BIOCHEMICAL JOURNAL LETTERS

Effect of cell age on erythrocyte choline transport: implications for the increased choline permeability of malaria-infected erythrocytes

Two papers published recently in this journal have arrived at different conclusions about the characteristics of choline transport into malaria-infected erythrocytes. Ancelin *et al.* (1991) demonstrated that erythrocytes isolated from monkeys (*Macaca fascicularis*) infected with *Plasmodium knowlesi* show an increased rate of choline uptake via a pathway with the same kinetic and pharmacological properties as the endogenous choline transporter but with a $V_{\max}$ more than 10 times that of cells from uninfected monkeys. They found no evidence for the transport of choline via parasite-induced, non-saturable permeability pathways of the sort that mediate the enhanced transport of a variety of other solutes in malaria-infected erythrocytes (e.g. Ginsburg, 1988) and concluded that choline entry into infected cells remains entirely under carrier control. By contrast, following on from an earlier study (Elford *et al.*, 1990), Kirk *et al.* (1991) demonstrated that human erythrocytes infected *in vitro* with *Plasmodium falciparum* show a new choline transport component which differs, both kinetically and pharmacologically, from the endogenous choline transporter. This component did not saturate at choline concentrations of up to 500 μM . The characteristics of the endogenous (saturable) component of choline influx into infected cells were not investigated in detail in our study; however, it is clear from the results presented that the saturable component did not show the 10-fold increase in $V_{\max}$ that was reported for *P. knowlesi*-infected erythrocytes. This discrepancy may relate to inherent species-specific dissimilarities between the two malarial and host erythrocytes involved. However, in this Letter we wish to suggest that it may be due, at least in part, to the different cell-age distribution of the infected cell populations used in the two studies. In our work parasites were cultured *in vitro* using normal human erythrocytes which had been stored for 1–5 days before malaria infection and which would therefore have contained a very low proportion of reticulocytes (< 1%). By comparison, Ancelin *et al.* (1991) collected heavily parasitized blood from splenectomized monkeys 6–9 days after infection with *P. knowlesi*. Both the splenectomy (Lewis, 1983) and the malaria infection are likely to have led to an increased proportion of circulating reticulocytes and it is therefore relevant to consider the effect of red cell age on the characteristics of choline transport.

It has been reported previously that in density-fractionated erythrocytes (suspended at an extracellular choline concentration of 2 μM) the rate of choline transport in the least-dense (younger) cells is greater than that in the most-dense (older) cells (Uney *et al.*, 1985). In preliminary experiments, in which a Percoll gradient technique (Wolowyk, 1982) was used to obtain density-fractionated erythrocytes from normal human volunteers, we obtained similar results for choline transport rates measured under $V_{\max}$ conditions ([choline] = 250 μM); the rate of choline uptake in the least-dense cell fraction was $152 \pm 13\%$ ($\pm \text{S.E.M.}$, $n = 4$) that in the most-dense cell fraction. In order to characterize

further the variation of choline transport rates with erythrocyte age, blood was obtained from two haemochromatosis patients with a high degree of reticulocytosis (5–8% reticulocytes) and a repeated-centrifugation technique was used to obtain reticulocyte-enriched and reticulocyte-depleted cell fractions (Tucker & Young, 1980). Briefly, the blood was washed by repeated suspension and centrifugation (800 g, 15 min) of the cells in about 5 vol. of ice-cold phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.4). Care was taken to remove leukocytes from the top of the cell pellet following each centrifugation step. Washed cells were suspended in an approximately equal volume of saline, then centrifuged for 1 h (800 g, 4 °C). The resulting packed cell column was divided into thirds; the top one-thirds from a number of tubes were combined, as were the bottom one-thirds. This procedure was then repeated three times. In a number of experiments a significant concentration of leukocytes persisted through the initial washes so the fractionated cells were passed down a column packed either with a combination of glass and polymer wool (Tucker & Young, 1980) or cellulose powder (CF11, Whatman, U.K.; Homewood & Neame, 1976; Ancelin *et al.*, 1991). The proportion of reticulocytes in the final top and bottom fractions was estimated using New Methylene Blue stain (Dacie & Lewis, 1975); it ranged from 10 to 23% in the top fraction and was consistently < 2% in the bottom fraction. Total erythrocyte concentrations in the two fractions were measured using a Coulter counter (Model D). Negligible leukocyte contamination of the washed cell fractions was confirmed from Giemsa-stained smears (Dacie & Lewis, 1975).

