LABORATORY AND FIELD COMPARISONS OF ADENOSINE INFLUX IN *PLASMODIUM FALCIPARUM* AND *PLASMODIUM VIVAX* INFECTED ERYTHROCYTES WITH GENETIC ABNORMALITIES FROM PATIENTS IN MYANMAR

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Abstract. Influx of the purine nucleoside, adenosine, was assessed in erythrocytes from both normal subjects and from subjects with a range of genetically determined erythrocyte disorders from Myanmar. The latter included α-thalassemia major (Myanmar variant), β-thalassemia major (Myanmar variant), β-thalassemia trait, HbEE and HbAE erythrocytes and two variants of glucose-6-phosphate dehydrogenase (G6PDH) deficiency. Significant reductions ($p < 0.01$) of adenosine influx were observed in erythrocytes from individuals with α-and β-thalassemia major and severe G6PDH deficiency. Abnormal erythrocytes infected with the malarial parasites, *Plasmodium falciparum* or *Plasmodium vivax*, demonstrated a reduction in adenosine transport which correlated with the proportion of abnormal erythrocytes present in the samples obtained. The effect of nitrobenzylthioinosine (NBMPR) on adenosine influx was explored in normal and abnormal erythrocytes. In all these cases, NBMPR completely inhibited the transport of adenosine. However, transport of adenosine into *P. falciparum* and *P. vivax*-infected normal erythrocytes and abnormal cells was only inhibited 50-60% by NBMPR. The combination of tubercidin and NBMPR completely blocked adenosine transport into both normal and abnormal erythrocytes infected with either *P. falciparum* or *P. vivax*.

INTRODUCTION

Malaria remains a serious health problem in tropical and subtropical regions. In terms of socio-economic impact, it is the most important of the transmissible parasitic diseases. It was observed some years ago that there appeared to be a correlation between the incidence of malaria and the inherited hemoglobinopathies such as thalassemia and sickle cell anemia (Haldane, 1949). This coincidence led to the development of the "malaria hypothesis" that the various heterozygote states of the major hemoglobinopathies are more resistant to malaria, the selective advantage being great enough to compensate for the continual loss of defective genes through the death of affected homozygotes (Weatherall and Clegg, 1981).

Red cell disorders are extremely common in Southeast Asia. Because of its high frequency in the area, many attempts have been made to obtain evidence for a connection between this abnormal hemoglobin and resistance to malaria. The overall incidence of a α-thalassemia is reported to be 10% in Myanmar (Aung-Than-Batu *et al,* 1971) and 8-9% in Thailand (Wasi *et al,* 1969). As in other malarious areas, there is also a high incidence of G6PDH deficiency in Myanmar (4-14% in non-endemic areas; 15-16% in malaria hyperendemic areas) (Aung-Than-Batu and Hla-Pe, 1969). The distribution of β-thalassemia trait is 4.3% in Myanmar (Aung-Than-Batu *et al,* 1968) and 3.5% in Thailand (WHO, 1966). However, there are considerable local variations and it has been estimated that in the central areas of Indochina, as few as 15% of the population may exist with completely normal hemoglobin (HbAA) (Sicard *et al,* 1979).

The most attractive rationale for the "malaria hypothesis" is that affected erythrocytes are insufficiently stable to support the reproduction of the parasite. The development of techniques for the *in vitro* cultivation of *P. falciparum* by Trager and Jensen (1976) has greatly extended the tools avail-

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able for the study of the malarial parasite, including the assessment of its ability to grow in other than normal erythrocytes (HbAA).

Studies in the University of New South Wales laboratory have been concerned with the transport of the essential nutrient adenosine and other purine nucleosides into the normal human erythrocyte and compared to malaria infected cells. It has been observed in laboratory strains of *P. falciparum* infected erythrocytes that significant changes occur in the mode of transport following infection. In particular, the differential effect of the purine analog nitrobenzylthioinosine (NBMPR) on nucleoside transport into the malarial infected cell with respect to the uninfected erythrocyte demonstrated a significant lack of inhibition in the former cells (Gero *et al,* 1988; Gero and Upston, 1992; Upston and Gero, 1995). These investigations were concerned with *P. falciparum* infected normal erythrocytes. In addition, we have recently described changes in adenosine transport with Australian subjects, substantially of Mediterranean origin, with β-thalassemia. There were significant decreases in the transport properties which appeared to correlate with the increased existence of abnormal erythrocytes (Myint-Oo *et al,* 1993).

These studies were carried out with laboratory strains that had been maintained in culture for many years. Noting that the aim of the studies was to demonstrate adenosine analogues as potential antimalarial agents, it was considered imperative to determine whether equivalent observations occurred on freshly obtained samples from infected individuals. Such a study, based on hospital admissions in Myanmar is described below.

