Clin. exp. Immunol. (1983) **53**, 127–134.

Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA

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(Accepted for publication 9 May 1983)

SUMMARY

An enzyme linked immunosorbent assay (ELISA) has been developed to estimate disease related antibodies in sera from malaria patients or individuals living in malaria endemic areas. As antigen, Percoll enriched fractions (mainly late trophozoites, schizonts) from *Plasmodium falciparum in vitro* cultures were used. An ELISA with ghosts from normal human red blood cells (RBC) was performed in parallel. One hundred and seventy-five sera were tested for their reactivity with either one of the two antigens. Seven sera from patients with acute *P. falciparum* infection were negative. Most of these had been taken very early in infection and consecutive samples taken later usually were positive. The antibodies reacting with the *P. falciparum* antigen had a high parasite specificity as indicated by inhibition and absorption experiments. Many sera also had elevated levels of antibodies specific for RBC antigens. A correlation, most pronounced in the IgM system, was also seen between the anti-RBC and the anti-*P. falciparum* antibody levels.

Keywords *Plasmodium falciparum* malaria human red blood cells ELISA

INTRODUCTION

Since its introduction, the enzyme linked immunosorbent assay (ELISA) has been applied frequently to determine antibody levels in the blood of patients with parasitic disease. In malaria, an ELISA for anti-Plasmodium antibodies in patients was first developed with *P. falciparum* antigen derived from Aotus monkeys (Voller *et al.*, 1974; Voller, Bartlett & Bidwell, 1976; Voller *et al.*, 1980). Later antigen preparations from human placentae (Quakyi, 1980), or from *in vitro* cultures (Spencer *et al.*, 1979a, 1979b, 1981) have been used.

In this paper we report on the development of an ELISA suitable for screening of human sera for anti-*P. falciparum* antibodies. As an antigen source we used sonicates of mature erythrocytic stages of the parasite, enriched from *in vitro* cultures of *P. falciparum* by fractionation on a Percoll gradient. The reactivity with this antigen of a large number of patients' and control sera was compared with that obtained with similarly processed normal erythrocytes.

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MATERIAL AND METHODS

P.falciparum *antigen.* A Tanzanian strain (F32) of this parasite, isolated in 1978, was used as antigen source. The parasites were cultured in red blood cells (RBC; group 0), according to Trager & Jensen (1976). The parasites were harvested when parasitaemias had reached 5–10%. After two washes in 0·01 M phosphate-buffered saline (PBS, pH 7·2), the RBC were suspended in PBS at a 10% haematocrite. Two millilitre fractions were placed in conical centrifuge tubes on top of 2·5 ml of 55% (vol./vol.) Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Centrifugation for 15 min at 1,500*g* gave a distinct band, containing 50–100% parasitized erythrocytes with late trophozoites and schizonts as well as free parasites. The bands containing parasitized RBC and free parasites were washed twice in PBS. For final processing of sufficiently large antigen batches the Percoll band fractions were pooled, suspended in a small volume of PBS and sonicated. The material was then aliquoted and stored at −70°C until use.

In some experiments, the parasites were metabolically labelled with ^{35}S-methionine. ^{35}S-methionine (10 μCi/ml, 1·4 Ci/mmol, Radiochemical Centre, Amersham, England) was added to the cultures when the parasites were in early ring stage. After 24 h culture, the medium was changed, fresh ^{35}S-methionine was added and the cultures were allowed to develop into late schizonts. Antigen was then prepared as described above.

Erythrocyte antigen. Normal RBC (group 0) were lysed and freed of haemoglobin according to Dodge, Mitchell & Hanahan (1963). The RBC ghosts were then sonicated and processed as described above for the parasite antigen.

Serum samples. Control sera were from 31 healthy Swedish blood donors and 12 healthy Colombians. The test sera were from three different groups. (1) Fifty-five were from 55 patients with proven *P. falciparum, P. ovale, P. vivax* or *P. malariae* malaria, hospitalized at Roslagstull Hospital, Stockholm. Some of these sera were from patients who had experienced their first malaria infection. (2) Thirty-four sera were from Liberia, West Africa. Of these, 11 sera were from adults not having acute malaria but living in a holoendemic area (Hedman *et al.*, 1979). For the others, living in both rural and urban areas, malaria experience was not defined. (3) Forty-three sera were from Colombian patients, who had experienced one or several *P. falciparum* infections (kind gift of Dr M. Patarroyo, Universidad Nacional, Bogotá, Colombia). All sera were stored at −70°C.

