells. Also, supernatant from a *P. falciparum* culture seems to be able to rigidify healthy donor red blood cells, but the exact mechanism remains to be elucidated (Unpublished observations). Although heat damages red blood cells, an increase in temperature up 41^0C did not reduce the RBC deformability of normal erythrocytes *in vitro* as measured by

LORCA, suggesting that fever was not a major contributor to this effect. The relative roles of systemic host factors or endothelial cell dysfunction in reducing RBC deformability is not known.

In conclusion, this study shows a strong predictive value of admission RBC deformability at a high shear level the severity of the anaemia that develops in the course of severe falciparum malaria. Since the reduction in RBC deformability is caused mainly by rigidification of nonparasitized erythrocytes, this correlation could be an explanation for the increased splenic clearance of noninfected erythrocytes.

Table 4.

Mean (SD) clinical and laboratory variables in patients with severe falciparum malaria according to the severity of anaemias* Group 1 - Severe anemia (Hb <9.0g/dl)(all patients), Group 2 - Severe anemia (Hb < 9.0 g/dl) (microcytosis excluded), Group 3 - Mild anemia (Hb > 9.0 g/dl)

	Group 1 (n = 17)	Group 2 (n = 12)	Group 3 (n = 19)	*P*
Age (years)	27 (13)	28 (14)	26 (10)	NS
Fatal cases (n)	3	3	3	NS
Artesunate (n)	9	6	8	NS
Quinine (n)	8	6	11	NS
Parasitaemia (%)	6.3 (5.1)	7.4 (5.4)	9.1 (7.5)	NS
Hb (g/dl)	9.1 (2.4)	9.4 (2.6)	13.9 (1.7)	0.03
MCV (fl)	84.4 (8.7)	88.5(6.1)	88.6 (6.6)	NS
Nadir Hb (g/dl)	7.1 (1.2)	7.3 (1.2)	10.9 (1.4)	0.001
Time after admission to nadir in Hb (hr)	41 (42)	53 (48)	83 (26)	NS
RCD at 30 Pa (EI)	0.574 (0.034)	0.564 (0.036)	0.597 (0.020)	0.002
RCD at 1.7 Pa (EI)	0.233 (0.032)	0.227 (0.032)	0.251 (0.031)	0.05
RCD (30 Pa) at time of lowest Hb (EI)	0.574 (0.032)	0.567 (0.033)	0.598 (0.025)	NS
LDH (mmol/L)	672 (153)	717 (137)	548 (364)	NS

Total bilirubin (μmol/L)	47.0 (46.0)	57.7 (53.7)	70.1 (114.3)	NS
Direct bilirubin (μmol/L)	27.4 (35.9)	35.9 (41.7)	46.2 (93.2)	NS
Haptoglobin (g/L)	0.2 (0.6)	0.0 (0.0)	0.1 (0.2)	NS
Serum iron (μmol/L)	10.4 (9.4)	12.7 (10.4)	6.4 (7.8)	NS
ASAT (U/L)	69 (27)	66 (12)	79 (94)	NS
Creatinine (μmol/L)	101 (35)	108 (42)	94 (59)	NS
Glucose mmol/L)	4.5 (1.8)	4.6 (2.0)	3.6 (0.6)	NS
Lactate (mmol/L)	4.8 (3.1)	5.0 (2.7)	3.9 (1.8)	NS

Hb = hemoglobin; MCV mean cell volume; RCD = red blood cell deformability; Pa Pascal; EI elongation index; LDH lactate dehydrogenase; ASAT aspartate aminotransferase *P* values for significance of difference between patients with severe and mild anemia, with the exclusion of patients with microcytic anemia.

Figure 7. Correlation between admission values of mean red blood cell deformability (RCD) at a shear stress (SS) of 30 Pa and the lowest hemoglobin (Hb) level reached during the time of hospitalization in patients with severe falciparum malaria. (n = 36, correlation coefficient = 0.49, $P = 0.002$). When patients with a microcytic anaemia (mean red blood cell volume < 80 fl, open circles) are excluded, the correlation coefficient between the two variables becomes much stronger (n = 31, correlation coefficient = 0.64, $P < 0.001$). MCV = mean cell volume.

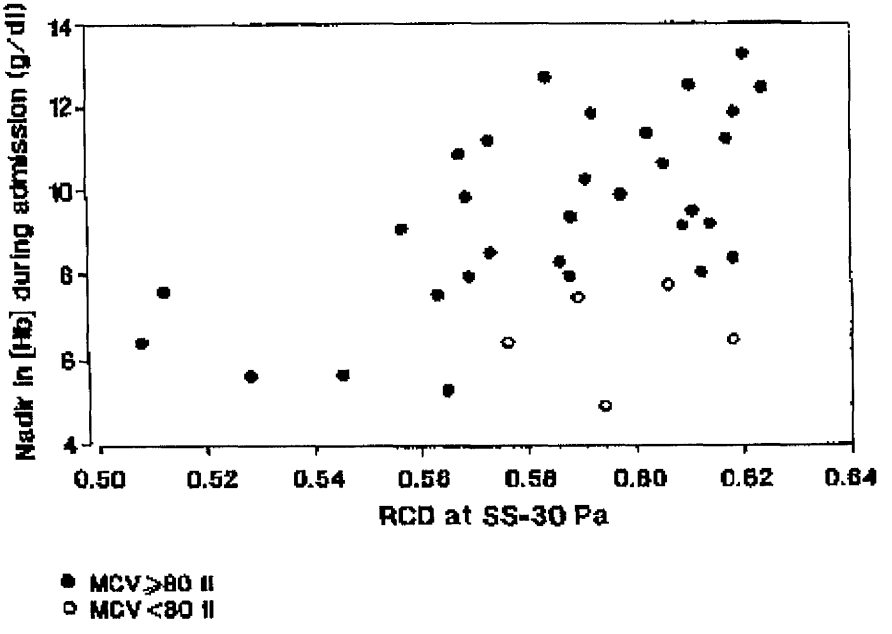
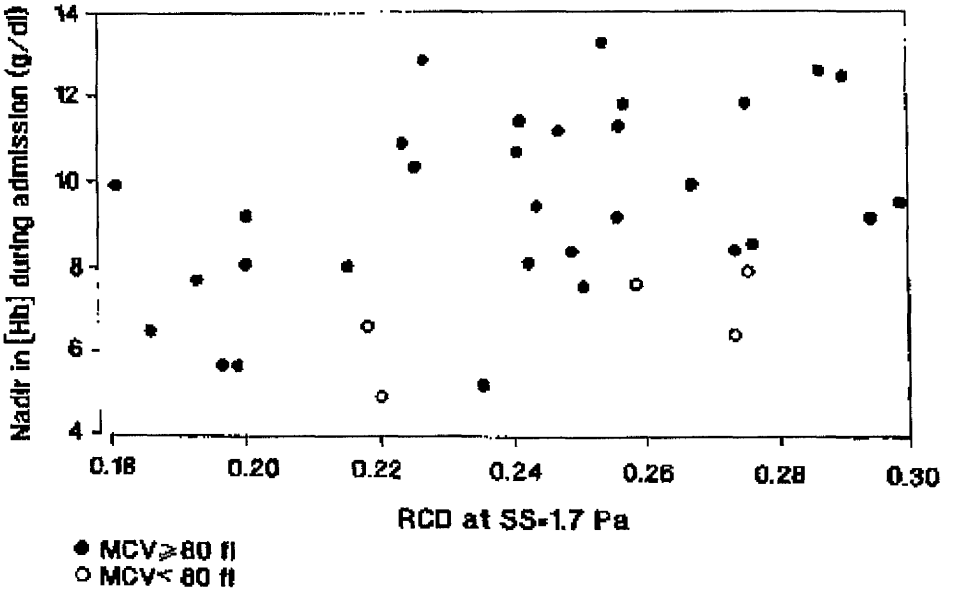


Figure 8. Correlation between admission values of mean red blood cell deformability (RCD) at a shear stress (SS) of 1.7 Pa and the lowest hemoglobin (Hb) level reached during the time of hospitalization in patients with severe falciparum malaria. (n = 36, correlation coefficient = 0.38, $P = 0.02$). MCV = mean cell volume.



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Plasma nitrogen oxides and blood lactate concentrations in Ghanaian children with malaria

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This study was approved by the Committee of Research, Publication and Ethics of the School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Informed consent was obtained from attendant relatives of enrolled patients.

Abstract

Nitric oxide is an important host defence molecule as well as being a mediator in many pathophysiological processes. To investigate its role in severe malaria, we measured plasma nitrate and nitrite concentrations in 70 children with malaria (54 with severe malaria) and 48 control subjects (33 with medical conditions and 15 surgical patients). We related these measurements to plasma lactate concentrations, an established marker of disease severity in malaria. Plasma lactate levels were significantly elevated in patients with deep coma (P=0·0007) and those with a fatal outcome, but mean nitrogen oxide concentrations were not significantly different in the 2 outcome categories and were not related to depth of coma (P>0·5). In patients whose cerebrospinal fluid (CSF) was examined, lactate concentrations were elevated in fatal cases (geometric mean 8·2 mmol/L, n=5) compared with survivors (3·4 mmol/L, n=13; P=0·032); corresponding CSF nitrogen oxide concentrations were 10·7 μM in fatal cases compared with 12·5 μM in survivors (P=0·5). Plasma nitrogen oxide concentrations were negatively correlated with admission parasitaemia (r=–0·41, n=70; P<0·0001). In our population, elevations of plasma lactate, but not nitrite or nitrate, reflected disease severity in malaria.

Introduction

Nitric oxide (NO) is an important host defence molecule synthesized from L-arginine in a reaction catalysed by the inducible isoform of nitric oxide synthase (NOS2). Expression of NOS2 in murine macrophages occurs after they are exposed to bacterial endotoxin, or to 'pro-inflammatory' cytokines including interleukin 1, tumour necrosis factor (TNF) and interferon γ. The NO synthesized by NOS2 is toxic

to many fungi and protozoa, and certain bacteria and viruses (Anggard 1994; Vallance and Moncada 1994). This potentially important defensive role has been supported by the observation that mice lacking a normal NOS2 gene cannot suppress the proliferation of *Leishmania* (Wei et al. 1995). Human monocytes, after appropriate activation, also express NOS2 and produce sufficient NO to kill intracellular *Leishmania* (Vouldoukis et al. 1995). The role of NO in the pathophysiology of human malaria, however, is still unclear. A recent study in Tanzanian children with malaria suggested that decreased NO synthesis may predispose to cerebral malaria and also to a fatal outcome (Anstey et al. 1996). This study also suggested that TNF or other proinflammatory cytokine synthesis in severe malaria infection may have been up-regulated because of relatively decreased NO production.

In contrast, others have argued that increased NO synthesis may contribute to cerebral symptoms in malaria (Clark, Rockett, and Cowden 1992), and have found that elevated concentrations of plasma reactive nitrogen intermediates are associated with coma (Al-Yaman et al. 1996). This latter role implies increased local (cerebral endothelial) synthesis of NO in patients with cerebral malaria, but does not exclude other potential roles for NO such as host defence.

We measured plasma nitrate and nitrite concentrations in Ghanaian children with uncomplicated and severe malaria, and related these measurements to elevations in plasma lactate concentration, a recognised marker of severe disease (Allen et al. 1996; Krishna et al. 1994b). We also compared these indices to those obtained from children recovering from elective surgery and other non-malarial disease processes. Plasma nitrate and nitrite are breakdown products arising from the short lived parent

compound NO, and increased concentrations in plasma reflect increased NO production through inducible pathways.

Material and Methods

Patients

Patients were studied as part of published and current studies on lactic acidosis in malaria carried out between October and December in 1993 and 1995 (Krishna et al. 1995). We defined severe malaria as asexual *Plasmodium falciparum* parasitaemia associated with hyperlactataemia (whole blood or plasma lactate when screened ≥5 mmol/L), and we included a further 5 patients with cerebral malaria (Blantyre coma score ≤2) and one with a fatal outcome. When our metabolic definition of severe malaria was compared with the World Health Organization classification of severe disease, all except one patient fulfilled at least one stipulated criterion and >80% fulfilled 2 or more criteria (World Health Organization 1990). Children with malaria without hyperlactataemia (screening lactate concentration <5 mmol/L) or cerebral malaria were classified as having non-severe malaria. The lactate values presented here were those obtained simultaneously with sampling for nitrogen oxides (NOx; nitrate plus nitrite). For baseline values, this was approximately 1 h after screening. Patients with hyperlactataemia and malaria were managed by the study team and their parasitological and clinical measures of recovery were estimated as reported previously (Krishna et al. 1995). In some children with severe malaria, measurements of plasma NO x were also carried out one and 12 h after admission.

Control subjects were children with other severe medical illnesses (meningitis, encephalitis, septicaemia, pneumonia, Burkitt's lymphoma, tetanus, or jaundice) or

children in a surgical ward (with head or limb injuries, appendicitis, or cellulitis) at the study site. Cerebrospinal fluid (CSF) samples obtained from children undergoing lumbar puncture when clinically indicated were also examined for lactate, glucose and nitrogen oxides.

Assay for plasma nitrite, nitrate and lactate

Plasma nitrate and nitrite concentrations were assayed using capillary electrophoresis with reported inter-assay coefficients of variation of 4·6% and 1·2% for nitrite and nitrate (50 μM), respectively (Leone et al. 1994). Lactate and glucose were assayed using YSI glucose and lactate analysers (model 1600) or a combined analyser (model 2300, YSI Instruments, Yellow Springs, Ohio, USA).

Statistical analyses

Statistical analysis was carried out using SYSTAT™ v. 5·2 (SYSTAT Inc., Evanston, Illinois, USA) or Statistica™ v. 3 (StatSoft Inc., Tulsa, Oklahoma, USA). Normally distributed variables were compared by Student's *t* test and linear regression analysis and non-normally distributed variables were normalized by log transformation before analysis by ANOVA and Bonferroni's post-hoc test. Correlations were analysed by Spearman's rank correlation. Non-normal measures were compared by the Mann–Whitney *U* or Wilcoxon tests. Trend analysis was carried out with SPSS™.

Results

One hundred and eighteen children were studied, 70 children with malaria and 48 control subjects; 54 children had severe malaria, 9 of whom (17%) died, and 16 had uncomplicated malaria, all of whom recovered. Fifteen control patients were either recuperating from surgical procedures (studied at least 5 d after surgery) or awaiting surgery. The remaining 33 control patients included 5 with bacteriologically confirmed meningitis (3 fatal cases), 4 with febrile convulsions, and 4 with Burkitt's lymphoma. The admission clinical and laboratory findings in the study groups are given in the Table 5.

Table 5.

Clinical and laboratory characteristics of patients with malaria and control subjects

	Malaria patients		Controls	
Variable a	Uncomplicated	Severe	Surgical	Medical
No.	16	54	15	33
Age (months)	63(53)	42(26)	98(50)	100(164)
Body mass index (kg/m^2)	0·154(0·037)	0·153(0·032)	0·137(0·032)	0·153(0·03)
Oral temperature (°C)	37·8(0·8)	38(1)	36·8(0·6)	37·8(1)
Pulse (per min)	122(20)	144(21)	83(6)	123(27)
Systolic blood	101(9)	102(22)	93(11)	102(19)

pressure (mm Hg)				
Diastolic blood pressure (mm Hg)	63(12)	57(14)	64(7)	64(15)
Respiratory rate (per min)	37(12)	51(15)	24(4)	42(17)
Packed cell volume (%)	29(8)	23(8)	32(12)	27(9)
Parasitaemia (per μL)b	66,171(10,050–443,300)	100,300(10–1,799,470)	–	–
Plasma NO 2 (μM)b	1·06(0·5–2·2)	1·06(0·4–2·63)	1·3(0·67–2·2)	1(0·5–2·4)
Plasma NO 3 (μM)b	39(14·7–183)	33·8(10·6–197)	42(12·6–153)	41(16–208)
Blood lactate (mM) b	2·3(0·9–3·8)	6·4(1·2–17·3)	–	3·87(1–14)
Blood glucose (mM) b	6·54(3·9–11·4)	6·5(3·7)	–	5·8(2)

a All values are means (with standard deviation in parentheses) except where shown. b Geometric mean (range in parentheses).

In the whole study population, there was no difference between survivors and the 14 fatal cases (12%) in individual or summed plasma NO x concentrations (for

survivors, geometric mean NO x value=37·3 μM; for fatal cases, it was 43 μM; P=0·4; Fig. 9). However, lactate concentrations were significantly elevated in fatal cases (geometric mean=8·2 mM) compared with survivors (geometric mean=4 mM; P<0·0001). Nitrate and nitrite levels were not correlated with each other (r=0·003, n=118; P=0·56) or with clinical and biochemical correlates of disease, including temperature, blood pressure, tachypnea, pulse rate, lactate and glucose.

Figure 9. Plasma concentrations of nitrogen oxides (NOx) in patients with malaria and control subjects. Clinical details are given in the Table 5.

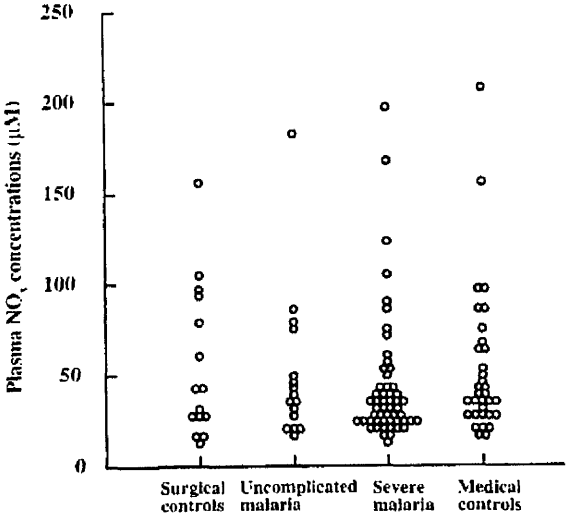


Figure 10. Relationship between admission parasitaemia and plasma concentrations of nitrogen oxides (NOx); the linear regression line is shown, with 95% confidence intervals.