During the course of the project, the opportunity of carrying out equivalent studies with *P. vivax* arose. *P. vivax* is a major cause of morbidity in Myanmar, and an increasing incidence has been observed recently, notably in Northern Shan State (Myint-Oo *et al,* 1992). The infection rates of *P. vivax* were found to be higher than those of *P. falciparum* in all groups of subjects living in this locality.

The study was further extended to erythrocytes from both normal subjects and from subjects with a range of genetically determined erythrocyte disorders from Myanmar (Myint-Oo *et al,* 1991) including patients with genetic disorders, who were also infected with *P. falciparum* or *P. vivax.* The conditions investigated included normal heterozygous hemoglobin E (HbAE), homozygous hemoglobin E (HbEE), β-thalassemia trait, α-and β-thalassemia majors and glucose-6-phosphate dehydrogenase (G6PDH) deficiency in individuals identified from a survey of 119 subjects (Myint-Oo *et al,* 1995).

MATERIALS AND METHODS

Chemicals

[2,5-8-^{3}H] Adenosine (26 Ci mmol^{-1}), [U-^{14}C] sucrose (540mCi mmol^{-1}) and [^{3}H] H_2O (5mCi mmol^{-1}) were obtained from Amersham International, Amersham, UK. Eosin and hematoxylin, for the cytochemical staining of fetal erythrocytes and NBMPR were purchased from Sigma Chemical Co, St Louis, MO, USA. Saturated solutions of NBMPR were obtained by stirring in phosphate-buffered saline (PBS), (NaCl, 0.15M; KH_2PO_4, 4.8mM, pH7.4) for 2 hours at room temperature. 2,5-Diphenyloxazole (PPO) was obtained from Packard Instruments International, Zurich, Switzerland. Silicone oils were obtained from Dow Corning, Blacktown, Australia. A silicone oil mixture of density 1.03 g ml^{-1} was prepared by mixing (w/w) oils of three grades; oil 702 (45 cSt viscosity, 80 parts), oil 200 (1.5 cSt viscosity, 14 parts), and oil 200 (5 cSt viscosity, 7 parts). RPMI-1640 medium (with glutamine, without $NaHCO_3$) was purchased from Flow Laboratories Inc, VA, USA. All other reagents were of the best available commercial grade. Deionized water was used in all. Deionised preparations.

Blood and parasite samples

Normal blood samples were collected from 119 subjects (blood donors) of various ethnic groups from the Blood Bank, Yangon General Hospital, Myanmar. Blood from clinically confirmed cases of α-and β-thalasaemia were collected from patients at the Children's Hospital, Yangon.

P. falciparum: Patients with *P. falciparum* infections, with at least 10 parasites/thick field and negative by the Haskin urine test (Bruce-Chwatt, 1986), were selected for the investigation. For all samples, 20ml of venous blood was collected from each patient and aliquoted into four parts: (i) 2ml with heparin, (ii) 4ml clotted blood, (iii) 4ml in acid-dextrose-citrate buffer (ACD) and (iv) the re-

maining 10ml in ACD which was used for short-term culture.

Whole blood in ACD isolated from patients with *P. falciparum* infection of 4.2 to 12.6% was centrifuged at 1,500*g* for 10 minutes. The pellet was resuspended in an equal volume of sterile PBS (pH 7.2) and passed through Whatman CF11 powder to remove leukocytes (Thaithong *et al,* 1989). The erythrocyte suspension was washed three times with PBS and the pellet of erythrocytes, containing 1-5% *P. falciparum* rings and trophozoites, suspended in 12 volumes of complete medium (RPMI-1640 with 10% group "O" human serum). The suspension was incubated at 37°C for 24-30 hours using the technique of Trager and Jensen (1976). For experiments, the cultures containing well developed parasites, were harvested by using a 78% Percoll cushion (Dluzewski *et al,* 1984). The resulting parasites, 70-80% trophozoite and schizonts -infected erythrocytes, were washed three times with RPMI-1640 and the cell suspension reduced to 2% with sterile PBS.

P. vivax: Blood samples with *P. vivax* schizonts were collected from subjects attending North Okkalapa General Hospital, Yangon and the Defence Services General Hospital, Yangon. Long term laboratory studies on *P. vivax* could not be carried out due to the difficulty of maintaining the parasite in culture. However, schizont infected erythrocytes were harvested from fresh venous blood using discontinuous (30-50%) Percoll gradients as described by Andrysiak *et al* (1986). Leukocytes were firstly removed from all blood and parasite samples by passage through Whatman CF11 cellulose powder and the suspension centrifuged at 1,000*g* for 10 minutes. Two ml of diluted blood (1:3 with RPMI-1640) were then pipetted gently on top of the 30% Percoll layer. The Percoll gradients were centrifuged at 1,450*g* for 10 minutes. The resultant bands were collected, washed twice with media and smears prepared to determine the percentage of infected erythrocytes. Mean *P. vivax* parasitemia levels from patients of 6.8 to 11.6% were concentrated to 70-80% trophozoite and schizonts -infected erythrocytes after harvesting with Percoll.

