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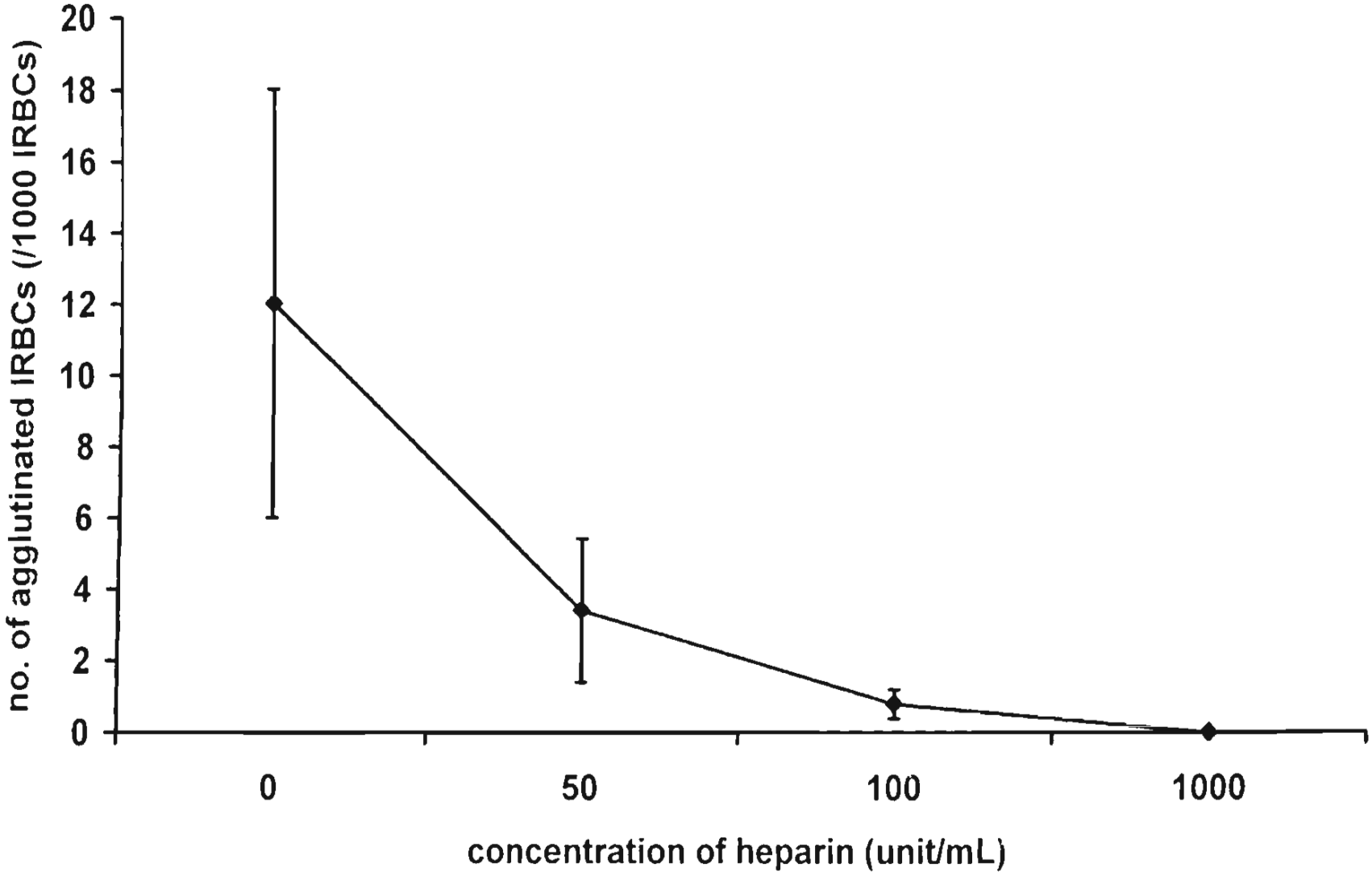
