Clin. exp. Immunol. (1983) **54**, 313–318.

Studies on the specificity of anti-erythrocyte antibodies in the serum of patients with malaria

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(Accepted for publication 24 June 1983)

SUMMARY

Sera from patients with *Plasmodium falciparum*, *P. vivax* or *P. ovale* malaria were selected according to their high levels of antibodies against human erythrocyte membranes as measured in a microELISA. The specificity of the anti-erythrocyte antibodies in these sera and two normal sera was investigated by means of an immunoblotting technique in combination with SDS-polyacrylamide gel electrophoresis. All the patients' sera as well as the control sera contained antibodies against several erythrocyte polypeptides. As compared with normal sera, most malaria sera showed elevated levels of antibodies against polypeptides of 80K, 70K, 40K and 28K molecular weights. Two sera reacted strongly against a polypeptide with an electrophoretic mobility similar to the α subunit of spectrin. One serum showed strong reaction and several other sera, including normal sera, showed weak reaction against a 45K molecular weight polypeptide corresponding to actin. No pervading differences were seen in the pattern of specificities of the anti-erythrocyte ghost antibodies between sera from patients with *P. falciparum*, *P. vivax* or *P. ovale* infections.

Keywords anti-erythrocyte antibodies immunoblotting malaria

INTRODUCTION

The appearance of various autoantibodies in sera of patients with malaria is well documented. Thus, elevated titres of antibodies to heart, thyroid and gastric parietal cells (Shaper *et al.*, 1968) as well as to lymphocytes (Wells *et al.*, 1980) and erythrocytes (Rosenberg *et al.*, 1973; Ronai, Avraham & Sulitzeanu, 1981; Zouali *et al.*, 1982) have been described to be correlated with plasmodial infections. Furthermore, high titres of rheumatoid factor (Shaper *et al.*, 1968; Greenwood, Muller & Valkenburg, 1971), anti-single stranded DNA and anti-nuclear antibodies (Greenwood, Herrick & Holborow, 1970; Adu *et al.*, 1982) have been shown to be associated with malaria infection.

Anti-erythrocyte antibodies in malaria patients' sera have been detected by means of haemagglutination (Zouali *et al.*, 1982), indirect immunofluorescence (Rosenberg *et al.*, 1973) and enzyme linked immunosorbent assay (Wahlgren *et al.*, 1983). In the present study we have used the 'western blotting' technique (Burnette, 1981) for determination of the molecular specificity of malaria-induced antibodies to human erythrocyte membrane polypeptides.

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MATERIALS AND METHODS

Serum samples. The test sera were from patients with either of *Plasmodium falciparum*, *P. vivax* or *P. ovale* malaria hospitalized at the Department of Infectious Diseases, Karolinska Institutet, Roslagstull Hospital, Stockholm, Sweden. All these sera were from patients who had experienced one or a few malarial infections. One serum (Y.2) of a blood donor from a malaria endemic area of Liberia was also included. Control sera were from healthy Swedish blood donors.

Erythrocyte ghosts. Human erythrocytes (group O) from out-dated blood of healthy blood donors were used to prepare haemoglobin free erythrocyte membranes according to the method of Dodge, Mitchell & Hanahan (1963). The protein concentrations were determined by means of the Lowry method.

ELISA (enzyme linked immunosorbent assay). For determination of the levels of anti-*P. falciparum* and anti-erythrocyte antibodies in the sera ELISA was performed as described by Wahlgren *et al.* (1983). Rabbit anti-human immunoglobulin coupled with alkaline phosphatase was used as conjugate (Wahlgren *et al.*, 1983).