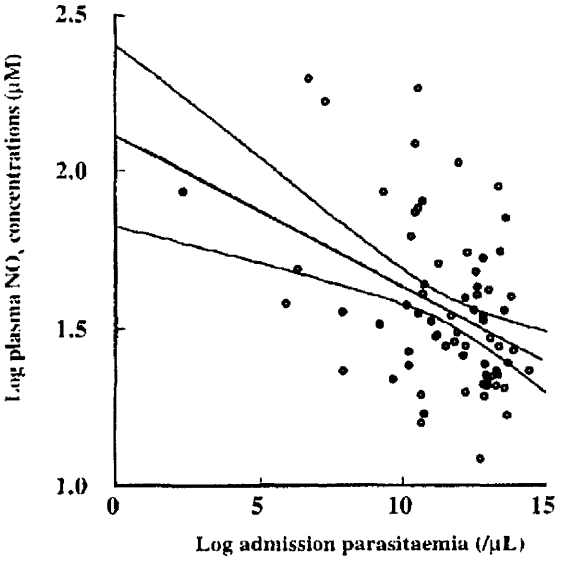
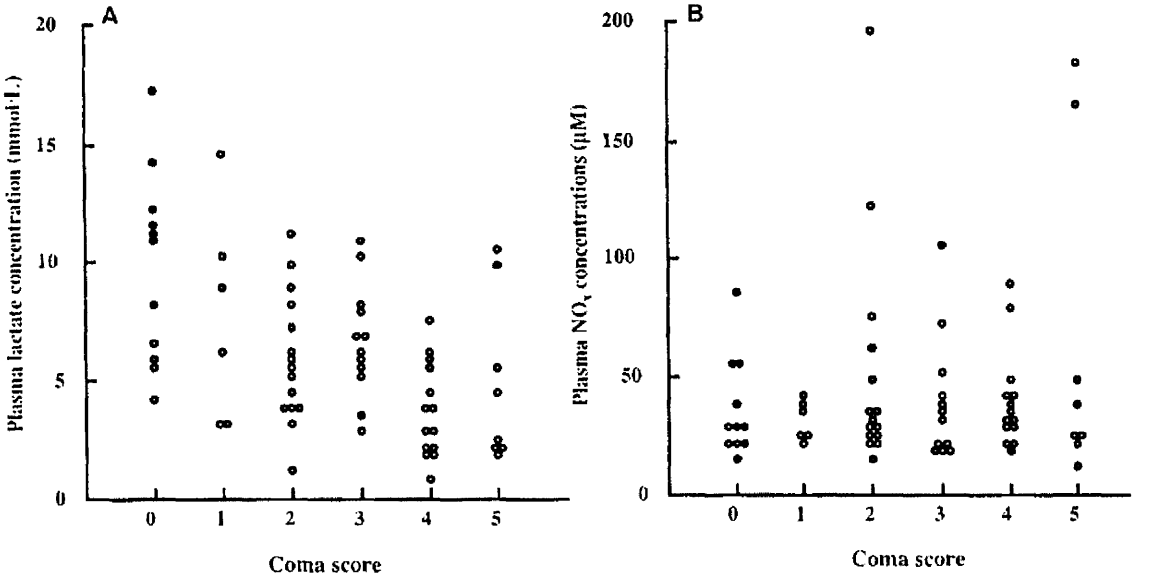


Figure 11. Relationship between (A) admission plasma lactate concentration, and (B) plasma concentration of nitrogen oxides (NO x), and the Blantyre coma score; open circles represent survivors and filled circles show fatal cases.



Patients with malaria

Patients with severe malaria had higher admission pulse rates (P=0·002) and respiration rates (P=0·002), and were more anaemic (P=0·019) than uncomplicated cases (Table 5). Parasitaemia was marginally higher in severe cases (P=0·05) but it was not correlated with lactate levels. Parasitaemia was significantly negatively correlated with summed NO x levels (r=—0·41, n=70; P<0·0001; Fig. 10). Thirty-four patients (49%) had coma scores ≤2 on admission and, overall, lactate levels were negatively correlated with coma scores (Fig. 11A, $\chi 2$ for trend=21·32, d.f.=5; P=0·0007) as well as fatal outcome. In contrast, NO x levels were not associated with admission coma scores (Fig. 11B; P>0·5) or fatal outcome.

For some patients studied in greater detail, there was no relationship between clinical measures of recovery such as time to recovery of consciousness (n=23), time

to eat (n=26), time to sit (n=21) and time to walk (n=18), parasitological indices of improvement (50%, 90% and 100% parasite clearance times; n=29–31) (White and Krishna 1989), fever clearance data (n=27) and the admission nitrite, nitrate or summed NO x levels. There was no significant difference between the 4 study groups (Table 5) in admission nitrite, nitrate or NO x levels (Fig. 1; P>0·5). One and 12 h following admission, there was no significant change in nitrate concentration in a subset of 19 patients with severe malaria.

Nitrite concentrations increased significantly at 12 h from a mean of 1·1 to a mean of 1·8 μM (n=13; P=0·002) in this group of patients.

Cerebrospinal fluid findings

In no patient was there a relationship between nitrite, nitrate or NO x levels in CSF and the corresponding plasma levels (r=0·32, n=19; P=0·19), nor any relationship between nitrite and nitrate concentrations in the CSF. In fatal cases, CSF lactate concentrations were significantly elevated compared with those of survivors (geometric mean CSF lactate concentration=8·2 mM (n=5) vs. 3·4 mM (n=13); P=0·032), but no difference was observed for corresponding CSF NO x levels between the 2 outcome categories (fatal cases, geometric mean NO x level=10·7 μM (range 5·7–17·7) vs. 21·5 μM (range 5·9–23·9); P=0·5). Neither was any difference in CSF NO x level between patients with cerebral malaria and those with other conditions (P>0·7). CSF nitrite levels were never above 3·4 μM in any patient.

Discussion

There was no significant difference between admission plasma nitrate or nitrate concentrations in malaria patients and control subjects. Neither was any relationship observed between clinical and laboratory markers of disease severity in malaria (apart

from parasitaemia, discussed below) and concentrations of NO x in the plasma. In contrast, blood lactate concentrations were clearly associated with disease severity and a fatal outcome in these patients. There are several potential confounding factors in interpreting measurements of plasma nitrate and nitrite in our patient population. Dietary ingestion of nitrate may lead to elevation of plasma nitrate for a few hours. However, this elevation does not exceed 50% of fasting nitrate levels (Leone et al. 1994) and it would have tended to increase levels in the children with uncomplicated malaria or the surgical controls, rather than those in severely ill children who had not eaten or drunk for some hours before admission. The median time when the 35 children severely ill with malaria for whom data were available had last eaten was 10h (range 2–72) previously. Nitrite levels are unaffected by fasting.

Renal impairment (assessed by a single measurement of plasma creatinine levels) is infrequent and relatively mild in West as well as East African children with malaria (Anstey et al. 1996; Waller et al. 1995). As nitrate is excreted by the kidneys, correction for minor elevations in plasma creatinine concentrations may have lowered our values for plasma NO x in severely ill children. We were not able to measure plasma creatinine, but the magnitude of the correction required would be small as none of the children developed clinical evidence of renal failure. No relationship was observed between plasma NO x concentrations and depth of malarial coma, and there was no difference in plasma NO x concentrations between patients with malaria and surgical control subjects. Our findings are consistent with observations from Yaoundé, where children with cerebral malaria had mean plasma NO x concentrations of 44·3±36·5 μM and no statistically significant relationship between depth of coma, disease severity and NO x level was noted (Cot et al. 1994). In our study, mean

concentrations of nitrogen oxides in the plasma (37·5±22 μM) were not elevated to the degree seen in septic adults (63·1±6·5 μM, *n*=72) (Ochoa et al. 1991) or septic neonates with shock (582±385 μM, range 242–1230) (Shi et al. 1993), even though our severely ill malaria patients were at high risk of mortality. In a small study in Viet Nam, higher plasma concentrations of NO x were reported in severe malaria, although levels in fatal cases did not differ from those in survivors (Nussler, Eling, and Kremsner 1994). More recently, in a series from Papua New Guinea, relatively elevated serum levels of reactive nitrogen intermediates were noted in children with cerebral malaria compared with those who were not comatose, and significantly higher levels in fatal cases compared with survivors (Al-Yaman et al. 1996). Peripheral parasitaemia and NO x were negatively correlated (*r*=–0·22) in that study, as they were in this one (*r*=–0·4, Fig. 1). Although parasitaemia correlates poorly with other indices of disease severity (Krishna et al. 1994b), this negative association between peripheral parasitaemia and NO x levels may suggest a protective role for NO in patients with malaria. In a separate study from Papua New Guinea, admission lactic acidosis, not coma, was predictive of a fatal outcome, and parasitaemia was considerably lower than in children in Africa presenting with disease of similar metabolic severity to our patients, highlighting geographical variation in the clinical features of malaria (Allen et al. 1996). The absence of a strong correlation between plasma nitrite and nitrate concentrations in our patients probably reflected the instability of nitrite in whole blood, where it is particularly susceptible to the action of haemoglobin (Leone et al. 1994). The inconsistent associations between circulating NO x and disease status and severity of malaria in several reports may reflect not only methodological differences but also differences between geographically distinct

patient populations. Control populations in some studies may themselves have been significantly exposed to malaria, and this may have influenced interpretation of NO x values, although this does not mitigate against examining the role of NO x in different malaria syndromes within a population (Al-Yaman et al. 1996; Anstey et al. 1996). Furthermore, plasma estimations of NO metabolites may not represent important changes in local concentrations of NO, and the precise cellular source of the NO detected in malaria is uncertain. Thus changes in endothelial or cerebral NO metabolism may still be undetected because of the insensitivity of plasma based assays. We therefore also measured CSF nitrate concentrations in a subset of patients with cerebral malaria and did not find any difference between fatal cases and survivors. Interestingly, the normal CSF concentration of reactive nitrogen intermediates has a mean of 5·1 μM (SD=0·8) (Milstien et al. 1994). In another series, mean CSF nitrate and nitrite levels were 6·3 μM and 0·5μM, respectively, in patients with noninflammatory neurological disease.

These levels were significantly elevated in patients with meningococcal meningitis to mean values for nitrate of 18·4 μM and nitrite of 25·8 μM (Visser, Scholten, and Hoekman 1994). In our patients, elevations of CSF nitrate were relatively modest, with geometric mean concentrations approximately twice those reported for normal individuals and no disproportionate increase in CSF nitrite in patients with cerebral malaria or meningitis. Disease severity in our study was mirrored by elevations in plasma or CSF lactate concentrations and not by changes in plasma or CSF NO x. Clearly, more sophisticated techniques designed to measure NOS2 expression (of both encoding messenger ribonucleic acid and protein) in cerebral endothelial cells obtained from patients dying of cerebral malaria will

provide a more sensitive method than measurement in CSF of examining the hypothesis that local derangements in NO metabolism are contributory to the cerebral syndrome. The role of changes in NO metabolism in malaria, and the effects of such changes on host defence or disease pathophysiology, clearly require further investigation.

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Nitric oxides in plasma, urine and cerebrospinal fluid in patients with severe falciparum malaria

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Abstract.

It has been suggested that nitric oxide (NO) plays an important role in the pathogenesis of severe falciparum malaria. Since NO has a very short half-life, nitrate and nitrite (NOx) levels, stable metabolites of NO, are used as measures of NO production. We measured plasma NOx levels in 24 adults with severe falciparum malaria on the Thai-Burmese border. After correction for renal function, there was no correlation between plasma NOx levels, or the total amount of NOx excreted in the urine, and disease severity. Plasma NOx levels decreased after the first 48 hr in all patients (P 0.007), suggesting decreased NO production. The NOx levels in cerebrospinal fluid (CSF) correlated well with plasma NOx levels, but these did not show a correlation with coma depth, and were not significantly different from those in a healthy control group. These findings do not support the hypothesis that excessive NO production contributes to the pathogenesis of severe falciparum malaria. However, local changes in NO production, e.g., in the central nervous system might not be reflected in the total NOx production or NOx levels in the CSF.

Introduction

Severe falciparum malaria remains an important cause of mortality in the tropical world with an annual global mortality of 1 - 2 million people and a mortality rate as high as 15 - 30% despite effective anti-malarial treatment (World Health Organization 1990; World Health Organization 1993). It has been proposed that nitric oxide (NO) plays a central role in the pathogenesis of severe, and in particular cerebral malaria (Clark, Rockett, and Cowden 1991). Nitric oxide is an important mediator of many homeostatic processes and host defense mechanisms, and may also

have a function as neurotransmitter (Moncada and Higgs 1993). The production of NO is increased by proinflammatory cytokines such as tumor necrosis factor α and interleukin-1, which reach high levels in severe falciparum (Rockett et al. 1992). Excessive synthesis of NO by cerebrovascular endothelial cells might alter neurotransmission and thus contribute to the pathogenesis of cerebral malaria (Clark, Rockett, and Cowden 1991). On the other hand, NO is thought to play a role in parasite killing (Green et al. 1994; Mellouk et al. 1994). It also reduces the induction of endothelium-bound adhesive molecules such as intercellular adhesion molecule-1, which are thought to play an important role in the intracerebral sequestration of parasitized erythrocytes (Decaterina et al. 1995).

Study of NO production *in vivo* is difficult because of its very short plasma half-life of less than 10 sec (Kelm and Schrader 1990). In plasma, NO is almost immediately oxidized to nitrite and subsequently converted to nitrate, and this seems to be the most important fate of the molecule (Kelm et al. 1992; Westfelt et al. 1995). As a consequence, all clinical information about the possible role of NO in falciparum malaria derives from the measurements of plasma and urine levels of nitrites and nitrates (Anstey et al. 1996; Cot et al. 1994; Kremsner et al. 1996; Nussler, Eling, and Kremsner 1994; Prada and Kremsner 1995). These studies have produced sometimes contradictory results. In the present study, we measured nitrate and nitrite (NOx) levels in patients with severe falciparum malaria, both in plasma and urine, to provide a better estimate of total body NOx production. In addition, we measured NOx levels in cerebrospinal fluid (CSF) in patients with cerebral malaria and compared these with NOx values obtained in CSF from controls.

Patients and Methods

Consecutive adult patients admitted to Mae Sot Hospital, Tak Province, Thailand with acute falciparum malaria were included in this study, provided that written informed consent was obtained from the patients or their attendant relatives. Malaria transmission is low in this area with a seasonal peak during the rainy season, which starts in late spring (Luxemburger et al. 1996). Disease severity was classified according to standard criteria (World Health Organization 1990). Exclusion criteria were an age less than 14 years, pregnancy, and previous antimalarial drug treatment within 24 hr of admission. Previous quinine treatment was checked in a baseline blood sample by a rapid quinine dipstick method in all patients (Silamut et al. 1995). A full clinical examination was performed on admission and all details were recorded on a standard form. Patients were randomly assigned to treatment with either intravenous quinine dihydrochloride or intravenous artesunate in a comparative study, the results of which will be published elsewhere. Full supportive care was given as described previously (World Health Organization 1990). If necessary, patients were transferred to an intensive care unit for mechanical ventilation, peritoneal dialysis, or haemodynamic support and monitoring. Postmortem examinations of the fatal cases were not performed. This investigation was part of studies approved by the Ethical and Scientific Review Subcommittee of the Ministry of Public Health, Thailand.

Laboratory methods.

Baseline blood samples were taken for a full blood count, glucose, lactate and routine biochemistry. Thick and thin films from peripheral blood were taken on admission and stained with Field's stain for parasite counting (White and Silamut 1989). Blood samples for plasma NOx and creatinine measurements were taken every

12 hr. Urine was collected every 12 hr from the time of admission for NOx and creatinine measurements. Chlorhexidine was added to the collection containers to prevent bacterial growth. In cases of cerebral malaria, a lumbar puncture was performed, and routine laboratory measurements were done to exclude other causes of altered consciousness. Five patients undergoing a lumbar puncture for anesthesia served as a control group for this part of the study. All samples were immediately stored at -20^{0}C for later analysis.

Table 6.

Admission clinical and laboratory variables in patients with severe malaria*

Variable	Survivors (n = 7)	Fatal cases (n=7)	Significance of difference (P)
Age (years)	26±8	28±14	NS
Pulse rate (per mm)	114 ± 11	109 t 15	NS
Blood pressure (mm of Hg)			
Systolic	109±12	104±14	NS
Diastolic	64±16	63±20	NS
Temperature (°C)	38.8 ±1.1	38.2 ± 1.4	NS
Median coma score	15	8	0.004 $
Packed cell volume (%)	34 ± 11	31±9	NS
Parasitaemia (%)	6±6	10± 11	NS
Plasma creatinine (μmol/L)	81 ± 18	148 ± 97	0.01
Plasma lactate	4.2 ± 2.6	13.1 ± 9.2	0.02

(mmol/L)			
Plasma glucose (mmol/L)	9.0 ± 6.1	8.8 ±3.5	NS
Plasma NOx (μmol/L) Median (range)	33.6 (15.2—151.1)	117.4 (20.2—935.0)	NS $
Plasma $NOx/creatinine_{plasma}$ (μmol/L/μmol/L) Median (range)	0.51 (0.16—1.78)	0.63 (0.26—3.08)	NS
Urine NOx (μmol) in first 12 hr after admission Median (range)	422.9 (204.3—2311.5) (n = 14)	85.3 (22.4—561.8) (n =7)	0.03 $
Urine $NOx/creatinine_{urine}$ (μmol/mmol) Median (range)	82.8 (27.7—360.7) (n = 14)	47.1 (38.5—179.3) (n = 7)	NS $
CSF NOx (μmol/L) Median (range)	4.8 (2.5—7.7) (n = 6)	13.8 (1.5—61.4) (n = 5)	NS $

* NS = not significant; NOx = nitrate and nitrite; CSF = cerebrospinal fluid.

Values, where indicated are the mean ± SD.

$ Comparison by Mann-Whitney U test; all other comparisons by Students t-test.

