Molecular and Cellular Biochemistry **91**: 167–172, 1989.

Invited Paper

Fluorescence studies on erythrocyte membrane isolated from *Plasmodium berghei* infected mice

Vishwanath Koppaka, Ravin Sharma and Anil K. Lala
Biosciences and Engg. Group, Department of Chemistry I.I.T. Powai, Bombay 400 076, India

Accepted 12 June 1989

Key words: fluorescence, erythrocyte membrane, *Plasmodium berghei*

Abstract

The erythrocyte host cell plays a key role in the well defined developmental stages of the malarial parasite growth and propogation in the erythrocyte cycle of malaria. The host cell serves the parasites by supplying metabolites and removing the catabolites produced by the obligatory parasites. It has been observed that the plasma membrane of the infected cells show a substantially higher fluidity probably due to the depletion of cholesterol content from the host cell. The protein component of the membrane is also modulated due to the insertion of new polypeptides of the parasitic origin, which confers upon it new antigenic properties. We have studied the membrane fraction isolated from mice erythrocytes infected with *Plasmodium berghei* using fluorescent probes like DPH, ANS and series of fluorenyl fatty acids, which permit depth dependent analysis of membrane. We have observed that there is a marked difference in the fluorescence emission wavelength maximum, the dissociation constant K_d of ANS when bound to normal and infected erythrocytes, though relatively small differences are observed in the fluorescence polarisation values of the two cell types. The fluorenyl fatty acids also show the differences when bound to normal and infected erythrocytes, indicating that either they are in a different environment or they have differing binding properties to the two cell types.

Abbreviations: DPH – 1,6-Diphenyl-1,3,5-Hexatriene, ANS – 8-Anilino-napthalene Sulfonic Acid, C2A-FL – 2-Fluorenyl-acetic Acid, C4A-FL – 2-Fluorenyl-butyric Acid, C6A-FL – 2-Fluorenyl-hexanoic Acid, C8A-FL – 2-Fluorenyl-octanoic Acid

Introduction

The erythrocyte cycle of the malaria is characterised by defined developmental stages of parasite growth and propogation. A key role in this development is played by the host cell, which is largely responsible for supplying metabolites and removing catabolites produced by the obligatory parasites [1]. The traffic of material between the infected cell and the external melieu is likely to be mediated by red blood cell membrane transport mechanisms. To what extent the normal constituents of the erythrocyte membrane can subserve the growing demands of the internal parasite is still unknown.

Although previous studies indicate the presence of structural and functional alterations of the host cell membranes [2–5] little is known about the cause of these alterations, let alone their physiological significance. Following defined red cell membrane properties through the various developmental stages of intraerythrocyte growth, it was recently demonstrated that marked abnormalities oc-

cured in the cell permeability barrier as *P. falciparum* parasites developed from ring to the trophozoite stage [6]. Infection of an erythrocyte by a malarial, parasite invokes profound structural and functional changes in the host cell, i.e., enhanced permeability of glucose, amino acids, and Ca^{+2}, decreased ATPase dependent Na^{+} transport, and increased osmotic fragility. These altered properties of the parasitised red cell are due, in part to modifications in the composition as well as the organisation of membrane lipids of the plasma membrane [7–9]. It was found that the fluidity of the plasma membrane of mouse erythrocytes infected with *P. Berghei* was increased over that of uninfected cells. In the latter work no evidence was found for the increased membrane fluidity in the remaining uninfected erythrocytes when these were separated from parasitised red cells [10]. In contrast Gupta and his coworkers [11] have found the phospholipid assymmetry in both parasitised and uninfected cells from monkeys infected with *P. Knowlesi* to be changed.

A very few fluorescent probes [12] have been employed to study the various aspects of the infected cell membrane. Merocyanine 540 (MC-540) is a negatively charged fluorescent dye that binds preferentially to relatively disordered or fluid domains in the outer leaflet of the lipid bilayer of intact cells. The fluorescent microviscosity probes, Anthroyloxy fatty acid probes [13], DPH and TMA-DPH [14] have been used to demonstrate the difference in the properties of the infected and the uninfected red blood cells. It was found that that there is atleast an 8% increase in the fluidity in the infected cells. Spin labels have been used by Smith *et al.* [15] to find out the difference between the normal and the diseased cells.