Screening of thalassemia traits

Hemoglobin concentration (Hb g%), packed cell volume (PCV%), erythrocyte count, mean corpuscular hemoglobin (MCH mg or pg), erythrocyte morphology (from Giemsa stained thin blood smears), osmotic fragility test (percentage lysis), HbA2 and serum iron (SI) determinations were carried out according to standard procedures (Dacie and Lewis, 1984). Classification of thalassemia was according to the protocols described by Modell and Berdoukas (1984).

Electrophoretic characterization of G6PDH variants

G6PDH enzyme assays and electrophoretic characterization of G6PDH variants were carried out according to the standardized methods recommended by the World Health Organization (WHO, 1967).

Erythrocyte morphology and content of abnormal cells

Erythrocyte morphology and counting of abnormal red cells (anisocytes, poikilocytes, elliptocytes and target cells) were assessed using Giemsa stained thin blood smears under oil immersion (100X). The determination of fetal hemoglobin (HbF) in erythrocytes was estimated by the acid-elution cytochemical method (pH 1.5) (Dacie and Lewis, 1984). Giemsa stained thick blood smears under oil immersion (100X) for 50 thick fields was used for examination of parasites. Counting and speciation was done against 5,000 erythrocytes in Giemsa stained thin blood smears.

Nucleoside transport assay

Adenosine transport over short time intervals was measured as reported previously (Gero *et al,* 1988; Myint-Oo *et al,* 1993). In brief, experiments were initiated by adding blood cells (2×10^8 ml^{-1} in 100 µl PBS) to Eppendorf tubes containing 150 µl [^{3}H]-adenosine (final concentration, 1µM) layered on top on the silicon-oil mixture. Transport intervals (2-30 seconds at 25°C) were ended by centrifugation at 16,000*g* for 20 seconds to pellet cells under the oil layer. Two seconds was the minimum time period for the cells to pass through the oil layer. Initial rates were calculated over the 2-7 seconds linear portion of each assay. No metabolism of adenosine occurred within the cells over this time period. For the inhibition assays, NBMPR or tubercidin was added to 100 µl permeant containing [^{3}H]-adenosine to a final concentration of 1µM before initiation of the transport assays. Any leukocytes which had not been removed, and any erythrocytes which were broken or lysed, did not

enter the oil layer during centrifugation. Background radioactivity due to the extracellular volume of permeant in the cell pellet was measured by control experiments containing [U-^{14}C] sucrose in the medium in place of the nucleoside permeant and [^{3}H]-H_2O used to determine the total water space of the pellet. The cell pellet was processed as described previously (Myint-Oo *et al,* 1993) and counted in a Beckmann Model LS6000 liquid scintillation spectrometer.

RESULTS

Erythrocytes with genetic abnormalities

Comparison of adenosine influx in normal erythrocytes (HbAA) and blood from patients with β-thalassemia major is shown in Fig 1. Adenosine (1μM) was transported into normal erythrocytes (HbAA) at an initial rate of 0.39 ± 0.02 pmoles/μl cell water/s (n = 10) whereas the transport into

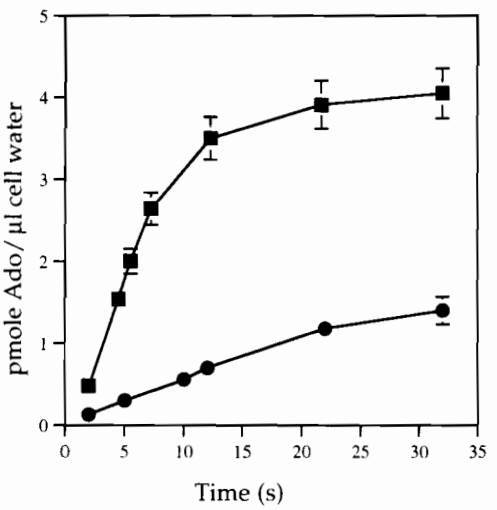


Fig 1– Transport of [^{3}H]-adenosine (1μM) into normal human erythrocytes, HbAA(■) and Myanmar subjects with β-thalassemia major (●). Erythrocytes were exposed to the permeant for time periods between 2 and 32 s, followed by centrifugation through an inert oil layer. The shortest time period, 2s, represented the time for the cells to be centrifuged from the permeant layer to beneath the oil. Total [^{3}H]-adenosine incorporated into the cells was determined by subtracting background radioactivity due to the extra cellular space as described in Materials and Methods. Samples are averages of triplicate assays.

erythrocytes from subjects with β-thalassemia major was significantly reduced with a rate of 0.061 ± 0.007 pmoles/μl cell water/s (n = 3).