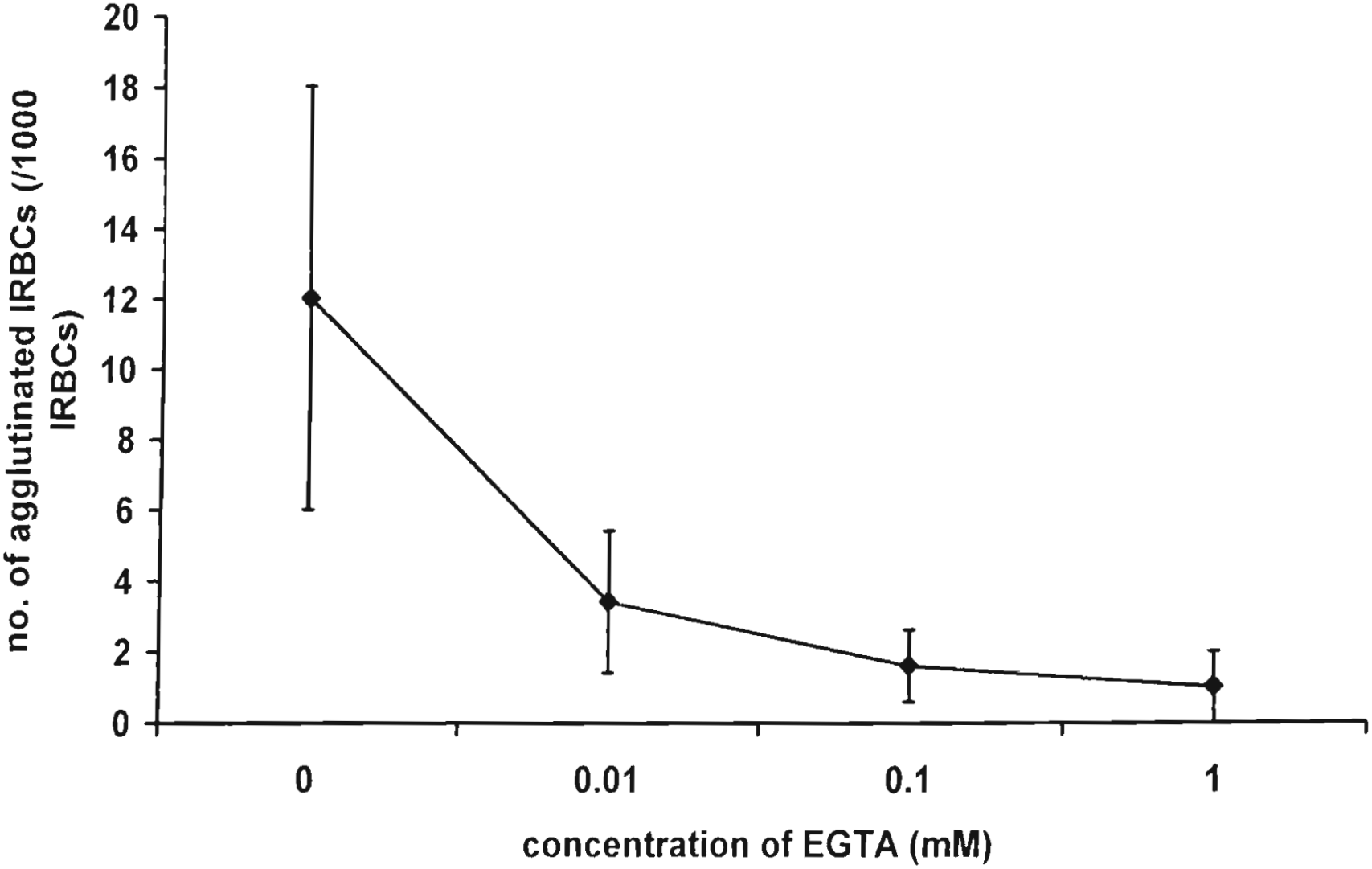
รูปที่ 4 ผลของ heparin ต่อการเกิด auto-agglutination



