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Rotational dynamics of the integral membrane protein, band 3, as a probe of the membrane events associated with *Plasmodium falciparum* infections of human erythrocytes

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Time-resolved phosphorescence anisotropy was used to study the molecular organisation of band 3 in the erythrocyte membrane. Three different rotational relaxation regimes of mobile band 3 were resolved. These populations may represent different aggregation states of band 3 within the membrane, or they may result from association of band 3 with other proteins at the cytoplasmic surface. The polycation spermine decreases the apparent mobility of band 3 by a mechanism that does not involve the underlying cytoskeleton. A monoclonal antibody directed against the cytoplasmic portion of band 3 can also cause an increase in the immobile fraction of band 3 molecules. This monoclonal antibody will inhibit invasion of erythrocytes by malaria parasites. Membranes prepared from erythrocytes infected with mature stages of the malaria parasite, *Plasmodium falciparum*, show altered dynamic properties corresponding to a marked restriction of band 3 mobility.

Introduction

The sequence of events whereby a malaria parasite attaches to and invades an erythrocyte has been delineated by a series of elegant electron microscopic and biochemical studies (see Refs. 1 and 2 for reviews). The erythrocyte membrane protein to which *Plasmodium falciparum* initially binds remains to be definitively identified. The most likely candidate seems to be glycophorin A [3] although band 3 has also been implicated [4]. The invasion process which follows seems to involve disruption of the normal interactions within the erythrocyte membrane to create a patch of membrane which is free of both integral membrane proteins and cytoskeletal proteins [5]. Moreover, as the parasite develops within the erythrocyte, marked changes are observed in properties of the erythrocyte membrane, such as permeability, deformability and cytoadherence [6]. These changes presumably reflect alterations in the molecular organisation of the erythrocyte membrane components.

To observe these processes at a molecular level, physical methods are required which are sensitive to alterations in the interactions between different erythrocyte membrane components. One reflection of such interactions is the diffusional mobility of band 3, the major integral membrane protein of human erythrocytes. The mobility of band 3 has been well-characterised and the rates of both rotational and lateral diffusion of band 3 have been shown to be restricted by interactions with other membrane proteins.

Restrictions of the lateral mobility are thought to be due to trapping of the cytoplasmic domain of band 3 in the interstices of the cytoskeletal protein network at the cytoplasmic surface of the membrane [7,8].

The rotational diffusion of band 3 does not appear to be affected by the presence of the spectrin-actin network, even though at least 10–20% of the band 3 molecules are linked via ankyrin to spectrin [9]. Both spectrin and the cytoplasmic domain of band 3 are probably quite flexible which, it has been inferred [10], is sufficient to allow effectively unconstrained rotation

Abbreviations: MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PNG, Papua New Guinea; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

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of band 3. The observed restriction of rotation is instead suggested to be due to participation of band 3 in large complexes with other integral and peripheral membrane proteins, such as glycophorin [11], ankyrin and proteins 4.1 and 4.2 [10], as well as with the glycolytic enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase [12].

In the present study, we have measured the rotational diffusion of band 3 in human erythrocyte membranes as a probe of the molecular events occurring during malaria infections. Band 3 has been labelled with eosin-5-maleimide and its rotational diffusion was followed using the technique of time-resolved phosphorescence anisotropy.

To examine the role of band 3 in the process of malarial invasion, we have examined changes in its rotational mobility brought about by agents that affect the efficiency of invasion of erythrocytes. We have studied two such species viz., a monoclonal antibody against the cytoplasmic domain of band 3 and the polycation, spermine. It has previously been shown that spermine, when incorporated inside resealed human erythrocytes, inhibits invasion of erythrocytes by *Plasmodium falciparum* [13]. We show that a monoclonal-band 3 antibody also inhibits invasion in this assay.

We have also examined the rotational mobility of band 3 in membranes prepared from erythrocytes infected with mature stages of the parasite, *Plasmodium falciparum*. The organisation of the erythrocyte membrane proteins appears to be altered in the mature parasite-infected cells, such that band 3 mobility is substantially restricted.

Materials and Methods

Materials. Glucose oxidase (type II) and papain were obtained from Sigma. Eosin-5-maleimide was purchased from Molecular Probes, U.S.A. Immulon ELISA plates were obtained from Dynatech. Concanavalin A-Sepharose was obtained from Pharmacia. Horse-radish peroxidase conjugated anti-mouse globulins, 2,2′-azino-di(3-ethylbenthiazoline) sulphonate and [^{14}C]methylated protein markers were obtained from Amersham.