[^{14}C]Choline (Amersham International) influx into both top and bottom cell fractions (at 37 °C and at an extracellular choline concentration of 2 μM) appeared linear with time for up to 10 min. Initial rates of choline transport were therefore estimated from the uptake of [^{14}C]choline during a fixed 5 min incubation period, measured using a standard influx technique with triplicate samples (Fervenza *et al.*, 1991). K_m and $V_{\max}$ values were estimated by fitting a simple Michaelis–Menten expression to the concentration-dependence of the initial influx rates using a numerical fit routine.

Fig. 1 shows the concentration-dependence of choline influx into the top and bottom cell fractions under ‘zero-trans’ conditions (i.e. with depleted intracellular choline). The results are representative of those obtained in four similar experiments carried out with blood from one patient with haemochromatosis (donated on four separate occasions over a 6 week period) and a further experiment with blood from a second patient. The mean K_m for choline uptake by the top fraction (averaged from the K_m values estimated from blood from the five experiments) was $10.5 \pm 0.5 \mu\text{M}$ and was not significantly different from that for the bottom fraction ($9.6 \pm 0.5 \mu\text{M}$, $P = 0.16$, paired *t*-test). By contrast, the mean value for the $V_{\max}$ estimated for the top fraction under zero-trans conditions ($3.05 \pm 0.21 \mu\text{mol} \cdot \text{h}^{-1} \cdot 10^{12} \text{ erythrocytes}^{-1}$) was significantly higher than that estimated for the bottom fraction ($0.69 \pm 0.14 \mu\text{mol} \cdot \text{h}^{-1} \cdot 10^{12} \text{ erythrocytes}^{-1}$, $P = 0.002$).

The effect of intracellular choline on the rate of choline influx

Fig. 1. Concentration-dependence of choline influx into reticulocyte-enriched (○) and reticulocyte-depleted (●) red cell fractions prepared from blood from a patient with haemochromatosis

The fractionated cells were depleted of endogenous choline by preincubation at 37 °C for 2 h in phosphate-buffered saline supplemented with 15 mM-Hepes and 10 mM-glucose, with replacement of the extracellular solution at 30 min intervals. The least-dense cell fraction contained 18% reticulocytes and the most-dense cell fraction less than 1% reticulocytes. The results shown are from a single experiment and are representative of those obtained in four other similar experiments.

was also investigated. In cells in which leakage of the endogenous intracellular choline was prevented by maintaining the cells at 4 °C prior to flux measurements, the rates of choline influx in the top and bottom cell fractions at an extracellular choline concentration of 100 μM (i.e. $\sim 10 \times K_m$) were 4.95 ± 0.15 and 1.10 ± 0.24 μmol · h⁻¹ · 10¹² erythrocytes⁻¹ respectively ($n = 4$). These values were significantly higher ($P < 0.015$) than those measured in cells from the same preparations but in which the intracellular choline had been depleted as described in Fig. 1 (3.11 ± 0.11 and 0.74 ± 0.17 μmol · h⁻¹ · 10¹² erythrocytes⁻¹ for the top and bottom fractions, respectively).

It is clear from these results that the V_{max} for choline uptake by the reticulocyte-enriched cell fraction is much higher than that for the reticulocyte-depleted fraction, whereas the K_m is essentially the same. The observation by Ancelin *et al.* (1991) that the malaria-infected cell fraction showed increased choline influx via a pathway having a similar K_m but a much higher V_{max} than that in erythrocytes from uninfected monkeys might therefore be explained if the infected cell fraction contained a higher proportion of reticulocytes and/or very young cells than the uninfected cell fraction. There are two ways in which this might have come about. The parasites may have invaded reticulocytes and/or young erythrocytes in preference to the older cells in the circulation. Some human, murine and avian malarias do show a marked preference for reticulocyte-invasion (Mons, 1990); however this is not known to be the case for *P. knowlesi*. An alternative (or additional) explanation is that the parasitized cell fraction from the *P. knowlesi*-infected monkeys may have been 'contaminated' by uninfected reticulocytes. In their work, Ancelin *et al.* (1991) used a density-centrifugation technique to enrich the infected cell fraction and any uninfected cells incorporated into the parasitized top layer were therefore likely to have been the youngest red cells in the circulation.