Adenosine transport was also assessed in erythrocytes from seven subjects with HbAE, six subjects with HbEE, three with β-thalassemia major and three subjects with α-thalassemia major. The ability of these various genotypes to transport lμM adenosine into erythrocytes is collected in Table 1. Statistical analysis (Student's *t*-test) indicated a significant reduction of normal adenosine transport in erythrocytes with HbEE ($p < 0.01$), α-thalassemia major ($p < 0.01$), and β-thalassemia major ($p < 0.01$). There were no significant differences between values for HbAA and HbAE. One subject with β-thalassemia trait had a value of 3.25 ± 0.24 pmoles/μl cell water; however, the distribution of abnormal red cells for this sample was not available.

Adenosine transport was also assessed in five subjects with G6PDH mild deficiency (Gd^{B-}) and three with a G6PDH severe deficiency ($Gd^{Myanmar}$) (Table 1). $Gd^{Myanmar}$ refers to an apparent severe deficiency of G6PDH detected in four subjects to date in Myanmar, who had less than 5% of the normal activity (Myint-Oo, 1993). Transport into $Gd^{Myanmar}$ erythrocytes (0.27 ± 0.021 pmoles/μl cell water/s) was significantly lower than that of normal erythrocytes (Gd^{B+}). However, although adenosine transport in the Gd^{B-} variant erythrocytes (0.320 ± 0.018 pmoles/μl cell water/s) appeared less than in normal cells, the differences were not statistically significant ($p < 0.10$).

The average distributions of erythrocytes with altered morphology in each blood sample with various genetic disorders are also collected in Table 1. The most significant differences in altered morphology were seen with the α- and β-thalassemias, though all of the genetic conditions varied somewhat from normal. The results showed an obvious correlation when the distribution of altered morphology was compared to the transport rates.

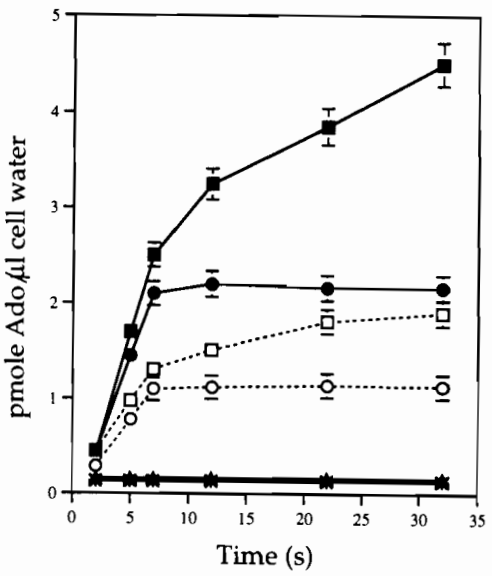
Adenosine transport into erythrocytes from all the above sources was measured in the presence of the transport inhibitor NBMPR (1μM). In all the different red cell species NBMPR completely inhibited the permeation of adenosine. This inhibition has been previously demonstrated in the Australian laboratories with normal and β-thalassemia cells (Myint-Oo *et al,* 1993; Gero *et al,* 1988).

Table 1

[^{3}H] Adenosine (1μM) uptake from subjects with red cell genetic abnormalities and the distribution of abnormal red cells in each blood sample.

Type of red cells	Adenosine transport pmole/μl cell H_2O/s (x10)	No. of samples	Percentages calculated from counts/500RBCs*				
			Anisocytes	Poikilocytes	Elliptocytes	Target	% normal RBCs
HbAA Normal	3.92 ± 0.21	10	2.8	0	0	1.2	96.0
β-thalassemia major	0.61 ± 0.04	3	23.4	8.4	10.4	9.8	48.0
HbAE (Homozygous)	4.12 ± 0.34	7	4.8	0	3.0	16.2	76.0
HbEE (Heterozygous)	2.85 ± 0.28 ($p < 0.01$)	6	4.6	5.2	4.6	24.6	55.0
α-thalassemia major	1.05 ± 0.04 ($p < 0.01$)	3	25.2	10.4	14.4	15.0	35.0
G6PD normal (Gd^{B+})	3.75 ± 0.14	15	3.2	0	0	1.8	95.0
G6PD deficient (Gd^{B-})	3.20 ± 0.18 (*NS)	5	5.2	0	4.4	4.4	86.0
G6PD deficient ($Gd^{-Myanmar}$)	2.66 ± 0.21 ($p < 0.01$)	3	4.8	0	4.6	7.6	83.0