Parasites. Plasmodium falciparum (FCQ27/PNG) was continuously cultured as described by Trager and Jensen [14]. Synchronisation was achieved using sorbitol treatment [15]. Trophozoite stages were isolated using a procedure based on the method of Aley et al. [15] involving centrifugation through a Percoll/sorbital gradient.

Labelling of band 3. Fresh human blood was obtained from the Blood Transfusion Service, Melbourne, Australia. Band 3 was labelled in intact erythrocytes by incubation with eosin-5-maleimide as described by Nigg and Cherry [16], with the following modifications. Eosin-5-maleimide (0.2 ml × 0.5 mg/ml) was added to 1 ml of packed erythrocytes in 130 mM NaCl, 18 mM sodium phosphate (pH 8.0) and incubated for 1 h at room temperature in the dark. Unreacted probe was removed by washing the cells three times with 160 mM NaCl, 5 mM sodium phosphate (pH 7.5). Ghosts were prepared by hypotonic lysis and washing in 5 mM sodium phosphate (pH 8.0). The final wash was with 10 mM NaCl, 10 mM Tris HCl (pH 7.6) unless otherwise specified.

Band 3 in infected erythrocytes was labelled in the same manner as in uninfected cells. During the preparation of ghosts from *P. falciparum*-infected erythrocytes, a haemozoin pellet collected underneath the membranes. Membranes were separated from this pellet by at least two centrifugation steps in Eppendorf tubes, with careful transfer of the more lightly sedimenting membranes to fresh tubes after each centrifugation.

The specificity of labelling of band 3 in membranes prepared from normal and malaria-infected erythrocytes was checked by SDS-PAGE (7.5% acrylamide). Eosin fluorescence was photographed through an orange filter, in unstained gels illuminated by UV light [16]. As has been shown previously by Nigg and Cherry [16], the major labelled component corresponds to band 3. Minor components run ahead of band 3 including a band of free eosin which runs at the position of the tracking dye. A labelling ratio of 0.7–1 eosin chromophores per band 3 subunit was determined spectrophotometrically for SDS-solubilised ghosts as described by Nigg and Cherry [16].

Purification of band 3. Band 3 was extracted from erythrocyte ghosts with 1% Triton X-100 and absorbed on concalavalin A-Sepharose as described by Findlay [17]. The resin was mixed with Laemmli sample buffer, heated (100 °C for 3 min) and applied directly to a preparative SDS-PAGE gel (8%, 1.5 mm thick). Following electrophoresis, the gel was lightly stained with Coomassie blue and the broad band 3 at 90 kDa was excised and electroeluted.

Hybridomas. Female Balb/c mice were immunised intraperitoneally with 50 μg of purified band 3 emulsified in Freund's adjuvant. Similar doses were re-administered after a further 2 and 4 weeks. Four days later, hybridomas were produced using the method of Galfe et al. [18] by fusing spleen lymphocytes with NS-1 myeloma cells. Culture supernatants from the various hybridoma clones were tested for binding of purified band 3 using an enzyme-linked immuno assay.

Immunoglobin purification and preparation of Fab fragments. The monoclonal antibody B-9 (IgG class) was affinity purified using protein A-Sepharose chromatography. Fab fragments were prepared by incubating 5 mg of MAb with 0.05 mg of activated papain in 2 mM EDTA, 1 mM DTT, 0.1 M Tris-HCl (pH 8.0) for 1 h at 37 °C. The reaction was stopped by addition of iodoacetamide to 20 mM. The resulting mixture was

dialysed overnight against 10 mM Tris-HCl (pH 8.0), then passed over a column of protein A-Sepharose to remove any undigested intact IgG and the Fc portion. The eluate of the Fab fragments was concentrated by Amicon filtration. Purity of each fraction was assessed using SDS-PAGE (12% acrylamide). The final preparations of B-9 MAb and Fab were judged to be > 95% pure.