In this paper we have examined the behaviour of fluorescent probes like Anilino napthelene sulfonic acid, Diphenyl hexatriene and a series of fluorescent fluorenyl fatty acids reported recently by us [16] in normal and *P. Berghei* infected mice red blood cells.

Material and methods

Normal mice were infected with the parasite *P. berghei* and after four or five days levels of parasitemia was determined at appropriate intervals by microscopic examination of the blood smear. On post infection, blood was obtained (1.5 ml) from normal and infected mice and was washed with mouse tonicity phosphate buffered saline (MTPBS) or citrate buffer pH 7.3 and centrifuged at 3000 rpm for 5 min. The composition of MTPBS was 20 mM Sodium phosphate, 0.149 M NaCl, pH 7.3 and the composition of citrate – Tris buffer was 150 mM citrate, 2 mM Tris Base, 2 mM Histidine and the pH was adjusted to 7.4 with 1 N HCl. After washing the cells with the buffer, plasma and the buffy coat of leucocytes were removed. Three washings with the buffer was given to the red blood cells so obtained. For seperation of the infected cells from the uninfected cells 55% Percoll gradient was used [17]. The seperated cells were given two washings with the required buffer and then we have the intact pure and infected mice red blood cells for further use. Counting of the cells was done after appropriate dilution using a Hemecytometer.

DPH stock solution in THF was prepared and an aliquot of this solution was added to the buffer at 70° C with stirring in dark so that the final concentration of the DPH in buffer is 2 μM. Normal and infected red blood cell samples (1.5 ml) containing 6×10^6 cells/ml were taken and 1.5 ml of the buffer (MTPBS) solution was added to it. The solutions were incubated for 1 h and fluorescence emission and polarisation spectrum were recorded using an excitation and emission wavelength of 360 and 430 nm respectively. RBC-ANS experiments were carried out by taking 10×10^6 cells/ml of intact and infected mice erythrocytes in citrate buffer (pH 7.4) and adding appropriate amount of ANS in ethanol to it so that the final concentration of ANS in the sample is 30 μM. In the case of the fluorenyl fatty acids the experiments were carried out in an identical manner by maintaining the probe concentration at 2 μM. The excitation wavelengths for ANS was 385 nm and fluorenyl fatty acids was 272 nm respectively.

RBC-ANS binding experiments were carried

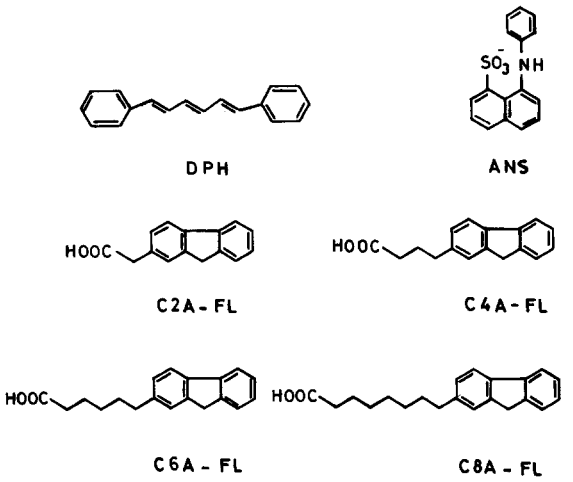

Fig. 1. Structures of the fluorescent probes used.

out by adding aliquots of red blood cells to a buffer solution containing 30 μM ANS. These experiments were carried out for both normal and infected red blood cells. Proper corrections were applied by taking the fluorescence of the buffer with only ANS and buffer with cells only. Increase in fluorescence was monitored after 5 min of addition of an aliquot of the cells. Scatchard plots were drawn for the above binding studies and the binding parameters were evaluated using the equation.

$$\frac{L_o}{xA_o} = \frac{1}{n} + \frac{K_d}{n}\left[\frac{1}{A_o - xA_o}\right]$$

where L_o = total cell concentration
A_o = the total ANS concentration
x = fraction of ANS bound which is calculated as the ratio of the fluorescence value and the reciprocal of the intercept of the double reciprocal plot of the fluorescence versus the cell concentration in the presence of a given concentration of ANS.
n = number of binding sites.
K_d = dissociation constant [M].

