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High titres of anti-T antibodies and other haemagglutinins in human malaria

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SUMMARY

The prevalence of antibodies against (i) human red blood cells (RBC) of A and B groups, (ii) trypsinized O Rh^+ RBC and (iii) neuraminidase treated O Rh^+ RBC were investigated both in sera of Africans from a malaria endemic area of Upper Volta and in sera of Europeans with acute malaria from a Paris hospital. An increased frequency of high titres of haemagglutinins was observed against A and B as well as O Rh^+ trypsinized human RBC, thus confirming previously published results. In addition, agglutination of neuraminidase treated RBC showed that the titres were increased in about 40% of Africans studied and in about 80% of patients with acute malaria. Using agglutination with a specific anti-T lectin and inhibition with two ligands, it was found that sera of malarious patients contain high titres of antibodies directed against the T antigen of neuraminidase treated RBC. The mechanisms of appearance of high titres of auto-haemagglutinins in malaria and their possible interference in the anaemia associated with this disease are discussed.

INTRODUCTION

Malaria parasites have been shown to affect directly or indirectly the host immune system through non-specific mechanisms which may lead to an immunosuppression (Houba *et al.*, 1980), a polyclonal lymphocyte activation (Greenwood & Vick, 1975) and auto-immune phenomenons (Rosenberg, 1978). Antibodies directed against parasites and self components—DNA, smooth muscle, thyroglobulin, etc—are responsible for the marked hyperimmunoglobulinaemia (Zuckermann, 1970). Some of these antibodies are thought to be involved in the pathogenesis of the anaemia associated with the disease (McBride, Micklem & Ure, 1977). Several reports have pointed out the prevalence of agglutinins against normal and trypsinized human RBC (Kano, McGregor & Milgrom, 1968) as well as RBC of animal origin (Adeinyi-Jones, 1967; Kano *et al.*, 1968; Greenwood, 1970; Houba, Page Faulk & Matola, 1974). It should be noted, however, that there could be some artifacts in the interpretation of these results because of the heterogeneity of the groups studied which were composed of Africans attained with malaria and usually with various other infections. Therefore, the positive tests reported in malaria may be due to other causes.

We designed a study to re-evaluate such haemagglutinins in primary infected Europeans in contrast to Africans exposed to many diseases. In addition, we investigated the antibodies against a cryptic auto-antigen of the RBC, namely the T antigen. In the present report we describe the prevalence of antibodies against (i) normal human RBC of A and B blood group, (II) trypsinized

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and (III) neuraminidase treated RBC of O group in Africans from an area of Upper Volta with endemic malaria compared to French patients with acute malaria and exempt of other diseases. This is the first report of increased titres of anti-T antibodies during malaria infection.

MATERIALS AND METHODS

Sera. Serum samples from 166 subjects were investigated for the presence of haemagglutinins. Specimens were obtained from three groups of subjects: (a) 50 blood donors from 'Centre de Transfusion Sanguine, Institut Pasteur de Paris' who were not previously exposed to malaria and presenting negative tests for syphilis and B hepatitis; (b) 75 individuals from a rural area of Upper Volta (Village of Donse) investigated during the course of a malaria survey and (c) 100 sera from 41 patients from 'Hôpital Salpêtrière, Paris' hospitalized during the course of an acute malaria attack, as proved by blood smears examination (species: *P. falciparum*, 71%; *P. vivax*, 17%; *P. ovale*, 10%; *P. malariae*, 2%; and exempt of any other infection. Anti-T activity of a reference positive serum was kindly determined by G. F. Springer (Immunochemistry Research, Evanston Hospital, Illinois, USA).

Serum samples were diluted 1/10 in phosphate-buffered saline solution (PBS), pH 7·4 and inactivated at 56°C for 30 min.

Treatment of red blood cells. Human O Rh^+ red cells were obtained from 'Centre de Transfusion Sanguine, Institut Pasteur, Paris'. Cells were washed three times in saline solution and a 1% solution was made in PBS. RBC were treated by addition of washed cells (0·10 ml) dropwise with constant stirring, to an equal volume of 0·05 M aceto–acetic buffer pH 5·5 containing five units of neuraminidase (Sigma Chemical Co., St Louis, Missouri, USA). Half a millilitre of saline was added and the mixture was then incubated for 60 min at 37°C. Trypsinization of the RBC was achieved by adding washed cells, dropwise with constant stirring, to an equal volume of trypsin (2·5 mg) followed by incubation for 10 min at 37°C (Kano *et al.*, 1968). The mixtures were centrifuged and the cells washed three times in PBS and finally a 0·8% suspension was made in 1% bovine serum albumin (BSA) in PBS.