Partial proteolysis, electrophoresis and Western blotting. Ghosts were auto-phosphorylated by incubation with [γ-^{32}P]ATP [19] and phosphorylated band 3 was purified as described above. Both cold ghosts and purified labelled band 3 were treated with α-chymotrypsin (25 μg/ml) in 1% ammonium bicarbonate for 30 min at 23° C, prior to treatment with Laemmli sample buffer (3 min at 100° C) and electrophoresis (SDS-PAGE, 8% acrylamide) of the partially proteolysed mixture. Immunoreactive zones were detected on Western blots using the anti-band 3 Mab supernatant (B-9) followed by ^{125}I-goat anti-mouse globulins [20]. For detection of phosphorylated fragments derived by partial proteolysis of the purified labelled band 3, the gel was dried and directly autoradiographed.

P. falciparum invasion assay. The ability of the MAb to inhibit invasion of released erythrocytes was determined using the reversible lysis procedure described by Dluzewski et al. [12]. Briefly, erythrocytes were suspended at 30% haematocrit in isotonic solution containing the anti-band 3 antibody and then lysed by dialysis against 5 mM phosphate, 1 mM MgATP (pH 7.4) for 100 min at 4° C. The red cells were resealed by dialysis against isotonic buffer for 45 min at 37° C. The resealed cells were washed in the culture medium (RPMI 1640, containing 10% human serum). Purified schizonts were then added to the resealed cells. Red cells lysed and resealed in the absence of antibody were the controls. Parasitemia levels were determined after about 20 h by staining thin smears with Giemsa reagent and counting 10^3 cells. Parasitemias were determined as invasion ratios, i.e., the observed parasitemia at a given time to the initial parasitemia at zero time, and are expressed as a percentage of the control.

Preparation of samples for spectroscopy. Eosin-labelled erythrocyte membranes were gently suspended at a concentration of about 0.8 mg/ml protein, which corresponded to an eosin concentration of approx. 2 μM. Deoxygenation was achieved enzymatically using 12 μg/ml glucose oxidase and 50 mM glucose, and this state was maintained by passing a gentle stream of argon over the sample during the measurement. Spermine and the anti-band 3 monoclonal antibody were added as small aliquots of concentrated, pH-adjusted solutions.

Instrumentation and data analysis. Time-resolved phosphorescence spectroscopy was carried out with an instrument described by Jovin et al. [22] and Tilley et al. [23]. A pulsed nitrogen laser was used to drive a coumarin dye laser (8–10 ns pulse width, 515 nm). The laser beam, vertically polarised with an intercavity prism, illuminated the sample contained in a 7 × 7 mm cross-section cuvette. Luminescence was collected at right angles to the incident beam and the phosphorescence isolated by a combination of RG695 and KV550 cut-off filters. A gated photomultiplier (EMI 9817 QBG) was switched on 1 μs after the laser pulse, thereby avoiding the intense spike of prompt fluorescence. Decays of the parallel ($I_{VV}(t)$) and perpendicular ($I_{VH}(t)$) components of the polarised transient phosphorescence were collected serially by 90° rotation of the emission polaroid after every 64 decays. A 1024-channel Nicolet 1170 signal averager was used to digitise the signal and accumulate 512 decays of each component. After subtraction of background signals, the decays were transferred to to a PDP 11/23 computer and the total intensity ($I(t)$) and the antisotropy ($r(t)$) curves generated according to the expressions:

$$I(t) = I_{VV}(t) + 2I_{VH}(t) \quad (1)$$

$$r(t) = (I_{VV}(t) - I_{VH}(t))/(I_{VV}(t) + 2I_{VH}(t)) \quad (2)$$

The generated curves were fitted to functions:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) + B \quad (3)$$

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j) + r_\infty \quad (4)$$

α_i representing the initial intensity of the decay component i having a lifetime τ_i, and β_j the partial anisotropy of component j associated with the rotational correlation time ϕ_j. For a hindered rotor, r_∞ is the limiting anisotropy, determined in part by the distribution function for the population of chromophores at times long relative to the correlation time. Both r_∞ and β_j are determined by the angles that the absorption and emission transition moments make with the axis of rotation (for review see Ref. 22). B is a fitting parameter that accounts for any offset in the total intensity decay and was close to zero in the experiments described below. These data were fitted to 1 to 4 exponentials using a Chesbyshev transformation procedure contained within a data aquisition operating system (DAOS) supplied by Labsoft Associates, Melbourne, Australia. Goodness-of-fit was determined from values of the reduced χ-squares or from plots of the weighted residuals [24].