Anti-immunoglobulin conjugates. For use in ELISA, purified antibodies from rabbit or goat anti-human immunoglobulin sera were conjugated with alkaline phosphatase (ALP, Sigma, St Louis, Missouri, USA) as described by Engvall & Perlmann (1971). Two kinds of conjugates were prepared. One was with antibodies from the serum of three rabbits immunized with human immunoglobulin (Cohn fraction II, Kabi, Stockholm, Sweden). This conjugate reacted primarily with IgG but was not class specific since it also contained antibodies to immunoglobulin light chains (designation: anti-Ig). The other conjugate (anti-IgM) was made from a goat anti-human IgM serum, adsorbed with cord serum to free it from antibodies to α_2-macroglobulin and with human IgA to eliminate anti-light chain reactivity. The antibodies were finally made isotype specific by purification on a Sepharose–IgM immunoadsorbent. The specificity of the conjugates was established by a microELISA in which the wells had been coated with human IgG or IgM myeloma proteins.

ELISA. The procedure for the enzyme linked immunosorbent assay was the microtitration test described by Voller *et al.*, (1974). Optimal dilutions of the reagents were determined by checker board titrations with a few known positive patients' sera and negative control sera (Quakyi, 1980). The optimal dilutions were 1:1,000 both for the *P. falciparum* antigen (stock solution 7 mg protein/ml) and for the test sera. The normal RBC antigen preparation was used at a protein concentration equivalent to that of the *P. falciparum* antigens.

Dynatech MicroELISA plates were coated with antigen by adding 0·1 ml of the appropriate dilution in 0·06 M bicarbonate buffer to each well. The plates were then incubated over night at 4°C. Non-adsorbed antigen was removed and replaced by 0·2 ml buffer containing 2% (wt/vol.) BSA to prevent non-specific protein binding to the plastic. After incubation for 5 h at 4°C the plates were washed three times for 3 min with saline containing 0·05% (vol./vol.) Tween 20. The plates were

drained and shaken to remove excess fluid. One tenth of a millilitre of the test sera, diluted in PBS containing 0·05% Tween 20 and 0·02% sodium azide (PBS-T) was added to the wells and the plates were incubated over night at 4°C. After washing as above, 0·1 ml of conjugate, diluted in PBS-T, was added to each well. The plates were incubated once more for 5 h at 4°C, washed and supplemented with 0·1 ml/well of *p*-nitrophenylphosphate (Sigma) diluted in enzyme substrate buffer (10% diethanolamine, 0·5 mM $MgCl_2$ and 0·02% NaN_3, pH 9·8). The plates were then incubated at room temperature in the dark for approximately 65 min. The exact time of incubation was adapted on the basis of the reaction of standard sera, included in each test in order to diminish intertest variations. The absorbance at 405 nm of the contents of each well was determined in a Titertek multiscan spectrophotometer. All sera were tested in duplicates.

Inhibition experiments. The test sera were incubated with different amounts of antigen prior to adding them to the ELISA plates. Briefly, 0·1 ml of 10-fold dilutions of the antigen (*P. falciparum* or RBC antigen), containing 10 ng to 1 mg protein in PBS-T were incubated for 30 min at 37°C in microtitre plates (with U shaped wells) with 0·1 ml of the test sera diluted 1:500. The plates were then centrifuged for ten minutes at 4°C and 750*g*. One tenth of a millilitre aliquots of the supernatants were tested in ELISA as described above.

Absorbtion of sera. Sera diluted 1:100 in PBS-T were incubated over night with an equal volume of the non-sonicated erythrocyte antigen (group 0, 2 mg/ml) on a roller drum at 4°C. The mixture was centrifuged for 15 min at 10,000*g*. The absorbed sera were stored at −20°C.

RESULTS

In order to determine the efficiency of the coating of the plates with Plasmodium material, ^{35}S-methionine labelled antigen preparations were adsorbed to the microplates under the same conditions as used in the assays. The radioactivity of the supernatants and that remaining adsorbed to the wells after three washes with PBS-T was determined separately in a scintillation counter. In parallel tests, the radioactivity remaining adsorbed to the polystyrene after a complete ELISA was also measured. After over night incubation and three washes, about 50% of the radioactivity added remained adsorbed to the wells (two experiments). After a complete ELISA, 32% of the total input radioactivity was recovered in the wells.