Micro-haemagglutination tests. For titrations, BSA diluted 1% in PBS was used as the serum diluent. Serial dilutions of inactivated serum were prepared in micro-agglutination plates (Technique Biologique, France) using microdropping tubes (Dynatech, Switzerland) and micro-diluters (Cooke Engineering, Virginia, USA) so that each well contained 0·025 ml. To each dilution was added 0·025 ml of the three times washed RBC suspension. A control was set up with 0·025 ml serum diluent and 0·025 ml red blood cells suspension. The plates were shaken to suspend the cells and then kept 45 min at 37°C and then 18 hr at 4°C. The reactions were assessed by the settling pattern formed by agglutinated cells on the bottom of the well. The titre was expressed as the reciprocal of the highest serum dilution at which definite agglutination was noted.

Treatment of sera with β-mercaptoethanol. Mercaptoethanol reduction was carried out by treatment of serum with an equal volume of 0·1 M mercaptoethanol in PBS at room temperature for 18 hr. Three volumes of 0·34 M iodoacetamide were then added and the serum dialysed against PBS (Osler, Mulligan & Rodriguez, 1966) and tested for anti-T activity.

Fractionation of immunoglobulins on ion exchange chromatography. IgM and IgG were purified from anti-T active serum by chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), according to the method of Kuga (1980).

Inhibition experiments. Lectin from *Arachis hypogaea* has been used for the clinical determination of T polyagglutinatility (Bird, 1964) of RBC since it gives a similar immunological reaction as the anti-T antibody of mammalian sera (Lotan *et al.*, 1975). It is specific for T antigen (Uhlenbruck, Pardoe & Bird, 1969). This agglutination of neuraminidase treated RBC is inhibited by specific ligands: β-D-galactose and derivates where the galactose must be in a non-reducing terminal position (Pereira *et al.*, 1976).

The effects of galactose and lactose (P.L. Biochemicals, Great Britain) on agglutination of neuraminidase treated red cells by anti-T lectin and by sera of malarious patients were examined. Saccharidic compounds were added in increasing amounts to active anti-T sera or to anti-T lectin

(Sigma Chemical Co.) and incubated for 1 hr at room temperature. The anti-T activity was then tested using neuraminidase treated RBC as indicated above.

RESULTS

Agglutination of A and B human red blood cells

Subjects of O blood group had elevated titres of anti-A and anti-B agglutinins. In the group of Africans 29% of individuals had increased anti-A antibody titres, whereas in the patients from Paris, only 18% had elevated titres. In addition, anti-B titres were higher in both groups: 47% in the Africans and 18% in the primary attacks (Fig. 1). Forty-five percent of african sera tested were found to contain elevated titres of anti-A (B group subjects) or anti-B (A group subjects) agglutinins. In primary attacks, only about 20% of the sera tested showed increase in titres of both agglutinins to 160 or more (Fig. 2).

The occurrence of increased titres of anti-A and anti-B haemagglutinins in blood group O sera suggested that these findings are due to elevated titres of 'cross-reacting' anti-A and anti-B antibodies. This latter assumption was examined by cross-absorption experiments. After absorption of the sera with RBC of the A blood group, not only their anti-A activity disappeared, but their anti-B titre was also reduced. Similarly absorption with RBC of the B blood group removed the anti-B activity and decreased the anti-A titre.

Agglutination of trypsinized red blood cells

The control sera gave consistently negative results whereas 15 out of 75 african sera under investigation agglutinated trypsinized human RBC of blood group O in low titres. More

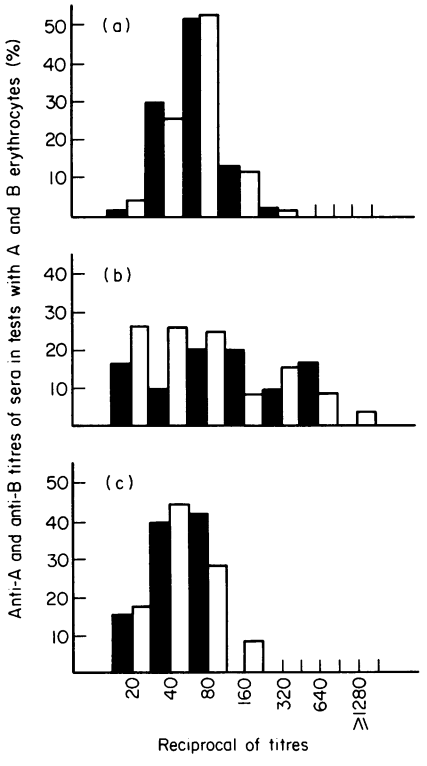

Fig. 1. Distribution of agglutination titres of sera (O group subjects) positive for A(□) and B(■) erythrocytes. (a) Acute malaria; (b) endemic malaria and (c) healthy blood donors.