It is also relevant to note that, in the study by Ancelin *et al.* (1991), although the erythrocytes obtained from uninfected monkeys were depleted of their intracellular choline by repeated washing in choline-free medium, erythrocytes from the infected animals were not choline-depleted in the same way. The intracellular concentration of free choline in infected cells is very low; however this is not true of uninfected cells present in the

parasitized-cell fraction (Ancelin & Vial, 1989). As has been shown here, endogenous intracellular choline levels give rise to significant *trans*-stimulation of choline influx in uninfected cells. The transport of choline into any uninfected cells present in the parasitized-cell preparation used by Ancelin *et al.* (1991) would therefore have been *trans*-stimulated by intracellular choline and this would have contributed further to the increase in V_{max} observed for this fraction.

Human erythrocytes infected *in vitro* with *P. falciparum* show a non-saturable choline transport component that is absent from uninfected cells (Elford *et al.*, 1990; Kirk *et al.*, 1991). The failure of Ancelin *et al.* (1991) to detect any such component in the uptake of choline by *P. knowlesi*-infected erythrocytes may reflect both the large size of the saturable influx component in their parasitized-cell fraction and the low choline concentration range over which their studies were carried out. The initial time-course experiments reported by Ancelin *et al.* (1991) (on the basis of which the presence of a new, malaria-induced permeability pathway was ruled out) were carried out at a choline concentration of 0.9 μM, well below the K_m of the native carrier. At this concentration the contribution of any non-saturable transport pathway to the total choline influx will have been relatively small. In order to assess the contribution of a non-saturable component it is usual to work at substrate concentrations sufficiently high to saturate any saturable pathway present (see e.g. Christensen 1975). However, the maximum choline concentration tested by Ancelin *et al.* (1991) (~ 17 μM) was approximately twice the reported K_m (8.5 μM), at which concentration the flux via the saturable pathway would have been only two-thirds of the V_{max} . In the absence of any measurements made at choline concentrations sufficient to saturate the native transporter we would suggest that the transport of choline by a new, non-saturable pathway in *P. knowlesi*-infected erythrocytes cannot be ruled out.

K.K. was supported by an Oxford Nuffield Medical Fellowship, C.E.P.de F. by C.A.P.E.S. and B.C.E. by the Medical Research Council. The authors are grateful to Dr Faith Holdsworth and Dr Catherine Edwards for supplying blood from patients with haemochromatosis. Ms Heather Horner and Mr Robin Parsons are thanked for expert technical advice and Dr David Roberts for helpful discussions.

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Received 29 August 1991

Saturable and non-saturable components of choline transport in *Plasmodium*-infected mammalian erythrocytes: possible role of experimental conditions

Studies on choline transport in normal erythrocytes were initiated by Askari [1]. He revealed the presence of two distinct mechanisms for the uptake of choline in normal erythrocytes: a facilitated uptake mechanism, which becomes saturated at low external choline concentration (K_m 20 μM), and a simple diffusion mechanism in which the uptake rate is proportional to the concentration detected at very high concentrations (in the millimolar range). Thorough studies were then carried out which led to characterization of the saturable component. It is mediated by an asymmetric carrier operating according to a cyclic model whose limiting step is the re-orientation of the free carrier inside the membrane (for review see [2]).

Until recently little attention has been paid to choline transport across the membrane of malaria-infected erythrocytes. However, two papers were published in 1991 which led to different conclusions concerning the nature of choline transport after malaria infection. Kirk *et al.* [3] found that human erythrocytes infected *in vitro* with *Plasmodium falciparum* incorporated choline mainly via a component differing kinetically and pharmacologically from the endogenous transporter and which did not saturate at choline concentrations up to 0.5 mM, whereas only the saturable component was observed in normal cultured erythrocytes (K_m 11 μM). By contrast, Ancelin *et al.* [4] found that *P. knowlesi*-infected simian erythrocytes showed an increased rate of choline transport via a pathway with the same kinetic properties as the endogenous choline transporter (K_m 9 μM), except that the V_{max} was more than 10-fold higher than that of uninfected erythrocytes. Non-saturable permeability pathways were not detectable at physiological choline concentration, *i.e.* up to 24 μM . In fact, we observed a non-saturable component both for infected and uninfected erythrocytes detected from 1.2 to 1.7 mM (our unpublished work), that is about 50–100-fold the physiological concentrations [5].