Western blotting (Burnette, 1981). Human erythrocyte ghost polypeptides were separated under reducing conditions in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5–15% gradient slab gels using the method of Neville (1971) as modified by Cohen *et al.* (1977). A part of the gel was stained for protein with Coomasie brilliant blue R (Sigma Chem Co., St Louis, Missouri, USA). The separated polypeptides in the remaining gel were transferred electrophoretically onto a nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad Labs, Richmond, California, USA) using a Bio-Rad Trans-Blot electrophoretic blotting apparatus (150mA, 40V for 16 h) and in the following buffer: 25 mM Tris–192 mM glycine, pH 8·3/20% (vol./vol.) methanol/0·1% (wt/vol.) SDS (Erickson, Minier & Lasher, 1982). The nitrocellulose sheet was then incubated over night in 3% (wt/vol.) bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7·4) containing 0·02% (wt/vol.) sodium azide. This and all the following incubations and washes were done at room temperature on a rocking platform. One cm broad strips were cut from the nitrocellulose and transferred to the wells of a LKB Ultra Rack II rack tray (LKB, Bromma, Sweden) and incubated for 16 h in 3 ml of test serum diluted 1/30 in PBS containing 1% BSA, 0·1% (vol./vol.) Triton X-100, 0·02% (wt/vol.) SDS and 0·02% NaN_3 (Fisher, Berrios & Blobel, 1982). The strips were washed in the incubation buffer with 0·2% BSA for about 4 h with five changes of buffer (5 ml each time) and were then incubated for 16 h with the same conjugate as used in the ELISA, diluted 1/500 in incubation buffer, but with SDS omitted. This latter buffer was also used for washing the strips as above. The bound alkaline phosphatase was visualized by incubation of the strips in 50 mM Tris-HCl buffer pH 8·6, containing 10 mM $MgCl_2$, 1mM 1-naphthylphosphate (Sigma) and 2 mM Fast blue B salt (Merck, Darmstadt, FRG) for 5–15 min (Allen & Hunter, 1960).

Human erythrocyte ghosts, dog muscle fibre proteins, urease, transferrin, BSA, human IgG and haemoglobin were used as molecular weight markers in the SDS-PAGE.

RESULTS

Sera from patients with malaria infection were selected according to their relatively high antibody reactivity against human erythrocyte ghosts as tested in ELISA. These ELISA values as well as the ELISA reactivity of the sera against a *P. falciparum* antigen are included in Table 1. The two control sera used in this study were among the control sera used by Wahlgren *et al.* (1983) and their reactivity in ELISA against both the *P. falciparum* antigen and the erythrocyte ghost antigen was less than OD_{405} 0·25.

Fig. 1 shows the patterns of reactivity of the different sera against erythrocyte ghost polypeptides as detected by means of western blotting. These results are summarized in Table 1. All the patients' sera contained antibodies against several human erythrocyte polypeptides. The control sera reacted also, but weakly, with some of the polypeptides. The reactivity of these two control sera is representative for the reactivity of all control sera tested so far (totally nine). Most malaria sera

Table 1. A summary of ELISA values and anti-erythrocyte ghost antibody reactivities of malaria patients' and control sera

		ELISA values*		Antibody reactivities†								
Patient	Type of malaria	*P. falciparum*	Erythrocyte ghosts	240K	94K	80K	76K	70K	45K	40K	37K	28K
B.J.	*P. falciparum*	1·40	0·95	+	+	+	+	+	(+)	(+)		+
B.W.	*P. falciparum*	0·70	0·70		+	(+)		+	(+)	(+)		+
H.P.	*P. falciparum*	1·15	1·10			+	(+)	+	(+)	+	+	+
K.N.	*P. vivax*	1·15	>2·0		(+)	+	+	+	(+)	+	+	+
K.J.	*P. vivax*	0·40	1·10			+	+	+		+		+
P.K.	*P. vivax*	1·00	1·00			+	+	+	+	+	(+)	+
U.S.	*P. ovale*	0·60	1·05	+		+	+	+		+	+	+
Y.2	Hyperimmune	1·10	0·35			+				+		(+)
C.1	—	<0·25	<0·25			(+)		(+)	(+)	+		(+)
C.2	—	<0·25	<0·25			(+)		(+)	(+)	(+)		(+)

* OD_{405} at a serum dilution 1/1,000 (for details see Wahlgren *et al.*, 1983).

† Antibody reactivities as detected in western blotting (see Materials and Methods). + = elevated reactivity as compared with the control sera. (+) = weak reactivity.

showed elevated levels of antibodies against polypeptides of approximate molecular weights 80K, 70K, 40K and 28K, having electrophoretic mobilities comparable with those of the erythrocyte ghost polypeptides 4·1, 4·2, 6 and 7, respectively (Fairbanks, Steck & Wallach, 1971; Lux, 1979). The 80K polypeptide appears here as a doublet and most sera react equally strong with both bands, while patient P.K. stains mainly the lower one of these bands.

Two of the sera, B.J. and U.S., showed reactivity to a 240K polypeptide, which comigrates on the gel with the erythrocyte ghost polypeptide 1, corresponding to the high molecular weight subunit of spectrin (Fairbanks *et al.*, 1971; Lux, 1979). One serum, P.K., reacted strongly and some other sera, including a control serum C2, reacted weakly with a 45K polypeptide, corresponding in electrophoretic mobility to erythrocyte ghost polypeptide 5, which is actin (Fairbanks *et al.*, 1971; Lux, 1979).