Tsuji et al. [8] have suggested that the different correlation times for the rotation of band 3 correspond to different populations of different aggregate size. They determined a value of $r_\infty/r_0 = 0.18$ for band 3 which is unrestricted by external constraints (i.e., for band 3 in membranes stripped of all peripheral proteins). Using this value the relative amplitudes for the anisotropy

decays can be expressed as fractions of the maximum possible decay amplitude:

$$f_j = (\beta_j / r_0)/0.82 \quad (5)$$

$$f_{im} = (r_\infty / r_0 - 0.18)/0.82 \quad (6)$$

where r_0 is the initial anisotropy and f_j and f_{im} are, respectively, the mobile and immobile populations of band 3.

Results and Discussion

Band 3 rotation in normal erythrocyte ghosts

The time-resolved phosphorescence anisotropy decay of eosin-labelled band 3, at 20°C, in ghosts prepared by hypotonic lysis of human erythrocytes is shown in Fig. 1. The anisotropy decay was determined at two different time resolutions. Panel B shows the decay of anisotropy over a 5 ms time period after excitation of the sample by a flash of light from the pulsed laser. Panel A gives a high resolution of the early time period, 0–0.5 ms. Each of these anisotropy decays has been fitted to two exponentials (see fitted parameters in Table I). Three decay events are resolved within the noise of the phosphorescence decay data. The event with a correlation time of about 200 μs can be resolved using either time base.

If the band 3 molecule is modelled as a cylinder in a planar bilayer, a freely rotating band 3 dimer would be expected to have two rotational correlation times, ϕ and $\phi/4$ where

$$\phi = 4\eta V/kT$$

η is the viscosity of the bilayer (assumed to be 6 P at 20°C), V is the molecular volume of the cylinder, k is Boltzmann's constant and T is the absolute temperature [25]. The values of $\phi/4$ and ϕ are expected to be 19 and 75 μs (assuming that the bilayer viscosity is 6 Poise at 20°C [26] and that 55% of band 3 is membrane-embedded [27]). For a heterogeneous population of band 3 aggregates, each population would be expected to contribute two exponential terms to the anisotropy decay. In practice, however, the number of components that can be resolved is limited by the noise of the data.

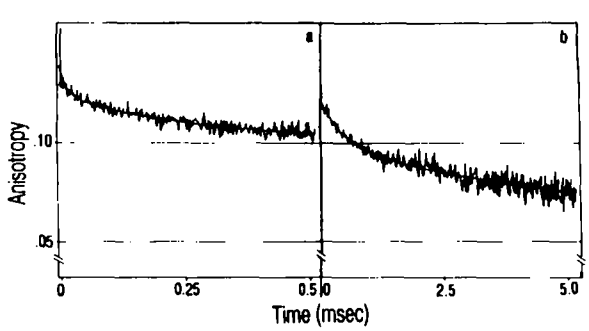

Fig. 1. Decay of phosphorescence anisotropy of eosin-5-maleimide labelled band 3 in human erythrocyte ghosts at 20°C. Data were collected in two different regimes of time resolution: (A) 0–0.5 ms, (b) 0–5.0 ms. Data are fitted to Eqn. 4. Concentrations of membrane protein and eosin were 0.8 mg/ml and 2 μM, respectively. The best-fit parameters are summarised in Table I.

TABLE I

Fitted parameters (using equation 4) for the anisotropy decays of eosin-band 3 in human erythrocyte ghosts at 20°C (see Fig. 1)

	ϕ_1 (μs)	ϕ_2 (μs)	β_1	β_2	r_∞
Panel A (0–500 μs)	16	220	0.012	0.021	0.100
Panel B (0–5000 μs)	200	3650	0.016	0.015	0.080

The correlation times determined in this study differ from each other by a factor that is much greater than 4 and are therefore assumed to represent band 3 populations of different association states rather than the ϕ and $\phi/4$ values for one or two species (see Ref. 28 for discussion). The shortest correlation time (16 μs) is consistent with the $\phi/4$ value for a freely rotating band 3 dimer and may represent a population of free dimers. Alternatively, this correlation time may arise from some degree of internal flexibility within the band 3 dimer. The longer correlation times presumably correspond to slowly rotating oligomers of band 3. These oligomers could be higher self-associated states or complexes of band 3 with other membrane proteins, such as ankyrin and bands 4.1, 4.2 and 6 [10].