The binding parameters were evaluated both for normal and infected mice erythrocytes.

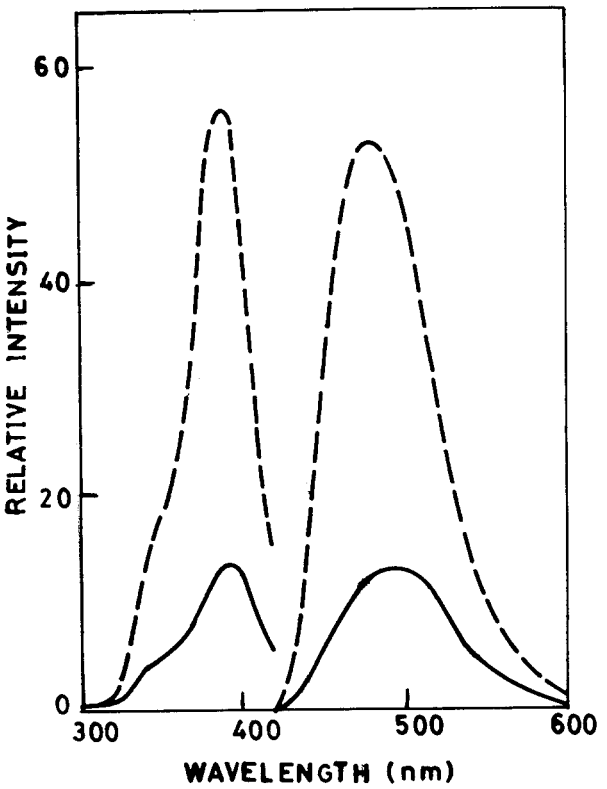

Fig. 2. Fluorescence excitation and emission spectrum of ANS in normal intact (—) and infected intact (- - -) mice red blood cells infected with *Plasmodium berghei*. 10×10^6 cell/ml were taken and the final ANS concentration was 30 μM. The excitation wavelength used was 385 nm and the wavelength of emission was 498 nm and 483 nm for normal and infected cells respectively. The excitation and emission slits were 5 and 10 nm respectively.

Results and discussions

Figure 1 shows the structures of the fluorescence probes used. The fluorescence spectrum of ANS (Fig. 2) shows an emission maximum at 498 nm in

Table 1. Fluorescence polarisation values of fluorescent probes DPH and ANS in normal and malaria infected mice erythrocytes

PROBE	Fluorescence polarisation values in intact mice erythrocyte	
	Normal cells	Infected cells
DPH	0.305	0.172
ANS	0.210	0.200

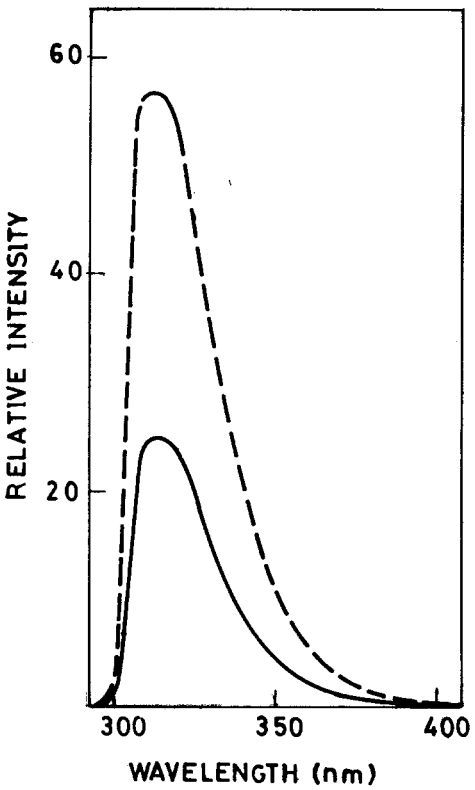

Fig. 3. Fluorescence emission spectrum of C4A-FL in normal intact (—) and malaria infected intact (- - -) mice erythrocytes. 20×10^6 cells/3 ml was used and the probe concentration was 2 μM. The excitation wavelength used was 272 nm. The excitation and emission slits used were 5 and 5 nm respectively.