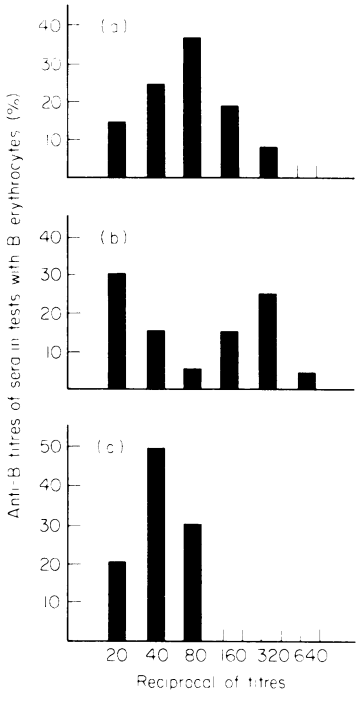

Fig. 2. Distribution of agglutination of sera (A group subjects) positive for B erythrocytes. (a) Acute malaria; (b) endemic malaria and (c) healthy normal donors.

interestingly was the observation that about 40% of the sera of patients with primary attacks were positive for such cells. Titres reached 1,280 in some patients (Fig. 3).

Agglutination of neuraminidase treated red blood cells

Intriguing results were obtained with agglutination of neuraminidase treated red cells. Indeed, the agglutinating titres were increased in about 40% of African subjects from an area where *P. falciparum* malaria is holoendemic, namely the 'Donse' village in Upper Volta. The prevalence of these haemagglutinins was even higher in French patients with acute malaria since 80% of them showed agglutination of neuraminidase treated RBC (Fig. 4). Most of these patients had been for a short period in a malarial endemic area and were apparently exempt of any other disease. However, their blood was collected during, or a short time after, the malaria attack.

Physicochemical nature of the agglutinin

The activity against neuraminidase treated RBC was found to be completely abolished by treatment of sera with β mercaptoethanol, suggesting that immunoglobulins of the IgM class were responsible for this activity. Three sera with high titres of this agglutinin were fractionated by ion exchange chromatography. In each case the agglutinin activity was found only in the fraction of the peak which was shown to contain IgM on polyacrylamide gel electrophoresis and immunodiffusion using specific anti-human immunoglobulins.

Demonstration of anti-T specificity in the sera of malarian patients

To determine whether the agglutinin detected in sera of patients with malaria was effectively directed against the T antigenic determinant of neuraminidase treated RBC, we examined the effect of two specific ligands, namely β-D-galactose and lactose, on the agglutination of the treated cells by three sera from malarious patients and by specific anti-T lectin. Fig. 5 and Fig. 6 present the anti-T activity titres after treatment with increasing amounts of lactose and β-D-galactose. Both of the two

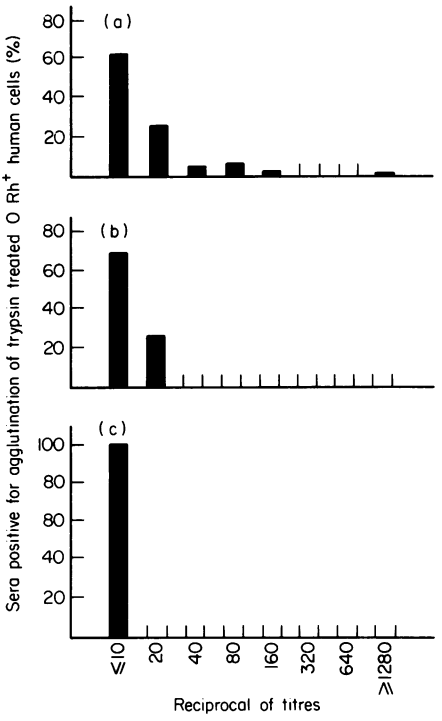

Fig. 3. Distribution of agglutination titres of sera positive for trypsinized blood group O erythrocytes. (a) Acute malaria; (b) endemic malaria and (c) healthy blood donors.

