Flow cytometric assessment of oxidant stress in age-fractionated thalassaemic trait erythrocytes and its relationship to *in vitro* growth of *Plasmodium falciparum*

A. C. SENOK[1], K. LI[1]*, E. A. S. NELSON[1], M. ARUMANAYAGAM[2] *and* C. K. LI[1]

[1] *Departments of Paediatrics and* [2] *Chemical Pathology, The Chinese University of Hong Kong, Shatin NT, Hong Kong*

(*Received 23 March 1997; revised 28 July 1997; accepted 1 August 1997*)

SUMMARY

The role of oxidant stress in mediating the protection against malaria in thalassaemic red blood cells (RBC) has been hypothesized. In this study we have assessed the relationship between oxidant stress, red cell age and malarial parasite activity in thalassaemic RBC. Using a flow cytometric method to assess lipid peroxidation, we have shown that the age-related increase in sensitivity to oxidative stress previously demonstrated in normal RBC also occurs in thalassaemic RBC. Invasion and growth of *Plasmodium falciparum* was also shown to deteriorate with increasing RBC age. This effect was more pronounced in thalassaemic RBC with associated schizont maturation arrest and abnormal parasite morphology. In addition, there was a slight but consistent inverse correlation between sensitivity to oxidant stress and parasite activity ($R = -0{\cdot}43$; $P = 0{\cdot}03$ for normal RBC and $R = -0{\cdot}42$; $P = 0{\cdot}01$ for thalassaemic RBC). Our findings indicate an association between red cell age, oxidant stress and *P. falciparum* growth, providing further support for the role of oxidant stress in mediating the protective effect against malaria in thalassaemic RBC.

Key words: thalassaemia, malaria, oxidant stress, cytometry.

INTRODUCTION

Increased sensitivity to oxidative stress is one of the anomalies associated with thalassaemic red cells (RBC) (Shinar & Rachmilewitz, 1990). This susceptibility has been suggested as a mechanism whereby these variant erythrocytes confer protection against malaria. This is particularly pertinent as it is known that the malaria parasite is microaerophilic in nature and highly susceptible to free-radical damage (Clark & Hunt, 1983; Clark, Chaudhri & Cowden, 1989). In addition to oxidant stress, other anomalies such as red cell size (and its confounder cell age), deformability changes and altered immune response have been alternatively hypothesized to mediate the protective effect (Teo & Wong, 1985). Supportive of a role for oxidant stress was work by Friedman (1979) which showed reduced parasite activity in thalassaemic RBC under conditions of oxidant stress. However, Luzzi and colleagues (1991) failed to demonstrate similar findings. We have used a model of fractionating normal and thalassaemic RBC to show that increased RBC age has a clear adverse effect on parasite growth (Senok *et al*. 1997). We also described a new phenomenon of schizont maturation arrest in malaria parasites growing in these older thalassaemic RBC.

There is evidence indicating that RBC age and sensitivity to oxidant stress are inversely related in normal RBC (Westerman, Pierce & Jensen, 1963; Nakai, Imanishi & Takino, 1984; Clark & Shohet, 1985; Jain, 1988). However, in spite of the higher sensitivity to oxidant stress in thalassaemic RBC, studies using assays of the antioxidant enzyme, gluthathione peroxidase, have failed to demonstrate an age-related increase in oxidative stress (Cellerino, Guidi & Perona, 1976; Prasartkaew *et al*. 1986). A recent report (Scott & Eaton, 1995) has shown that the high levels of free intraerythrocyte iron in these variant RBC initiate a chain reaction of oxidation of cellular components with subsequent increased oxidant stress and membrane damage. In addition, there is an indication that this redox effect could be age related.

In this study, we evaluate the relationship between red cell age, oxidative stress and *P. falciparum* growth in thalassaemic red cells. Sensitivity to oxidant stress in both fractionated and whole blood samples was assessed by determining the relative susceptibility to lipid peroxidation using flow cytometry. This technique of flow cytometry represents a sensitive tool for the measurement of the degree of susceptibility to oxidant stress. Our findings indicate that an association exists between oxidant stress and red cell age as possible mechanisms mediating the protection against malaria in thalassaemic RBC.