Tsuji et al. [8] have described a method of data analysis (see Eqns. 5 and 6) which allows the different populations of band 3 to be expressed as a percentage of the total population. Table II shows an analysis of the anisotropy decay components based on a model which assumes three mobile populations of band 3 and an apparently immobile fraction. It should be stressed, however, that, as for the correlation times, the extracted amplitudes depend on the constraints which are imposed on the fitting procedure (see discussions by Mühlebach and Cherry [29] and Morrison et al. [30]). The most meaningful measure of band 3 mobility is probably the percentage of immobilised band 3. Our

TABLE II

Analysis of the anisotropy decays from Fig. 1 using Equations 5 and 6

The model assumes one immobile and three mobile populations of band 3.

	Correlation time (ϕ) (μs)	% of total population
f_1	16	12
f_2	210	18
f_3	3650	15
$f_{(immobile)}$	-	55

value of 50–55% for the immobile band 3, at 20°C, is in reasonable agreement with the work of Tsuji et al. [8].

Effect of spermine on band 3 rotation

Addition of the polyamine, spermine, further restricts the mobility of band 3. Fig. 2 shows the decay of phosphorescence anisotropy for eosin-band 3 in erythrocyte ghosts suspended in 10 mM NaCl, 10 mM Tris-HCl (pH 7.6) with and without 1.2 mM spermine, at 20°C. The best-fit parameters for the anisotropy decays are given in Table III. The short correlation time is least affected by the treatment with spermine. This may suggest that this correlation results from internal flexibility within the band 3 rather than global motion. The immobilisation of band 3 by spermine can be reversed by addition of salt (Table III), indicating that electrostatic interactions may be responsible for the spermine effect on band 3 mobility. The spermine effect is consistent with the reported immobilisation of band 3 by divalent cations and by basic peptides such as polylysine [10]. Polycations, such as spermine, are also known to stabilise the erythrocyte membrane cytoskeleton [17]. However, the effect of spermine on band 3 does not seem to be mediated through an effect on the cytoskeleton, because the immobilisation is also observed in membrane vesicles depleted of spectrin and actin (data not shown).

Characterisation of the anti-band 3 monoclonal antibody

Cationic agents such as spermine may cause aggregation of band 3 molecules by cross-linking the acidic cytoplasmic domains. However, they may also interact with the negatively-charged sugars on the extracellular domain of band 3 or indeed with associated phospholipid. An agent was therefore sought which would cross-link band 3 only at the cytoplasmic face. We have used a monoclonal antibody (B-9), which recognises an epitope within the 43 kDa cytoplasmic domain of band

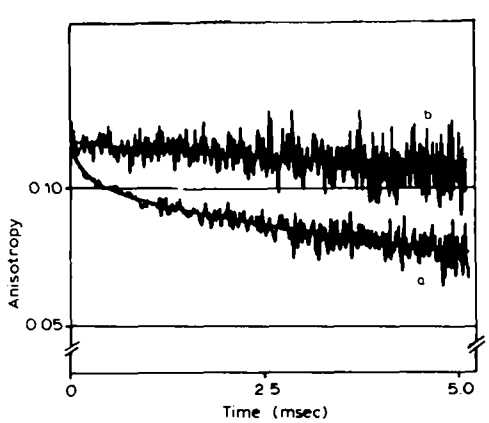

Fig. 2. Decay of phosphorescence anisotropy for eosin-labelled band 3 in erythrocyte ghosts at 20°C in the absence (a) and presence (b) of 1.2 mM spermine. Spermine was added as a small aliquot to membranes in 10 mM Tris HCl (pH 7.5), 10 mM NaCl.

TABLE III

Analysis of the effect of spermine on the phosphorescence anisotropy decays for eosin-labelled band 3 in erythrocyte ghosts

Decays were fitted to Eqn. 4 and the amplitudes were analysed using Eqns. 5 and 6. Data represent the average ± S.D. for at least three measurements.