The Liberian serum Y.2 gave a relatively weak reaction against erythrocyte ghosts in ELISA and showed anti-erythrocyte ghost antibodies mainly against the 80K and 40K polypeptides.

Furthermore, several sera reacted with polypeptides of approximate molecular weights 94K, 76K, 47K, 37K. The identity of these polypeptides is not known. With most of the sera weak staining of some other polypeptides than those mentioned was also seen. The appearance of a 220K band with all sera is, however, artefactual, as it also stained when no test serum was added.

DISCUSSION

The present study demonstrates the presence in malaria patients' sera of elevated levels of antibodies against certain human erythrocyte membrane polypeptides. The weak reactivity of the normal sera against some of the polypeptides is probably not due to artefactual background staining, but rather due to the presence in the sera of naturally occurring autoantibodies. Antibodies reacting with the cell surface of enzyme treated or aged autologous erythrocytes have been demonstrated in the serum of most normal individuals (Alderman, Fudenberg & Lovins, 1980; Kay, 1975; Lutz & Kay, 1981). These antibodies, however, react mainly with a 100K molecular weight polypeptide and its 62K proteolytic fragment (Lutz, 1981) which does not correspond to any of the antigens detected in our tests. The naturally occurring anti-erythrocyte antibodies described

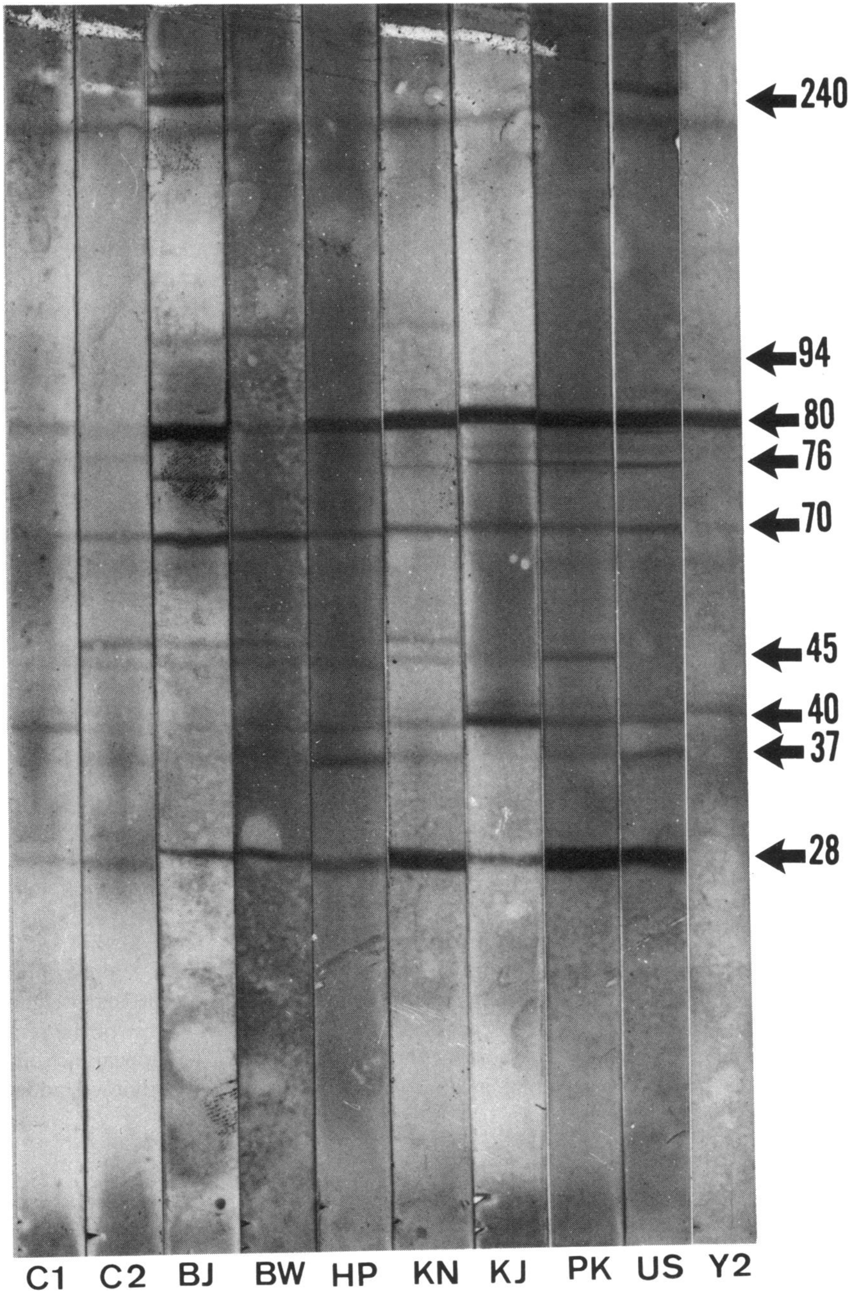

Fig. 1. Western blotting of malaria patients and control sera for detection of antibodies to human erythrocyte ghost polypeptides. Letters at the bottom indicate the patients and the numbers to the right indicate approximate molecular weights $\times 10^{-3}$.

herein include antibodies against intracellular polypeptides such as cytoskeletal components (band 4.1 and actin) and may thus correspond to autoantibodies similar to those demonstrated by Guilbert, Dighiero & Avrameas (1982).

Non-infected erythrocytes of patients with *P. falciparum* malaria have been shown to have antibodies on their surface (Facer, Bray & Brown, 1979; Facer, 1980). The schizont reactivity of some of these antibodies indicated that they were from immune complexes which had been adsorbed to the erythrocytes, rather than being antibodies with erythrocyte specificity (Facer, 1980). However, anti-erythrocyte autoantibodies reacting with the surface of normal or acetone fixed human erythrocytes have also been reported to occur in *P. falciparum* patients' sera (Rosenberg *et al.*, 1973; Zoulai *et al.*, 1982) and are thought to be at least in part responsible for the anaemia frequently seen in these patients. Furthermore, elevated anti-erythrocyte ghost antibody levels have recently been demonstrated to be associated with human malaria infections (Wahlgren *et al.