* Corresponding author: Department of Paediatrics, 6th Floor, Clinical Sciences Building, Prince of Wales Hospital, Shatin NT, Hong Kong. Tel: +852 26322859. Fax: +852 26360020.
E-mail: b247773@mailserv.cuhk.edu.hk

https://doi.org/10.1017/S0031182097001911 Published online by Cambridge University Press

MATERIALS AND METHODS

Sample collection

Alpha and beta thalassaemic subjects ($n = 8$ for each group) and normal healthy volunteers ($n = 10$) were recruited for the study. Thalassaemic trait subjects were asymptomatic and the alpha thalassaemic trait subjects had double gene deletion. The beta thalassaemic trait subjects were identified on the basis of elevated Hb A_2. None of the subjects had received blood transfusion within 12 weeks preceding sample collection and glucose-6-phosphate dehydrogenase deficiency was ruled out by quantitative assays (Beutler, 1984). Informed consent was obtained from volunteers and samples were collected in acid citrate dextrose and used within 4 h.

Red cell fractionation and age assessment

All chemicals used were obtained from Sigma Chemical Co, Mo, USA. Red cell fractionation was carried out using the Percoll density-gradient method (Rennie *et al*. 1979; Senok *et al*. 1997). Red cell age was assessed in both whole blood and fractionated samples by red cell creatine assays using the diacetyl-α-naphthol reaction method (Li *et al*. 1982). All assays were done in duplicate using the Cobas-Bio Centrifugal analyser (Roche Diagnostica, Basle, Switzerland). Three age-distinct fractions identified on the basis of red cell creatine assays were used in the study. These fractions were designated as young, intermediate and old RBC.

Assessment of sensitivity to oxidant stress

This was carried out using the flow cytometric method described by Hammouda & Fakeir (1995), with a few modifications. Briefly, the red cells were washed 3 times in cold (4 °C) azide-containing buffered saline at 1000 ***g*** for 5 min. The azide-containing buffered saline was made up of sodium azide (7·8 mM), NaCl (150 mM), Na_2HPO_4/NaH_2PO_4 (3·4 mM), pH 7·4. A 1:10 suspension of washed cells was allowed to equilibrate for 10 min at 37 °C in azide-containing buffered saline. Twenty μl of the red cell suspension was added to 0·1 mM hydrogen peroxide (H_2O_2) (pre-incubated at 37 °C) and allowed to stand in a 37 °C shaking water bath for 2 h. Signals for 30000 events were acquired at a rate of not more than 600 cells/sec on a FacScan flow cytometer with the Lysis 2 software (Becton Dickinson, Mountain View, Ca, USA). The red cell population was gated on a forward-scatter versus side-scatter dot plot using logarithmic scale on both axes (Pattanapanyasat *et al*. 1992). Fluorescence 1 (FL 1) histogram was generated on the gated population to determine the red cell mean channel fluorescence (MCF) indicative of basal autofluorescence. The relative degree of lipid peroxidation which had occurred was determined by calculating the difference between the MCF in control (without H_2O_2 treatment) and test samples (with H_2O_2 treatment) (Hammouda & Fakeir, 1995).

Parasite culture

The FC27 chloroquine-sensitive strain of *P. falciparum* was maintained in continuous culture using conventional methods (Trager & Jensen, 1976). Schizont-stage parasites used as inoculum were obtained by Percoll gradient concentration (Rivadeneira, Wasserman & Espinal, 1983) of synchronous cultures. To correlate parasite activity in prolonged cultures with degree of sensitivity to oxidant stress, erythrocytes from whole blood and fractionated samples were inoculated using schizont-stage parasites at a starting parasitaemia of 0·5 % with 4 % haematocrit. Parasite invasion and growth over 3 erythrocytic cycles (144 h) were studied by slide microscopy. Paired thin blood smears were prepared daily to determine levels of parasitaemia and changes in parasite morphology.