Control sera

All 31 sera from Swedish blood donors gave low ELISA values with the *P. falciparum* antigen and either one of the conjugates. On the basis of these results, an absorbance of 0·25 or higher was considered as a positive reaction. When assayed with normal RBC antigen, only three of the sera gave positive ELISA values (0·35) with the anti-Ig conjugate but not with the anti-IgM conjugate (Fig. 1). Four of the 12 Colombian controls gave weakly positive reactions with *P. falciparum* when assayed with the anti-Ig conjugate. One of these was also positive when tested for anti-RBC activity (Fig. 1).

Swedish malaria patients

Twenty-five of 32 sera from patients with *P. falciparum* malaria reacted positively when assayed with either the anti-Ig or the anti-IgM conjugates (mean reactivity 0·66 and 0·44 respectively). Titration of positive sera in the anti-Ig/*P. falciparum* assay indicated that the antibody titres were sometimes very high, giving positive reactions (OD_{405} > 0·25) at serum dilutions up to 1/50,000. Twenty-two of these sera also reacted positively with the normal RBC antigen in either the anti-Ig or the anti-IgM assay (mean 0·45 and 0·35, respectively) (Fig. 1).

The seven sera from *P. falciparum* malaria patients which were negative in the *P. falciparum* assays were taken either very early or late in the infection. When fresh serum samples from the first group were taken consecutively during infection, they generally turned positive as well. A typical example are serum samples, taken at eight different occasions during a period of 20 weeks from a patient who had acquired chloroquine resistant malaria in Kenya (Aronsson *et al.*, 1981) (Fig. 2). In this case, the anti-RBC reactions correlated well with parasitaemia by showing an early peak at the