The NOx levels were assessed by ion-pair chromatography. To precipitate proteins in plasma or CSF, 500 μA of water, 100 μl of 0.35 M $ZnSO_4$, and 100 μ1 of 0.75 M NaOH was added to a 100-μl sample. Urines were treated in the same way after a 1:4 dilution in water. The precipitates of protein were removed by centrifugation at 1,500 X g for 10 min. Standards were processed in the same way as the samples. A volume of 20 μ1 of the deproteinized sample was injected on a 100 x 3.0 mm Chromsphere c18 column (Chrompack, Bergen op Zoom, The Netherlands). Separation was achieved by ion-pair chromatography with 0.01 M n-octylamine as eluent (Stein and Classen 1988). The pH of the eluent was brought to 6 with sulfuric acid and then adjusted to pH 6.5 with 2 mmol/L of ammonium acetate. Detection was measured at 215 nm. The flow rate was 0.65 ml/min. The coefficient of variation of duplicate determinations was less than 2%. Plasma NOx levels were corrected for kidney function by taking the ratio of NOx concentration and plasma creatinine concentration. Similarly total urinary NOx values were corrected for renal function by taking the ratio of NOx and total urinary creatinine values in the same sample (Anstey et al. 1996). Fractional NOx excretion (the fraction of filtered NOx that is excreted) was calculated with the formula total NOx in urine (μmol/12 hr)/[NOx] in plasma (μmol/L) divided by total creatinine in urine (μmol/l 2 hr)/[creatinine] in plasma (μmol/L).

In one patient who died 2 hr after urine collections were started, the total amount of NOx excretion in the urine in the first 12 hr was obtained by extrapolation of the excretion in the first 2 hr after admission.

Statistical methods.

Statistical analyses were carried out using SPSS 6.1 statistical programs (SPSS

Corporation, Chicago, IL). Normally distributed data were analyzed using Student's t-test. The Mann-Whitney test was used to compare non-normally distributed variables. Correlations were assessed by the method of Pearson for normally distributed variables, and by the method of Spearman for the remainder. The Wilcoxon matched pairs rank test was used to compare changes in NOx levels between two time points.

Results

Twenty-four consecutive patients with severe falciparum malaria, 11 of whom had cerebral malaria, were studied. Seven patients died. One patient who was included initially because of severe anemia accompanying her malaria was excluded subsequently when it became apparent that her anemia resulted from thalassemia. Complete, sequential, 12-hr urine samples were collected from 21 of these patients.

Clinical and laboratory findings are summarized in Table 6. There was no significant difference between fatal cases and survivors in the NOx levels in plasma or CSF, although values tended to be lower in survivors. This tendency disappeared when plasma nitrate levels were corrected for renal function by dividing plasma NOx by plasma creatinine. There was a significant correlation between plasma nitrate and plasma creatinine levels at admission ($r = 0.47$, $P = 0.03$). The total amount of NOx excreted in the first 12 hr following admission was greater in survivors than in the fatal cases (P 0.03. by Wilcoxon rank test). This difference also disappeared when the NOx levels in the urine were corrected for renal function by dividing them by the total creatinine level in the same sample. The mean fractional clearance of NOx in the survivors was 17% compared with 12% in the fatal cases (not significant). Admission parasitaemia did not correlate significantly with either plasma, urine, or CSF nitrate levels.

Figure 12.

Plasma nitrate and nitrite (NOx) levels over time corrected for kidney function in patients with severe falciparum malaria in survivors and fatal cases. The two patients with elevated plasma NOx levels in the group with fatal cases both had severe kidney insufficiency, with calculated creatinine (creat) clearances of 4.0 ml/min (*) and 8.0 ml/min (*), respectively, at admission. Despite the correction of plasma NOx for kidney function by taking the ratio with plasma creatinine, this measure is still likely to be an overestimation of NOx production since fractional NOx excretion (F-NOx) decreases with deteriorating kidney function (Westfelt et al. 1995). In these patients, F-NOx at admission was 1.3% (*) and 10.5% (k), respectively, versus a mean F-NOx of 15.5% for all patients. h = hours.

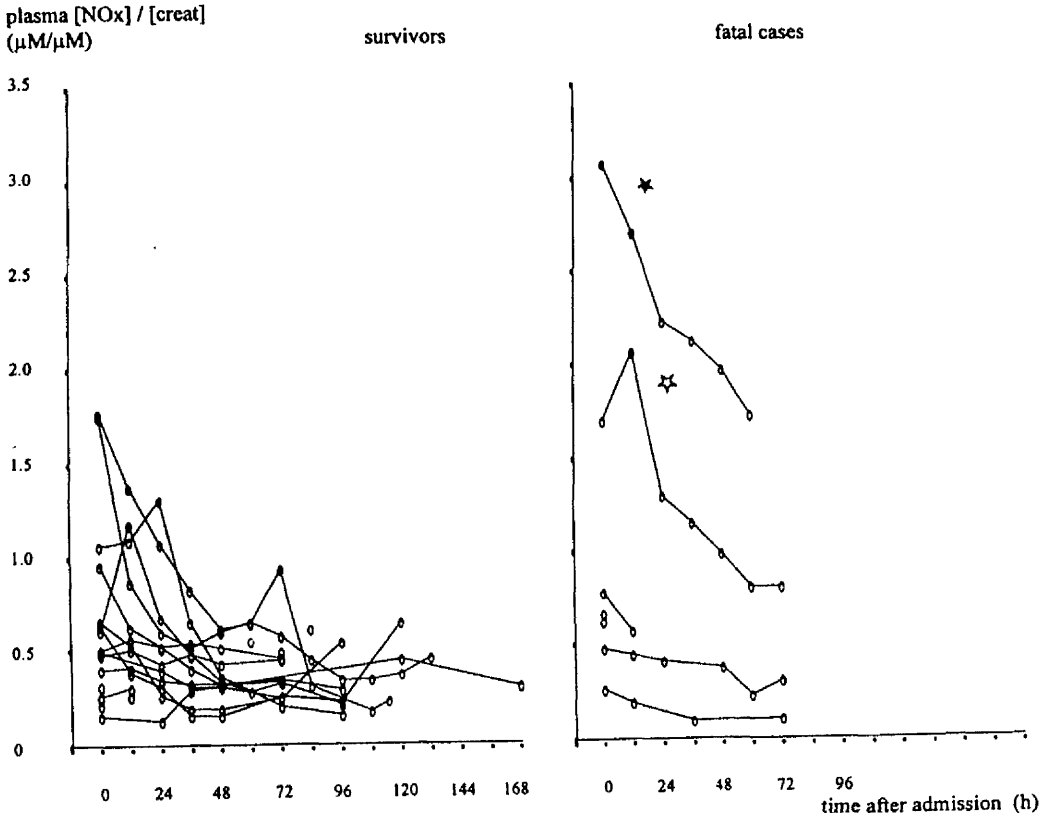
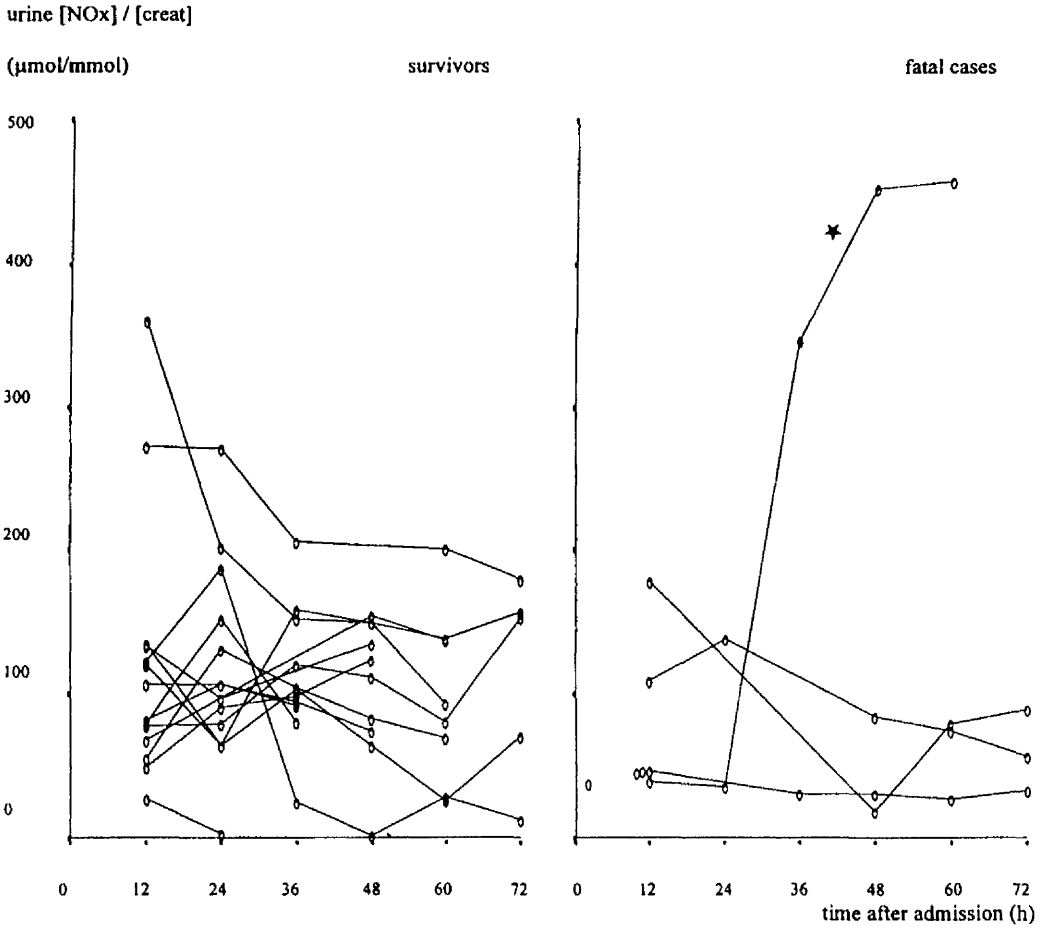


Figure 13.

Urinary nitrate and nitrite (NOx) levels over time corrected for kidney function in patients with severe falciparum malaria in survivors and fatal cases. The sharp increase in urinary NOx in one patients in the fatal group (*) could be attributed to a urinary tract infection. creatinine; h hours.



Plasma NOx levels were lower 48 hr after admission (Figure 12) (median value = 0.63 μM/μM versus 0.38 μM/μM; $P = 0.007$, by Wilcoxon matched pairs rank test) and also if the data were not corrected for kidney function (median value = 38.4

μmol/L versus 32.4 μmol/L; *P*= 0.04). The two patients with elevated plasma NOx levels in the group with fatal cases both had renal failure, with an estimated creatinine clearance on admission of < 5.0 mL/min and 8.0 mL/min, respectively. Correction of plasma NOx levels for renal function by taking the ratio with plasma creatinine may still overestimate NOx production since fractional NOx excretion (F-NOx) decreases with deteriorating kidney function (Anstey et al. 1996). In these patients, admission F-NOx values were 1.3% and 10.5%, respectively, versus a mean F-NOx of 15.5% for all patients.

Urinary NOx tended to decrease following admission (Figure 13). One patient who died showed a sharp increase in urinary NOx, but this was attributed to a coincident urinary tract infection. There was a significant correlation between nitrate levels in the CSF and the plasma. (Spearman correlation coefficient = 0.59, *P* = 0.045). We did not find a significant correlation between nitrate levels in the CSF and the Glasgow coma scale, nor with coma recovery time or survival. The CSF nitrate levels did not differ significantly between healthy controls (n = 5) and patients with severe malaria (n = 11), with median values of 2.4 μmol/L (range = 0 - 8.9 μmol/L) and 4.8 μmol/L (range 1.5 - 61.4 μmol/ L), respectively.

Discussion

In this study, we did not show a correlation between either plasma NOx levels or the total amount of NOx excreted in the urine, and disease severity or outcome in patients with severe falciparum malaria after correction of the values for renal function. From studies with inhaled NO in humans, it has become clear that about 70% of the NO is converted to NOx, and a smaller amount to methaemoglobin and

other molecules (Kelm et al. 1992; Westfelt et al. 1995). The NOx levels are therefore taken as a measure for total NO production, since NO itself has a very short half-life (Kelm and Schrader 1990). Although the sample size was rather small, the present study shows that overall NO production does not seem to be a predictor of disease outcome or severity in nonimmune adult patients. This study group differs from those in several recent studies performed on malarial patients in highly endemic areas. In these latter studies, the highest serum NOx levels were found in healthy controls or patients with asymptomatic parasitaemia, and it was concluded that high NOx levels might be associated with malarial tolerance rather than with disease severity (Anstey et al. 1996; Clark et al. 1996; Kremsner et al. 1996).

In our study, we showed a significant correlation between plasma NOx and creatinine levels. Patients with renal failure showed very high plasma NOx levels with only little NOx excreted in the urine. Because NOx excretion is dependent on renal function and severe malaria is associated with renal impairment, severe malaria can be associated with higher levels of plasma NOx. As F-NOx secretion decreases as renal function deteriorates, correction of plasma NOx levels by taking the ratio with plasma creatinine may still overestimate NOx production in patients with renal insufficiency (Anstey et al. 1996). Our results contrast with those of the study of Al-Yaman and others, who did not find a correlation between kidney function and plasma NOx levels in children with cerebral malaria in Papua New Guinea (Al-Yaman et al. 1997). However, of the 41 patients in their study who were evaluated for both plasma NOx levels and plasma creatinine, only four children had a slightly disturbed kidney function.

Nutritional status may also confound the interpretation of plasma NOx levels

(Granger, Miller, and Hibbs 1996; Mitchel, Shonl, and Grindley 1916). In our study, bias from exogenous nutritional sources of NOx also cannot be excluded, but most patients had probably eaten very little before admission. Since gastric or parenterally feeding is never started immediately, patients with severe malaria, such as those reported here, almost invariably have a period of starvation during the first days after admission. This period of starvation was associated with a decrease in plasma NOx levels. Since the plasma half-life of nitrates is approximately 8 hr when kidney function is normal, a much sharper decrease in NOx levels would be expected if levels on admission were only elevated because of NOx derived from food taken before admission (Jungertsen et al. 1993; Radomski, Palmiri, and Hearn 1978). It is likely that a decrease in endogenous NO production after treatment contributed to the decrease in plasma NOx levels.

The NOx levels in the CSF were all much lower than blood levels, suggesting that NO production in the central nervous system is much lower than in the blood compartment. There was a clear correlation between plasma and CSF levels of NOx. This can be interpreted either as reflecting free diffusion of nitrites and nitrates across the blood-brain barrier or that cerebral NO production is a reflection of NO production elsewhere in the body. Moreover, NOx levels in CSF did not correlate with the coma depth in our patients. This is in accordance with the findings in Ghanaian children, where transmission of falciparum malaria is high (Agbenyega et al. 1997).These findings do not support the hypothesis that high levels of intracerebral NO production impair neurotransmission in the fatal cases brain in severe falciparum malaria (Clark, Rockett, and Cowden 1991; Kremsner et al. 1996). This hypothesis was formulated without data on NOx levels in the CSF. The CSF levels of NOx,

however, might not be a good indicator of the local NO concentrations at the cerebral tissue level. For instance, in Alzheimer's disease, increased activity of inducible nitric oxide synthase (iNOS) can be measured in postmortem—obtained brain microvessels, without evidence of increased NOx levels in the CSF (Dorheim et al. 1994; Milstien et al. 1994). Similarly, micro vascular iNOS in the brain could be increased in cerebral malaria, thus influencing nearby synapses, without increased NOx levels in the CSF, but this hypothesis will be difficult to prove *in vivo*.

In conclusion, there was no correlation between NOx levels in plasma, urine, or CSF and disease severity in nonimmune patients with severe falciparum malaria in this study. Plasma NOx levels are dependent on renal function. High NOx levels in CSF did not correlate with coma depth in cerebral malaria and the CSF-levels were much lower than those found in the blood compartment. These findings do not support a pivotal role for NO in the pathogenesis of cerebral malaria. However, local overproduction of NO might not be reflected in total NOx production or CSF levels of NOx. Therefore, the role of NO in severe falciparum malaria remains to be elucidated.

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Pharmacokinetics and pharmacodynamics of dichloroacetate in children with lactic acidosis due to severe malaria

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Abstract

Lactic acidosis frequently complicates severe malaria in African children, and is a strong independent predictor of mortality. We tested the hypothesis that sodium dichloroacetate (DCA), an activator of pyruvate dehydrogenase, rapidly reduces hyperlactataemia in this patient population. Eighteen children with severe malaria and capillary plasma lactate > 5 mM were randomised to receive either intramuscular quinine plus a single 50 mg/kg intravenous infusion of DCA in saline, or quinine plus intravenous saline alone. Two patients in each treatment group died following randomisation. Thirty minutes after treatment, the mean plasma lactate was 28% below pre-treatment baseline values in the DCA group, but was unchanged in the placebo group. Throughout the first 4 h after treatment, mean plasma lactate in the DCA-treated patients was significantly less than that in controls (p= 0.003). Thereafter, mean plasma lactate declined in both groups and was <2 mM 10 h after treatment. DCA was well tolerated and did not alter quinine pharmacokinetics. A single intravenous dose of DCA rapidly improved lactic acidosis in African children with severe malaria, suggesting that DCA may be a useful adjunct in the initial treatment of these patients, and may increase their chance of survival by improving a major complication of their illness.

Introduction

Lactic acidosis is frequently associated with severe malaria and independently predicts a mortality of over 30% (Krishna et al. 1994b; Taylor, Borgstein, and Molyneux 1993). The treatment of lactic acidosis complicating *P. falciparum* infection relies on the rapid initiation of antimalarial therapy and correction of

exacerbating factors such as intravascular volume depletion, seizures and anaemia. No specific treatment of lactic acidosis has been assessed in children with malaria, although African children are the largest group at risk of death from this complication (Taylor, Borgstein, and Molyneux 1993).

Sodium dichloroacetate (DCA) stimulates aerobic consumption of lactate by activating pyruvate dehydrogenase, the mitochondrial enzyme that catalyses the rate-limiting step in the oxidation of lactate (via pyruvate) to acetyl CoA2 (Stacpoole 1993). The usefulness of DCA in children with malaria-associated lactic acidosis was anticipated on the basis that it rapidly corrects lactic acidosis in adults with severe malaria (Krishna et al. 1994a). The objectives of this study were therefore to carry out a detailed pharmacokinetic and pharmacodynamic study of DCA in children with lactic acidosis due to malaria, using a study design appropriate to circumstances prevailing in many African hospitals.

