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THE RED CELL CYTOSKELETON AND INVASION BY MALARIA PARASITES

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The role of the red cell cytoskeleton, in particular spectrin, in the invasion process by P. falciparum *merozoites has been investigated. When normal red cells were made more rigid with the oxidant diamide, then invasion was markedly reduced. Similarly, by increasing the percentage of spectrin dimers in the membrane by treatment of normal erythrocytes with N-ethyl maleimide, a decrease in invasion was again observed. Cells from a patient with hereditary pyropoikilocytosis, in which there is a genetic defect in spectrin dimerisation, were refractory to invasion by merozoites. All cell types are much less deformable than normal and it is probably this feature that limits endocytosis by the parasite.*

Several distinct steps are involved during the entry of the malaria merozoite into a red cell. Following accidental contact with a red cell the merozoite re-orientates so that its apical end is perpendicular to the red cell surface and the first recognition step is established. Recognition and attachment are via epitopes on the transmembrane proteins glycophorin (Pasvol, Wainscoat & Weatherall, 1982; Facer, 1983) and band 3 (Friedman, Fukuda & Laine, 1985). Following attachment there is a transient distortion of the red cell followed by entry of the merozoite into the cell. An electron dense zone forms at the point of contact of the merozoite and is thought to represent aggregation of intramembranous particles (IMP) within the erythrocyte membrane. This electron dense zone migrates to the edges of the area of initial contact of the merozoite and a bare patch is left at which the invagination originates (McLaren et al., 1979). This redistribution of IMP suggests that significant changes in cytoskeletal configuration induced by the merozoite may be a necessary condition for penetration of the parasite into the cell. How these changes may be initiated in relation to the major erythrocyte skeletal protein, spectrin, have been investigated by either chemically modifying normal spectrin or by examining invasion into human red cells with defined genetic defects in spectrin self-association.

MATERIALS AND METHODS

Parasites and invasion assays – A modification of the method of Trager & Jensen (1976) was used for the continuous culture of the Wellcome/Liverpool West African strain of *Plasmodium falciparum* (Facer, 1983). Sorbitol synchronisation of cultures was performed according to the technique of Lambros & Vanderberg (1979).

Invasion assays were carried out as previously described using fluorescein isothiocyanate-labelled red cells (Facer, 1983). Results are expressed as the ratio of invasion into normal erythrocytes to the invasion into abnormal cells. Alternatively, invasion into the variant cells is expressed as a percentage of invasion into normal red cells.

HPP and HE2 erythrocytes – Cells from a Greek cypriot patient with mild hereditary elliptocytosis (type HE2) were obtained by venepuncture at The London Hospital. Blood was drawn from a black patient (NE) with hereditary pyropoikilocytosis (HPP) and from her asymptomatic (HPP carrier) mother (CE) at Queen Elizabeth's Hospital, Boston, and sent on ice to London where the samples were tested for susceptibility to invasion within 24h of receipt (four days after the sample was taken).

Incubation with oxidants and sulfhydryl reagents – Group O erythrocytes were obtained from healthy volunteers by venepuncture using sterile acid-citrate-dextrose (ACD) as anticoagulant. Cells were washed and the buffy coat removed. Erythrocytes were suspended to 10% haematocrit in 5mM phosphate buffer, pH 7.4, containing 150mM NaCl alone or plus 1.0mM diamide. Cells were also incubated with either the permeant sulphydryl reagent N-ethyl maleimide (NEM) at 0.2mM and 2.0mM concentrations or the impermeant sulfhydryl reagent monobromotrimethyl-ammoniobimane (MQ) at the same molarities. Incubation was for 15 min at 37°C and the cells washed three times with buffer. All manipulations were performed under sterile conditions.

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Membrane protein analysis – Hypotonic lysis of red cells and extraction of spectrin from the ghost membranes was carried out according to the method of Smith & Palek (1983). Spectrin extracts were analysed for spectrin species by non-denaturing agarose-acrylamide gel electrophoresis (Liu et al., 1981).

Quantitation of spectrin dimers in the membrane – Spectrin dimers (Sp-D), tetramers (Sp-T) and higher oligomers (Sp-O) were measured by the relative intensity of Coomassie blue staining of the bands using a Beckman Lasar Densitometer.

Statistical analysis of data – The significance of differences between invasion into normal and treated or variant red cells was assessed using unpaired t-tests.

RESULTS

Table I shows that invasion into diamide-treated normal erythrocytes was significantly reduced in the two samples tested. 1.0mM diamide effectively reduced invasion to 33% of normal in sample CP and 26.2% of normal in sample CH.

TABLE I

P. falciparum invasion into red cells treated with 1.0 mM diamide

Invasion	Donor			
	CP		CH	
	Control	+ diamide	Control	+ diamide
$\bar{X}$ ratio * (± I SD)	1.15 ± 0.33	0.50 ± 0.30	1.03 ± 0.33	0.27 ± 0.18
Significance	$p < 0.01$		$p < 0.05$	

* Ratio of invasion into FITC-labelled test red cells (normal variant or chemically altered) to non-labelled (normal) red cells.