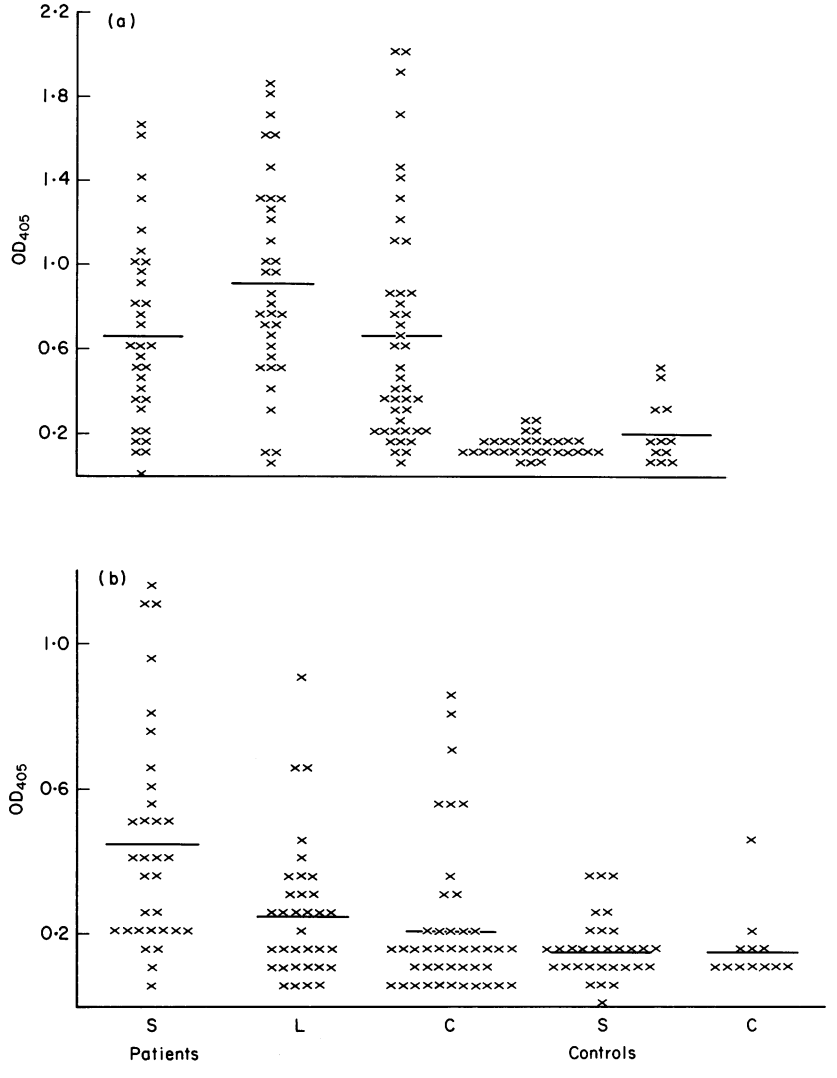


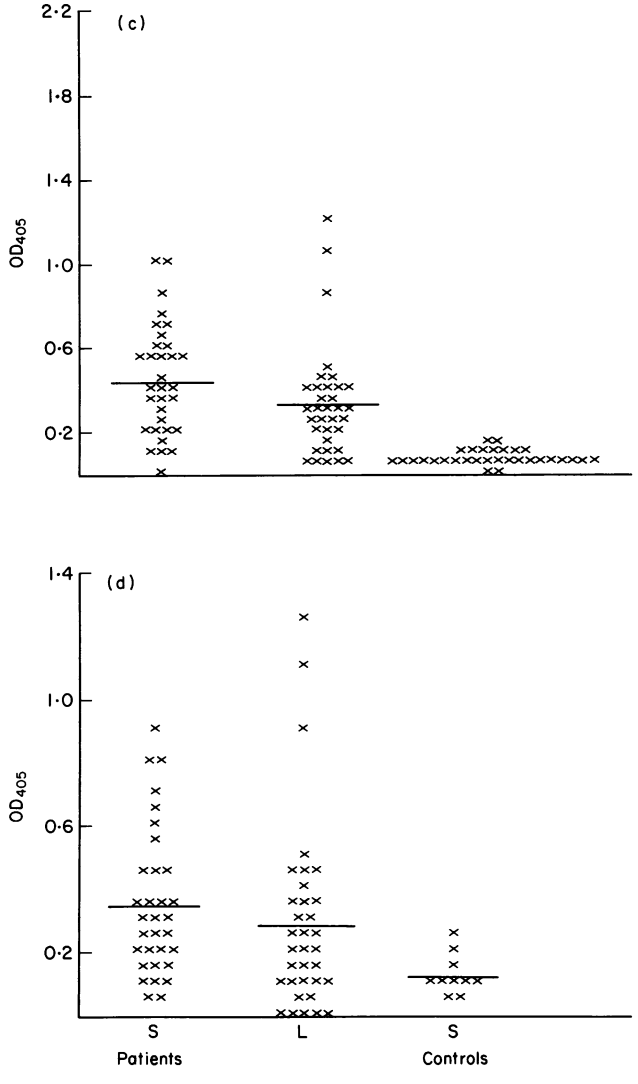
Fig. 1. ELISA values (OD_{405}) of sera (1/1,000) in (a) the anti-Ig/*P. falciparum* assay; (b) ELISA values (OD_{405}) of sera (1/1,000) in the anti-IgM/*P. falciparum* assay; (c) ELISA values (OD_{405}) of sera (1/1,000) in the anti-Ig/RBC ghost assay; (d) ELISA values (OD_{405}) of sera (1/1,000) in the anti-IgM/RBC ghost assay. Patients: S = sera

onset of the disease but no significant rise at relapse. In contrast, the anti-Ig/*P. falciparum* reaction showed both an early peak and a significant second peak at the second relapse. This rise was much less pronounced in the anti-IgM/*P. falciparum* assay.

The sera from 23 patients with *P. vivax*, *P. ovale* or *P. malariae* were also tested with either one of the antigens. Seventeen of these exhibited positive reactions in the anti-Ig/*P. falciparum* assay but most of them gave relatively weak reactions. In contrast, some of these sera reacted very strongly in the anti-Ig/normal RBC assay (mean 0·73).

Liberian sera

When tested with the anti-Ig conjugate, the sera from all 11 Liberians living in holoendemic areas reacted positively with the *P. falciparum* antigen. Most of them also gave positive reactions with the anti-IgM conjugate. Similar results were obtained with the sera from 23 Liberians with unknown



from 32 Swedish patients with *P. falciparum* malaria. C = sera from 43 Colombian patients with *P. falciparum* malaria. L = sera from 34 Liberians with mixed malaria background. Controls: S = sera from 31 (10 in d) healthy Swedish blood donors. C = sera from healthy Colombians, said not to have had malaria. —— = means of OD_{405} readings.

malaria background but the reactions were generally slightly weaker. Nevertheless, even when the results were pooled (Fig. 1a & b), the mean of the anti-Ig/*P.falciparum* reactivity for the Liberian group tended to be higher than for any of the other groups (mean 0·91). Sixteen of the 34 Liberian sera reacted positively with the RBC antigen in either the anti-IgM assay. However, the anti-RBC reactivity appeared generally to be less pronounced for the Liberians than for the Swedish patients.