รูปที่ 5 ผลของ EGTA ต่อการเกิด auto-agglutination



## OUTPUT ที่ได้จากโครงการ

1. ผลงานวิจัยที่ได้จากโครงการนี้ ได้รวบรวมและนำเสนอต่อ วารสารนานาชาติเพื่อพิจารณา ตีพิมพ์ใน British Journal of Haematology
2. ได้นำเสนอผลงานวิจัยนี้ในงานประชุมวิชาการนานาชาติ ได้แก่
   2.1 International conference on malaria: current status and future trend. 16-19th February 2003. Bangkok Thailand
   2.2 Oxford Network Meeting. 31st March – 4th April 2003. Oxford. United Kingdom.
   2.3 Joint Malaria and BSP Spring Meeting. 6th – 9th April 2003. Manchester. United Kingdom.
3. ผลงานวิจัยนี้ชี้นำไปสู่การศึกษาในแง่ต่างๆ เช่น คุณสมบัติ autoagglutination ในเชื้อมาลาเรียชนิด อื่นๆ ( *P.vivax, P. malariae , P. ovale*) และแรงยึดเกาะระหว่างเม็ดเลือดแดงติดเชื้อ รวมทั้ง การเกิดกลุ่มก้อน auto-agglutination ในสภาวะที่มีแรงดัน และแรงหนืดที่เกิดขึ้นในเส้นเลือด

# ภาคผนวก

# *P. falciparum* infected red cells platelet-induced autoagglutination phenotype and disease severity in Thailand.

Heading: *Autoagglutination Phenotype of P. falciparum*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Summary

The relationship of platelet mediated autoagglutination of *P. falciparum* infected red cells to disease severity was investigated in 182 Thai patients with falciparum malaria. 50% of parasite isolates showed the agglutination phenotype. Autoagglutination was reversed by both heparin ($EC_{50}$; 50 units/ml) and EGTA ($EC_{50}$; 0.01 mM). The proportion of parasites showing the agglutination phenotype in patients with uncomplicated malaria (43%) was similar to with severe malaria (41%), whereas all 15 isolates from patients with cerebral malaria showed autoagglutination (P=0.001). The median (range) number of infected red cells (IRBC) in agglutinates per 1000 IRBC was significantly higher in cerebral malaria (6; 3-42) compared to severe (0; 0-52) and uncomplicated malaria (0; 0-24) (P=0.01). Agglutination was associated positively with admission parasitaemia (P=0.001), and blood urea nitrogen concentration (P=0.013) but negatively with plasma $HCO_3$ (P=0.01). In multivariate analyses, high parasitaemia and cerebral malaria were associated significantly and independently with parasite agglutination.

**Keywords**: *P. falciparum*, autoagglutination, severe malaria, platelets, *P. falciparum*-infected red cells

## INTRODUCTION

*P.falciparum* is the most virulent of the human malaria parasites and accounts for nearly all the malaria attributable mortality in the world. The severity of the infection is determined by both host and parasite factors. Several human genetic polymorphisms has been associated with susceptibility or protection from severe malaria, notably those involving the genes related to haemoglobin synthesis. The haemoglobinopathic red cells e.g. thalassemic red cells, ovalocytes and sickle cells are associated either with restricted parasite invasion, or poor intraerythrocytic growth, and these red cells therefore attenuate parasite expansion (Friedman, 1978; Pasvol *et al* , 1980, Mohandas *et al* , 1984; Vernes *et al*, 1986; Bunyaratavej *et al* , 1997; Senok *et al*, 1997; Pattanapunyasat *et al*, 1999). On the parasite side, *P. falciparum* isolates from patients with severe malaria show higher multiplication rates at high densities than isolates obtained from patients with uncomplicated malaria (Chotivanich *et al* , 2000).

Sequestration resulting from the adherence of red cells containing mature forms of *P. falciparum* to vascular endothelium is the essential pathological feature of severe falciparum malaria. The adhesion properties of parasitized red cells (cytoadherence and rosette formation) have been associated with disease severity in some studies but not in others (Carlson *et al* , 1990; Ho *et al*, 1991; Udomsangpetch *et al*, 1992; Al-Yaman *et al*, 1995). Cytoadherence is confined to *P. falciparum*, whereas rosetting is a property of all human malarias. *In vivo* cytoadherence occurs in all *P.falciparum* infection whereas only some isolates show rosetting. Recently a new parasite adhesive phenotype has

been recognised and associated with disease severity in Kenyan children. This is platelet mediated autoagglutination of parasitized red cells (Roberts *et al*, 2000). In this study we have investigated the association between autoagglutination phenotype of *P. falciparum* isolates from Thailand and disease severity.