Statistics

One-way ANOVA and Pearson's correlation coefficient were used to test for significant difference and correlation respectively.

RESULTS

Sensitivity to oxidant stress

Fig. 1 is a flow cytometry printout showing the change in the MCF peak before and after H_2O_2 treatment in a thalassaemic sample. The higher the degree of change in basal autofluorescence or MCF (between control and test samples), the higher the relative susceptibility to lipid peroxidation. In each red cell type (normal, α and β thalassaemic), the degree of susceptibility to H_2O_2-induced lipid peroxidation was significantly higher with increasing RBC age ($P < 0·05$; one-way ANOVA). For the fractionated samples, the respective degrees of lipid peroxidation were significantly higher in thalassaemic trait red cells (α and β) when compared to their age equivalents for normal RBC (Fig. 2). There was no significant difference between α and β thalassaemic trait RBC. Similarly, in unfractionated whole blood, the MCF changes in α and β thalassaemic trait red cells ($53·0 \pm 9·9$ and $52·1 \pm 7·5$ respectively) were higher when compared with that seen in normal red cells ($47·4 \pm 6·5$), although the differences were not statistically significant ($P = 0·4$). In all experiments, the flow cytometric pattern of forward scatter and side scatter (measurements of cell size and granularity respectively) remained unchanged after H_2O_2 treatment (data not shown).

https://doi.org/10.1017/S0031182097001911 Published online by Cambridge University Press

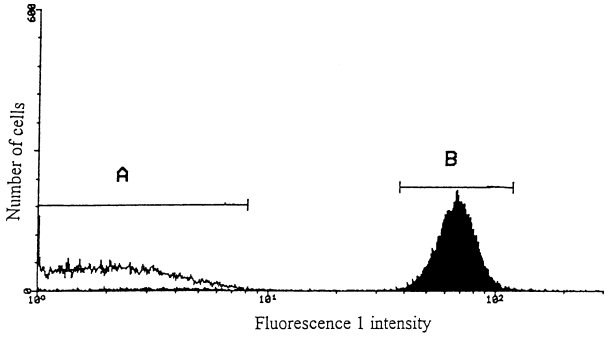

Fig. 1. Computer print out of histogram analysis of FL1 (basal autofluorescence) from the flow cytometer using the Lysis 2 software. Fluorescence intensity was assessed by flow cytometry before and after hydrogen peroxide (H_2O_2) challenge in control and test samples respectively (basal autofluorescence FL1 on *x*-axis). The degree of change in the mean channel fluorescence (MCF) of the basal autofluorescence with H_2O_2 treatment indicates the degree of susceptibility to H_2O_2-induced lipid peroxidation. The first peak (A) shows the MCF before H_2O_2 challenge. The second peak (B) which is for the test sample, shows the increase in basal autofluorescence that occurs after H_2O_2 challenge. The MCF for the first peak (A) was similar in all fractions of both normal and thalassaemic RBC with variations seen only in the second peak (B).

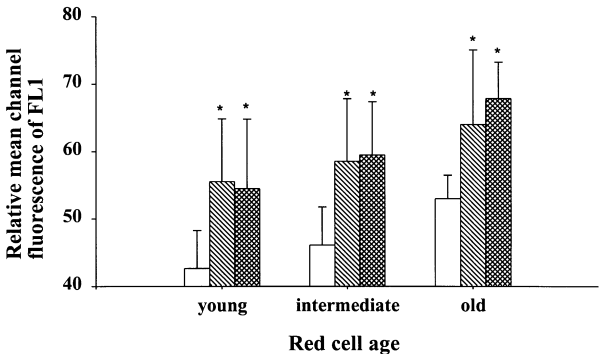

Fig. 2. Sensitivity to oxidant stress was assessed by measuring the degree of susceptibility to lipid peroxidation using flow cytometry. This was significantly higher in fractions from α and β thalassaemic RBC compared to age equivalent normal RBC. There was no significant difference between age equivalent fractions of α and β thalassaemic RBC. $^*P < 0{\cdot}05$. (□) Normal, $n = 10$; (▨) β thal, $n = 8$; (▩) α thal, $n = 8$.