*Mean values
*NS = not significant
n = number of subjects



Adenosine transport in *P. falciparum*-infected erythrocytes with genetic abnormalities

Transport of adenosine into *P. falciparum* infected normal erythrocytes (HbAA) which had been collected from patients in Myanmar and the effect of 1μM NBMPR is illustrated in Fig 2. Normal

Fig 2– Adenosine transport into *P. falciparum*-infected erythrocytes from both normal and β-thalassemia subjects. Conditions were as described for Fig 1 for the following samples: *P. falciparum*-infected normal erythrocytes, HbAA (■); *P. falciparum*-infected β-thalassemia trait erythrocytes (●); *P. falciparum*-infected normal erythrocytes + (1μM) NBMPR (□); *P. falciparum*-infected normal erythrocytes + (1μM) NBMPR + (1μM) tubercidin (▲); *P. falciparum*-infected β-thalassemia trait + (1μM) NBMPR (○); *P. falciparum*-infected β-trait erythrocytes + (1μM) NBMPR + (1μM) tubercidin (*). Values are the average of triplicate experiments.

erythrocytes (HbAA) from 3 subjects were used as controls. Adenosine transport in normal (Fig 1) and infected erythrocytes (Fig 2) (concentrated to 70-80% parasitemia as described in Materials and Methods) showed no significant differences. The transport in uninfected normal red cells was completely inhibited by 1μM NBMPR whilst there was approximately 60% reduction in transport by the same concentration of NBMPR in *P. falciparum* infected normal erythrocytes (Fig 2). When NBMPR (1μM) was combined with tubercidin (1μM), complete inhibition of transport was observed in *P. falciparum* infected normal erythrocytes. The addition of 1μM tubercidin alone reduced the transport in infected normal erythrocytes by approximately 20% (data not shown).

Adenosine transport into *P. falciparum* infected erythrocytes, which contained a population of β-thalassemia cells (parasitemia 70-80%) is also shown in Fig 2. Adenosine (1μM) transport was significantly reduced in these samples compared to that of normal erythrocytes infected with *P. falciparum*. However, transport in *P. falciparum* infected erythrocytes containing a population of β-thalassemia cells was inhibited by 1μM NBMPR to a similar extent (approx 60%) as were the *P. falciparum* infected cells in the presence of normal erythrocytes. Complete inhibition of adenosine transport was observed in *P. falciparum* infected β-thalassemia erythrocytes when 1μM tubercidin was combined with 1μM NBMPR.

The above experiment was repeated with *P. falciparum* infected bloods containing the abnormal red cells HbAE, G6PDH mild deficiency (B-), β-thalassemia trait and HbAE/ G6PDH (B-). The data from adenosine transport analysis of (a) normal and these abnormal *P. falciparum* infected cells (b) in the presence of NBMPR (1μM) and (c) with the combination of 1μM NBMPR and 1μM tubercidin is summarized inTable 2. Only in the

Table 2

Inhibitory effect of NBMPR and tubercidin on adenosine transport into *P. falciparum*-infected erythrocytes with genetic abnormalities.

	Red Cell	No. of* subjects	Drug	pmole Ado/μl cell water/s (times 10)
(a)	HbAA	5	Nil	3.74 ± 0.14
	HbAE	2	"	3.98 ± 0.16
	G6PDH $^{(B-)}$	2	"	3.51 ± 0.21
	β-trait	1	"	2.16 ± 0.11^{+}
	HbAE/ G6PDH $^{(B-)}$	1	"	3.22 ± 0.24
(b)	HbAA	5	NBMPR	1.67 ± 0.11
	HbAE	2	"	1.64 ± 0.08
	G6PDH $^{(B-)}$	2	"	1.32 ± 0.13
	β-trait	1	"	1.23 ± 0.06
	HbAE/ G6PDH $^{(B-)}$	1	"	1.64 ± 0.05
(c)	HbAA	5	NBMPR^{+} tubercidin	0.21 ± 0.04
	HbAE	2	"	0.16 ± 0.03
	G6PDH $^{(B-)}$	2	"	0.13 ± 0.03
	β-trait	1	"	0.15 ± 0.02
	HbAE/ G6PDH $^{(B-)}$	1	"	1.13 ± 0.02

* Three replicates for each sample were carried out.

** Original parasitemias were in the range 4.2-12.6%. Following culturing and Percoll centrifugation, the experiments were out with parasitemias of 70-80%.

\+ Significant at the $p < 0.01$ level.