## PATIENTS AND METHOD

### Patients

Adult patients were admitted to Mae Sot Hospital or the Hospital for Tropical Diseases in studies of antimalarial drugs treatment which will be published elsewhere. Severe malaria and cerebral malaria were defined using the W.H.O. criteria (World Health Organization, 1990). Patients or attendant relatives gave fully informed consent to providing a 5 mL blood sample for parasite culture. These studies were approved by the Ethics committees of the Faculty of Tropical Medicine and the Ethical and Scientific review subcommittee of the Thai Ministry of Public Health.

### Parasite culture

*P. falciparum*-infected blood samples were collected in heparinized tubes and centrifuged at 800 g for 5 minutes. Buffy coat and plasma were removed, packed red cells were washed three times then resuspended in a standard malaria culture-medium (Trager & Jensen, 1976) of RPMI-1640 supplemented with 25 mM HEPES, 10% human AB serum and 40 µg/mL gentamicin to achieve a final concentration of 5% haematocrit. After 30 hours of cultivation at 37$^{o}$C, 5% $CO_2$, thin blood films were prepared and the stage of parasite maturation was examined by microscopy (Silamut & White, 1993). The

*P. falciparum* agglutinating clone C10 was maintained in culture and used as positive control.

Platelet preparations

Five mL of whole blood from volunteer donors, who had not been exposed to malaria, was obtained and centrifuged at 250g for 10 minutes. The plasma fraction was transferred to a new tube and platelet rich plasma (PRP) was separated by further centrifugation at 250 g for 15 minutes. To obtain platelet poor plasma (PPP) 15 minutes centrifugation at 1500 g was used. The density of platelets in the plasma was measured by flow cytometry (FACS). PRP and PPP were kept at $4^{o}C$ and used within 2 weeks.

Autoagglutination assay

Only mature trophozoite-infected red cells (estimated mean age 30-32 hours old) were used for this assay. An aliquot of the parasite culture was taken and centrifuged to remove the culture medium. The packed red cells were resuspended at 10% haematocrit with 10% PRP resuspended in RPMI (final concentration $1X10^{7}$ platelets/ml). Ethidium bromide 200 µg/mL, was added to identify the infected red blood cells. This mixture was rotated gently (10 rpm) for 15 and 30 minutes. After rotation, 10 µL of sample was taken and placed on a glass-slide and examined by fluorescent microscopy. Autoagglutination was defined as clumping of three or more (but less than 20) infected red cells. The frequency of autoagglutination is the percentage of infected red cells in the agglutinating clumps in the total 1000 infected red cells counted.

Two or more uninfected red cells aggregated with an infected erythrocyte were classified as a rosette. The number of infected red cells in rosettes was also counted for 1000 infected red cells.

Disruption of agglutination of fresh clinical isolates

Fifty microliters of the agglutinated *P. falciparum* culture was mixed with a) standard heparin (Leo® Denmark) in various concentrations (range from 50 to 1000 unit/ml) and b) EGTA (ethylene-bis (oxy-ethylenenitrilo)tetra-acetic acid) in veronal buffer, pH 7.2 (range from 0.01 to 1 mM) into the autoagglutination IRBCs. After gentle rotation for 15 minutes, samples were taken to assess the number of the autoagglutinated IRBCs in 1000 red cells as described above and compared with controls (without heparin or EGTA).

Statistical analysis

The frequency of agglutination and frequency of rosette formation were compared between the uncomplicated, severe and cerebral malaria patient groups using the Kruskal-Wallis test. The association with other patient characteristics (parasitaemia, $HCO_3$, serum BUN, creatinine, total bilirubin) was examined using the Spearman Rank Correlation Coefficient. The number of agglutinated infected red cells and number of rosette forming infected red cells were categorized prospectively into four groups (0, 0-10, 10-20, >20), and for each of them ordered logistic regression was used to determine the association with patient characteristics. Characteristics associated independently with the outcome were identified by using the forward variable

selection method with a significance level of 0.05 for retention in the model. The final model is presented, together with cutoffs for linear predictor (LP = $\beta_0+\beta_1x_1+....+\beta_kx_k$) corresponding to the categories of the outcome variable. The data were analyzed with STATA program version 7.