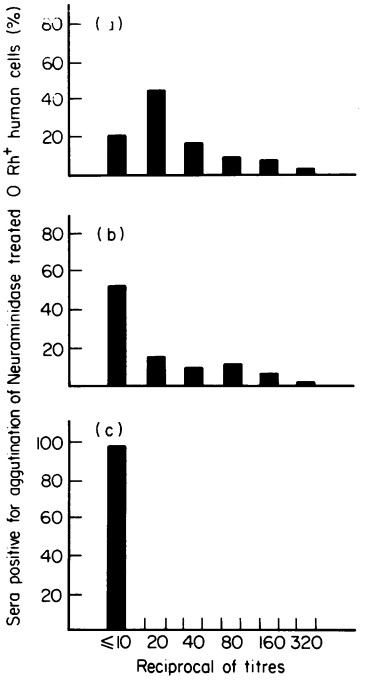

Fig. 4. Distribution of agglutination titres of sera positive for neuraminidase treated O Rh+ human erythrocytes. (a) Acute malaria; (b) endemic malaria and (c) healthy normal donors.

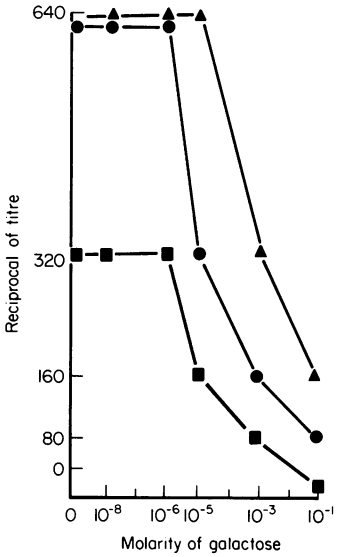

Fig. 5. Effects of galactose on anti-T activity of three sera of malaria patients on red blood cells treated with neuraminidase. (●—●) Patient 176 E, (■——■) Patient 101 A, (▲——▲) Patient 101 B.

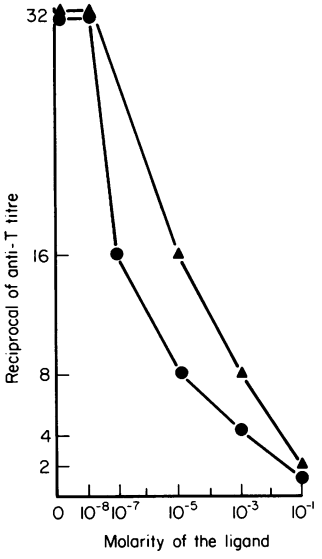

Fig. 6. Effects of two specific ligands on anti-T activity of lectin from *A. hypogaea* on red blood cells treated with neuraminidase. Galactose (▲——▲); lactose (●——●). Anti-T titre $= n \times 10^{-3}$.

ligands inhibited the anti-T activity of the anti-T lectin, and also significantly decreased the anti-T titres of treated sera. These data clearly demonstrate that the antibody detected in the sera of malarian patients examined in our study, is directed against the T antigen of neuraminidase treated RBC.

DISCUSSION

The data presented here clearly indicate that, both in African and in European patients, the titres of anti-A and anti-B natural haemagglutinins are increased. The absorption experiments led support

to the hypothesis that, at least in the samples tested, sera contained 'cross-reacting' anti-A and anti-B antibody. These data are in agreement with results of Kano *et al.* (1968). The finding of haemagglutinins with activity against human RBC treated with trypsin is as interesting as it is enigmatic. Since these antibodies do not react with untreated RBC, they are probably not directed against antigenic determinants of the intact cell surface. Kano *et al.* (1968) found agglutinins of this type in 29% of Gambians, whereas we obtained 20% of positive sera in Africans from Upper Volta and 40% in European patients. It is of special importance that our latter group was composed of patients who were exempt of other infections. Therefore, the haemagglutinins combining with trypsinized human RBC are very likely to be due to malaria.

One of the aims of this study was to evaluate the titre of antibodies directed against neuraminidase treated RBC in malaria. The titres of these haemagglutinins were increased in about 40% of Africans and 80% of Parisian patients. The physicochemical nature of the antibodies has been investigated. They belong to the IgM class of immunoglobulins. Their specificity was further examined using inhibition experiments (see Materials and Methods). We showed that antibodies detected at a high level in sera of malarious patients were directed against the T antigen of the treated RBC which is known to be characterized by the β-D-galactosido-(1-3)-*N*-acetyl-D-galactosamine immunodominant disaccharide structure (Kim & Uhlenbruck, 1966).