Methods

This study was approved by the Committee of Research, Publication and Ethics of the School of Medical Sciences, University of Science and Technology, Kumasi, Ghana. Fully informed written consent was obtained from attending relatives of enrolled patients. This was an open, randomized comparison of DCA vs. saline placebo given as an adjunct to conventional treatment in children with severe malaria aged 18 months to 10 years. Initially, 12 children were randomized in pairs to receive either one dose of DCA or an equivalent volume of normal saline. To ensure comparability between DCA- and placebo-treated groups, the six remaining patients were stratified for treatment on the basis of entry lactate concentrations. Criteria for

admission to the study were a confirmed diagnosis of severe falciparum malaria (asexual parasitaemia in a patient who either had strictly defined cerebral malaria or who looked severely ill, was unable to sit unaided, and required parenteral treatment) and an admission capillary blood lactate concentration ~5 mmol/l. To exclude confounding causes of coma, hypoglycaemia was corrected, a blood culture was taken and a lumbar puncture was performed to examine the cerebrospinal fluid.

Management

Patients admitted to the Department of Paediatrics at Komfo-Anokye Teaching Hospital Kumasi, Ghana, in November and December 1993 with the suspected diagnosis of severe malaria were referred to the study team. All patients received intravenous fluids while the diagnosis of malaria was confirmed by blood film examination. Hypoglycaemia was excluded by capillary glucose measurement (BM stix) and the patient was weighed. Two fine-gauge (22 or 23G) intravenous cannulae were inserted, one for the administration of intravenous fluids and DCAI placebo, and the other for sampling purposes.

Antimalarial and DCA treatment

After a rapid history and examination, and taking of baseline venous blood samples (250 µl for the immediate measurement of glucose, lactate and PCV), antimalarial treatment was begun (Waller et al. 1990). In patients who had not received previous quinine treatment, 20 mg salt/kg of quinine dihydrochloride (BP8O, Rotexmedich) was injected intramuscularly, half into each thigh, after dilution (1:1 v/v with sterile water for injection). Patients who had been pretreated with quinine received a maintenance dose (10 mg salt/kg diluted as before). Subsequent doses of quinine (10mg salt/kg i.m., also diluted as before) were repeated every 12 h in

alternate thighs from the commencement of the first dose until the patient recovered sufficiently to tolerate oral quinine, which was continued for a total of 7 days. DCA (Tokyo Kasei Kyogo) was formulated as a 10% solution (w/v) in saline and sterilized by filtration (Stacpoole et al. 1992). DCA was administered by intravenous infusion (50 mg/kg, over 10 mm) or an equivalent volume of saline was administered to those subjects randomized to receive placebo. DCA and placebo were administered immediately after quinine treatment had begun and venous access was established.

Supportive treatment

An infusion of 5% glucose was administered at a rate of 3 mg/kg/mm for a minimum of 24 h. All patients received a single dose of intravenous thiamine (100 mg, Northwick Park Hospital, Harrow) and comatose patients received a single intramuscular dose of phenobarbitone (7 mg/kg, Biomedicine SPRI) at the start of the study. Seizures occurring after admission were treated with intravenous diazepam (0.3 mg/kg, Diazemuls, Dumex), high temperatures (>38.5 ^{0}C) were treated with rectal paracetamol (120 mg paracetamol suppositories) and severe anaemia (PCV <15%) was treated with transfusions of HIV- and hepatitis-B-virus-screened packed cells given with frusemide (1 mg/kg, Antigen Pharmaceuticals). Secondary bacterial infections were managed with gentamicin (20 mg/2ml, Roussel 2 Labs) and ampicillin (Berk Pharmaceuticals).

Monitoring and sampling

Vital signs (respiratory rate, pulse, blood pressure) and the coma score assessed on the Blantyre scale (Molyneux et al. 1989) were monitored every 5 mm during the DCA infusion, then at 15 mm, 30 mm and 60 mm, followed by every 2 h for 12 h, and then at 16, 20 and 24 h after admission. Subsequent monitoring of vital signs was

every 6 h until patients recovered, and other clinical measures, such as time to sit and time to drink, were also noted as endpoints. Temperature was monitored at 30 and 60 mm, at 4 h and then with the other vital signs. The packed cell volume (PCV) and parasitaemia were monitored every 4—6 h for the first 24 h and then every 6 h until resolution of infection.

Whole blood (400 μl) was collected into chilled heparinised (10 IU) microcentrifuge tubes each time vital signs were obtained, and was transported on ice before separation and processing of plasma. Plasma glucose and lactate assays (50 μl) were carried out on site immediately after collection using YSI glucose and lactate analysers, as reported previously (Krishna et al. 1994a). The lactate assay uses lactate dehydrogenase, and is stereospecific for L(±)-lactate. Plasma for DCA and quinine measurements (200 μl) was stored at —20 °C, and transported on dry ice for assay.

Quinine and DCA assays

DCA in plasma was analysed by gas chromatography, as described (Curry et al. 1991). Briefly, plasma (100 μl) and trichloracetic acid (TCA, 50 μl of a 50 μg/mL solution as an internal standard) were mixed in a 16 x 100mm screw cap culture tube (Klimax), and BF_3 (1 ml, 14% solution in methanol) was added. The tube was vortexed, heated to 100^0C for 15 mm and cooled to room temperature. Cyclohexane (1 ml) and water (1 ml) were added to the mixture and the tube was agitated vigorously for 10 mm. After centrifugation (10 mm, 2500 g), the organic layer (top, 1 μl) was injected into a Varian 3600 gas chromatograph equipped with a 6 ft x 2 mm i.d. Chromosorb 101 column, a ^{63}Ni electron capture detector, and a PE Nelson Turbochrom 3 chromatographic integrator. The column temperature was 160 ^{0}C. DCA and TCA had retention times of 1.96 and 3.18 mm, respectively. The peak areas

obtained from the integrator were used in the determination of the ratio DCA:TCA. Every batch of DCA analysis involved calibration with known mixtures of DCA in plasma (2.5 to 200 μg/ml) and TCA. The coefficients of variation on the assay were 2.02% (intraday) and 3.52% (interday), and the calibration graph was linear. DCA in the treatment vials was also measured by gas chromatography, after 1000-fold dilution with deionised water.

Quinine was measured using high performance liquid chromatography as described previously (Mhoru et al. 1991). An aliquot of plasma (200 μl) with internal standard added (quinidine, 200 ng) was mixed with ammonia (0.5 ml) and incubated at room temperature (5 mm). Samples were extracted by vortexing (30 s) with a mixture of hexane:ethylacetate (5 ml, 1:1 v/v). After centrifugation (5 mm, 2000 g) the organic phases were discarded, and contents of the tubes evaporated under N_2 to dryness at 37 ^{0}C. After reconstitution in the mobile phase (150 μl), 20 μl was injected into the column. The inter- and intra-assay coefficients of variation for the assay were <8%.

Data analysis

One- and two-compartment pharmacokinetic analysis of DCA was carried out using PCNONLIN (v4, Statistical Consultants), and the most appropriate model selected on the basis of the Aikake Information Criterion and simplicity. Quinine profiles were analysed using Topfit (Schering) and a one-compartment model.

Statistical analysis used SYSTAT (v5.2, Evanston) or SAS programmes (SAS System Release 6.09, SAS Institute). Normally distributed data were analysed by Student's t test, and non-normally distributed data were analysed by the Kruskall-Wallis test or logarithmically transformed to a normal distribution before analysis.

Relative changes in lactate from baseline were analysed using the equation:

$$d_{tij} = \log_2(y_{tij}/y_{oij}) \quad (1)$$

where y_{ti} is the venous plasma lactate concentration measured at time t for patient i. For patients who received quinine only, (j=0) and for patients who received DCA and quinine (j = 1).

To estimate and test the effects of adding DCA to quinine therapy, repeated measures data (for lactate and vital signs) were analysed using the randomized coefficient regression modelling methods in Proc Mixed in the SAS system. We used maximum likelihood estimation without assumption of the pattern for the structure of the common within-group covariance matrix.

These models tested the effects of treatment (DCA plus quinine vs. quinine alone), the effects of time and the effects of interactions between treatment category and time. The early phase of changes in lactate concentrations (Phase 1, up to 4 h, see Results) required the addition of a quadratic component to the model to achieve an adequate goodness of fit, and the model is represented in the following equation:

$$d_{tij} = (\alpha + a_{ij}) + (\beta_j + b_{ij})\log_2\text{time} + \gamma_j(\log_2\text{time} - 0.5)^2 + e_{ij} \quad (2)$$

where $\Sigma_i = a_{ij} = 0$, $\Sigma_i = b_{ij} = 0$

The following simpler mixed linear model describes the later changes in lactate concentrations (Phases II and III, 4 h and subsequently):

$$d_{tij}=(\alpha + a_{ij}) + (\beta_j + b_{ij})\log_2 time + e_{ij} \quad (3)$$

In these models, the fixed effects for group j are the intercept α_j, the slope β_j, and the quadratic coefficient γ_j. The random effects, a_{ij} and b_{ij} allow individual patients to have intercepts and slopes that deviate from their groups' fixed values. A test of the primary hypothesis (that the addition of DCA produces differences in fixed effects from the recipients of quinine alone), was tested with standard log-likelihood X^2 tests comparing the above models with their null counterparts. For example, for the model described in (2), the null counterpart would be:

$$d_{tij}=(\alpha + a_i) + (\beta_j + b_i)\log_2 time + \gamma_j(\log_2 time - 0.5)^2 + e_{tij} \quad (4)$$

From these models we also computed estimates and 95% Cls for the differences between the groups' means on d at each time point.

Results

Patients

During the study period, 72 children suspected of having severe malaria were referred to the study team. The diagnosis of severe malaria was confirmed in 43 (60%), and 18 (42%) with capillary venous lactate concentrations >5 mM were entered into this study. Fourteen patients (78%) presented with a history of vomiting. The median(range) duration of fever *(n*= 18) before admission was 48(1 —96) h, the time after the last meal was 14(2—72) h, and time after the last drink was 4(2—72) h. Fourteen patients (78%) had a history of convulsions, 8(44%) in the DCA-treated

group. Five patients (28%) had opisthotonus, and one patient was hypoglycaemic (blood glucose ~2.2 mM) on admission. The two treatment groups were comparable for these variables (Table 7).

Table 7. Clinical characteristics of patients

Variable	Placebo (n=9)	DCA (n=9)
Sex (M/F)	5/4	4/5
Age (months)	49 ±29	39 ± 22
Weight (kg)	14.9 ± 5.7	11.7 ± 3.4
Pulse (/min)	148+11	150 ± 25
Respirations (1mm)	44 ± 11	55 ± 17
Blood pressure (mmHg) Systolic	122 +37	111 +16
Diastolic	62 ± 14	62 ± 11
Temperature ("C)	38 ± 1.0	37.8 ± 1.2
Liver (cm)s*	3.5(2—5)	3.5(0—5.5)
Spleen (cm)**	0.5(0—3)	1.0(0—3.5)
Cerebral malaria	5/9(56%)	4/9(44%)
Parasitaemia* (/μl) range	41 520 10—778 500	63215 10—758 600
Haematocrit (^{0}/o)	21+9	22+7

Data are median (range), proportion or mean ± SD, except

* geometric mean.

** Measured from the costal margin in the midclavicular line.

Clinical course of infection

Two patients in each treatment group died, all within 24 h of admission, giving an overall mortality of 22%. Eleven patients (61%) had seizures after admission which were often recurrent, partial motor in character, and sometimes difficult to distinguish from opisthotonus and decerebrate rigidity. Nine patients (50%) received blood transfusions, and in three patients (2 DCA, 1 placebo) a partial exchange transfusion was carried out because of high parasitaemia and/or severe anaemia. Clinical and parasitological measures of recovery in the two treatment groups are summarized in Table 8. There were no significant differences in these measures between treatment groups, except for the time to sit, which was slightly shorter in the placebo group (p=O.O48) because of one patient with particularly prolonged measures of recovery (Table 8).

Table 8. Clinical measures of recovery

Variable (h)	Placebo (n=5—8)	DCA (n=5—8)
Time for parasitaemia to fall by 50% of baseline (PC5O)	18(2.5—34)	21(2.5—36)
Time for parasitaemia to fall by 90% of baseline (PC9O)	23(19—36)	28(15—46)
Parasite clearance time (PCT)	37.5(20—48)	48(36—60)
Fever clearance time (FCT_B)	26(5—44)	36(5—54)

Time to drink	18(10—42)	32.25(2—68.4)
Time to eat	18(12—44)	40.8(5—112)
Time to sit unaided	42.5 (23.3—66.3)	67 (34—112)
Time to stand unaided	49(37.5—119.5)	114(49—191)

All values are medians (ranges).

Efficacy of dichloroacetate

The baseline geometric mean venous plasma L(+)-lactate concentrations in the two treatment groups were 8.2 mM in the placebo group, and 7.6 mM in the DCA group. Corresponding $\log_2$lactate mean $\pm$ SD values were 3.03 $\pm$ 0.72 and 2.92 $\pm$ 1.06 ($p>0.1$). Because venous lactate concentrations were analysed after $\log_2$transformation (equation 1), a difference of 1 is a two-fold difference in concentration, and values of d=0, -0.5, -1, -1.5, and -2 correspond to reductions from baseline of r=0%, 29%, 50%, 65%, and 75% ($r=2^d - 1$). Inspection of profile plots of the means of d (changes in lactate from baseline) relative to $\log_2$time in the two treatment groups revealed a nonlinear pattern characterized by phases (Figure 13 and Table 9). The effects on lactate profiles of adding DCA to quinine therapy were analysed separately for these phases, as described in Methods. These procedures used all the available data, and assumed that censoring due to death is not informative.

Phase 0 (time=5 min, 10 min) was too soon after the DCA infusion to discern differences between the DCA and control groups, and was therefore not analysed further. Phase I (t=30 min, 1 h, 2 h, 4 h) describes the clinically important reductions

in lactate concentrations in the two treatment categories because in this phase overall geometric mean lactate concentrations exceeded 4.5 mM (i.e. patients were grossly hyperlactataemic). There were no deaths in Phase I, and DCA recipients had significantly greater lactate reductions than controls (p=0.003, two-tailed), especially at 30 min and 1 h.

In Phase II (t=6 h, 8 h, 10 h, 12 h), geometric mean lactate concentrations fell below 3 mM , and the rate of fall in lactate concentrations increased compared with Phase I (i.e. the slope of decline in lactate concentrations was steeper). In this phase, lactate concentrations in the control group (quinine alone) declined more rapidly than DCA-treated patients (p=0.021). However, lactate concentrations had already fallen considerably at the start of Phase II, and mean lactate at 8 h was 2 mM in controls, and 2.6 mM in DCA recipients.

Phase III (t = 16 h, 20 h, 24 h) contained groups whose mean lactate concentrations were below 1.8 mM. There was considerable inter-patient variation in lactate profiles, without any apparent overall reductions, and no significant differences between groups (p=0.09). The four patients who died had the highest baseline venous lactate concentrations (p<0.001). A fatal outcome was associated with both higher lactate concentrations (p=0.001) and smaller reductions in lactate from baseline (p= 0.05) for the lactate measurement taken immediately prior to death (see footnote Table 9).

There was no evidence of toxicity with DCA. Using the same mixed linear models described above, no DCA effects were inferred for changes in glucose, pulse, respiratory rate and blood pressure.

Figure 14. Lactate profiles in a DCA-treated and b placebo-treated children with severe malaria.

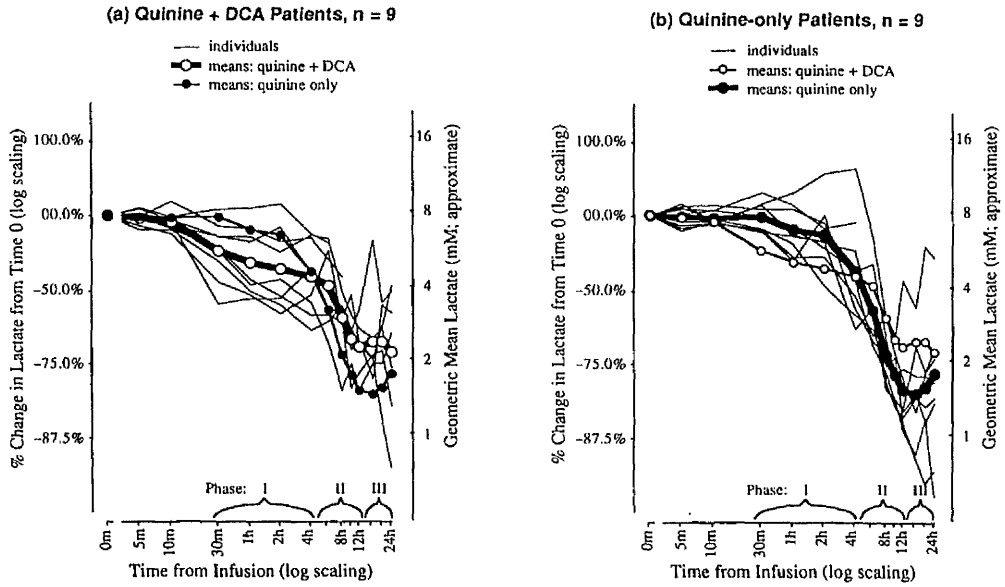

Figure 1. Lactate profiles in **a** DCA-treated and **b** placebo-treated children with severe malaria.