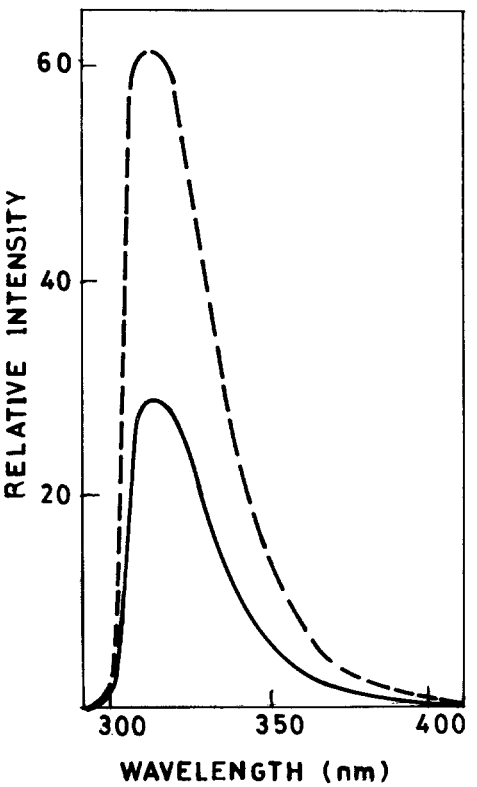

Fig. 4. Fluorescence emission spectrum of C6A-FL in normal intact (—) and malaria infected intact (- - -) mice erythrocytes. 20×10^6 cells/3 ml was used and the probe concentration was 2 μM. The excitation wavelength used was 272 nm. The excitation and emission slits used were 5 and 5 nm respectively.

the normal erythrocytes and at 483 nm in infected erythrocytes. It is also observed that for an identical quantity of ANS the fluorescence intensity of ANS is higher in the sample containing the infected cells than that containing the normal cells. The fluorescence of normal human erythrocyte ghosts on treatment with ANS is reported extensively [18–20]. The wavelength of the emission maxima of ANS is known to be very sensitive to the polarity of the environment and so is the intensity. The difference in the wavelength of emission maxima in the normal and infected cells may thus indicate that ANS occupies a more nonpolar environment in the infected cells then the normal cells and also since intensity of emission is more in the infected cells this fact supports the non-polar nature of the bind-

Table 2. Binding parameters for the binding of ANS to normal and malaria infected mice erythrocytes

Binding parameters	Intact mice erythrocytes	
	Normal	Infected
n, number of moles of ANS bound per rbc	24×10^{-15}	60×10^{-15}
K_d: dissociation constant, M	19×10^{-5}	51.4×10^{-5}
Number of molecules of ANS bound per rbc	14.5×10^{9}	39.1×10^{9}

ing site in infected cells. Further the intensity increase is not due to the rigidity of the ANS molecule when bound at a particular site in the infected cell is supported by the fact that no change is observed in the fluorescence polarisation values between the normal and the infected cell. It has been observed both in the case of intact as well as the ghost cell membranes of the normal and the infected mice red cell membrane that the polarisation value of the fluorescent microviscosity probe DPH is higher in the case of the normal cell compared to the infected cell membrane thus indicating that there is an increase in fluidity as a result of infection. No difference in the wavelengths of excitation and emission of DPH was observed between the two cell types. The values of fluorescence polarisation using DPH as a probe is compared to that obtained by using ANS in Table 1. While DPH shows a difference in the polarisation values ANS shows no difference in the polarisation values between the normal and the infected cells. This may be due to the fact that while ANS monitors the membrane around the glycerol moiety DPH monitors deeper around the centre of the bilayer.