	Control	1.2 mM spermine	1.2 mM spermine + 0.15 M NaCl
ϕ_1 (μs)	19 ± 7	21 ± 3	16 ± 2
f_1 (%)	13	10	13
ϕ_2 (μs)	260 ± 120	–	230 ± 150
f_2 (%)	12	–	10
ϕ_3 (μs)	4040 ± 710	2690 ± 430	4470 ± 940
f_3 (%)	20	11	22
$f_{(immobile)}$ (%)	55	78	55

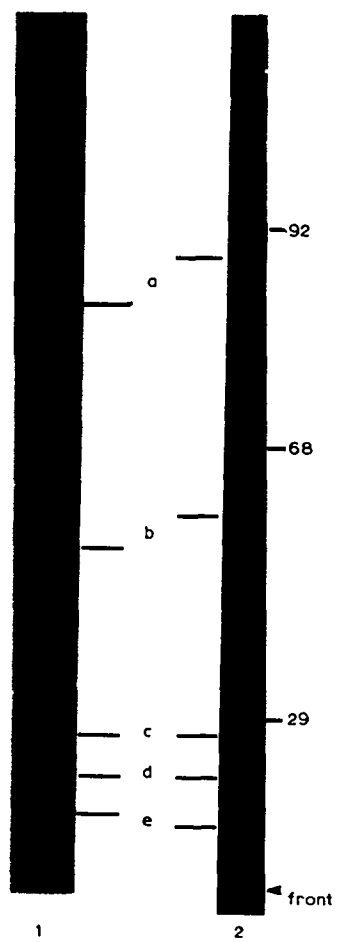

Fig. 3. Partial α-chymotryptic digestion of band 3. Lane 1: Ghosts were treated with α-chymotrypsin and electrophoresed in 8% SDS gels. Proteins were transferred electrophoretically to nitrocellulose sheets [20] and probed with an anti-band 3 monoclonal antibody (B-9) followed by ^{125}I-goat anti-mouse globulin. Lane 2: Purified phosphorylated band 3 was treated with α-chrymotrypsin and the cleavage products electrophoresed. The gel was dried and directly auto radiographed to reveal the phosphorylated fragments. Lower case letters a, b, c, d, e, show the position of zones detected by the anti-band 3 Mab which were also phosphorylated.

3. When used to probe immunoblots of erythrocyte ghosts, MAb B-9 recognised the intact band 3 molecule at about 90 kDa and also its phosphorylated proteolytic fragments (Fig. 3). Band 3 is phosphorylated exclusively within the N-terminal cytoplasmic domain of the molecule and predominantly at Tyrosine-8 [32]. Therefore the epitope of MAb B-9 must be contained within the N-terminal region of band 3.

Effect of the anti-band 3 antibody on band 3 rotation

Incubation of erythrocyte membranes, at 20 °C, for 1 h, in the presence of MAb B-9 resulted in a significant decrease in band 3 mobility (Table IV). By contrast, Fab fragments of the B-9 antibody had no effect on rotation of band 3 (see Table IV). The immobilisation of band 3 by the intact MAb B-9 was found to be unaffected by depletion of spectrin and actin by low ionic strength extraction (data not shown). These data suggest that the intact MAb B-9 is directly cross-linking band 3 dimers into larger aggregates.

Effect of the anti-band 3 antibody on malarial invasion of erythrocytes

Intracellular spermine concentrations of 1 mM have been shown to cause a 50% inhibition of invasion of erythrocytes by the malaria parasite, *Plasmodium falciparum* [13]. To examine whether MAb B-9 had an effect on invasion, the antibody was resealed into erythrocytes at a concentration range similar to that used for the measurements of band 3 rotational diffusion. As shown in Table V, MAb B-9 caused a decrease in efficiency of invasion compared with controls. These results support a previous finding that the resealing of a polyclonal anti-band 3 antibody into erythrocytes decreases the efficiency of invasion compared with controls [33]. Control pre-immune serum has no effect on invasion [33]. The invasion process evidently involves disruption of the normal organisation of the erythrocyte membrane to create a patch of membrane free of both integral and cytoskeletal proteins. Cross-linking of band 3 into homo-oligomers or aggregates containing several membrane proteins may affect the ability of the parasite to create a protein-free area on the erythrocyte surface. It should be noted that higher concentrations of B-9 antibodies (> 1 B-9 Mab: 1.5 band 3 dimer) caused echinocytosis of the erythrocytes (data not shown) which may reflect more extensive reorganisation of the membrane components.

TABLE IV

Effect of monoclonal antibody (B-9) on the rotation diffusion of eosin-labelled band 3 in human erythrocyte membranes

B-9 MAb and Fab were incubated with erythrocyte membranes in 50 mM NaCl, 5 mM sodium phosphate (pH 7.6) for 1 h at 20 °C.