## RESULTS

### Patients

A total of 182 patients were studied. The clinical and laboratory variables on admission are shown in Table1. In total 63 patients had uncomplicated malaria, and 119 patients had severe malaria, of whom 15 had cerebral malaria (Glasgow coma score less than 11). None of the patients died.

As expected, the geometric mean parasitaemia, serum creatinine, total bilirubin, and transaminases were significantly higher and haematocrits and platelet counts were significantly lower in patients with severe malaria compared with uncomplicated malaria ($P\leq0.001$). The haematocrit, serum urea, total bilirubin and SGPT values differed significantly between patients with severe and cerebral malaria (Table 1).

### Autoagglutination of parasite isolates

Of the 182 isolates, 51% (93/182) showed agglutination of infected red cells and, 93% (169/182) showed rosette formation. The proportion of agglutination phenotypes in the uncomplicated group was similar to that in the conscious severe malaria group i.e. excluding isolates from patients with cerebral malaria (43% and 41%

of isolates, respectively). All of the isolates (15/15) from the cerebral malaria group showed autoagglutination (P=0.001).

The median (range) number of autoagglutinated infected cells was significantly higher in samples from cerebral malaria (6 ; 3 to 42 per 1000 infected red cells, p=0.001) than from severe malaria (0; 0 to 52 per 1000 infected cells ) and from uncomplicated malaria (0; 0 to 24 per 1000 infected cells) (P=0.003).

Patients with the agglutination phenotype had higher values of admission parasitaemia (P<0.001), BUN (P=0.013) and lower plasma $HCO_3$ (P=0.01). To determine the strength of independent associations between autoagglutination phenotype and the clinical and laboratory variables, multivariate analysis was performed. As in the univariate analysis, high parasitaemia and cerebral malaria were associated with parasitized erythrocyte agglutination and these were independent associations. There was no association between rosette formation and autoagglutination.

Rosette formation (median, range) was significantly higher in isolates from the cerebral malaria group (10, 1-26 per 1000 infected cells) than in severe malaria (4, 0-20 per 1000 infected cells) or uncomplicated malaria (3, 0-18 per 1000 infected cells) groups (P=0.002). There was a positive correlation between rosette formation and parasitaemia ($r_s$=0.52, P<0.001), HCO3 ($r_s$ =0.38, P<0.001), BUN ($r_s$ =0.22, P=0.010), creatinine ($r_s$ =0.21, P=0.008), total billirubin ($r_s$ =0.19, P=0.022) and a negative

correlation with albumin ($r_s$ =0.21, P=0.022). In the multivariate analysis, only admission parasitaemia was associated independently with rosette formation (P< 0.001).

Disruption of autoagglutination

20 isolates showing the positive autoagglutination phenotype were tested further in this assay. Agglutination was reversed by both heparin and EGTA (in a dose dependent manner). EGTA strongly and dose dependently disrupted already existing auto-agglutination. 50% of autoagglutinating infected red cells were reversed by a concentration of EGTA 0.01 mM and complete disruption was obtained with EGTA concentrations higher than 1mM. (Fig 1A). The $EC_{50}$ for heparin was 50 unit /ml and all agglutinates were disrupted with a heparin concentration of 1000 unit /ml.(Fig 1B).