Binding characteristics of ANS in normal and infected mice red blood cells were studied and are given in Table 2. It is observed that the number of ANS molecules bound per cell is higher in case of infected cell then the normal cells. The K_d value seem to be higher in the case of infected cells. The reason for a higher *n* value for ANS in the infected cell compared to the normal cell may be due to the fact that the lipid composition in the former is different from the latter. There is also a depletion of cholesterol and the presence of new polypeptides in the infected cell membrane which helps in increasing the number of molecules of ANS to be bound. It was reported that hydrophobic probes such as DPH can pass through the plasma membrane and label internal as well as external membranes, whereas Toluidinyl napthelene-6-sulfonate tend to be non-permeant and so remain on the surface of the plasma membrane [21]. We therefore have used DPH, ANS and the negatively charged fluorenyl fatty acids as probes to compare their results in normal as well as infected cells. Fig. 3. and Fig. 4 shows the emission spectrum of C4A-FL

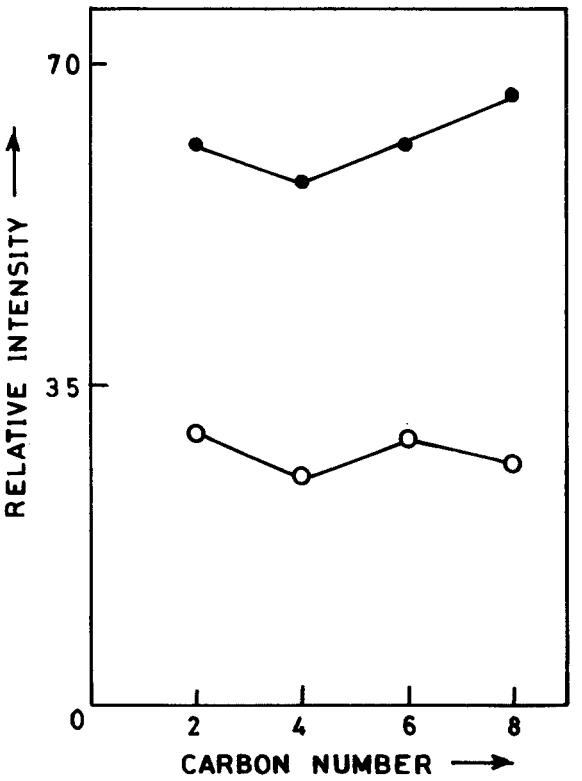

Fig. 5. Plot showing the change in fluorescene intensity of the fluorenyl fatty acids: C2A-FL, C4A-FL, C6A-FL and C8A-FL denoted by the carbon number of the attachment of the fluorene along the fatty acid chain in normal (○) and malaria infected (●) intact mice erythrocytes.

and C6A-FL in normal and infected mice erythrocytes. It is clearly observed that the fluorescence intensity is more in the case of the infected cells. A plot showing the change in fluorescence intensity of the fluorenyl fatty acids denoted by the carbon number of the attachment of the fluorene along the fatty acid chain in normal and infected mice erythrocytes is given in Fig. 5. The increase in intensity in the infected cells may be due to the increase in probe molecules partitioning into the infected cells rather than the normal cells. A plot of fluorescence polarisation of the fluorenyl fatty acids in the normal and infected cells is given in Fig. 6. It is observed that as in the case of ANS there is no substantial change in the polarisation value between the two cell types and that in fact there is a slight increase in the polarisation values in the infected cells as monitored by the fluorenyl fatty acid probes. In conclu-