	Control	B-9 MAb (1:1.7 band 3)	B-9 MAb (1:0.85 band 3)	B-9 Fab (1:1 band 3)
ϕ_1 (μs)	17 ± 5	10 ± 1	14 ± 2	17 ± 2
f_1 (%)	12	21	18	14
ϕ_2 (μs)	410 ± 170	350 ± 130	-	480 ± 120
f_2 (%)	12	6	-	15
ϕ_3 (μs)	4530 ± 1100	3760 ± 2000	1590 ± 520	3880 ± 390
f_3 (%)	25	10	4	21
$f_{(immobile)}$ (%)	51	63	78	50

TABLE V

Inhibition of malarial invasion of resealed ghosts by intracellular monoclonal antibodies against band 3

Invasion was measured as the ratio of the number of parasitised cells (ring-stage) after 19 h to the number (trophozite- and schizont-stages) in the initial inoculum. This quantity was compared to the corresponding value for the controls prepared by resealing ghosts in the presence of all reagents except the antibody. Data are the mean ± S.D. for three or mean ± mean deviation for two experiments.

Intracellular B-9 MAb (mg/ml)	MAb: Band 3	Invasion (% control)
0	0	100
0.04	1:14	94 ± 10 (2)
0.08	1:7	42 ± 5 (2)
0.16	1:3.5	23 ± 7 (3)

Band 3 mobility in membranes from Plasmodium falciparum-infected erythrocytes

We have also studied the mobility of band 3 in membranes prepared from erythrocytes, infected with mature stages of *P. falciparum*. Infected cells were enriched on a sorbitol/Percoll gradient. Band 3 was labelled in intact infected erythrocytes under the same conditions as for normal cells. The specificity of band 3 labelling was shown to be indistinguishable in membranes from normal and infected erythrocytes (Fig. 4). Similarly, the lifetimes for the phosphorescence intensity decay for band 3 (determined using Eqn. 3) were the same in membranes prepared from normal and infected erythrocytes.

Fig. 5(b) shows the decay of phosphorescence anisotropy of eosin-labelled band 3 in membranes prepared by hypotonic lysis of cells infected to a greater than 90% extent with trophozoite stage parasites. Table VI gives the analysis of the fitted data for several such measurements and reveals a substantial increase in the apparently immobile fraction of band 3. The control membranes (Fig. 5(a), column 1 in Table VI) were prepared from erythrocytes that had been kept in culture medium for 48 h in the absence of parasites. Table VI (column 3) gives results for band 3 mobility in membranes prepared from erythrocytes that had been recovered from the bottom of the Percoll sorbitol gradient. This fraction contained mainly uninfected erythro-

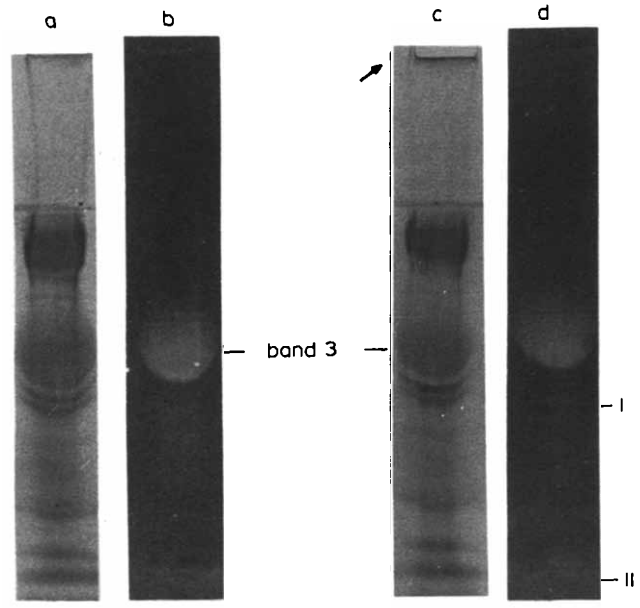

Fig. 4. SDS-polyacrylamide gels (stacking gel: 3.5% acrylamide, running gel: 7.5% acrylamide) of membranes prepared from uninfected (a,b) and trophozoite-infected (c,d) erythrocytes. Eosin fluorescence is detected under UV light. The major labeled species is band 3, although further faint fluorescent bands (I and II) can be detected. Coomassie blue staining of the gels (a,c) reveals no obvious differences between membranes prepared from infected and uninfected erythrocytes, except that the membranes from trophozoite-infected erythrocytes contain a small amount of high molecular weight material which does not enter the 3.5% acrylamide stacking gel (arrow).

cytes with less than 10% rings and less than 3% mature stages. Band 3 mobility in these membranes was not significantly different from that in the controls.