## DISCUSSION

Autoagglutination of *P. falciparum* infected red cells mediated by platelets was observed in 50% of isolates from patients admitted with acute falciparum malaria in North-Western Thailand. Autoagglutination was associated particularly with the parasites causing cerebral malaria. Autoagglutination occurs when *P. falciparum* parasites develop to the trophozoite and schizont stages. The mechanism underlying autoagglutination is a ligand-receptor interaction, possibly mediated by CD36 on platelets acting as a receptor for the variant surface protein PfEMP-1 on infected red cells (Pain *et al*, 2001). Autoagglutination is therefore analogous to cytoadherence to CD 36 on vascular endothelium. The findings in this study are generally consistent with the previous report from a study in Kenya (Roberts et al, 2000; Pain *et al*, 2001). The

association of agglutination and cerebral malaria observed in these studies might be explained by the clumping of IRBC in cerebral venules causing increased resistance to blood flow or occlusion. This would contribute to packing of infected red cells in the microvasculature in cerebral compared with non-cerebral malaria (Macpherson et al, 1985; Turner, 1997). Alternatively attachment to other parasitized cells may contribute to the unequal distribution of sequestration in the microvasculature (Silamut *et al*, 1999). The relative contributions of adhesion to endothelium, to other parasitized red cells, and to uninfected red cells, and the role of rigidity of the uninfected red cells in microvascular obstruction needs to be elucidated further.

Parasite density was an important factor determining autoagglutination frequency. High parasite densities were associated with more IRBC-agglutination. Thrombocytopenia is usual in malaria. The number of platelets used in this study ($1x10^7$/ml) corresponded with the value in malaria patients. The density of platelets may also contribute to agglutination *in vivo*. We also showed that agglutinating-infected red cells could be disrupted by EGTA and heparin. A dose dependent disruption of already formed auto-agglutination was obtained with heparin, and magnesium and calcium chelation with EGTA. Similar findings have been reported previously for rosetting (Carlson *et al*, 1990; Carlson & Wahlgren, 1992). Platelet induced auto-agglutination may involve a lectin-like interaction which could be blocked by the glucosaminoglycan, or result from electrostatic effects countered by the negatively charged heparin. Autoagglutination also appears to depend on divalent cations ($Ca^{2+}$ and $Mg^{2+}$). If

autoagglutination is mediated by the interaction between the variant surface parasite derived protein (PfEMP-1) and CD 36 on platelets, then the effects of heparin and calcium chelators are different to the effects on adherence to vascular CD 36, but similar to those involved in rosette formation. The level of heparin needed to disrupt the clumping of agglutinated IRBC is within the anticoagulant range (1000 unit /L). If autoagglutination of IRBC is causally linked to disease severity and can reversed, then this could be beneficial. However heparin has been used in severe falciparum malaria, and was associated with a very high rate of serious bleeding (Punyagupta *et al*, 1972).

ACKNOWLEDGMENTS

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Table 1. Admission clinical and laboratory characteristics

| Variable | Uncomplicated | Severe | Cerebral malaria |
|---|---|---|---|
| | n = 63 | n= 104 | n= 15 |
| Glasgow coma score # | 15(14-15) | 15(11-15) | 7(4-10) |
| Haematocrit(%)* | 38.3 (31.7-44.7) | 34.7 (28.2-41.1) | 29.5 (27-36) |
| Parasitaemia (µL)˙ | 68770 (67141-116564) | 242156 (220372-350953) | 199192 (96441-468722) |
| Serum creatinine (µmol/L)* | 110 (88-132) | 139 (107-170) | 135 (71-198) |
| Serum BUN (mmol/L)# | 17.8 (11.5-20) | 44.5 (15-79) | 51.8 (45-58) |
| Plasma glucose (mmol/L)* | 11.7 (8.7-14.7) | 7.7 (4.2-11.2) | 9.6 (5-11.5) |
| Platelets x$10^{9}$# (/L) | 129.7 (26-233) | 39 (14-121) | 83 (52-144) |
| Serum total bilirubin (µmol/L)# | 13 (5-61) | 32 (17-380) | 93 (50-690) |
| Serum bicarbonate ($HCO_3$)* (mmol/L) | 19 (16-21) | 17 (14-19) | 13 (9-16) |
| Serum alkaline phosphatase* | 71 (58-84) | 95 (62-128) | 124 (90-320) |
| Serum AST# | 41 (29-52) | 104 (52-156) | 152 (127-575) |
| Serum ALT# | 19 (10-28) | 37 (14-61) | 172 (122-217) |

- *mean (95% CI), ˙ geometric mean (95% CI), # median (range)
- AST = aspartate aminotransferase ; ALT= alanine aminotransferase