1μM of NBMPR and 1μM of tubercidin were used throughout.

case of infected erythrocytes from a β-thalassemia subject was there a significant ($p < 0.01$) reduction in the rate of transport (Table 2a). In all cases, NBMPR partially inhibited transport in the infected cells (Table 2b). This was totally consistent with the results demonstrated with laboratory strains of *P. falciparum* infected cells in Australia (Gero *et al*, 1988). However, the combination of NBMPR and tubercidin (Table 2c) completely inhibited transport, which had not been demonstrated previously with the laboratory strains of *P. falciparum* infected cells in Australia.

Adenosine transport in *P. vivax* infected erythrocytes

Equivalent studies with *P. vivax* infected cells were carried out, including assessment of the effect of 1μM NBMPR and 1μM tubercidin on eight isolates of *P. vivax* infected erythrocytes with HbAA, HbAE and G6PDH deficiency. Adenosine transport into *P. vivax* infected normal erythrocytes (n = 5), and the effect of either 1μM NBMPR alone or in combination with 1μM tubercidin is shown in Fig 3. Normal uninfected erythrocytes served as controls. Adenosine transport in *P. vivax* infected normal erythrocytes (also concentrated to 70-80% parasitemia) was reduced to approximately 50% by NBMPR. Complete inhibition of adenosine transport was observed when the permeant contained both 1μM NBMPR and 1μM tubercidin, as observed for the *P. falciparum* infected cells.

Essentially identical results were observed in *P. vivax* infected erythrocytes with HbAE (n = 2) and G6PDH (Gd^{B-}) (n = 11). As before NBMPR (1μM) partially blocked adenosine transport in both types of *P. vivax* malaria infected cells. The combination of 1μM NBMPR with 1μM tubercidin led to complete inhibition of adenosine transport in *P. vivax* infections from Myanmar, identical to the effects described above for *P. falciparum* from field strains in Myanmar.

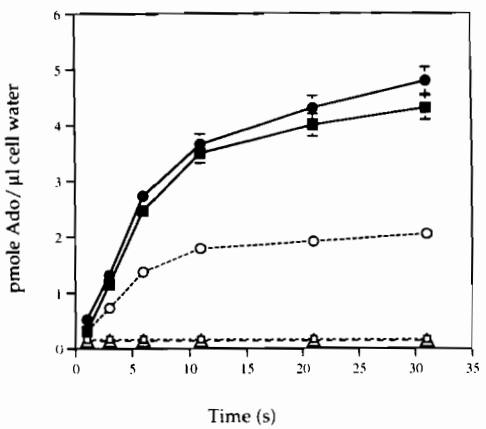


Fig 3– Adenosine transport into *P. vivax* infected erythrocytes from normal subjects. Conditions were as described for Fig 1 for the following samples. Normal erythrocytes, HbAA, (■); normal erythrocytes + 1μM NBMPR (□); *P. vivax* infected erythrocytes (●); *P. vivax* infected erythrocytes + NBMPR (1μM) (○); *P. vivax* infected erythrocytes + NBMPR (1μM) + tubercidin (1μM) (Δ). Values are the average of triplicate experiments.

DISCUSSION

The results presented suggest that a number of red cell genetic disorders give rise to alterations to the effectiveness of adenosine transport. Decreases were observed with subjects with α-thalassemia and there were also significant differences between HbAA and subjects with HbEE and a severe deficiency of G6PDH. It was postulated that this decrease in permeability corresponded to the presence of abnormal red cells in the various samples. An assessment of the proportion of normal cells in each blood sample allowed the calculation of the contribution of abnormal cells to the observed transport rates (Fig 4). With the assumption that the

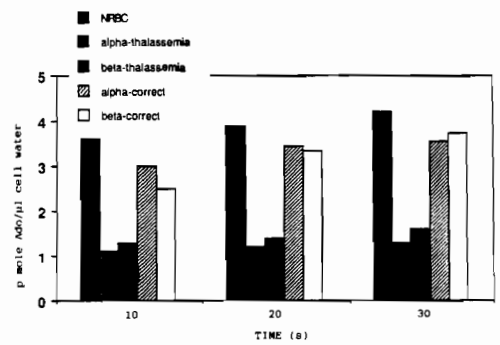


Fig 4– Comparison of adenosine transport into normal erythrocytes (NRBC, ■), erythrocytes from subjects with α-thalassemia major (αRBC, ▨), and β-thalassemia major (βRBC, ▩), and adjusted values taking into account only the percentages of normal erythrocytes in blood samples with ∝-thalassemia (αRBC corrected, ▨) and β-thalassemia (βRBC corrected, □).

normal erythrocytes in the α- and β-thalassemia samples transported adenosine at the same rate as erythrocytes from normal patients, data from each sample were recalculated, based on the number of abnormal cells in each sample from Table 1. In all cases, correlation between the variety of abnormal cells present and the rate of adenosine transport led to the conclusion that the abnormal cells contributed little if any of the overall transport of adenosine. This conclusion was reinforced by the fact that NBMPR was capable of totally inhibiting adenosine transport in both normal erythrocytes and all those with genetic abnormalities. The results with Myanmar subjects with β-thalassemia were substantially similar to those obtained with a group of patients analysed in Australia, principally of Mediterranean origin, described in a previous study (Myint-Oo *et al,* 1993).