Caution is required in assigning the cause of the apparent restriction of band 3 motion in membranes prepared from trophozoite-infected erythrocytes. Membranes prepared from these cells were invariably contaminated to a small extent by haemozoin, as judged by

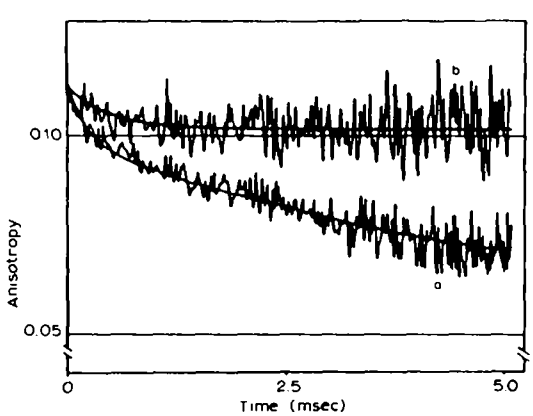

Fig. 5. Decay of phosphorescence anisotropy of eosin-labelled band 3 at 20 °C in membranes prepared from (a) control erythrocytes and (b) erythrocytes infected with trophozoite stages of the malaria parasite, *P. falciparum*. Trophozoite-stages were enriched using Percoll/sorbitol gradient centrifugation of infected erythrocytes. Membranes were prepared by hypotonic lysis of eosin-labelled erythrocytes.

TABLE VI

Analysis of anisotropy decays for eosin-labelled band 3 in membranes prepared from either control or trophozoite stage-infected erythrocytes

The data have been analysed in terms of one immobile and three mobile populations of band 3.

	Control (uninfected)	Trophozoite-infected	Control (<10% rings)
ϕ_1 (μs)	21 ± 5	22 ± 4	17 ± 5
f_1 (%)	12	16	15
ϕ_2 (μs)	270 ± 130	540 ± 70	340 ± 160
f_2 (%)	13	8	13
ϕ_3 (μs)	3400 ± 500	-	3280 ± 1140
f_3 (%)	21	-	22
$f_{(immobile)}$ (%)	53	76	50

the slightly greyish appearance of the membrane pellet and by the presence of high molecular weight material which does not enter SDS-polyacrylamide gels (Fig. 4c). Haemozoin from *P. falciparum* has been shown to be consist mostly of haemin together with a 14 kDa protein of parasite origin [34]. However, there may also be some denatured globin [34]. Denatured haemoglobin has been reported to interact more strongly than native haemoglobin with band 3 [35]. There are conflicting reports regarding clustering of band 3 by membrane-associated denatured haemoglobin [35,36]; it is clearly possible that a direct interaction of haemozoin with band 3 may be responsible for the immobilisation.

Alternatively, the observed immobilisation may be due to aggregation of band 3 to form 'knobs' (electron-dense protuberances) on the surface of erythrocytes infected with mature *P. falciparum* parasites. It has been suggested [37] that aggregation of band 3 molecules into clusters may serve as a recognition signal for auto-antibody binding to the external domain of band 3 (which carries the senescence antigen). Winograd et al. [38] have quantitated the binding of auto-antibodies to the senescence antigen in erythrocytes infected with *P. falciparum*. They found that erythrocytes infected with a knobless variant of *P. falciparum* bound twice as much anto-antibody as controls, whereas 'knobby' variants bound 30 times more.

Knobs have been observed on erythrocytes infected with the *P. falciparum* isolate (FCQ27/PNG) used in this study [39]. It is possible that proteins of malarial origin may be cross-linking band 3 molecules into aggregates in these knobs and that the restriction of band 3 rotation in membranes from trophozoite-infected erythrocytes may reflect the same processes that promote auto-antibody binding and knob formation. It should be noted that only a fraction of the total population of band 3 molecules would be present in the knobs, however, aggregation of band 3 on a less dramatic scale

could occur over the whole surface of the erythrocyte. Experiments are in progress to assess band 3 rotation in cloned knobby and knobless cultures of *P. falciparum*.

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