Parasite growth and oxidant stress

Parasite growth was significantly lower in thalassaemic RBC compared to equivalent age groups of normal RBC ($P < 0{\cdot}05$; Fig. 3). This poor parasite activity in thalassaemic RBC was associated with significantly lower invasion (at 24 h) and re-invasion rates (at 72 and 120 h). In the old thalassaemic RBC, maturation arrest of schizont-stage parasites and the presence of morphologically abnormal moribund schizonts were noted during the third cycle (96–144 h) of culture. Comparison of age equivalent α and β thalassaemic RBC showed that the pattern of parasite activity was similar in both red cell types. These are in keeping with our previously reported findings (Senok *et al.* 1997). Furthermore, levels of parasitaemia correlated inversely with the degree of sensitivity to oxidative stress in all cell types ($R = -0{\cdot}42$; $P = 0{\cdot}03$ for normal RBC and $R = -0{\cdot}43$; $P = 0{\cdot}01$ for thalassaemic RBC) (Fig. 4).

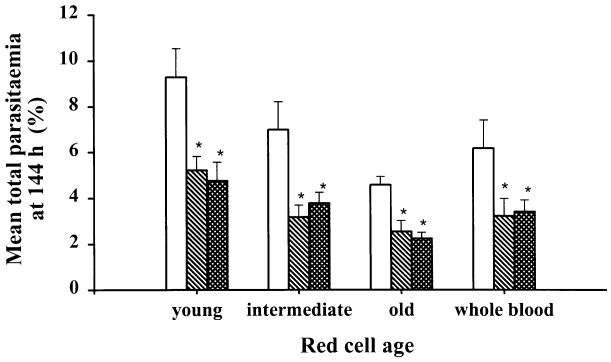

Fig. 3. The parasitaemia at the end of the culture duration (144 h) was compared in age equivalent fractions of normal and thalassaemic RBC. Parasite levels were found to be significantly higher in normal RBC compared to age equivalent thalassaemic RBC. $^*P < 0{\cdot}05$. (□) Normal, $n = 10$; (▨) β thal, $n = 8$; (▩) α thal, $n = 8$.

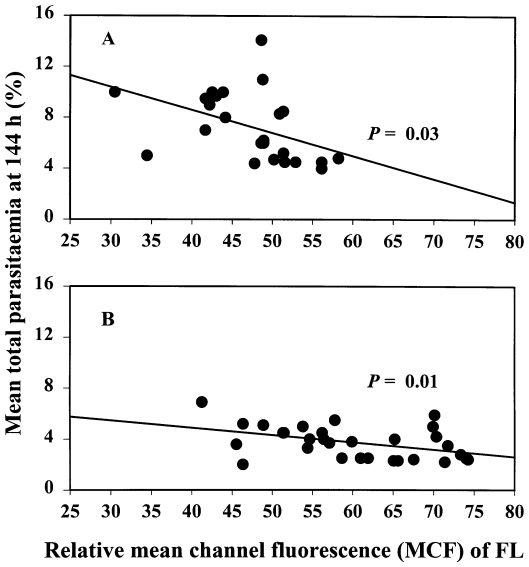

Fig. 4. Analysis for correlation between the relative mean channel fluorescence of FL1 and the parasitaemia at the end of the culture duration (144 h) was carried out. There was a significant inverse correlation between levels of parasitaemia and degree of sensitivity to H_2O_2-induced lipid peroxidation in both normal (A) and thalassaemic RBC (B).