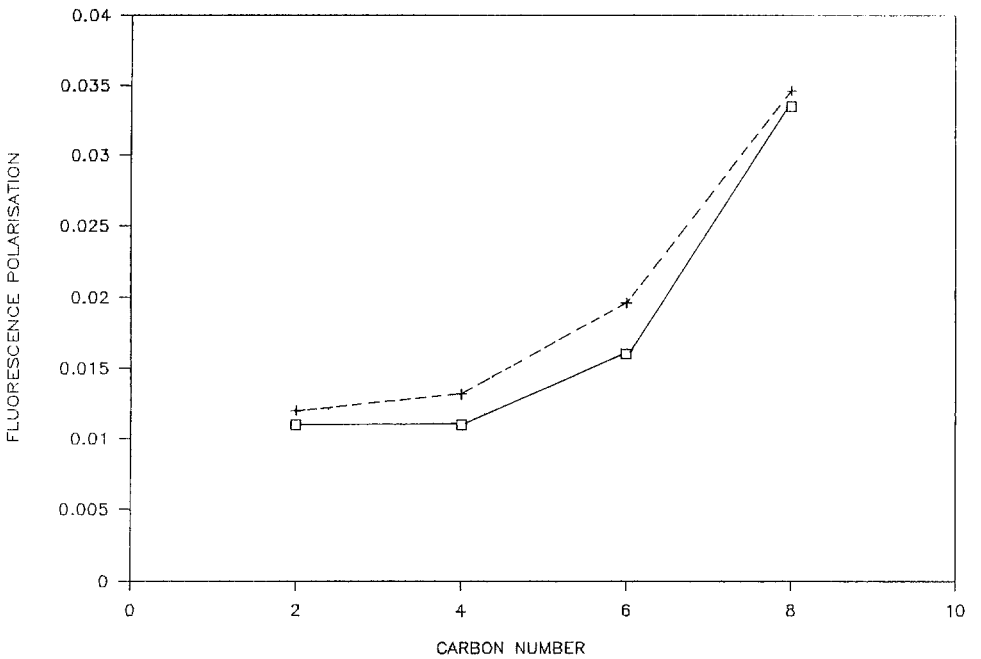

Fig. 6. Plot showing the values of the fluorescence polarisation of the fluorenyl fatty acids indicated by the carbon number of the attachment of the fluorene along the fatty acid chain in normal (—□—) and malaria infected (—+—) intact mice erythrocytes.

sion use of fluorescent probes can go a long way for differentiating a normal and a malaria infected red blood cell membrane both in terms of the membrane function and its composition.

References

1. Sherman IW: Microbiol Rev 43: 453–495, 1979
2. Hadley TJ: Ann Rev Microbiol 40: 451–77, 1986
3. Ullrich SR, Wallach DFH: Proc Natl Acad Sci USA 75: 4949–4953, 1978
4. Smith HRJK, Smith PM, Mitchell GF: Ann Trop Med Parasitol 81: 273–298, 1978
5. Howard RJ: Immunol Rev 61: 67–107, 1982
6. Kutner S, Brauch D, Ginsberg H, Cabantchik ZI: Biochim Biophys Acta 687: 113–117, 1982
7. Beach D, Sherman I, Holz G: J Parasitol 63: 62–75, 1983
8. Alfred D, Sterling G, Morse P: Mol and Biochem Parasitol 7: 27–39, 1983
9. Van der Schaft PH, Beaumelle B, Vial H, Roelofsen B, Op den Kamp JAF, Van Deenen LLM: Biochim Biophys Acta 901: 1–14, 1987
10. Mathur AA, Dutta G: Nature 229: 259–261, 1982
11. Gupta CM, Mishra G: Science 212: 1047–1049, 1981
12. Schlegel R, Phelps B, Waggonner R, Terada L, Williamson P: Cell 20: 321–328, 1980
13. Russel HJ, Sawyer W: Parasitology 80: 331–342, 1980
14. Deguercy A, Duportail SG, Lanstrait G, Kuhry JG: Biochem Int 12: 21–31, 1986
15. Deslaurieus RG, Yves B, Keith W, Smith ICP: Q Rev Biophys 18: 65–110, 1985
16. Lala AK, Dixit RR, Koppaka V, Patel S: Biochemistry 27: 8981–8989, 1988
17. Kutner S, Breuer WV, Ginsburg H, Aley SB, Cabantchik ZI: Cell Physiol 125: 521–527, 1985
18. Collier HB: Experientia 26: 184, 1969
19. Slavik J: Biochim Biophys Acta 694: 1–25, 1982
20. Fischer H: Biochim Biophys Acta 241: 770–778, 1971
21. Pagano RE, Ozato K, Ruysschaert F: Biochim Biophys Acta 465: 661–666, 1977

Address for offprints:
A.K. Lala,
Biosciences and Engg. Group,
Department of Chemistry,
I.I.T. Powai,
Bombay 400 076, India