A major objective of the current study was the opportunity to correlate studies carried out on long-term laboratory cultured isolates of *P. falciparum* in normal erythrocytes in the Australian laboratory (Gero *et al,* 1988) with the field situation in South-east Asia, particularly Myanmar. Thus transport in *P. falciparum* and *P. vivax* infected erythrocytes from Myanmar, selectively collected from normal subjects and from subjects with certain genetic abnormalities, including HbAE, β-thalassemia and G6PDH deficiency were investigated. The results suggested substantial similarities between the transport properties of the laboratory cultured strains of *P. falciparum* (FCQ_{27} having been in culture for over 20 years) and those obtained from fresh infections and cultured for only 24 hours. Thus, in both cases infection by *P. falciparum* of either normal erythrocytes or those from subjects with genetic abnormalities, led to an NBMPR-insensitive component of adenosine transport. The overall study reported here was obviously limited by access to appropriate patient material. However, the results are consistent with the data found for the laboratory *P. falciparum* FCQ_{27} strain (Upston and Gero, 1995; Gero and Upston, 1992) and occurred irrespective of the source of the cells.

There were differences, however, in the combined effects of NBMPR and tubercidin between the laboratory and the indigenous infections. There are at least two components of adenosine transport in cultured *P. falciparum* infected cells; the endogenous host nucleoside transporter which is fully inhibited by NBMPR and a second "parasite induced component" totally insensitive to NBMPR which comprises approximately 50% of the total nucleoside transport in parasite infected cells (Upston and Gero, 1995). The results presented here indicate that tubercidin inhibited this second component, as the combination of NBMPR and tubercidin fully inhibited transport (Table 2, Fig 2). In addition, previous *in vitro* data on FCQ_{27} *P. falciparum* had shown also that the combination, NBMPR and tubercidin, produced synergistic antimalarial activity (Gero *et al,* 1988). In contrast, complete inhibition of tubercidin and NBMPR observed with the samples collected in Myanmar, suggested a greater sensitivity of the "field" strains to transport inhibition than the laboratory adapted strains. The possibility that this could be exploited in the development of potential antimalarials could be explored.

In addition, the influx data suggested that either the parasite only infected the normal erythrocytes within the population of genetically abnormal red cells, or that the parasite was also able to alter the permeability properties of the abnormal cells. It is possible also that the apparent decrease in adenosine transport in the abnormal erythrocytes could confer a nutrient deficiency that contributed to potential resistance to *P. falciparum* infection (Myint-Oo *et al,* 1993). Some evidence consistent with this hypothesis has been obtained from an epidemiological study of the relationship between genetic red cell disorders and *P. falciparum* infection in Myanmar (Myint-Oo *et al,* 1995). In this study mean parasitemia levels of infected subjects with β-thalassemia, α-thalassemia and severe glucose-6-phosphate dehydrogenase deficiency were lower than those of individuals with hemoglobin HbAA.

Studies of nutrient transport have not previously been carried on with *P. vivax* infected cells. Thus, the extension of the study to *P. vivax* infections was of considerable relevance (Myint-Oo, 1986) including assessment of the effect of NBMPR and tubercidin on eight isolates of *P. vivax* infected erythrocytes with HbAA, HbAE and G6PDH deficiency which showed similar effects to those determined with *P. falciparum.* The results indicate that strategies developed for *P. falciparum* may also be relevant to other malarial species.

The data contained in this paper reveals for the first time, that erythrocytes infected by strains of both *P. falciparum* and *P. vivax* were similar in their ability to transport and be inhibited by purine

nucleosides. In addition, it appeared that genetic abnormalities in erythrocytes reduced transport in a population of malaria infected erythrocytes. The correlation between a variety of red cell genetic polymorphisms and a degree of protection against malaria infection is well established. It has been suggested recently that specific interference with trafficking channels could substantially reduce the impact of parasitism (Deitsch and Wellems, 1996). The reduction of adenosine transport reported here and previously (Myint-Oo *et al,* 1993) may be a factor in the reduction of the severity of *P. falciparum* infections in subjects with erythrocytic abnormalities.