DISCUSSION

The findings presented here demonstrate that when compared to age-equivalent normal red cells, thalassaemic erythrocytes have an increased susceptibility

https://doi.org/10.1017/S0031182097001911 Published online by Cambridge University Press

to H_2O_2-induced lipid peroxidation. Although previous work has been done to assess lipid peroxidation in thalassaemic RBC, to our knowledge there is no known report using a flow cytometric method. With this technique, we have shown for the first time, that an increase in the degree of susceptibility to lipid peroxidation with red cell ageing occurs in thalassaemic RBC. In both normal and thalassaemic RBC there is a slight but consistent inverse correlation between sensitivity to oxidant stress and the ability to support malarial parasite growth.

In performing their function of oxygen transport, red cells generate reactive oxygen species. In a bid to escape oxidant damage they are equipped with various mechanisms for maintaining the redox equilibrium. Due to the anomalies associated with thalassaemic RBC, this ability to maintain a redox balance is altered leading to a situation of increased oxidant stress. Thus the increased lipid peroxidation seen in thalassaemic RBC may be due to an interplay of high membrane lipid, increased generation of free oxygen radicals and inadequate antioxidant defence mechanisms.

There is an indication that the intraerythrocytic parasite exerts increased oxidant stress on the host RBC due to intracellular generation of reactive oxygen species by the malaria parasites. Although this notion has previously been challenged (Hunt & Stocker, 1990), murine models have shown an increased production of H_2O_2, O_2^-, lipid peroxides and methaemoglobin during schizogony (Etkin & Eaton, 1975; Mohan *et al*. 1992; Nakornchai & Anantavara, 1992). In addition, reduced erythrocyte vitamin E content as well as an increased lipid peroxidation of ghost RBC membranes have been shown with *P. falciparum* (Simoes *et al*. 1992). More recent work (Atamna & Ginsburg, 1993) using *P. falciparum* has clearly demonstrated that the production of reactive oxygen species originates in the parasite food vacuole during the digestion of host cell cytosol. These free radicals diffuse into the host compartment and may thus disturb the redox equilibrium of the host erythrocyte. Thalassaemic RBC, which are already oxidant stressed, might be unable to cope with this intracellular generation of reactive oxygen species. This could further jeopardize the ability of these variant erythrocytes to support parasite growth and thus contribute to the protective effect. We have shown that compared to age-equivalent normal erythrocytes, thalassaemic RBC with greater susceptibility to H_2O_2-induced oxidant stress were also less able to support parasite activity. However, the increased susceptibility to oxidant stress in thalassaemic whole blood was not significantly higher when compared to normal whole blood. This is probably due to the fact that the whole blood represents a more heterogeneous population and is thus less sensitive in demonstrating subtle differences between the normal and thalassaemic RBC. Nevertheless the overall correlation between the degree of sensitivity to induced oxidant stress and parasite activity shown in this study is in keeping with a contributory role for free radicals in the protective mechanism.

Although free radicals have the potential of a direct parasiticidal effect (Allison & Eugui, 1982; Clark & Hunt, 1983; Dockrell & Playfair, 1984), the diverse cellular changes associated with lipid peroxidation could also have deleterious effects on the parasite activity in a number of ways. The peroxidative damage and cross-linking of membrane lipoproteins which occurs results in altered membrane deformability and permeability (Chiu, Kuypers & Lubin, 1989). Parasite invasion is affected by deformability changes (Bunyaratvej *et al*. 1992) and the reduced invasion which we have demonstrated in the thalassaemic RBC (Senok *et al*. 1997) could be due to this anomalous rheology. Furthermore, as a result of the membrane anomalies, thalassaemic RBC are predisposed to increased splenic clearance *in vivo* (Yuthavong *et al*. 1988) and this could potentially result in lower parasite load. Altered membrane permeability affecting ionic and nutrient transport could also have deleterious consequences on parasite growth (Friedman, 1979; Rachmilewitz & Kahane, 1980). Thus, the diverse effects of the increased sensitivity to oxidant stress and its enhancement with RBC ageing in these variant RBC strongly indicate that free-radical damage may serve as the primary mediator of the protective effect.