*, 1983). Most of the antigens recognized by the malaria-induced anti-erythrocyte antibodies in our test system are known intracellular components. Thus, spectrin, band 4.1 and actin constitute the major part of the erythrocyte cytoskeleton (Lux, 1979). At this stage of the study we do not known if some of the anti-erythrocyte membrane antibodies we detect are reactive with antigens on the erythrocyte surface.

The appearance of different kinds of autoantibodies in the serum in association with malaria infections has been ascribed to a polyclonal B cell activation due to a plasmodial mitogen (Greenwood & Vick, 1975; Greenwood, Oduloju & Platts-Mills, 1979). While this may well be true for the anti-erythrocyte antibodies seen in this study, it can not be excluded that these antibodies have been elicited specifically by antigens exposed due to the extensive erythrocyte damage occurring during the acute phase of the malaria infection.

The appearance of anti-erythrocyte antibodies is not restricted to *P. falciparum* malaria, but occurs also in the *P. ovale* and *P. vivax* malarias. In fact the latter plasmodial infections tend to give a higher frequency of sera with high levels of anti-erythrocyte antibodies than *P. falciparum* malaria as measured by ELISA (Wahlgren *et al.*, 1983).

The major contaminants in the parasite antigen preparations used for measurements of levels of anti-malaria parasite antibodies are erythrocyte membrane components. Thus, it is important to assess the influence of such contaminants in the test system to avoid false high readings of anti-parasite antibodies due to the presence of anti-erythrocyte antibodies (Wahlgren *et al.*, 1983).

This work was supported by grants from the Swedish Medical Research Council and the Rockefeller Foundation. We thank Inga Pontén and the staff at Roslagstull Hospital for their kind help and cooperation.

REFERENCES

Adu, D., Williams, D.G., Quakyi, I.A., Voller, A., Anim-Addo, Y., Bruce-Tagoe, A.A., Johnson, G.D. & Holborow, E.J. (1982) Anti-ssDNA and anti-nuclear antibodies in human malaria. *Clin exp. Immunol.* **49,** 310.

Alderman, E.M., Fudenberg, H.H. & Lovins, R.E. (1980) Binding of immunoglobulin classes to subpopulations of human red blood cells separated by density-gradient centrifugation. *Blood,* **55,** 817.

Allen, J.M. & Hunter, R. (1960) A histochemical study of enzymes in the epididymis of normal, castrated and hormone replaced castrated mice separated by zone electrophoresis in starch gels. *J. Histochem. Cytochem.* **8,** 50.

Burnette, W.N. (1981) 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112,** 195.

Cohen, R.S., Blomberg, F., Berzins, K. & Siekevitz, P. (1977) The structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. *J. cell Biol.* **74,** 181.