Colombian malaria patients

Thirty out of 43 patients with *P. falciparum* malaria displayed positive reactions, frequently quite strong, in the anti-Ig/*P. falciparum* assay (Fig. 1c). No IgM antibody estimations were performed with the Colombian sera.

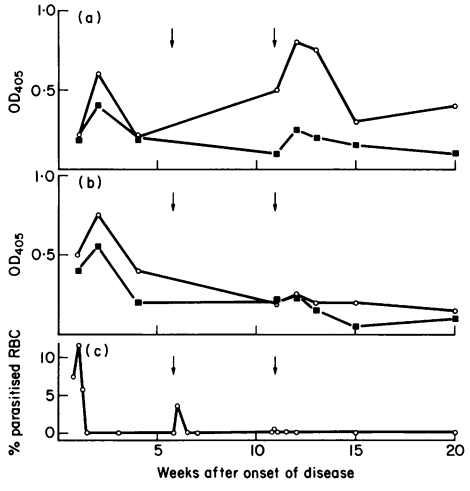


Fig. 2. ELISA values (a, b) and parasitaemia (c) of eight serum samples from patient H.P. taken during a 20 weeks period. The patient had chloroquine resistant *P. falciparum* malaria with parasite relapses (indicated by arrows). (a) OD_{405} in the *P.falciparum* assay. (b) OD_{405} in the RBC ghost assay. O—O = anti-Ig conjugate; ■—■ = anti-IgM conjugate.

*Correlations between anti-*P. falciparum *and anti-RBC antibody levels*

When the intensity of the anti-*P.falciparum* reaction of individual sera was compared with that of their anti-RBC reaction in the anti-Ig system certain correlations were found for both the pooled Liberian and the Swedish groups ($r = 0{\cdot}48$, $P < 0{\cdot}005$ and $0{\cdot}47$, $< 0{\cdot}01$, respectively). The IgM antibody reactivities against the *P. falciparum* and RBC-antigens showed an even stronger correlation. Similar results were obtained for both the Swedish and the Liberian groups ($r = 0{\cdot}59$, $< 0{\cdot}001$ and $0{\cdot}42$, $< 0{\cdot}05$ respectively, non-parametric Spearman Rank test).

Specificity tests

In order to establish the parasite specificity of the anti-*P. falciparum* reactions, five positive sera were absorbed with normal RBC ghosts. After absorbtion, the reactivity of the sera with the RBC antigen was completely abolished in the anti-Ig ELISA. When tested with the *P. falciparum* antigen in the anti-Ig assay, the reactivity remained essentially unchanged in all cases.

Fifteen sera were also tested in a competitive inhibition assay. Fig. 3a shows the results of a typical experiment in which the anti-Ig/*P. falciparum* reaction was efficiently inhibited by *P. falciparum* antigen, 3 μg antigen/ml giving 50% inhibition. In contrast, no significant inhibition was

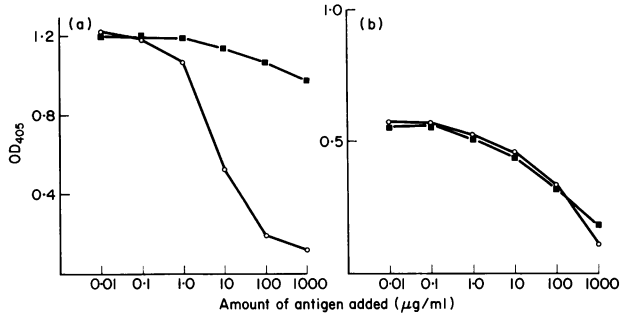


Fig. 3. Inhibition experiments. (a) Inhibition of anti-Ig/*P.falciparum* ELISA. (b) Inhibition of anti-IgM/RBC ghost ELISA. Inhibiting antigen; ■—■ = RBC antigen; O—O = *P. falciparum* antigen.