Considering that the transport measurements in the two studies were carried out using essentially similar techniques, Kirk *et al.* (1992) (the preceding Letter) investigated a possible effect of cell age on normal red blood cell choline transport and showed that the V_{max} for choline influx decreased with cell age. Similar results have already been demonstrated for other transport systems such as nucleosides [6,7], amino acids and sugars [8]. Nevertheless, Kirk *et al.* (1992) attempt to benefit from this difference by suggesting that the wide difference in the V_{max} we observed in cells from malaria-infected and uninfected monkeys might have been due to reticulocyte contaminants in our parasite preparation.

The presence of reticulocytes can be ruled out. First, monkey splenectomies were performed 4–7 years before and could not reasonably have been involved in an increased proportion of circulating reticulocytes. Secondly, in parasites like *P. knowlesi*

there is not any real restriction for reticulocytes as observed in *P. vivax* ([9] and B. Mons, personal communication). Furthermore, since reticulocytes comprise only 1–3 % of the total red blood cell population at any given moment, most rings of the non-restricted parasites will end up in mature cells (B. Mons, personal communication). In our experiments, parasitaemia was 10–30 % (late stages) on days 6–9. A simple calculation reveals that the majority of these parasites cannot conceptually be anywhere else than in mature cells. Lastly, we never observed induced reticulocytosis during infection (our observations) and reticulocyte-restricted parasites can never provide such fulminating parasitaemia without first inducing reticulocytosis (B. Mons, personal communication). The last possibility involving contamination by uninfected reticulocytes of the Percoll parasite-enriched fraction can be easily ruled out since smears always showed parasitaemia of more than 95 %, which seriously reduces the possibility of any reticulocyte involvement in the 10-fold increase in V_{max} of choline influx in the *P. knowlesi*-enriched fraction.

On the other hand, several technical differences in the transport measurements between the two studies should be detailed. They notably concern the method of obtaining infected and uninfected erythrocytes, the composition of washing, incubation and stopping media, the mode of stopping the influx reaction, and nonspecific determination.

Concerning the *P. falciparum* measurements [3], human erythrocytes were sorted for 1–5 days before being infected by *P. falciparum*, then maintained in culture, synchronized and later enriched (parasitaemia 46–93 %). Noninfected control cells were obtained from the same donor and cultured at the same time. The culture medium consisted of RPMI 1640, whose choline concentration is 21 μM [10], plus 10 % human serum. The washing and incubation media were phosphate- and Hepes-buffered saline and the medium for stopping the reaction was Mops-buffered MgCl_2 solution.

Concerning the *P. knowlesi* experiments [4], fresh *P. knowlesi*-infected simian erythrocyte suspensions and noninfected erythrocytes from a healthy monkey were removed from the leukocyte contaminants by using a cellulose-powder column. The highly enriched parasite fractions (parasitaemia > 95 %) were separated from uninfected erythrocytes by using a Percoll gradient. All fractions (containing infected and uninfected cells from the same monkey, or noninfected cells from a healthy monkey) were then treated in parallel, under the same conditions, *i.e.* extensively washed (for 2–3 h) with large volumes of choline-free RPMI 1640. This notably excludes any possible transactivation by the few uninfected cells present in the parasitized preparation in contributing to the V_{max} increase, as suggested by Kirk *et al.* for whom preincubation at 37 °C for 2 h, in phosphate-buffered saline supplemented with 14 mM-Hepes and 10 mM-glucose, was sufficient to deplete cells of endogenous choline (see Fig. 1 of Kirk *et al.*, 1992). Choline-free RPMI 1640 was also used for the choline influx measurements and to stop the reaction.

Blood storage preservation and culture conditions have been reported to be responsible for various modifications of several transport systems. Thus, the nucleoside transporter has kinetic properties consistent with a symmetrical carrier mechanism in fresh erythrocytes, but is asymmetrical in erythrocytes from outdated blood [11,12]. The difference between human and guinea pig erythrocytes in the degree of mobility of the empty and uridine-loaded carrier is highly accentuated during blood storage [13]. Storage of red cells in blood bank conditions or cultivation of the cells for 4–5 days in medium required for the propagation of *P. falciparum* parasites were demonstrated to increase (up to 5-fold) the non-saturable component of tryptophan transport (L system), and also the saturable component (T system) without any modification of K_m . Culture conditions