Table 9. Lactate levels and changes from baseline in children with severe malaria treated with quinine-only (Q) or DCA plus quinine (DCA + Q)

Table 3 Lactate levels and changes from baseline in children with severe malaria treated with quinine-only (Q) or DCA plus quinine (DCA+Q) (both $n=9$)

	Time	Lactate (mM) (geometric mean)		Change from baseline (d: mean ± SD)		% Change from baseline		Mean DCA effect on d (from mixed model)	
		Q	DCA+Q	Q	DCA+Q	Q	DCA+Q	Estimate	95% CI
Baseline	0 min	8.2	7.6						
Phase 0	5 min	8.2	7.4	−0.02 ± 0.14	−0.03 ± 0.10	−1	−2		
(not tested)	10 min	7.2	7.2	−0.05 ± 0.09	−0.09 ± 0.14	−3	−6		
Phase I	30 min	8.1	5.5	−0.02 ± 0.19	−0.47 ± 0.39	−1	−28	−0.43	[−0.74, −0.12]
($p=0.003$)	1 h	7.1	4.9	−0.20 ± 0.31	−0.63 ± 0.45	−13	−35	−0.49	[−0.83, −0.16]
	2 h	6.8	4.6	−0.27 ± 0.42	−0.71 ± 0.52	−17	−39	−0.38	[−0.79, +0.03]
	4 h	4.9[1]	4.3	−0.75 ± 0.68[1]	−0.82 ± 0.49	−41	−43	−0.09	[−0.61, +0.42]
Phase II	6 h	3.1	3.9[2]	−1.27 ± 0.53	−0.95 ± 0.52[2]	−59	−48	+0.36	[−0.12, +0.83]
($p=0.021$)	8 h	2.0	2.6[3]	−1.87 ± 0.39	−1.37 ± 0.46[3]	−73	−61	+0.48	[+0.13, +0.83]
	10 h	1.7	1.8	−2.15 ± 0.34	−1.66 ± 0.42	−77	−68	+0.57	[+0.19, +0.96]
	12 h	1.5	1.7	−2.35 ± 0.67	−1.76 ± 0.37	−80	−70	+0.65	[+0.17, +1.13]
Phase III	16 h	1.4	1.8	−2.40 ± 0.69	−1.71 ± 0.62	−81	−69	+0.72	[+0.04, +1.40]
($p=0.093$)	20 h	1.5[4]	1.8	−2.32 ± 0.92[4]	−1.70 ± 0.56	−80	−69	+0.55	[−0.19, +1.28]
	24 h	1.5	1.6	−2.14 ± 0.88	−1.83 ± 0.90	−77	−72	+0.40	[−0.52, +1.33]

[1,2,3,4] For each patient who died, the baseline lactate (rank/18, where 1/18 = minimum value), the time of final measurement, final lactate (rank/N, where 1/N = minimum value among N survivors at that time), and final d value (rank/N) are: [1] 17.4 mM (15/18), 4 h,16.4 mM (18/18), d = −0.09 (17/18); [2] 19.4 mM (17/18), 6 h, 15.0 mM (17/17), d = −0.37 (15/17); [3] 19.8 mM (18/18), 8 h, 11.2 mM (16/16), d = −0.82 (16/16); [4] 18.6 mM (16/18), 20 h, 2.8 mM (14/15), d = −2.73 (4/15).

Table 10. Pharmacokinetic parameters for DCA and quinine

Variable	DCA (n=8)	Quinine *(n*= 13)
Volume of distribution (V_d) (l/kg)	0.323 ± 0.097(0.1 6—0.5)	1.07 ± 0.27(0.75—1.71)
Area under the concentration-time curve (mg/l.h)	378 ± 65(237—441)	517 + 360(1 50—1 630)
Peak concentration (C_{min}) (mg/I)	170 ± 52(93—264)	15.5 + 3.26(9.6—21.6)
Half-life ($t_{1/2\beta}$) (h)	1.8±0.4(1.4—2.5)	23.5 ± 16.97(6.6—69.1)
Clearance (CL) (ml/min/kg)	2.68 ± 0.83(1.87—3.88)	0.725 + 0.43(0.17—1.84)
Mean residence time (h)	2.11 ±0.73(1.45—3.72)	—

Pharmacokinetics of DCA

Table 10 summarizes the pharmacokinetic parameters from eight patients who received DCA, and Figure 14 summarizes the changes in mean plasma concentrations of DCA with time. Spearman-correlations were computed between the individual slope values (b_{ij}) in Phases I and II for patients' lactate kinetics, and the following pharmacokinetic parameters: plasma half-life ($t_{1/2\beta}$)), apparent volume of distribution (V_d), area under the plasma concentrations curve (AUC), and peak plasma DCA concentration (C_{max}). The strongest relationship was between Phase I slope and AUC (r= -0.71), p=0.046). However, this result is not significant, given that only eight correlations were tested, and in this small study, no relationship was found between the derived pharmacokinetic and pharmacodynamic parameters for DCA.

Figure 15. Mean and individual DCA concentration-time profiles in eight patients ***a*** and quinine profiles ***b*** in 16 patients with severe malaria.

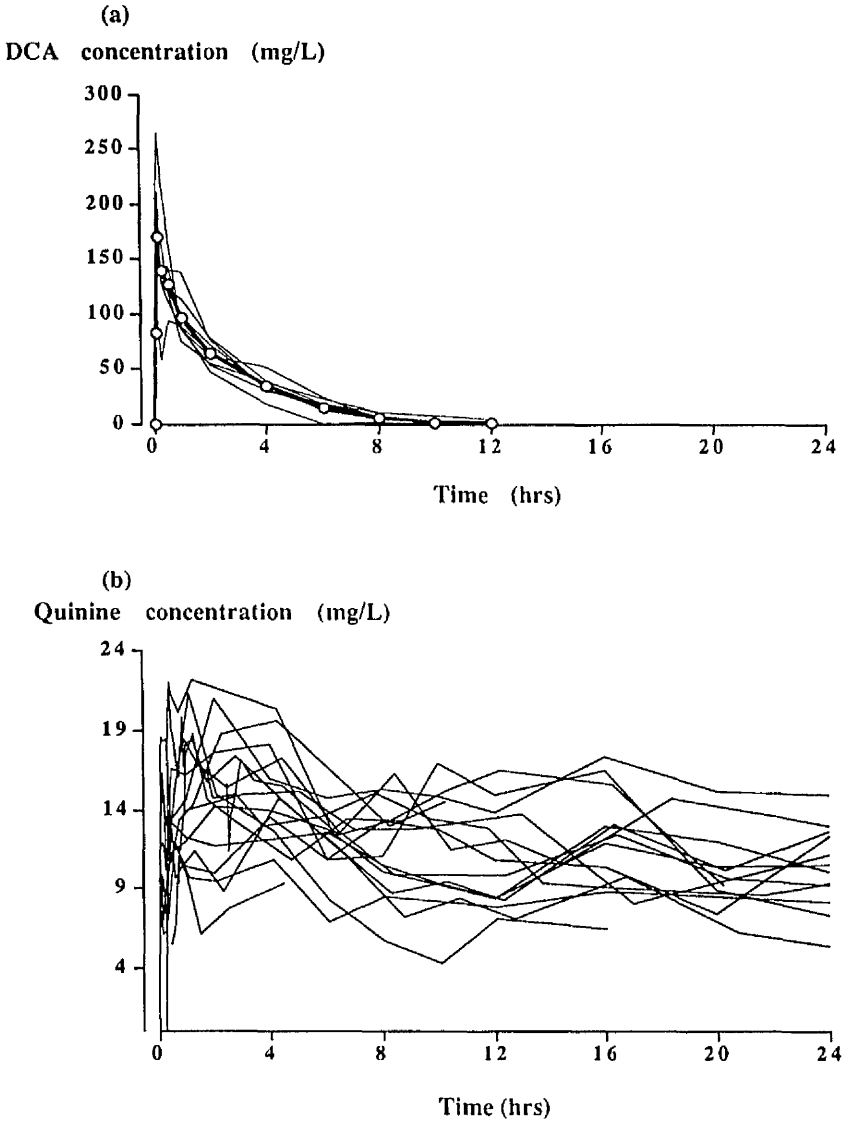

Figure 2. Mean (bold line with circles) and individual DCA concentration-time profiles in eight patients **a** and individual quinine profiles **b** in 16 patients with severe malaria.

Pharmacokinetics of quinine

Quinine was administered a mean ± SD of 16±4 mm before the DCA infusion was begun. Detailed pharmacokinetic evaluation was only available for 13 patients, and absorption kinetics on six patients, because in others the sampling schedule was

infrequent. The mean absorption half-time was 10.4 $\pm$ 9 mm. Quinine was absorbed relatively rapidly, without evidence of excessively high peak levels, and therefore gave satisfactory overall profiles (Figure 14). There were no significant differences in quinine pharmacokinetic parameters between DCA *(n= 7)* and placebo (n=6) recipients (p>O.l). The pharmacokinetic properties of this regimen of intramuscular quinine are summarized in Table 10. On discharge, three patients had small, non-tender, non-infected indurated lesions at the injection sites, and in one child similar lesions were tender to palpation. There was no other evidence of local toxicity.

Discussion

Lactic acidosis vies in frequency with cerebral malaria as a potentially lethal complication of severe *P. falciparum* infection (Waller et al. 1995). In children who survive an episode of severe malaria, lactic acidosis resolves rapidly with antimalarial and supportive treatment. In fatal cases, however, systemic lactic acidosis often persists, or worsens, up to the time of death (Krishna et al. 1994b). This sustained hyperlactataemia is the measurable endpoint of many processes that probably vary in their relative contributions from patient to patient. Such processes include diminished oxygen delivery to vital organs because of anaemia (Krishna et al. 1983; Krishna et al. 1994b) and microvascular obstruction by infected red cells (White and Ho 1992), increased tissue output of lactate due to seizures (Taylor, Borgstein, and Molyneux 1993), decreased lactate clearance by the liver (Pukrittayakamee et al. 1991), and the effects of increases in endogenous mediators, such as 'pro-inflammatory' cytokines (e.g. TNF and IL-lα) (Krishna et al. 1994b).

There is no established treatment for lactic acidosis associated with malaria. In this context, the use of DCA is based on the premise that it may prevent early deaths

from lactic acidosis and therefore allow antimalarial treatment time to reverse the underlying cause(s) of acidosis. Our studies have therefore focused on DCA as an adjunct to quinine therapy. In preliminary work, DCA attenuated hyperlactataemia in a *P. berghei*-infected/young Wistar rat model of severe malaria (Holloway, Krishna, and White 1991), and DCA was also effective in correcting lactic acidosis in Thai adults with severe malaria (Krishna et al. 1994a). To evaluate the efficacy of DCA in prolonging survival when lactic acidosis complicates malaria, DCA was added to quinine therapy in a large prospective study using the previously characterized rodent model of severe infection. A single dose of DCA given with quinine significantly prolonged survival of rats with severe malaria, compared with controls receiving quinine alone (Holloway et al. 1995). These initial studies have established the efficacy of DCA in severe malaria and suggest that it is a promising drug for further studies.

Most deaths in children with severe malaria occur in the first 24 h after admission. The first 4—8 h are therefore crucial in stabilizing a sick child, instituting antimalarial treatment, and treating complications (Waller et al. 1995). The current study confirms that within the first 4 h of admission, DCA corrects hyperlactataemia in children with lactic acidosis due to malaria more rapidly than supportive therapy alone. Hyperlactataemia predicted death in this study as well as in a previous larger investigation (Krishna et al. 1994b). The magnitude of the fall in lactate concentrations from baseline was smaller in patients who died than in survivors.

Four hours after admission, mean lactate concentrations had also fallen in the placebo group to relatively low levels (Phase II). Although the differences between placebo and DCA-treated patients were statistically significant during this period, they were small and not considered to be clinically important, as plasma lactate

concentrations were no longer grossly elevated. From 12 h after admission, differences in plasma lactate were no longer discernible between the treatment and placebo groups.

A single 10 mm infusion of DCA gave reproducible plasma concentration—time profiles (Figure 15). DCA was easily administered, rapidly effective and, when given in conjunction with a glucose infusion, non-toxic in this study. A glucose infusion was considered to be necessary, because glucose concentrations may also fall as pyruvate dehydrogenase is activated by DCA (Stacpoole 1989). The mean apparent volume of distribution (Vd=0.32 l/kg) of DCA in this study is lower than that observed in adults with severe malaria (Vd= 0.75 l/kg and Vd=0.44 l/kg in a further 11 adult patients with very severe malaria, unpublished observations) and the mean elimination half-life ($t_{1/2\beta}$= 1.8 h) is shorter (compared with adults $t_{1/2\beta}$= 2.3 h and $t_{1/2\beta}$= 3.4 h, unpublished). A single dose of DCA is probably sufficient to lower blood lactate in children with severe malaria, as conventional antimalarial and supportive treatment tend to normalize derangements in lactic acid metabolism within a few hours after admission. We have also studied two doses of DCA (46 mg/kg each) given 12 h apart to adults with very severe malaria, and shown that no significant additional metabolic improvement is observed when the effects of the second dose are compared with the hypolactataemic effects observed after the first dose (Krishna et *al.*, submitted).

In a previous study, undiluted intramuscular quinine (300 mg/ml, administered as an intramuscular loading dose of 20 mg/kg quinine salt, followed by 10 mg/kg every 12 h) produced acceptable plasma concentration-time profiles (Waller et al. 1990). In this study, similar profiles were observed for quinine given in the same dose

and frequency after 1:1 dilution. There were no significant differences between the mean elimination half-lives ($t_{1/2\beta}$), peak quinine concentrations (C_{max}) and clearances estimated in this study and those in the earlier one which used undiluted quinine ($p>0.5$ for all three pharmacokinetic parameters). The absorption half-times of quinine from the intramuscular site were, however, significantly more rapid when compared with undiluted quinine ($t_{1/2abs}$= 10.4±9 mm vs. 38+25 mm, respectively; p= 0.0031) and were similar to those observed after 1:5 (v/v) dilution of quinine ($t_{1/2abs}$=8.7+7.8 min (Winstanley et al. 1993). There was no evidence of major toxicity with quinine, or of interaction with DCA (Figure 15). Thus quinine diluted in an equal volume of sterile water and given intramuscularly is an appropriate regimen for children with severe malaria complicated by lactic acidosis. In summary, a single intravenous dose of DCA is an effective adjunct therapy to conventional antimalarial treatment for children with severe malaria complicated by lactic acidosis. As lactic acidosis frequently accompanies severe malaria and contributes to a fatal outcome, and DCA ameliorates lactic acidosis more rapidly than supportive treatment, DCA should now be evaluated in larger studies designed to assess its impact on mortality.

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Glucose and lactate kinetics in children with severe malaria

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Abstract

Children with severe malaria often present with lactic acidosis and hypoglycaemia. Although both complications independently predict mortality, mechanisms underlying their development are poorly understood. To study these metabolic derangements we sequentially allocated 21 children with falciparum malaria and capillary lactate concentrations ≥5mmol/l to receive either quinine or artesunate as antimalarial therapy, and dichloroacetate or saline placebo for lactic acidosis. We then administered a primed infusion (90 min) of L-[3-$^{13}C_1$] sodium lactate and D-[6,6-D_2] glucose to determine the kinetics of these substrates. Nineteen surviving children were comparable at entry to the study. The mean (SD) glucose disposal rate in all patients was 56(16) μmol/kg/min and geometric mean (range) lactate disposal rate was 100(66-177) μmol/kg/min. Glucose and lactate disposal rates were positively correlated (r=0.62, p=0.005). Artesunate was associated with faster parasite clearance, lower insulin/glucose ratios and higher glucose disposal rates than quinine. Lactate disposal was positively correlated with plasma lactate concentrations (r=0.66; p=0.002) and time to recovery from coma (r=0.82, p<0.001; n=15). Basal lactate disposal rates increased with dichloroacetate. Elevated glucose turnover in severe malaria mainly results from enhanced anaerobic glycolysis. Quinine differs from artesunate in its effects on glucose kinetics. Increased lactate production is the most important determinant of lactic acidosis.

Introduction

Malaria kills approximately 2 million African children a year. Many of these children die with metabolic complications of infection. Lactic acidosis complicates 35% of severe childhood malaria (Krishna et al. 1994b) and hypoglycaemia is present in 20%

of children with cerebral malaria (Newton and Krishna 1998). Both metabolic derangements commonly coexist, and each independently defines severe disease and predicts fatality in children (Allen et al. 1996; Krishna et al. 1994b; Newton and Krishna 1998; Taylor, Borgstein, and Molyneux 1993) and in adults (Hien et al. 1996; Krishna et al. 1994a; White et al. 1983).

The causes of hypoglycaemia in African children with malaria are incompletely understood. Hyperinsulinaemia rarely accompanies hypoglycaemia in children presenting to hospitals with severe malaria (Krishna et al. 1994b; White et al. 1987; White et al. 1983) although hyperinsulinaemia can complicate the treatment of children who receive quinine (Hensbroek et al. 1996; Krishna and White 1996). The kinetics of glucose has not been investigated in these populations despite the high risk of hypoglycaemia and the metabolic consequences of quinine have not been defined.

Although quinine is the preferred treatment for severe malaria in most geographic areas, artemisinin derivatives, such as artesunate, are an increasingly valuable therapeutic option (Hensbroek et al. 1996; Pukrittayakamee et al. 1994a). Artesunate is currently being investigated as an alternative to quinine in the management of African children with severe malaria, particularly when it is given by the intrarectal route in primary health care settings. However, artesunate's effects on intermediary metabolism and insulin dynamics in children with severe malaria have not been investigated.

Sustained lactic acidosis may be the only clinically attributable cause of death in some children (Krishna et al. 1994b), but its causes and management are not established. Dichloroacetate (DCA) improves lactic acidosis in children with severe malaria (Krishna et al. 1994a) and increases survival in a rodent model of lactic acidosis caused by

Plasmodium berghei infection (Holloway et al. 1995). However, the mechanism of action of DCA has not been studied in children with severe malaria.

We used stable isotopes to examine the kinetics of glucose and lactate in African children with malaria-associated lactic acidosis, and examined the effects of drugs used in the management of severe malaria. Children received quinine or artesunate as antimalarial therapy, and either DCA or placebo for hyperlactataemia. We hypothesised that quinine would have important effects on glucose metabolism, and that DCA would influence lactate kinetics.