Dodge, J.T., Mitchell, C. & Hanahan, D.J. (1963) The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100,** 119.

Erickson, P.F., Minier, L.N. & Lasher, R.S. (1982) Quantitative electrophoretic transfer of polypeptides from SDS polyacrylamide gels to nitrocellulose sheets: A method for their re-use in immunoautoradiographic detection of antigens. *J. Immunol. Meth.* **51,** 241.

Facer, C.A. (1980) Direct Coombs anti-globulin reactions in Gambian children with *Plasmodium falciparum* malaria. II. Specificity of erythrocyte bound IgG. *Clin. exp. Immunol.* **39,** 279.

Facer, C.A., Bray, R.S. & Brown, J. (1979) Direct

Coombs anti-globulin reactions in Gambian children with *Plasmodium falciparum* malaria. I. Incidence and class specificity. *Clin. exp. Immunol.* **35,** 119.

FAIRBANKS, G., STECK, T.L. & WALLACH, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochem.* **10,** 2606.

FISHER, P.A., BERRIOS, M. & BLOBEL, G. (1982) Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of *Drosophila melanogaster. J. cell. Biol.* **92,** 674.

GREENWOOD, B.M., HERRICK, E.M. & HOLBOROW, E.J. (1970) Speckled anti-nuclear factor in African sera. *Clin. exp. Immunol.* **7,** 75.

GREENWOOD, B.M., MULLER, A.S. & VALKENBURG, H.A. (1971) Rheumatoid factor in Nigerian sera. *Clin. exp. Immunol.* **8,** 161.

GREENWOOD, B.M., ODULOJU, A.J. & PLATTS-MILLS, T.A.E. (1979) Partial characterization of a malaria mitogen. *Trans. R. Soc. Trop. Med. Hyg.* **73,** 178.

GREENWOOD, B.M. & VICK, R.M. (1975) Evidence for a malaria mitogen in human malaria. *Nature,* **257,** 592.

GUILBERT, B., DIGHIERO, G. & AVRAMEAS, S. (1982) Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation and characterization. *J. Immunol.* **128,** 2779.

KAY, M.M.B. (1975) Mechanism of removal of senescent cells by human macrophages *in situ*. *Proc. Natl. Acad. Sci. USA.* **72,** 3521.

LUTZ, H.U. (1981) Elimination alter Erythrozyten aus der Zirkulation: Freilegung eines zellalter-spezifischen Antigens auf alternden Erythrozyten. *Schweiz, Med. Wochenschr.* **111,** 1507.

LUTZ, H.U. & KAY, M.M.B. (1981) An age-specific cell antigen is present on senescent human red blood cell membranes. A brief note. *Mech. Ageing Dev.* **15,** 65.

LUX, S.E. (1979) Dissecting the red cell membrane skeleton. *Nature,* **281,** 426.

NEVILLE, D.M. (1971) Molecular weight determinations of protein–dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. biol. Chem.* **246,** 6328.

RONAI, Z., AVRAHAM, H. & SULITZEANU, D. (1981) Autoantibodies to red blood cells in rats infected with *Plasmodium berghei. J. Parasitol.* **67,** 351.

ROSENBERG, E.B., STRICKLAND, G.T., YANG, S.-L. & WHALEN, G.E. (1973) IgM antibodies to red cells and autoimmune anemia in patients with malaria. *Am. J. Trop. Med. Hyg.* **22,** 146.

SHAPER, A.G., KAPLAN, M.H., MODY, N.J. & MCINTYRE, P.A. (1968) Malarial antibodies and autoantibodies to heart and other tissues in the immigrant and indigenous peoples of Uganda. *Lancet,* **i,** 1342.

WAHLGREN, M., BERZINS, K., PERLMANN, P. & BJÖRKMAN, A. (1983) Characterization of the humoral immune response in *Plasmodiun falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of micro-ELISA. *Clin. exp. Immunol.* **54,** 127.

WELLS, R.A., PAVANAND, K., ZOLYOMI, S., PERMPANICH, B. & MACDERMOTT, R.P. (1980) Anti-lymphocytotoxic antibodies in sera of Thai adults infected with *Plasmodium falciparum* or *Plasmodium vivax. Clin. exp. Immunol.* **39,** 663.

ZOUALI, M., DRUILHE, P., GENTILINI, M. & EYQUEM, A. (1982) High titres of anti-T antibodies and other haemagglutinins in human malaria. *Clin. exp. Immunol.* **50,** 83.