The T antigen is responsible for the Hübener–Thomsen–Friedenreich phenomenon discovered in 1927. Meanwhile, it has been shown that, after removal of *N*-acetyl neuraminic acid from glycoproteins of the M and N human blood group the T receptor is uncovered in the form of an alkali labile disaccharide (Mäkelä & Cantell, 1958; Dahr *et al.*, 1977; Vaith & Uhlenbruck, 1978). The T antigen attracted much interest since Springer, Desal & Banatwala (1974) and Springer & Desal (1977) found this receptor on human tumour cells of breast, colon and gastric carcinoma. This crypto-antigenic marker of cell surfaces, masked in most cases by sialic acid, is now being taken into consideration in immunotherapy of certain tumours and leukaemias (Sedlacek, Seiler & Schwick, 1977). On the other hand, antibodies to the T antigen are found at a low level in human and animal normal sera. They usually belong to the IgM class of immunoglobulins although some authors found an anti-T activity in the IgG and IgA class (Springer *et al.*, 1974). The titres are increased in cirrhosis, in breast cancer and in chronic granulocytic leukaemia, but decreased in chronic lymphocytic leukaemia (Eyquem & Faucon, 1953). In infections with neuraminidase positive viruses and bacteria (*Ortho-* and *Para-myxovirus*, *Diplococcus pneumoniae*, *Streptococcus* . . .) the haemolytic anaemia observed is associated with a transient disappearance of anti-T activity and with T polyagglutinability.

Although the mechanisms mediating the production of large amounts of these auto-antibodies in malaria have not been established, several speculations can be made. It could be hypothesized that they may result from some infections—especially in individuals from the endemic area—or from ingested antigens. Considering the agglutinins combining with trypsinized human RBC and neuraminidase treated human RBC, it is tempting to suggest that they are directed against hidden antigens which are uncovered as a result of digestion of normal RBC with some enzymes (trypsin-like and neuraminidase-like) of parasitic origin. Another possibility is that excessive destruction of RBC leads to the exposure of normally cryptic antigenic determinants. Biochemical interactions between blood group substances of the host and the parasite metabolism products could result in appearance of neo-antigens or modification of antigens of the normal RBC. These haemagglutinins may be directed against 'cross-reactive' antigens of parasitic origin; they may also result from a non-specific polyclonal activation induced by mitogenic products of protozoa.

In human malaria, the number of parasitized RBC cannot explain the importance of the anaemia observed in many patients (Adner, Altstatt & Conrad, 1968). Recent studies have focused on immunologically mediated reactions involving non-parasitized erythrocytes and which could best explain the severe haemolysis observed (Jerusalem, 1978). Positive anti-globulin Coombs tests in malarious patients suggest a type III complex-mediated hypersensitivity (Ree, 1976; Facer, Bray & Brown, 1979). Cold agglutinins of the IgM class, with I blood group specificity (Harboe, 1971)—although inconsistently present on the surface of the RBC—may contribute to the removal of non-infected red blood cells if they lead to fixation of C3d component of complement. However, these mechanisms do not seem to play a major role in the pathogenesis of the anaemia associated

with malaria since (i) insignificant titres are often found, (ii) several investigators failed to detect such antibodies and (iii) most of the studies included Africans suffering of endemic malaria but also of other tropical infections.

If the above anti-T antibodies, present in large amounts in sera from malarious individuals, play a role in the non-specific anaemia induced by Plasmodium, a modification of the non-infected RBC exhibiting the T antigenic structure may be an additional mechanism required. In this connection, Howard & Day (1981) demonstrated that glycoproteins sialic acid of uninfected as well as infected RBC of mouse inoculated with *P. berghei* is modified during the infection. Similarly, another report indicated that lectin agglutinability of *P. knowlesi* infected Rhesus monkey RBC was reduced indicating a general modification of accessible sugars on the RBC surface (Vincent & Wilson, 1980). A separate study on *P. luphurae* infected ducks (Sherman & Jones, 1979) supports the above results indicating sialoglycoprotein modification in experimental malarias. Such investigations have been performed in 17 *P. falciparum* infected patients (Howard, 1982). The authors concluded to an absence of modifications of sialic acid of uninfected RBC in human malaria. This discrepancy, between experimental models and human malaria, is probably due to absence of *P. falciparum* schizonte infected RBC from peripheral blood—since they are usually sequestrated in deep—so that it does not exclude the possibility of sialoprotein modifications in human malaria. Nevertheless in *P. falciparum* a number of enzymes have been already described; some of them could modify the surface of infected as well as uninfected erythrocytes. Anti-T antibodies at a high titre may then account for destruction of normal RBC by providing receptors for immune effectors or by contributing to the agglutination and then removal of red cells. This latter point needs further investigation.

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