In this study, lipid peroxidation was assessed by flow cytometric measurement of fluorescent products (Hammouda & Fakeir, 1995). This method is based on the assessment of fluorescent chromolipids formed by the interaction of malonyldialdehyde and the amino groups of phospholipids and proteins during lipid peroxidation (Bidlack & Tappel, 1973). It has been suggested that the measurement of fluorescent products is probably an easy and reliable way to determine lipid peroxidation in biological systems (Tappel, Fletcher & Deamer, 1973; Goldstein & McDonagh, 1976; Goldstein, Rozen & Amoruso, 1979). The assessment of individual cells by the flow cytometer further enhances the sensitivity of this method. This is in contrast to other methods such as the thiobarbituric acid test (TBA) and its various modifications which deal with the cells as a bulk of oxidizing material (Stocks & Dormandy, 1971; Poli *et al*. 1986). In addition, the volume of H_2O_2 used is about 100 times less than that used for the TBA test. There was no indication of lysis as the cell size and cell granularity pattern on flow cytometry remained unchanged.

The findings of increased susceptibility to H_2O_2-induced lipid peroxidation with ageing in normal RBC is in keeping with results obtained with other methods (Jain & Hochstein, 1980; Glass & Gershon, 1984). This phenomenon has been associated with

https://doi.org/10.1017/S0031182097001911 Published online by Cambridge University Press

the decline in the red cell metabolic and defence capabilities with ageing (Kurata, Suzuki & Agar, 1993). In this study, we have shown that thalassaemic erythrocytes also have a similar age-related pattern whereas previous work based on the assay of antioxidant enzymes did not reveal any significant change with cell ageing (Cellerino *et al*. 1976; Prasartkaew *et al*. 1986).

Our study has demonstrated a relationship between red cell age, sensitivity to oxidant stress and *P. falciparum* growth, thus providing further evidence to support the hypothesis that the innate resistance against malaria in thalassaemic RBC could be mediated through free-radical damage.

REFERENCES

ALLISON, A. C. & EUGUI, E. M. (1982). A radical interpretation of immunity to malaria parasites. *Lancet* **2**, 1431–1433.

ATAMNA, H. & GINSBURG, H. (1993). Origin of reactive oxygen species in erythrocytes infected with *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **61**, 231–242.

BEUTLER, E. (1984). *A Manual of Biochemical Methods*, 3rd edn. Grune and Stratton, Orlando, Fl.

BIDLACK, W. R. & TAPPEL, A. L. (1973). Fluorescent products of phospholipids during lipid peroxidation. *Lipids* **8**, 203–207.

BUNYARATVEJ, A., BUTTHEP, P., SAE-UNG, N., FUCHAROEN, S. & YUTHAVONG, Y. (1992). Reduced deformability of thalassemic erythrocytes and erythrocytes with abnormal hemoglobins and relation with susceptibility to *Plasmodium falciparum* invasion. *Blood* **79**, 2460–2463.

CELLERINO, R., GUIDI, G. & PERONA, G. (1976). Plasma iron and erythrocytic glutathione peroxidase activity. A possible mechanism for oxidative haemolysis in iron deficiency anaemia. *Scandinavian Journal of Haematology* **17**, 111–116.

CHIU, D., KUYPERS, F. & LUBIN, B. (1989). Lipid peroxidation in human red cells. *Seminars in Hematology* **26**, 257–276.

CLARK, I. A., CHAUDHRI, G. & COWDEN, W. B. (1989). Some roles of free radicals in malaria. *Free Radical Biology and Medicine* **6**, 315–321.

CLARK, I. A. & HUNT, N. H. (1983). Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infection and Immunity* **39**, 1–9.

CLARK, M. R. & SHOHET, S. B. (1985). Red cell senescence. *Clinics in Haematology* **14**, 223–257.