obtained with as much as 1 mg of RBC antigen (highest amount used for inhibition). Very similar results were obtained with the other sera tested. The amount of *P. falciparum* antigen required for 50% inhibition ranged from 3 to 100 μg/ml. Similar results were also obtained in the anti-IgM/*P. falciparum* assay. The amount of *P. falciparum* antigen required for 50% inhibition here ranged from 1 to 20 μg/ml. However, in this system, a slightly more pronounced inhibition was sometimes obtained with the RBC antigen as well. In the RBC ELISA, the anti-Ig reactions were completely inhibited by the RBC antigen but the *P. falciparum* antigen also gave significant inhibition. This was even more pronounced in the anti-IgM assay in which the RBC and *P. falciparum* antigens gave equal and complete inhibition of the anti-RBC reactions in all instances. A typical example is shown in Fig. 3b.

DISCUSSION

In this report we describe a microELISA for measuring the levels of anti-*P. falciparum* or anti-RBC antibodies in the blood of malaria patients or individuals living in malaria endemic areas. The test antigen, obtained from trophozoite/schizont enriched *in vitro* cultures, recorded antibody reactions which were highly parasite specific in spite of the fact that it also contained red cell components. Thus, the *P. falciparum* antigen efficiently inhibited the parasite ELISA but also gave significant inhibition in the RBC ELISA. In the reverse experiments, normal RBC antigen did not inhibit in the *P. falciparum* ELISA but abolished all antibody reactivity in the RBC ELISA. This parasite specificity was primarily true for antibodies of IgG isotype as detected by the anti-Ig conjugate. In contrast, some of the IgM-dependent reactions obtained with the *P. falciparum* test antigen may have been directed against erythrocyte antigens since normal RBC material sometimes gave inhibition in this system. In general, however, the IgM-dependent reactions also had a good parasite specificity. Confirmatory results were also obtained by absorbing the sera with normal RBC ghosts before testing them in ELISA. The assay is also highly sensitive as positive reactions were observed at serum dilutions of 1/50,000. Similar results have recently been obtained by Avraham *et al.* (1982) who used a radioimmunoassay and a similar antigen.

Comparison of the anti-*P. falciparum* reactivity of sera from different parts of the world confirmed that antibody levels reflect repeated infection or prolonged exposure to the parasite (Perrin & Dayal, 1982). This was also evident from studies of consecutive serum samples taken from Swedish patients under different phases of their first infection or with relapsing disease. The reactions showed a considerable degree of species specificity which, however, was not absolute. Thus, sera from patients experiencing *P. malariae*, *P. ovale* or *P. vivax* malaria reacted weakly with the *P. falciparum* antigen although several of them displayed strong reactions with normal RBC. The nature of the *P. falciparum* antigens in our assay system is under study.

Our studies also provide good evidence that malaria infection gives rise to very strong antibody responses directed to normal RBC components. This was true both for acutely infected patients who had experienced one or a few infections and for chronically infected patients living in malaria endemic areas. The anti-RBC antibodies were formed in response to infection and were of both IgG and IgM isotype but with the latter predominating as suggested by the highly significant correlation of the anti-*P. falciparum* and anti-RBC reactivity of individual sera in the anti-IgM assay. This IgM response may be particularly pronounced during a first infection as seen from comparing the anti-RBC reactions displayed by different groups of sera (e.g. Swedish and Liberians). The consecutive studies of a few Swedish patients also suggested that the IgM anti-RBC response decreases during a more chronic phase of infection. However, it remains to be established if this decrease was real or merely reflected a competition between IgG and IgM antibodies at the antigen level.

The presence of anti-RBC autoantibodies, correlated with parasitaemia and suspected to be in part responsible for the malaria associated anemia, has previously been described in acutely infected *P. falciparum* patients (Rosenberg *et al.*, 1973; Zouali *et al.*, 1982). The results reported in this paper confirm and extend these findings and support the notion of a possible role of anti-RBC antibodies in the induction of anaemia in patients with acute malaria infection.

This work was supported by grant No. B83-16x-06252 from the Swedish Medical Research Council, the Rockefeller Foundation and the Swedish Agency for Research Cooperation with Developing Countries (SAREC). It is also a part of a collaboration between the Department of Immunology, University of Stockholm, Sweden, and the Immunology Section, Department of Medicine, Universidad Nacional, Bogotá, Colombia.

The skillful technical assistance of Mrs V. Stoehrel is gratefully acknowledged. We also wish to thank Inga Pontén and the staff at Roslagstull Hospital for their kind help and cooperation.

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