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REFERENCES

Andrysiak PM, Collins WE, Campbell GH. Concentration of *Plasmodium vivax*-infected erythrocytes from non-human primate blood using Percoll gradients. *Am J Trop Med Hyg* 1986; 35 : 251-4.

Aung-Than-Batu, Khin-Kyi-Nyunt, Hla-Pe. The thalassemias in Burma. *Union of Burma J Life Sci* 1968; 1 : 241-7.

Aung-Than-Batu, Hla-Pe. Glucose-6-phosphate dehydrogenase deficiency in Burma. *Union of Burma J Life Sci* 1969; 2 : 59-61.

Aung-Than-Batu, Hla-Pe, Khin-Kyi-Nyunt. Haemoglobinopathies in Burma. The incidence of α-thalassemia trait. *Trop Geogr Med* 1971; 23 : 23-5.

Bruce-Chwatt LJ. Chemotherapy of malaria. Geneva: World Health Organisation. 1986; 196-98.

Dacie JV, Lewis SM. Practical Haematology, 6th ed. Edinburgh: Churchill Livingstone, 1984.

Deitsch KW, Wellems TE. Membrane modifications in erythrocytes parasitized by *Plasmodium falciparum. Mol Biochem Parasitol* 1996; 76 : 1-10.

Dluzewski AR, Ling IT, Rangachari K, Bates PA,Wilson RJM. A simple method for isolating viable mature parasites of *Plasmodium falciparum* from culture. *Trans R Soc Trop Med Hyg* 1984; 78 : 622-4.

Gero AM, Bugledich EMA, Paterson ARP, Jamieson GP. Stage specific alteration of nucleoside membrane permeability and nitrobenzylthioinisine insensitivity in *Plasmodium falciparum*-infected erythrocytes. *Mol Biochem Parasitol* 1988; 27 : 159-70.

Gero AM, Upston JM. Altered membrane permeability: A new approach to malarial chemotherapy. *Parasitol Today* 1992; 8 : 283-6.

Haldane JBS. Disease and evolution. *Ric Sci* 1949; 19 (suppl) : 68-76.

Modell B, Berdoukas V. The Clinical Approach to Malaria. London : Grune and Stratton, 1984.

Myint-Oo. University of New South Wales, 1993. PhD thesis.

Nyint-Oo. Isozyme variation in schizonts of *Plasmodium vivax* from Burrna. *Trans R Soc Med Hyg* 1986; 80 : 1-4.

Myint-Oo, Than-Swe, Ye-Htut, *et al.* The changing incidence of *Plasmodium vivax* infections with malaria. *Myanmar Health Sci Res J* 1992; 4 : 53-5.

Myint-Oo, Tin-Shwe, Marlar-Than, O'Sullivan WJ. Relationship of genetic red cell disorders and severity of falciparum malaria infection in Myanmar. *Bull WHO* 1995; 73 : 659-65.

Myint-Oo, Yuthavong Y, O'Sullivan WJ. Malaria in South East Asia. *Today's Life Sci* 1991; 3 : 42-6.

Myint-Oo, Upston JM, Gero AM, O'Sullivan WJ. Reduced transport of adenosine in erythrocytes from patients with β-thalassemia. *Int J Parasitol* 1993; 23 : 303-7.

Sicard D, Lieurzo Y, Lapoumeroulie C, Labie D. High genetic polymorphism of haemoglobin disorders in Laos. Complex phenotypes due to associated thalassemic syndromes. *Hum Genet* 1979; 50 : 327-36.

Thaithong S, Siripoon N, Seugorn N, Bunnag D, Beale GH. Electrophoretic variants of enzymes in isolates of *Plasmodium falciparum, P. malariae* and *P. vivax* from Thailand. *Trans R Soc Med Hyg* 1989; 83 : 602-5.

Trager W, Jensen JB. Heman malaria parasites in continuous culture. *Science* 1976; 193 : 673-5.

Upston JM, Gero AM. Parasite induced permeation of nucleosides in *Plasmodium falciparum* infected erythrocytes. *Biochem Biophys Acta* 1995; 1236 : 249-58.

Wasi P, Na-Nakorn S, Pootrakul S, *et al.* Alpha and beta thalassemia in Thailand. *Ann NY Acad Sci USA* 1969; 165 : 60-75.

Weatherall DJ, Clegg JB. In: The Thalassaemia Syndromes. 3rd ed. Oxford : Blackwell Scientific 1981.

WHO Technical Expert Committee. Haemoglobinipathies and allied disorders. *WHO Tech Rep Ser* 1966; 338.

WHO Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group, Geneva. *WHO Tech Rep Ser* 1967; 366 : 125.