DOCKRELL, H. M. & PLAYFAIR, J. H. L. (1984). Killing of *Plasmodium yoelii* by enzyme-induced products of the oxidative burst. *Infection and Immunity* **43**, 451–456.

ETKIN, N. L. & EATON, J. W. (1975). Malaria-induced erythrocyte oxidant sensitivity. In *Erythrocyte Structure and Function* (ed. Brewer, G. J.), pp. 219–232. Alan R. Liss, New York.

FRIEDMAN, M. J. (1979). Oxidant damage mediates variant red cell resistance to malaria. *Nature, London* **280**, 245–247.

GLASS, G. A. & GERSHON, D. (1984). Decreased enzymic protection and increased sensitivity to oxidative damage in erythrocytes as a function of cell and donor aging. *The Biochemical Journal* **218**, 531–537.

GOLDSTEIN, B. D. & MCDONAGH, E. M. (1976). Spectrofluorescent detection of *in vivo* red cell lipid peroxidation in patients treated with diaminodiphenylsulfone. *Journal of Clinical Investigation* **57**, 1302–1307.

GOLDSTEIN, B. D., ROZEN, M. G. & AMORUSO, M. A. (1979). Relation of fluorescence in lipid-containing red cell membrane extracts to *in vivo* lipid peroxidation. *Journal of Laboratory and Clinical Medicine* **93**, 687–694.

HAMMOUDA, A. M. A. & FAKEIR, A. (1995). Flow cytometric evaluation of erythrocyte response to oxidant stress. *Cytometry* **20**, 19–22.

HUNT, N. H. & STOCKER, R. (1990). Oxidative stress and the redox status of malaria-infected erythrocytes. *Blood Cells* **16**, 499–526.

JAIN, S. K. (1988). Evidence for membrane lipid peroxidation during the *in vivo* aging of human erythrocytes. *Biochimica et Biophysica Acta* **93**, 205–210.

JAIN, S. K. & HOCHSTEIN, P. (1980). Polymerisation of membrane components in aging red blood cells. *Biochemical and Biophysical Research Communications* **92**, 247–253.

KURATA, M., SUZUKI, M. & AGAR, N. S. (1993). Antioxidant systems and erythrocyte life-span in mammals. *Comparative Biochemistry and Physiology* **106B**, 477–487.

LI, P. K., LEE, J. T., LI, C. S. & DESHPANDE, G. (1982). Improved method for determining erythrocyte creatine by the diacetyl-alpha-naphthol reaction: elimination of endogenous glutathione interference. *Clinical Chemistry* **28**, 92–96.

LUZZI, G. A., MERRY, A. H., NEWBOLD, C. I., MARSH, K. & PASVOL, G. (1991). Protection by α-thalassaemia against *Plasmodium falciparum* malaria: modified surface antigen expression rather than impaired growth or cytoadherence. *Immunology Letters* **30**, 233–240.

MOHAN, K., GANGULY, N. K., DUBEY, M. L. & MAHAJAN, R. C. (1992). Oxidative damage of erythrocytes infected with *Plasmodium falciparum*. An *in vitro* study. *Annals of Hematology* **65**, 131–134.

NAKAI, T., IMANISHI, H. & TAKINO, T. (1984). Separation of human red cells by ultracentrifugation in a discontinuous density gradient and relationship between red cell age and lipid peroxidation. *Acta Haematologica Japonica* **47**, 1230–1234.

NAKORNCHAI, S. & ANANTAVARA, S. (1992). Oxygen free radicals in malaria. In *Lipid Soluble Antioxidants: Biochemistry and Clinical Applications* (ed. Ong, A. S. H. & Packer, L.), pp. 355–362. Birkhäuser Verlag, Basel.