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Legends

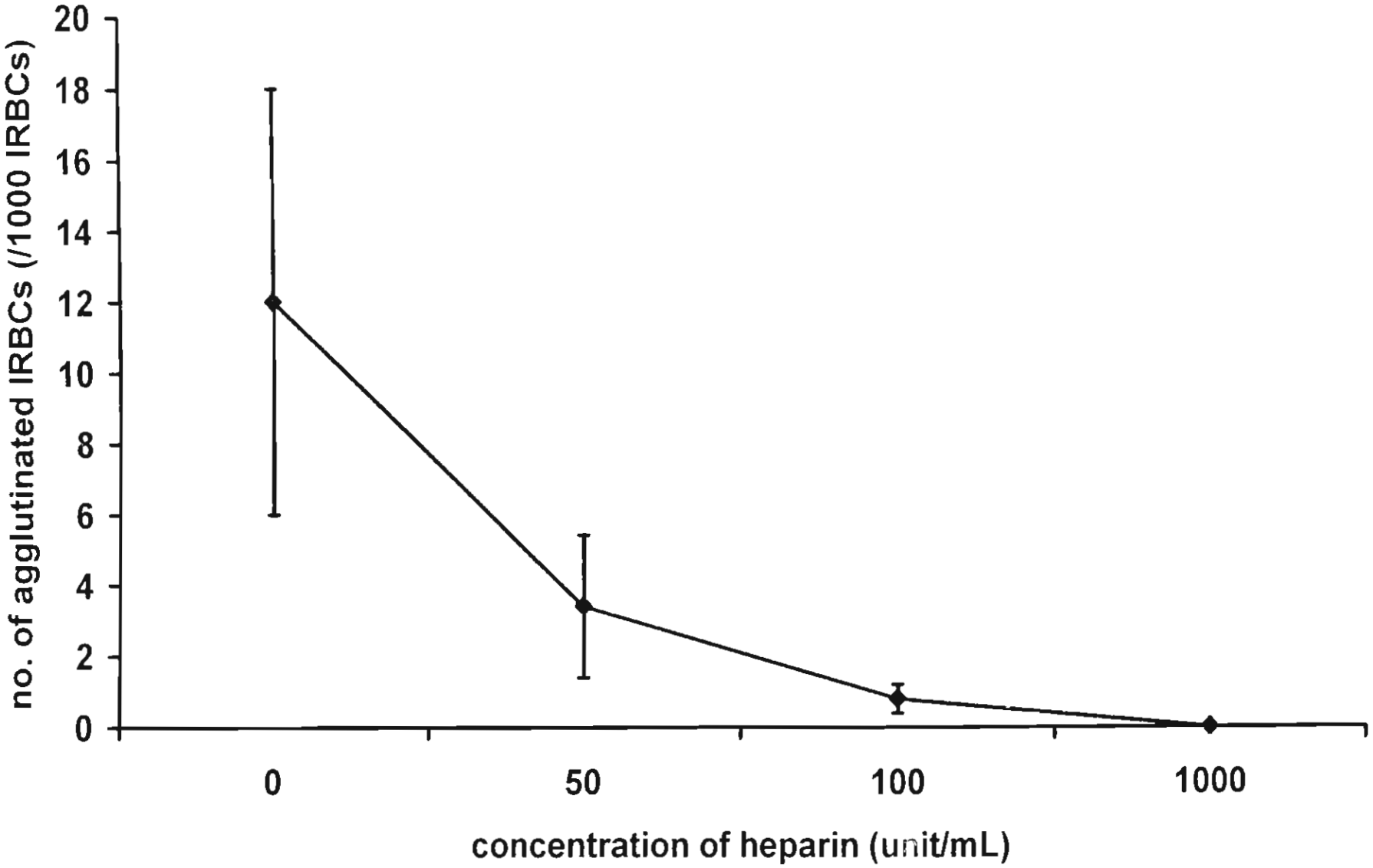
Table

Table 1 Admission clinical and laboratory characteristics

Figure

Figure 1A Cation dependency of platelet-mediated agglutination of the infected red cells. The vertical lines are the standard deviation of the mean