Materials and Methods

The Committee of Research, Publication and Ethics of the School of Medical Sciences, University of Science and Technology, Kumasi, Ghana and the Institutional Review Board of the University of Florida approved this study. Informed consent was obtained from relatives of enrolled patients. This was a parallel, open-label comparison of quinine versus artesunate as antimalarial treatment, and DCA versus saline placebo given as an adjunctive treatment to children aged 11 months to 10 years who had severe malaria. Twenty-one children were allocated in strict sequence to one of four treatment categories: quinine and saline placebo (n=6), quinine and DCA (n=5), artesunate and placebo (n=5), or artesunate and DCA (n=5) if they had severe falciparum malaria (defined as asexual parasitaemia in a patient who either had strictly defined cerebral malaria or who looked severely ill, was unable to sit unaided, and required parenteral treatment (Waller et al. 1995)) and a capillary blood lactate concentration equal to or greater than 5mmol/l at the time of screening (Krishna et al. 1995). To exclude

confounding causes of other illnesses, a blood culture was obtained in all subjects and a lumbar puncture was performed in comatose patients.

Management

Patients who were referred for study were admitted to the Department of Paediatrics at Komfo-Anokye Teaching Hospital, Kumasi, Ghana, in October-November 1995 with the suspected diagnosis of severe malaria. The diagnosis of malaria was confirmed by examination of a blood film. Hypoglycaemia (glucose $\leq$2.2 mmol/l) was excluded by capillary glucose measurement (BM stix™) at the time of screening but all glucose estimations were confirmed as described below. Two intravenous cannulae were inserted. One peripheral line was to administer intravenous fluids and either DCA or placebo, and a second central line (5Fr 2 lumen or 5.5Fr multi-lumen catheter, Arrow-Howest™, Arrow International) was to administer 50% glucose to treat hypoglycaemia, to monitor circulating intravascular volume and to administer fluids and obtain samples for analyses.

Antimalarial and dichloroacetate treatment

Antimalarial treatment was begun immediately (Waller et al. 1990) after venous blood was obtained to measure glucose, lactate and haematocrit. In previously untreated patients who were randomised to receive quinine, 20 mg salt/kg of quinine dihydrochloride (BP1980, Martindale Pharmaceuticals Ltd, Romford, Essex, UK) was injected intramuscularly, half into each thigh after dilution 1:1 (v/v) with water or normal saline (Krishna et al. 1995). Patients pre-treated with quinine received a maintenance dose (10 mg salt/kg diluted as before) thereafter. Subsequent injections of quinine (10 mg salt/kg, diluted) were repeated every 12 hours in alternate thighs from the commencement of the first dose until the patient recovered sufficiently to tolerate oral quinine (quinine

sulphate suspension, 50 mg/5ml, Edikay Pharmacy Ltd, Kumasi, Ghana), which was administered as a 10mg/kg dose every eight hours for a total of seven days.

Patients who were randomised to receive artesunate (Guilin No 2 Pharmaceutical Factory, Guangxi, China) were given an intramuscular injection of 2.4 mg/kg (60 mg/ml) that was made up immediately before use in a 5% sodium bicarbonate solution that was supplied with drug. This dose was followed by intramuscular injections of 1.2 mg/kg artesunate 12 hours and 24 hours after admission, and subsequently 1.2 mg/kg daily, for a total of seven days (total dose of artesunate, 10.8 mg/kg). Treatment was changed to oral artesunate (nearest 1/4 tablet equivalent; 50 mg/tablet) as soon as patients could swallow oral medication. Sodium dichloroacetate (Tokyo Kasei Kyogo Ltd., Tokyo, Japan) was formulated as described previously (Stacpoole et al. 1992) and 50 mg/kg was administered by intravenous infusion over 10 minutes. Normal saline instead of DCA was administered to subjects who were randomised to receive placebo.

Supportive treatment

To minimise the risk of hypoglycaemia, all subjects received an infusion of 5% glucose in water, using a syringe pump, at a rate of 1 mg/kg/min for the duration of the stable isotope study. All patients received a single dose of intravenous thiamine (100 mg, Northwick Park Hospital, Harrow, UK) to correct possible sub-clinical thiamine deficiency (Krishna et al. 1999)(17). Patients who were comatose patients or who had seizures received a single intramuscular dose of phenobarbitone (7 mg/kg, Biomedicine SPRI, Brussels, Belgium) at the start of the study. Seizures after admission were treated with intravenous diazepam (0.3 mg/kg, Diazemuls, Dumex Ltd., Princes Risborough, Bucks, UK), high temperatures (≥38.5°C) were treated with rectal paracetamol (120 mg paracetamol suppositories, WHA, South West Thames Pharmacy Services Production

Unit, St. George's Hospital, London, UK) and severe anaemia (haematocrit less than or equal to 15%) was treated with transfusions of packed cells screened for HIV– and hepatitis B virus. Frusemide (1 mg/kg, Antigen Pharmaceuticals Ltd., Rosivia, Ireland) was also administered during each transfusion. Patients with secondary bacterial infections received gentamicin (20 mg/2ml, Roussel 2 Labs Ltd., Uxbridge, UK) and ampicillin (Berk Pharmaceuticals Ltd., Eastbourne, UK). Intravascular volume depletion was monitored measuring central venous pressure and corrected with intravenous saline infusions.

Assay for antimalarials

Previous treatment with antimalarials was determined in pre-treatment plasma samples by dipstick ELISA tests that detect the presence of chloroquine, quinine, mefloquine and pyrimethamine (Silamut et al. 1995)(18).

Infusion of L-[3-$^{13}C_1$] lactate and D-[6,6-D_2] glucose and sampling for stable isotopes

Stable isotope solutions were formulated in sterile water (15 ml) by mixing L-[3-$^{13}C_1$] sodium lactate and D-[6,6-D_2] glucose to final concentrations of 2.22% and 2.78% respectively (i.e. 0.333 g sodium lactate, and 0.417 g glucose per bottle). Patients were resuscitated and stabilised, and antimalarial and supportive therapies were begun. Baseline venous blood samples were obtained to determine the natural abundance of stable isotopes, and to quantitate glucose, lactate and insulin concentrations (-10 min, -5 min and just before DCA or placebo infusion was begun). An intravenous priming bolus of 0.135 ml/kg of stable isotope was then injected after the DCA/placebo infusion had stopped and an infusion of 0.404 ml/kg/h of isotope solution was administered by syringe pump (Graseby 3200, Graseby Medical Ltd., Herts. UK) immediately after the loading

dose of isotope was given. This infusion was continued for 90 minutes (13.48 mg/kg of lactate and 16.8885 mg/kg of glucose were infused for the duration of the study). Sixty minutes after the end of the DCA infusion, four samples (2 ml each) were taken at 10 minute intervals (i.e. 60, 70, 80 and 90 minutes after isotope infusion had begun) to measure glucose, lactate and insulin concentrations, and an aliquot of each sample at the last three time points was used to determine enrichment with stable isotopes. The study protocol is illustrated in Fig 14.

Monitoring and sampling

Vital signs (respiratory rate, pulse, blood pressure) and coma score (Blantyre scale (Molyneux et al. 1989)), were monitored every five minutes during the DCA infusion, then at 15, 30, 60, 70, 80 and 90 minutes during stable isotope infusion, then every six hours until patients recovered. Other clinical measures, such as time to sit and time to drink, were also noted as endpoints. Temperature was monitored at 30 and 60 minutes, then with the other vital signs. Haematocrit and parasitaemia were quantified every six hours until parasites were undetectable by blood smear examination.

Whole blood (400 μl) was collected in chilled, heparinised (20 IU) microcentrifuge tubes each time vital signs were obtained, and was transported on ice and centrifuged to isolate plasma. Venous glucose and lactate (50 μl whole blood) were measured immediately after sample collection, using YSI 2300 glucose and lactate analysers (Yellow Springs Instruments, Youngtown, OH). For consistency, aliquots of plasma samples collected for isotope analysis were reassayed on a YSI 2300 STAT combined glucose/lactate analyser. These data were used for analyses of glucose and lactate kinetics. Plasma (600 μl) was stored at -20°C and transported on dry ice for stable isotope measurements.

Analysis of stable isotopes

To determinate lactate enrichment of blood, plasma (100 μl) was made acidic by adding 50 μl of 0.1M HCl. The mixture was passed through a Dowex AG-50 x 8 exchange column (Sigma, St. Louis, MO), washed with 0.5 ml distilled water and collected in a silianized test tube. The solution was reduced under vacuum until almost dry and drying was completed under nitrogen. After preparing the t-butyldemethylsilyl derivative (Guyton et al. 1993) the lactate enrichment was determined using a Hewlett Packard 5790 gas chromatograph/5790 MSD fitted with a 30 mm x 0.25 mm x 0.25 I.D. x 0.25 μm SPB fused silica capillary column (Supelco, Bellefonte, PA). The derivatised lactate had a molecular mass of 318 amu. The electron impact spectrum at 70 ev had a mass spectrum with a weak M-15 peak at 303 amu (without a methyl group) and a strong M-57 peak at 261 (without a t-butyl group). Using selective ion monitoring, we monitored ion channels 261 and 262 for lactate enrichment. Each sample was analysed in triplicate. The coefficient of variation was less than 1.5%.

To determine glucose enrichment, 50 μl plasma samples were made acidic by adding 25 μl of 0.1M HCl. The mixture was passed through a Dowex AG-50 x 8 column, washed with distilled water, collected and dried completely under vacuum. The pentaacetate derivative of glucose was prepared by mixing acetic anhydride (200 μl) and pyridine (200 μl) and by incubating at room temperature overnight. The derivatised glucose was analysed using the same gas chromatograph/mass spectrometer as for lactate, under electron impact conditions. The derivatised glucose produced two peaks in the mass spectrometry analysis: α- and β–D-glucopyranose pentaacetate. The spectrum of each compound had a fragment of mass 242 amu, due to the loss of C-1 carbon of glucose (Biemann, DeJongh, and Schnoes 1963). Using selective ion monitoring of the α-D-

glucopyranose pentaacetate, three ion channels, 242, 243 and 244, were monitored for glucose enrichment. Each sample was analysed in triplicate. The coefficient of variation was less than 3%. The results were confirmed by also monitoring β -D-glucopyranose pentaacetate.

Calculations

The lactate mole percent excess (MPE) was calculated using the 262/261 ratio for samples obtained after 60 minutes of infusion (Rs) and the time zero (Ro), as shown in the following equation.

$$MPE = \frac{Rs - Ro}{1 + (Rs - Ro)}$$

For D-[6,6-D_2] glucose, the 244/242 tracer ratio was corrected for contribution of singly enriched molecules, as suggested by Rosenblatt and Wolfe (Rosenblatt et al. 1992). Lactate and glucose disposal rates were calculated by a formula (Rosenblatt and Wolfe 1988) that corrects for the change in pool size from the exogenous lactate or glucose, ^{13}C natural abundance, and the enrichment of infusate. The rate of gluconeogenesis was obtained by assuming that, in fasted children such as those with severe malaria, the rate of appearance of glucose from glycogenolysis was negligible (White et al. 1987). Hence, gluconeogenesis becomes the glucose disposal rate minus the rate of exogenous glucose administration.

Data analysis

SYSTAT (v5.2, Evanston, Il) and Stata (v5, College Station, TX) was used for statistical analysis. Normally distributed data (assessed by Schapiro-Wilk *W* test) were

analysed by Student's t test or ANOVA and Pearson's correlation coefficient. Non-normally distributed data were analysed by the Wilcoxon Rank sum test, and correlations were determined by Spearman's rho. The assumption of steady state was examined by a test for trend. Repeated measures data were analysed using MANOVA with specified linear general hypotheses. Insulin levels below the level of detection (<10 pmol/l) were coded as 1 pmol/l for purposes of statistical analysis.

Results

Patients

During the study period 86 consecutive children were referred to the study team, and 54 of these had *P. falciparum* infection. The majority did not meet one of the study's entry criteria (screening lactate $\geq$ 5mM) leaving 24 with lactate concentration $\geq$ 5 mM. Twenty-one of these 24 children were subsequently entered into this study. Of the remaining 3, 1 child's parents refused consent and 2 children could not be studied because of difficulties with venous access. The clinical and laboratory characteristics of children in different treatment categories were comparable (Table 11). For all children, the median (range) duration of fever before admission was 48 (0.5-96) h, the time after food was last taken was 8.5 (4-24) h and the time after the last drink was 6 (2-15) h. Fifteen children (71%) presented with cerebral malaria (unrousable coma for at least 0.5 h following a seizure), five (24%) children were hypoglycaemic (glucose $\leq$2.2 mmol/l) on admission and five children (24%) presented with or subsequently developed retinal haemorrhages. All blood cultures and cerebrospinal fluid samples obtained on admission to the study were negative for pathogenic organisms.

Clinical course

Two patients (one who received quinine and placebo, and one who received artesunate and DCA) died soon after randomisation, so 19 patients were included in metabolic studies. Another patient who received artesunate and DCA died 120 hours after entry into the study (overall mortality = 14%). Ten patients (48%) required transfusion, six patients (29%) had seizures after admission and two boys (10%) developed haemoglobinuria. At the time of admission, three patients had chloroquine detectable in plasma samples, but no subject had detectable plasma levels of quinine, mefloquine or pyrimethamine. Patients received DCA a mean (range) of 35 min (8 min-75 min) after antimalarial treatment began, and after correction of hypoglycaemia, when appropriate. Table 12 summarizes the clinical and parasitological measures of recovery for the two different antimalarial treatments.

Despite the relatively small number of patients in each category, parasite clearance (estimated as time to fall to 50% and 90% of admission values, Table 12) was significantly more rapid with artesunate than with quinine. However, more rapid parasite clearance was not reflected in median times to recover from coma, to eat, to sit unaided, to be discharged from hospital or to become afebrile. Parasite clearance times were not related to admission parasitaemia, hepatomegaly, haematocrit or fever.

The admission geometric mean plasma lactate concentration in patients who died was higher than in patients who survived (12.4 mmol/l vs. 6.0 mmol/l; $p<0.0001$), and were also higher in those who presented with hypoglycaemia (geometric mean 10.7 mmol/l vs. 5.7; $p=0.013$). The parasite clearance time (PCT, Table 12) and the admission plasma lactate concentration were correlated ($r=0.54$, $p=0.017$; $n=19$), but parasitaemia and plasma lactate were not ($r=0.35$; $p=0.12$). Admission glucose levels were positively correlated with parasitaemia ($r=0.501$; $p=0.02$). There was no relationship between other

clinical or parasitological estimates of recovery (Table 12) and admission lactate concentrations, and there were no effects of DCA on these variables when DCA was included as a covariate with either antimalarial drug.

Metabolic changes

To establish that kinetic measurements were carried out under steady state conditions, linear regression analysis of data points obtained at sampling times after 60 min was used to estimate slopes for lactate and glucose disposal for each patient. The mean (95%CI) values of slopes for lactate and glucose disposal were -0.12(-0.62 to 0.38) and -0.08(-0.65 to 0.50), respectively, and were not significantly different ($p > 0.1$) from zero. Admission plasma glucose and lactate levels were not correlated with each other ($r=0.15$, $p=0.52$), but glucose and lactate disposal rates were positively correlated ($r=0.62$, $p=0.005$). The mean (SD) Ra for glucose was 56(16) μmol/kg/min and the geometric mean (range) Ra for lactate was 100 (66-177) μmol/kg/min.

In all patients, glucose disposal was not related to admission glucose values ($r=0.35$; $p=0.15$) or admission insulin concentrations ($r=0.04$; $p=0.86$) or age of patient ($r=0.025$; $p=0.92$). By contrast, lactate disposal was positively correlated with admission lactate concentrations ($r=0.66$; $p=0.002$). Lactate disposal was also positively correlated with the time to recovery from coma in patients with cerebral malaria ($r=0.84$, $p<0.001$; $n=15$). There was no relationship between admission parasitaemia or temperature and measures of glucose or lactate kinetics (not shown).

Thirteen patients had sustained hyperlactataemia (lactate concentrations $\geq$ 5mmol/l), both at screening and at the time of admission to the study (six in the quinine or DCA treatment categories and seven in the artesunate or placebo categories) and represented children with the most severe metabolic derangement. The following results

are therefore presented for 19 patients who were studied and separately for the subgroup of 13 more severely ill patients. Because the subgroup analysis was not specified prospectively, the results are exploratory.

Effects of antimalarials

Glucose

The plasma glucose and lactate concentrations obtained at the time of admission and during kinetic investigations were similar in patients who received quinine or artesunate (Table 11). Insulin levels at the start of the kinetic study were slightly higher in quinine-treated compared with artesunate-treated patients ($p= 0.14$) and were correlated with insulin levels measured after 60 min in the quinine group ($r=0.99$; $p<0.001$) but not the artesunate group ($r=0.30$; $p=0.41$). Insulin/glucose ratios were significantly higher in quinine-treated patients compared to subjects treated with artesunate during the study ($F=4.45$; $p=0.05$, with repeated measures analysis, Fig 16).

Glucose disposal was significantly lower in quinine-treated patients compared with those who received artesunate (Table 13, $p=0.04$). This difference was more obvious in the subgroup of patients who remained hyperlactataemic from the time of screening to the start of the study. In these individuals the mean ± SD glucose disposal was 49.2 ± 6.6 for subjects treated with quinine and was 70±15 μmol/kg/min for subjects administered artesunate ($p=0.011$, Fig 17). Gluconeogenesis, calculated from glucose disposal, was also significantly lower after treatment with quinine, compared with treatment with artesunate (Table 12), and accounted for a median (range) of 89.9% (83.7-93.6%) of the Ra values for glucose.

Lactate

There were no differences in lactate disposal between the two antimalarial treatment groups as a whole (Figure 18), nor in the more severely ill patients with sustained acidosis.

Effects of dichloroacetate

Glucose

Glucose disposal was not significantly influenced by DCA, either in the group as a whole or in those patients with sustained hyperlactataemia.