PATTANAPANYASAT, K., WEBSTER, H. K., UDOMSANGPETCH, R., WANACHIWANAWIN, W. & YONGVANITCHIT, K. (1992). Flow cytometric two-color staining technique for simultaneous determination of human erythrocyte membrane antigen and intracellular malarial DNA. *Cytometry* **13**, 182–187.

POLI, G., RAMENGHI, U., DAVID, O., BIASI, F., CECCHINI, G., CARINI, R., CHIARPOTTO, E. & DIANZANI, M. U. (1986). Lipid peroxidation in red blood cells obtained from

https://doi.org/10.1017/S0031182097001911 Published online by Cambridge University Press

thalassaemic subjects and exposed to oxidative stress. In *Free Radicals, Cell Damage and Disease* (ed. Rice-Evans, C.), pp. 186–199. Richelieu Press, London.

PRASARTKAEW, S., BUNYARATVEJ, A., FUCHAROEN, S. & WASI, P. (1986). Comparison of erythrocyte antioxidative enzyme activities between two types of haemoglobin H disease. *Journal of Clinical Pathology* **39**, 1299–1303.

RACHMILEWITZ, E. A. & KAHANE, I. (1980). The red blood cell membrane in thalassaemia. *British Journal of Haematology* **46**, 1–6.

RENNIE, C. M., THOMPSON, S., PARKER, A. C. & MADDY, A. (1979). Human erythrocyte fractionation in 'Percoll' density gradients. *Clinica Chimica Acta* **98**, 119–125.

RIVADENEIRA, E. M., WASSERMAN, M. & ESPINAL, C. T. (1983). Separation and concentration of schizonts of *Plasmodium falciparum* by Percoll gradients. *Journal of Protozoology* **30**, 367–370.

SCOTT, M. D. & EATON, J. W. (1995). Thalassaemic erythrocytes: cellular suicide arising from iron and gluthathione-dependent oxidation reactions? *British Journal of Haematology* **91**, 811–819.

SENOK, A. C., LI, K., NELSON, E. A. S., YU, L. M., TIAN, L. P. & OPPENHEIMER, S. J. (1997). *P. falciparum* activity inhibited in fractionated thalassaemic erythrocytes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **91**, 138–143.

SHINAR, E. & RACHMILEWITZ, E. A. (1990). Oxidative denaturation of red blood cells in thalassaemia. *Seminars in Hematology* **27**, 77–82.

SIMOES, A. P. C. F., VAN DEN BERG, J. J. M., ROELOFSEN, B. & OP DEN KAMP, J. A. F. (1992). Lipid peroxidation in *Plasmodium falciparum*-parasitized human erythrocytes. *Archives of Biochemistry and Biophysics* **298**, 651–657.

STOCKS, J. & DORMANDY, T. L. (1971). The autoxidation of human red cell lipids induced by hydrogen peroxide. *British Journal of Haematology* **20**, 95–111.

TAPPEL, A., FLETCHER, B. & DEAMER, D. (1973). Effect of antioxidants and nutrients on lipid peroxidation fluorescent products and aging parameters in the mouse. *Journal of Gerontology* **28**, 415–424.

TEO, C. G. & WONG, H. B. (1985). The innate resistance of thalassaemia to malaria: a review of the evidence and possible mechanisms. *Singapore Medical Journal* **26**, 504–509.

TRAGER, W. & JENSEN, J. B. (1976). Human malaria parasites in continuous culture. *Science* **193**, 673–675.

WESTERMAN, M. P., PIERCE, L. E. & JENSEN, W. N. (1963). Erythrocyte lipids: a comparison of normal young and normal old populations. *Journal of Laboratory and Clinical Medicine* **62**, 394–400.

YUTHAVONG, Y., BUTTHEP, P., BUNYARATVEJ, A., FUCHAROEN, S. & KHUSMITH, S. (1988). Impaired parasite growth and increased susceptibility to phagocytosis of *Plasmodium falciparum* infected alpha-thalassemia or hemoglobin Constant Spring red blood cells. *American Journal of Clinical Pathology* **89**, 521–525.