Figure 1B Effects of heparin on platelet- mediated agglutination of the infected red cells. The vertical lines are the standard deviation of the mean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12
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6
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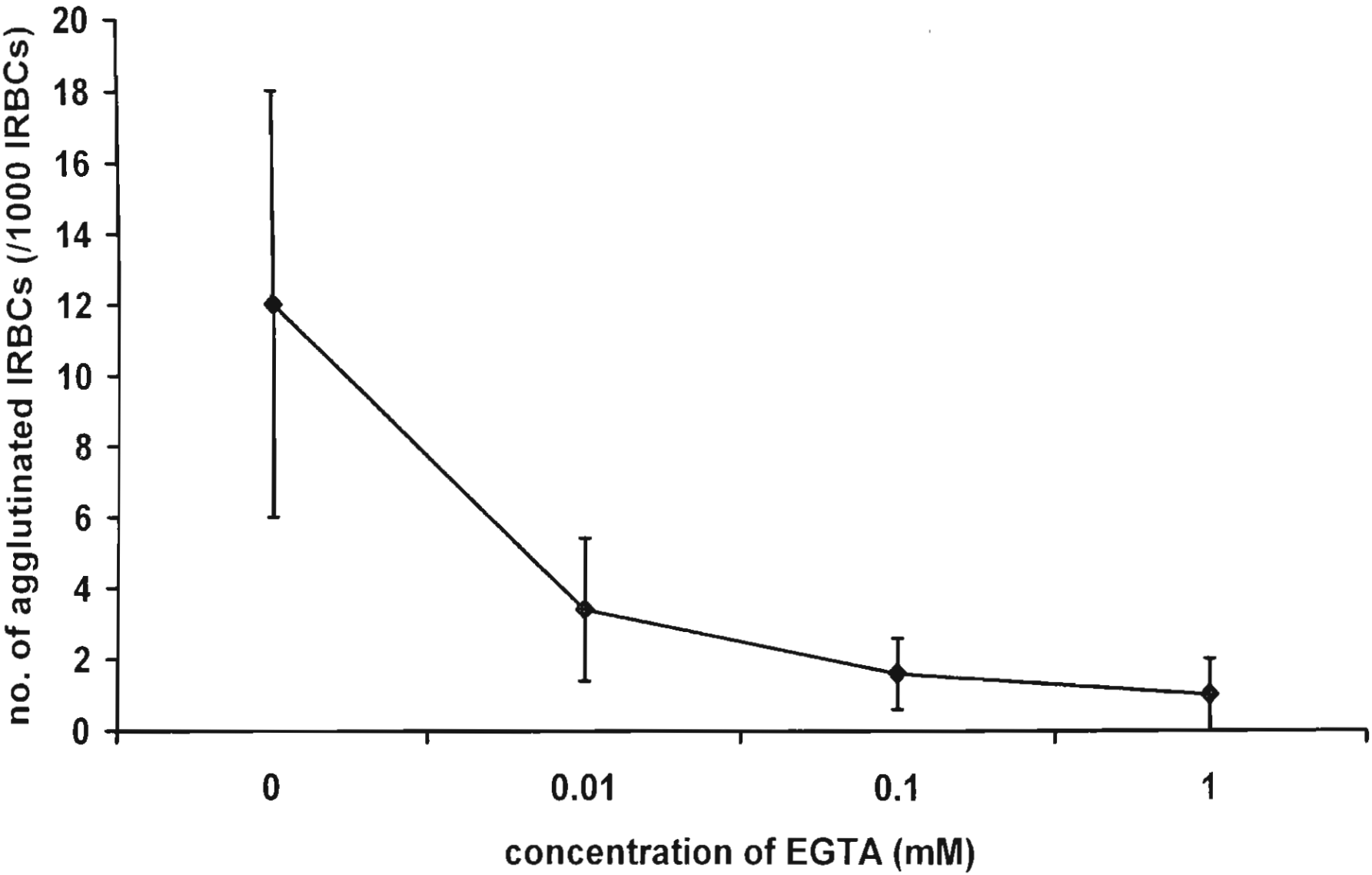
no. of agglutinated IRBCs (/1000 IRBCs)
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