Lactate

The mean (95% CI) change in venous plasma lactate concentrations following DCA was -39(-55 to -22)% at the end of the study period (90 min) and was greater than the fall in plasma lactate after placebo administration -15(-55 to +50)%. However, within group variances obscured statistically significant differences in these two treatment groups. Lactate disposal was positively correlated with admission lactate levels ($r=0.66$, see above) and was not significantly different between patients who received DCA and those who did not (Table 12, $p=0.6$). However, in the 13 more severely ill patients with sustained hyperlactataemia, there was a highly significant difference in mean (95%CI) lactate disposal between DCA-treated patients (134(109-160) μmol/kg/min) and placebo-treated patients (97(80-114) μmol/kg/min; $t=-3.2$, $p<0.01$, Fig 18).

Discussion

To optimise the management of hypoglycaemia and lactic acidosis in children with severe malaria, and to devise new treatment regimens, it is critical to understand the mechanisms that underlie these complications (White et al. 1987; White and Ho 1992). Studies in southeast Asian adults (White and Ho 1992), and in African children with uncomplicated malaria (Dekker et al. 1997a; Dekker et al. 1997b; Dekker et al. 1996) cannot be extrapolated to children with severe disease. Unravelling pathophysiological mechanisms in severely ill children is particularly complicated because both malaria itself and antimalarial drugs, such as quinine, contribute to metabolic derangements.

The Ra for glucose in our patients is approximately five times the value estimated in adults with severe malaria treated with quinine (Ra glucose~10 μmol/kg.min (Davis et al. 1993)). Glucose turnover is also much higher in children with severe malaria (Ra in this study = 56 μmol/kg.min), compared to children suffering uncomplicated infections with *P. falciparum* (Ra glucose ~ 26μmol/kg.min, (Dekker et al. 1997a)). Normal children have approximately threefold higher rates of glucose production (~35 μmol/kg.min) than do adults, and age influences these estimates significantly (Bier et al. 1977). We were unable to detect any influence of age on our glucose turnover estimates, as in our patients, the range of ages was relatively limited. To assess the metabolic effects of antimalarials, we allocated children to receive quinine or artesunate and evaluated their effects on glucose and lactate metabolism.

Quinine use is associated with higher mean insulin/glucose ratios and lower glucose turnover rates (by ~30%) compared to artesunate (Table 12). In adults with severe malaria, there is a similar decrease (by ~40%) in glucose turnover following treatment with quinine (Davis et al. 1993). Although insulin increases glucose utilisation by

peripheral tissues and decreases gluconeogenesis by the liver, adults with severe malaria demonstrate peripheral resistance to insulin's action (Binh et al. 1997). This suggests that the predominant effect of the relative hyperinsulinaemia (*i.e.* higher insulin/glucose ratios) in children who received quinine in our study was to decrease hepatic glucose production. Alternatively, there may be a significant non-insulin mediated component to glucose disposal in these patients, as was suggested to occur in adult patients with severe malaria who received quinine (Binh et al. 1997). Plasma levels of glucose did not fall to hypoglycaemic levels ($\leq$ 2.2 mmol/l) partly because a constant infusion of glucose supplied approximately 10% of the total glucose requirements in these closely monitored individuals.

Our findings provide the first evidence that there are important differences in the effects of quinine and artesunate on glucose metabolism. Taken together with results from two large prospective studies (Hensbroek et al. 1996; Hien et al. 1996) that found artemether to be associated with fewer episodes of post-admission hypoglycaemia compared to quinine, our data indicate that wherever monitoring of plasma glucose is difficult, an artemisinin derivative may offer important advantages over quinine for children who are severely ill with malaria.

The fact that glucose and lactate kinetics are positively correlated in our subjects suggests that glucose is consumed predominantly through anaerobic pathways. This interpretation is consistent with our current understanding of the pathophysiology of lactic acidosis in severe malaria (Newton and Krishna 1998) and with the positive correlation we observed between Ra for lactate and plasma lactate concentrations. These results also suggest that increased production of lactate (rather than decreased disposal) is the most important determinant of hyperlactataemia in severe malaria in African children.

Increased anaerobic glycolysis in severe malaria may be due to tissue hypoxia, to severe anaemia, enhanced skeletal muscle activity during seizures, the metabolic demands of parasitised erythrocytes, inhibition of mitochondrial glucose oxidation by host cells due to thiamine deficiency (Krishna et al. 1999), fever and associated increased cytokine production (Krishna et al. 1994b) and diminished clearance of lactate by the liver due to decreased hepatic blood flow (Pukrittayakamee et al. 1991; Pukrittayakamee et al. 1994b).

The positive correlation between duration of coma and the lactate disposal rates is consistent with the hypothesis that microvascular obstruction, due to sequestration of infected erythrocytes, is an underlying mechanism that is common to the development of both cerebral malaria and lactic acidosis.

Overall, there was an elevated turnover of lactate in our study group when compared with similar measurements in other patient populations (*e.g.* 36 μmol/kg.min in healthy adults (Cowett and Wolfe 1991)). Values for lactate kinetics in our children were comparable to those obtained in non-acidotic adults with severe malaria who were treated with quinine (85μmol/kg.min, (Davis et al. 1996)).

DCA rapidly lowers plasma lactate concentrations in both adults and children with severe malaria (Krishna et al. 1995; Krishna et al. 1994a; Krishna et al. 1996). DCA treatment in patients who had sustained hyperlactataemia was also associated with a higher Ra for lactate. This suggests that basal lactate disposal is still capable of being augmented in these patients.

DCA stimulates pyruvate dehydrogenase and thus accelerates the oxidative removal of glucose, pyruvate and lactate (Krishna et al. 1996). It improved survival in an animal model of lactic acidosis due to malaria, when it was administered together with quinine

(Holloway et al. 1995). DCA did not affect glucose concentrations or kinetics in our patients. This suggests that it is unlikely to cause hypoglycaemia in children with severe malaria who receive glucose supplementation. These effects, together with DCA's stability and ease of use, make it a potentially important intervention to improve mortality in patients severe malaria complicated by lactic acidosis.

Figure 16. Insulin/glucose ratios in patients receiving artesunate or quinine

The study protocol and sampling schedule are shown at the top of the graph. Mean (+ or - SEM) insulin/glucose ratios are given for patient who received artesunate (open circles) or quinine (filled squares) during the course of the study.

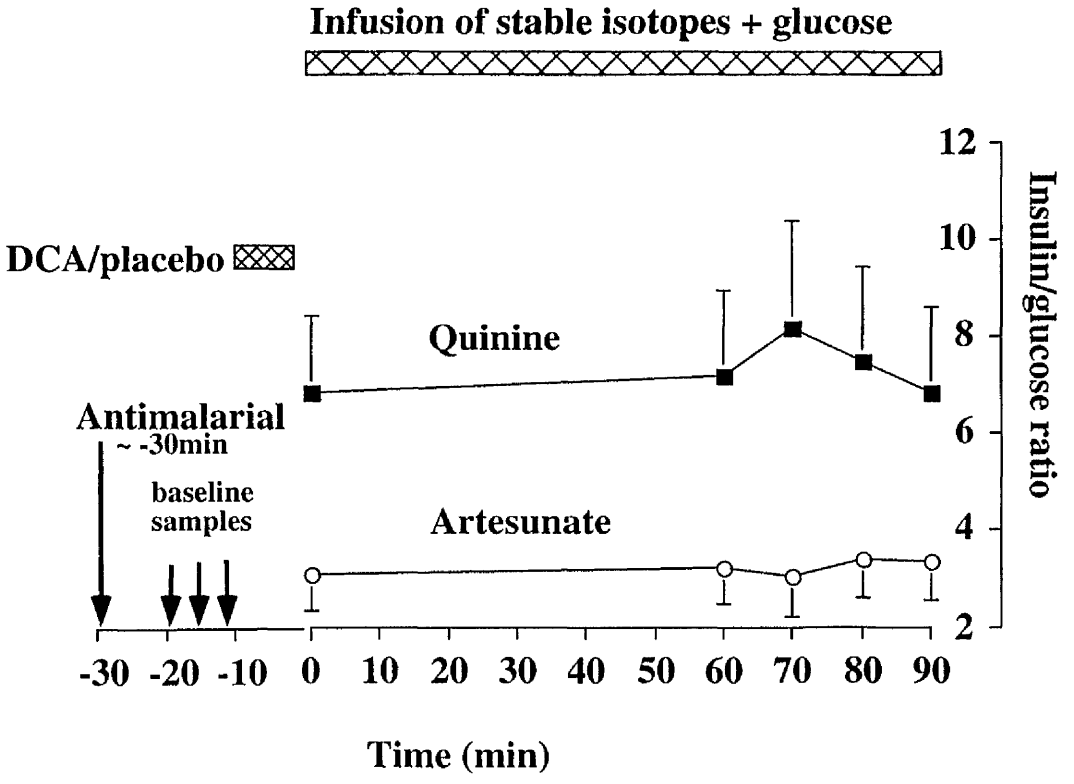

Figure 17. Glucose disposal after treatment with artesunate and quinine

Glucose disposal rates were determined for patients who were hyperlactataemic (≥ 5mmol/l) on admission to the study (filled circles) and for those whose lactates had fallen to <5mmol/l from the time of screening to the time of admission to study (open circles).

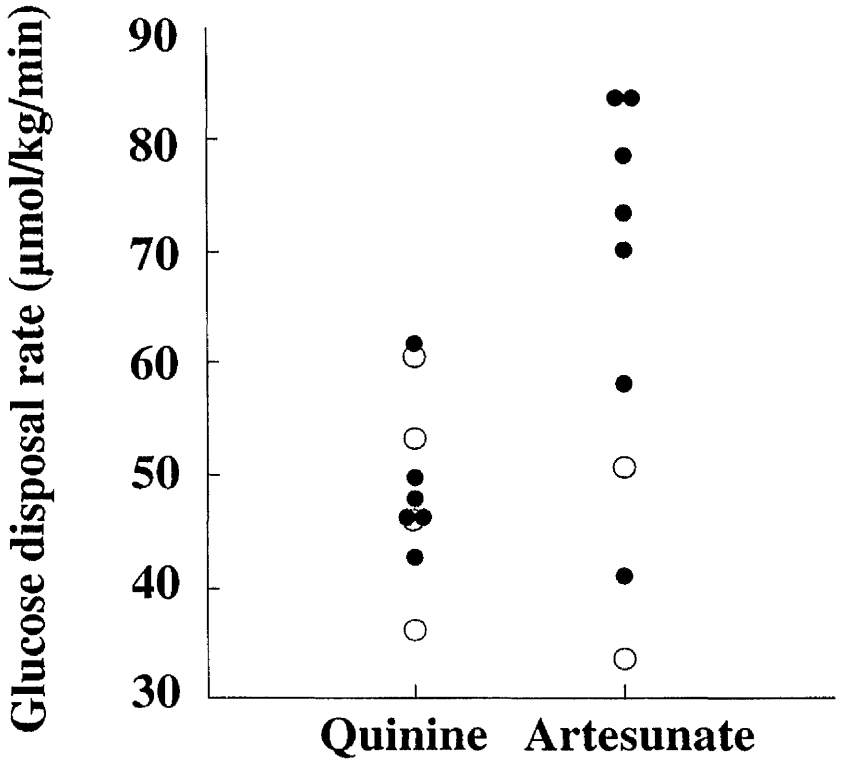

Figure 18. Lactate disposal after treatment with DCA or placebo

Lactate disposal rates were determined for patients who were hyperlactataemic (≥ 5mmol/l) on admission to the study (filled circles) and for those whose lactates had fallen to <5mmol/l from the time of screening to the time of admission to study (open circles).

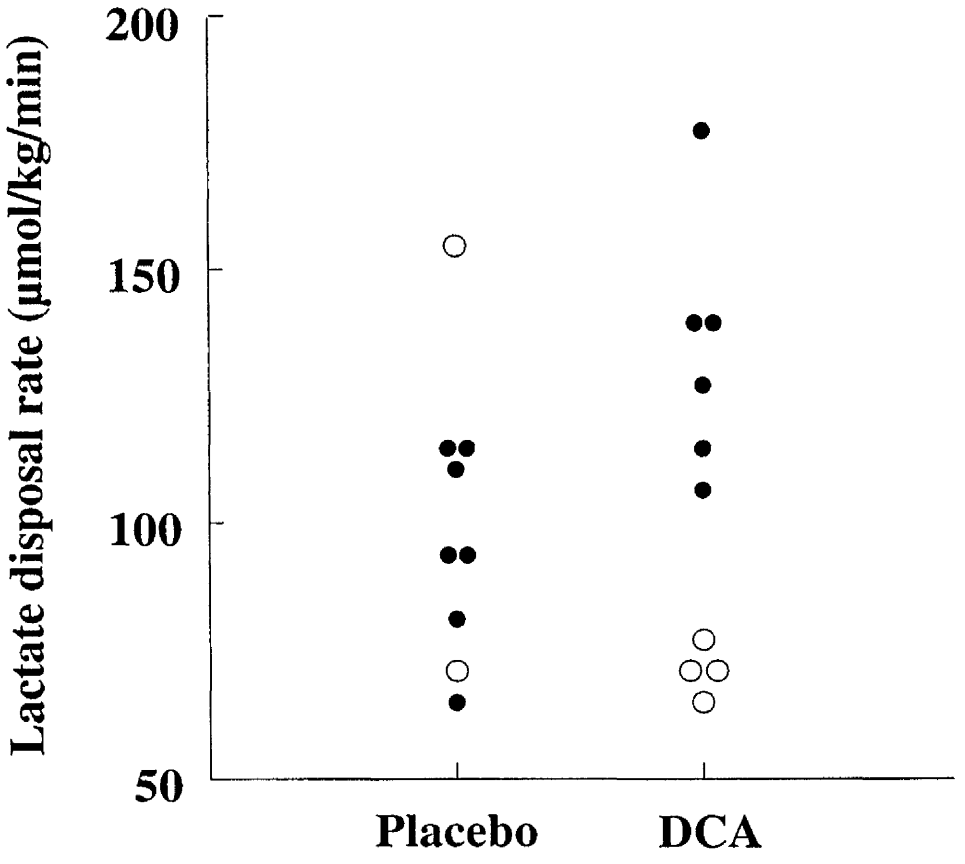

Table 11. Admission clinical and laboratory variables in patients.

Variable	Quinine (n = 6)	Quinine + DCA (n = 5)	Artesunate (n = 5)	Artesunate + DCA (n = 5)
Sex (F/M)	5F/1M	2F/3M	2F/3M	2F/3M
Age (months)	39 (18 – 72)	48 (18 – 84)	18 (11 – 69)	36 (18 – 72)
Body Mass Index (kg/m^2)	15.7 (4.4)	15.1 (1.2)	15.5 (1)	15.8 (1.2)
Heart rate (beats/min)	149 (16)	161 (13)	146 (16)	140 (28)
Respiratory rate (breaths/min)	56 (7.5)	50 (21	58 (15)	47 (12)
Mean arterial blood pressure (mmHg)	69 (16)	73 (5)	63 (13)	72 (22)
Axillary temperature (°C)	37.8 (1.6)	38.2 (0.3)	37.2 (0.8)	38.3 (0.6)
Liver (cm)[a]	3.5 (3 – 4.5)	4 (0 – 6)	3 (0 – 5)	3 (0 – 4)
Spleen (cm)[a]	0 (0 – 3)	1 (0 – 7)	1.5 (0 – 6)	3 (0 – 8)
Cerebral malaria	3	3	4	5
Parasitaemia (no./µl)	602,050 298,930-	236,930 38,180-	168,380 42,700–411,470	153,060 26,380–960,590

Range	1,798,840	761,140		
Haematocrit (%)	23 (9)	23 (7)	19 (6)	25 (5.5)

Table 11 (continued). Admission clinical and laboratory variables in patients.

Variable	All Quinine (n = 11)	All artesunate (n = 10)	All placebo (n = 11)	All DCA (n = 10)
Sex (F/M)	10F/1M	4F/6M	7F/4M	7F/3M
Age (months)	48 (18 – 84)	30 (11 – 72)	30 (11 – 72)	48 (18 – 84)
Body Mass Index (kg/m^2)	15.4 (3.2)	16(1)	16 (3)	15 (1.2)
Heart rate (beats/min)	155 (16)	143 (22)	148 (16)	151 (23)
Respiratory rate (breaths/min)	54 (15)	52 (14)	57 (11)	49 (16)
Mean arterial blood pressure (mmHg)	71(12)	68 (18)	66 (14)	72 (15)
Axillary temperature (^oC)	38 (1.2)	37.7 (0.9)	37.6 (1.3)	38.2 (0.4)
Liver (cm)[a]	4 (0 – 6)	3 (0 – 5)	3 (0 – 5)	3.3 (0 – 6)
Spleen (cm)[a]	0 (0 – 7)	2.5 (0 – 8)	0 (0 – 6)	1.5 (0 – 8)
Cerebral malaria	6	9	7	8
Parasitaemia (no./μl)	394,040 38,180–	159,740 26,380–	337,360 42,700-1,798,840	189,470 26,380-960,590

Range	1,798,840	960,590		
Haematocrit (%)	23 (8)	22 (6)	21 (8)	24 (6)

All data are presented as the mean (SD) or the median (range) values unless otherwise specified

[a] Measured from the intercostals margin in the mid-clavicular line.

Table 12. Clinical and parasitological measures of recovery after quinine and artesunate

Variable (h)	Quinine (n = 11)	Artesunate (n = 10)	Difference (*P*)
PC_{50}	19.5 (7.8)	10.9 (7.9)	0.025
PC_{90}	28.6 (5.6)	18.7 (7.1)	0.003
PCT	48 (30 – 72)	36 (24 – 72)	0.057
FCT	36.5 (0 – 116)	18 (0 – 89)	0.35
Coma recovery[a]	8 (3 – 72)	12 (1.5 – 72)	0.70

All data are the mean (SD) or the median (range) values. PC_{50} and PC_{90}, Times for parasitaemia to fall to 50% and 90% from baseline values; PCT, parasite clearance time (*i.e.* the first time point at which blood films become negative for malarial parasites); FCT, time for axillary temperature to fall and remain below 37 °C.

[a] The numbers of patients with cerebral malaria are given in Table 13.

Table 13. Metabolic data in patients according to treatment.