Treatment of erythrocytes with the permeant sulfhydryl reagent NEM (2.0mM) significantly altered spectrin self-association in the membrane and raised the percentage of Sp-D within the membrane from 8% to 26% of total spectrin. Conversely, when the cells were treated with the impermeant reagent, MQ, no alteration in spectrin dimerisation was apparent. Invasion into NEM and MQ-altered cells is shown in Table II. Both 0.2mM and 2.0mM NEM significantly ($p < 0.001$) reduced invasion, but no effect was seen with the MQ-treated cells ($p < 0.06$).

TABLE II

P. falciparum invasion into normal red cells treated with NEM or MQ

	Treatment of cells				
Invasion	PBS (control)	0.2 mM NEM	2.0 mM NEM	0.2 mM MQ	2.0 mM MQ
Invasion (% of control)	100	33	23	98	93

Cells from the patient with HE2 were fully susceptible to invasion by *P. falciparum* (Table III). In contrast, cells from the patient with HPP (NE) were markedly, although not totally, resistant to invasion (Table III) showing only 33% of the invasion observed into normal cells ($p < 0.001$). Interestingly, cells from her asymptomatic (HPP carrier) mother showed a smaller (68% of normal) but nevertheless significant resistance to invasion. The ability to resist invasion was correlated with the degree of spectrin dimerisation in the red cells membranes of the patient and her mother (Sp-D were 38% and 22% respectively) as shown by Liu et al. (1981).

TABLE III

P. falciparum invasion into HPP and HE2 variant erythrocytes

	Donor			
Invasion	Control	HE2	HPP (NE)	HPP carrier (CE)
(% of control)	100	110	33	68

DISCUSSION

The entry of a merozoite into a red cell depends on its ability to bring about deformation of the red cell membrane. How this process is achieved is uncertain although it is becoming clear that significant alterations in the red cell cytoskeleton are necessary for such a relatively large (in comparison to the red cell) parasite to successfully endocytose. The erythrocyte membrane presents a barrier to the merozoite. The cytoplasmic face of the membrane is laminated by a protein skeleton that is involved in stabilising the membrane and controlling cell shape, deformability and lateral mobility of the transmembrane proteins, glycophorin and band 3 (Sheetz, 1983). Any alteration in the self-association of the major skeletal protein, spectrin, dramatically effects the stability and deformability of the membrane (Smith & Palek, 1983). Self-association can be decreased with sulfhydryl reagents (eg NEM) or increased by oxidants such a diamide and both processes, although having opposite molecular effects, both produce undeformable red cells. Diamide X-links spectrin by intramolecular disulfide coupling and spectrin becomes a very high molecular weight complex ($> 10^8$ daltons). NEM reduces disulfide bonding on spectrin and increases the Sp-D : Sp-T ratio in the membrane (Smith & Palek, 1983).

The results of the effects of diamide-treatment of normal erythrocytes on invasion by *P. falciparum* merozoites confirms earlier reports on this effect Miller et al. (1984). More recently, Dluzewski et al. (1983) have X-linked spectrin with anti-spectrin antibodies and produced the same effect as diamide. These antibody-loaded cells were markedly resistant invasion and their decreased deformability was the feature which appeared to make them resist invasion.

We have shown in the present studies, that an increase in Sp-D in the erythrocyte membrane makes that cell relatively refractory to invasion. Thus normal cells treated with the sulfhydryl reagent NEM, and HPP erythrocytes, both containing a high percentage of Sp-D, resist invasion. Here, as in diamide-treated red cells, the integrity of the erythrocyte cytoskeleton is lost and linkages between the transmembrane and cytoskeletal proteins become defective. This may be relevant to the process of merozoite endocytosis should transmembrane signalling events be important prior to entry of the parasite. Alternatively, it has recently been shown that defective Sp-D self-association leads to an increase in the number of mechanical subunits of the skeletal matrix and an increase in membrane rigidity results (Chabanel, personal communication). This membrane rigidity may present a formidable barrier to merozoite endocytosis.

The HE2 erythrocytes were, as expected, not resistant to invasion since this sub-group of elliptocytes have a normal percentage of Sp-D in the membrane when compared to the HE1 group which have raised Sp-D (Palek, 1985).

Recent epidemiological and experimental evidence indicates that stomatocytic elliptocytes (ovalocytes) are resistant to invasion by a variety of malarial parasites (Kidson et al., 1981) and suggests that a high gene frequency in Melanesians is due to natural selection. The molecular explanation for this resistance is uncertain although it is probable that the reduced deformability of ovalocytes may provide the explanation. Similarly nearly all HPP patients described are Black (Prchal, personal communication) so it is possible that the HPP carrier state may confer some selective advantage in protection against malaria infections. Future work will concentrate on testing more HPP and HE1 cells with defined Sp-D abnormalities for resistance to invasion by malaria merozoites.

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