Variable	Quinine (n = 6)	Quinine + DCA (n = 5)	Artesunate (n = 5)	Artesunate +DCA (n = 5)
- 5 min				
Plasma glucose (mmol/L)	6.79 ± 4.01 6.12(4.38–14.8)	7.32 ± 5.15 6.19(3-16.1)	5.59 ± 0.5 5.57(4.93-6.21)	7.03 ± 2.27 6.76(4.54-10.7)
Plasma lactate (mmol/L)	9.12 ± 4.3 8.23(4.38-14.5)	5.93 ± 3.06 5.43(3.66-11.2)	5.47 ± 1.94 5.15(2.66-8.06)	8.92 ± 4.02 8.07(3.51-14.3)
Plasma insulin (pmol/L)	26 (16-56)	44 (16-230)	10.7 (1-36)	8.9 (1-52)
Plasma insulin/ glucose ratio	4.9 ± 2.6	9.1 ± 7	3 ± 2.2	3.2 ± 2.8
60 min				
Plasma glucose (mmol/L)	6.57 ± 2.86 6.15(4.34-11.2)	6.02 ± 3.4 5.4(3.17-11.8)	6.1 ± 0.28 6.08(5.6-6.93)	6.48 ± 3.32 5.95(3.85-12.2)
Plasma lactate (mmol/L)	7.62 ± 4.41 6.61(3.58-12.7)	4.52 ± 3.97 3.52(1.5-11.4)	4.49 ± 2.41 4(1.95-7.17)	7.25 ± 4.61 6(2.42-13.5)
Plasma insulin (pmol/L)	32 (1-72)	38 (13-201)	23 (12-42)	20 (1-38)
Plasma insulin/ glucose ratio	4.8 ± 4.1	9.1 ± 7	4.1 ± 1.6	2.4 ± 2.8
70 – 90 min				
Lactate disposal (μmol/kg.min)	106 ± 35 101 (67-155)	89 ± 28 86 (66-129)	94 ± 16 93 (73-113)	128 ± 38 123 (72-177)

Glucose disposal (μmol/kg.min)	50 ± 7.6 50 (42-62)	49 ± 9.3 48 (36-62)	57 ± 16 56 (42-79)	69 ± 21 65 (33-84)
Glucose production (μmol/kg.min)	45 ± 7.2 45 (39-56)	43 ± 9.6 42 (30 – 57)	51 ± 16 49 (36 – 73)	63 ± 21 59 (27-78)

Table 13 (continued). Metabolic data in patients according to treatment.

Variable	All quinine (n = 11)	All artesunate (n = 10)	All placebo (n = 11)	All DCA (n = 10)
- 5 min				
Plasma glucose (mmol/L)	7.03 ± 4.33 6.15 (3–16.1)	6.31 ± 1.73 6.14 (4.54-10.7)	6.3 ± 2.9 5.86 (4.38-14.8)	7.2 ± 3.8 6.47 (3-16.1)
Plasma lactate (mmol/L)	7.67 ± 3.97 6.82 (3.66-14.5)	7.19 ± 3.49 6.45 (2.66-14.3)	7.46 ± 3.8 6.65 (2.66-14.5)	7.42 ± 3.7 6.63 (3.51-14.3)
Plasma insulin (pmol/L)	33 (16-230)	9.8 (1-52)	17.4 (1-56)	19.7 (1-230)
Plasma insulin/glucose ratio	6.8 ± 5.3	3.1 ± 2.4	4.1 ± 2.5	6.1 ± 6
60 min				
Plasma glucose	6.3 ± 3	6.29 ± 2.25	6.34 ± 2	6.3 ± 3.2

(mmol/L)	5.8 (3.17-11.8)	6.01(3.85- 12.2)	6.12 (4.34-11.2)	5.67 (3.17-12.2)
Plasma lactate (mmol/L)	6.07 ± 4.28 4.82 (1.55-12.7)	5.87 ± 3.76 4.87 (1.95-13.5)	6.06 ± 3.7 5.12 (1.95-12.7)	5.89 ± 4.3 4.59 (1.55-13.5)
Plasma insulin (pmol/L)	36 (1-209)	22 (1-42)	26 (1-72)	30 (0-209)
Plasma insulin/glucose ratio	7.2 ± 6	3.2 ± 2.3	4.4 ± 2.7	5.7 ± 6.1
70 – 90 min				
Lactate disposal (μmol/kg.min)	98 ± 31 94 (66-155)	113 ± 34 108 (72-177)	101 ± 27 98 (67-155)	109 ± 38 103 (66-176)
Glucose disposal (μmol/kg.min)	49 ± 8[b] 49 (36-62)	64 ± 19[b] 61 (33-84)	53 ± 12 52 (42-79)	59 ± 19 56 (33-84)
Glucose production (μmol/kg.min)	44 ± 8[b] 44 (30-57)	58 ± 19[b] 55 (27-78)	48 ± 12 47 (36-73)	53 ± 19 50 (27-78)

Data are given as the mean ± SD and as the geometric mean (range) where appropriate. DCA, dichloroacetate.

[a] Five patients in the quinine and four in the artesunate group completed kinetic studies. [b] $P < 0.005$ between treatment groups.

Conclusions

The mortality in severe malaria has remained stubbornly high at 10 – 20% despite the use of intensive care (Warrell 1999). This is in spite of increasingly effective and rapidly acting anti-malarial drugs such as the artemisinin derivatives (Hensbroek et al. 1996; Hien et al. 1996) and has prompted the investigation of adjuvant therapies such as cytokine modifying drugs such as anti-TNF antibodies (van Hensbroek et al. 1996) and pentoxifylline (Looareesuwan et al. 1998). These unfortunately have proved as ineffective as did steroids and heparin some 20 years earlier (Warrell et al. 1982). New ideas are needed, based on what is known of the pathophysiology of severe malarial disease. My recent work has examined a few of these newer pathophysiological avenues with a view to developing new approaches to the care of the severely ill patient with malaria.

The pathophysiology of the anaemia of severe falciparum malaria is complex and multifactorial, and results in a condition that is a major cause of mortality and morbidity in patients living in malarial endemic areas. Falciparum malaria was the primary cause in 46 cases (46%) of children with severe anemia admitted to hospital in Kenya (Newton et al. 1997). The importance of anaemia as a cause of death in malaria may well be underestimated. The mortality rate in the children with severe malarial anemia was 8.6% compared with 3.6% in children with severe anemia due to other causes (Newton et al. 1997). There is undoubtedly an interaction between anaemia and acidosis (English et al. 1997; Marsh and Snow 1997). Some of the work detailed here has attempted to elucidate the steps in infected and uninfected erythrocyte destruction. The finding of erythrocytes that had malaria antigens incorporated into the red cell membrane but without plasmodial DNA suggested that

there were methods for removal of parasites *in vivo* that did not necessarily result in the immediate loss of the erythrocyte (Angus et al, 1997). This may be a mechanism for salvaging some erythrocyte function until new erythrocytes can be generated. Other studies involving measurement of red cell deformability showed that changes in elongation index relate to the development of anaemia and implicate splenic clearance indirectly as the main mechanism for this (Dondorp et al, 1999).

Rosetting was thought to be important in the process of microvascular obstruction in falciparum malaria and it may well be a contributing factor. Anti-rosetting antibodies were described as being disease modifying (Carlson et al. 1990a). Our reports of rosetting in other species of plasmodia such as *P. ovale* (Angus et al, 1997) and *P. vivax* (Udomsangpetch et al. 1995) put this in doubt. Since this report rosetting has also been described in *Plasmodium malariae* (Lowe, Mosobo, and Bull 1998), a finding that we have ourselves confirmed (unpublished data).

Exchange blood transfusion (ET) has been and is still used in many parts of the world for the treatment of severe falciparum malaria (Aquinas et al. 1996; Gulprasutdilog et al. 1999; Mathon et al. 1999). In general the consensus opinion is that exchange transfusion should be reserved for patients with hyperparasitaemia of >10% and with complications of multi-organ failure or with hyperparasitaemia >30% without complications. Since no randomised trials have been done the evidence for the indications, application and efficacy of its use are based on case reports and retrospective studies. Recent reports have been of automated exchange that may allow more rapid and predictable therapy (Macallan et al. 1999; Mainwaring et al. 1999). In the largest retrospective non-randomised study so far a 20% reduction in mortality was claimed (Hoontrakoon and Suputtamongkol 1998) although the control group

mortality was high at 69%. The rationale put forward for this form of therapy is based on (1) rapid reduction in parasite load by exchange transfusion, (2) removal of toxic substances and (3) reducing microcirculatory sludging (Pasvol and Jacobs 1999; White 1999). There are some problems with these theories. It is the sequestrated parasites that are causing the damage and these are not removed by exchange transfusion. However mathematical modeling of the reduction of parasitaemia by exchange transfusion predicts a 25% smaller reduction in parasitaemia than is observed (Van den Ende et al. 1994; Wilkinson et al. 1994) implying that some sequestered parasites may be removed. At lower parasitaemias however the reduction in parasite count must follow an exponential curve and therefore be of increasingly diminishing returns. TNF has not been shown to be reduced by exchange transfusion (Chuncharunee et al. 1997). ET has been shown to increase oxygen delivery and improve cardiovascular work by an unknown mechanism (Beards, Joynt, and Lipman 1994). In practice, the amount of compatible blood required for total exchange is rarely available in areas endemic for malaria and the risks of the procedure including transfusion-related infections are high. Our recent work has suggested that changes in red cell deformability may be a further rationale for its use. There have been no randomized controlled trials of exchange transfusion and indeed these would be difficult to perform and need large numbers to show a significant effect on mortality. Our finding that red cell deformability in a subset of patients with severe disease was strongly predictive of death allows further studies to focus on this group. Power calculations for a further study to give a 25% reduction in mortality would require around 30 patients only. These studies are currently being done in Kenya in African children. This has several logistical advantages in that there are more patients with

severe disease and they require less blood for the exchange. The other area that is still being pursued is the nature of the rigidifying factor that we have shown to cause changes in red cell deformability from the serum of severely ill patients that is also present in used malaria culture medium.

There has been a huge interest in the role of inducible nitric oxide in malaria recently. There is, however, still no consensus on the clinical significance as far as measuring serum or urinary nitrates are concerned. The lack of any difference between the groups studied in our two studies may be due to the inherent problems involved in measuring total nitrite excretion (Agbenyega et al, 1997; Dondorp et al, 1998). The hypotheses about associations between the pathophysiological effects of nitric oxide and severe malarial disease continue to be published predicting either benefit or harm (Al-Yaman et al. 1996; al-Yaman, Genton, and Clark 1998; Anstey et al. 1996; Burgner et al. 1998; Clark, Rockett, and Cowden 1991; Clark, Rockett, and Cowden 1992; Clark, Cowden, and Rockett 1993; Clark, Cowden, and Rockett 1995; Kremsner et al. 1996; Kun et al. 1998; Rockett et al. 1992; Taylor-Robinson and Looker 1998). This has more recently been extended to measuring inducible nitric oxide synthetase mRNA in patients but with similarly confused results (Burgner et al. 1998; Chiwakata, Hemmer, and Dietrich 2000; Kun et al. 1998). Given that the role of nitric oxide is probably dichotomous, that is it is simultaneously protective and harmful (Taylor-Robinson and Smith 1999) this is not surprising and reminiscent of the situation with TNF (Tracey 1995). Previous clinical studies of nitric oxide synthesis inhibitors have not been encouraging (Avontuur et al. 1998) and so this does not look like a promising avenue for therapeutic intervention.

Acidosis is an important contributor to mortality in malaria accounting for 43% of cases admitted to hospital in a study in Kenya (English et al. 1997). We have measured both lactate and glucose turnover in severe malaria and then have studied the utility of using dichloracetate as adjunctive therapy in severe malarial acidosis. Other approaches have been reported recently. In Vietnamese adults dopamine reduced whereas adrenaline infusion increased acidosis in severe disease (Day et al. 1996) In animal models infusion of cross-linked haemoglobin reduced acidosis in mice again highlighting the interaction between anaemia and acidosis in malaria (Freilich et al. 1999). There is no doubt that another factor contributing to the generation and maintenance of acidosis is hypovolaemia (English et al. 1997). Fluid replacement using whatever is available be it blood or crystalloid is a priority in severe malaria and it was striking that even in the control groups that we studied the replacement of volume even if only by crystalloid caused great improvement in acidosis. The studies presented here have been used as the basis for a large 5-year study of the effect of DCA on mortality in African children at this study site funded by the National Institutes of Health.

The drug, artesunate, was used in the studies to measure lactate and glucose turnover. This is one of a group of new drugs called the artemisinin derivatives. These drugs, the sesquiterpene lactones have been shown to be of a unique structure, have a unique mechanism of action and are very potent *in vitro* (Klayman 1985). They have been widely used in South East Asia particularly China and Vietnam and have been well tolerated. Artesunate is the water soluble hemi-succinate form which is rapidly hydrolysed to the active compound dihydroartemisinin (DHA) with good bioavailablity, a rapid onset of action and a short half-life (around 45 minutes). It can

be given as an intravenous or intramuscular injection as well as orally or per rectum. Recent studies comparing a sister compound the more lipid soluble artemether given intramuscularly with quinine have not shown a difference in mortality but have shown the artemisinin derivatives to be safe and well tolerated (Hensbroek et al. 1996; Hien et al. 1996; Pittler and Ernst 1999). In particular the main difference was in the occurrence of hypoglycaemia. Quinine is well known to induce hypoglycaemia (Taylor et al. 1988) while artesunate does not and these differences were highlighted in our studies. Since hypoglycaemia is itself a consequence of malaria obviously exacerbation of this is not a good thing particularly in situations where monitoring of the patient may not be all it should be. In view of the increasing resistance to commonly used antimalarial drugs including quinine the artemisinin derivatives are critical to "averting a malaria disaster" (White et al. 1999).

In conclusion, my studies have concentrated on pathophysiology in severe falciparum malaria. They have opened new avenues for trials for new adjunctive therapies in this disease. Red cell deformability may be the key to validating exchange transfusion in malaria, there is still no evidence for the exact role of nitric oxide as a mediator of disease and dichloroacetate has been shown to be safe and effective in reducing lactic acidosis in African children.

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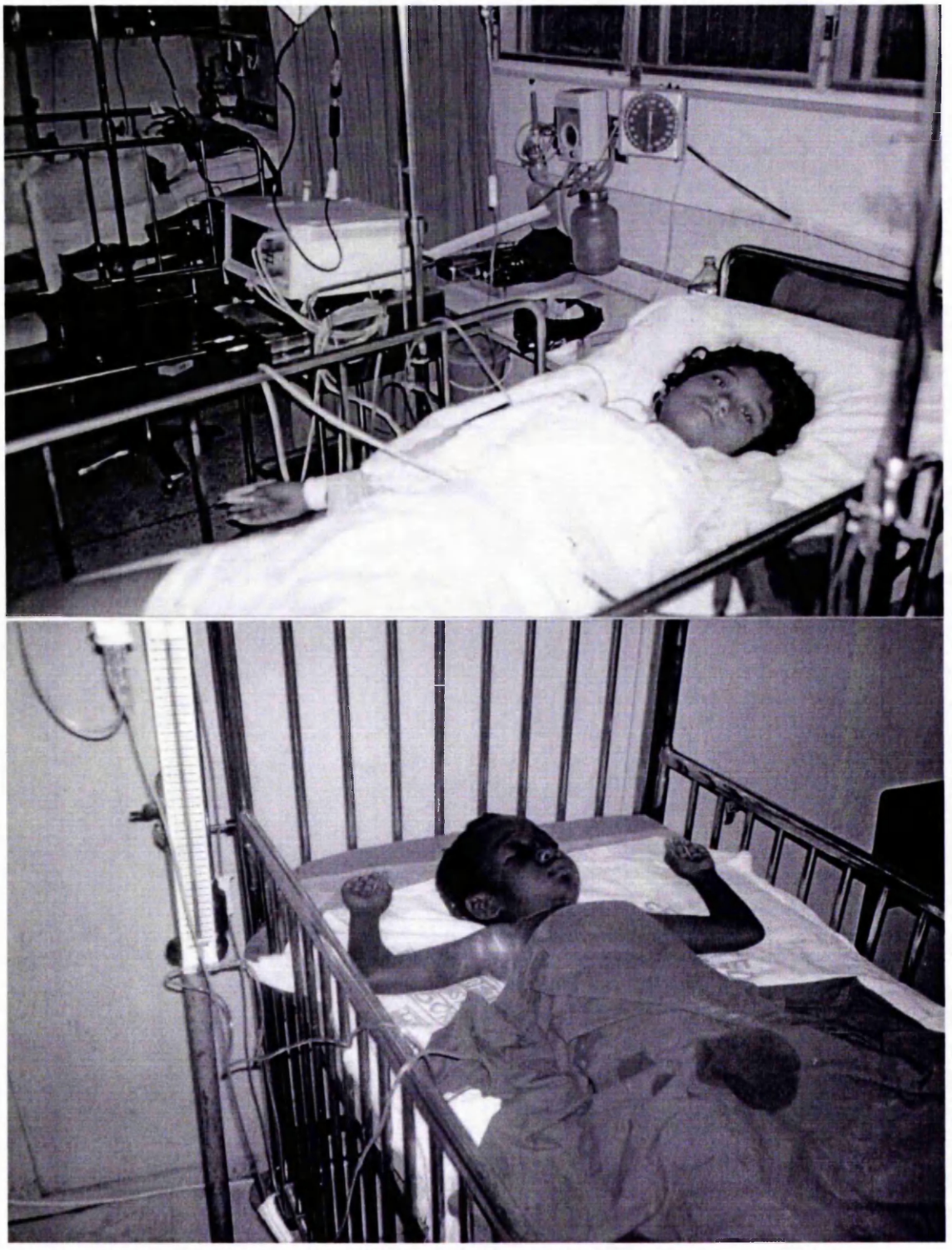

Appendix 1. The intensive care units in Thailand (top) and Ghana (bottom). Central venous cannulation was performed for exchange transfusion.

Appendix 2. A Ghanaian patient with typical features of cerebral malaria, comatose, fitting with extensor posturing and hepatomegaly. The patient recovered completely with no clinically detectable neurological deficit.

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