Survival of human carrier erythrocytes in vivo

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ABSTRACT

Erythrocytes offer the exciting opportunity of being used as carriers of therapeutic agents. Encapsulation within erythrocytes will give the therapeutic agent a clearance equivalent to the normal life of the erythrocyte therefore maintaining therapeutic blood levels over prolonged periods and also giving a sustained delivery to the monocyte–macrophage system (reticulo-endothelial system). Both the dose and frequency of therapeutic interventions could thus be reduced. Ensuring a near-physiological survival time of carrier erythrocytes is essential to their successful use as a sustained drug delivery system, and this has not been demonstrated in man. In this study we assessed the survival in vivo of autologous unloaded energy-replete carrier erythrocytes in nine volunteers, using a standard $^{51}$Cr erythrocyte-labelling technique. Within 144 h after infusion there was a 3 to 49% fall in circulating labelled cells, followed thereafter by an almost complete return to initial circulating levels; surface counting demonstrated an initial sequestration of erythrocytes by the spleen and subsequent release. Mean cell life and cell half-life of the carrier erythrocytes were within the normal range of 89 to 131 days and 19 to 29 days respectively. These results demonstrate the viability of carrier erythrocytes as a sustained drug delivery system.

INTRODUCTION

Erythrocytes have been proposed as carriers of encapsulated therapeutic agents. Their major potential applications are 2-fold: (i) the sustained and targeted delivery of drugs or enzymes to the monocyte–macrophage system (reticulo-endothelial system of liver, spleen and bone marrow, the sites of erythrocyte destruction) for the treatment of disorders associated with this cell lineage, e.g. lysosomal storage diseases; and (ii) the sustained maintenance in the circulation of therapeutic agents, for example of enzymes for the degradation of pathologically elevated tissue and plasma metabolites which are able to permeate the erythrocyte membrane. Encapsulation of therapeutic agents within erythrocytes with a normal mean cell life range of 89 to 131 days (normal half-life of 19 to 29 days) would limit the vascular clearance of the administered drug thus reducing the dose and frequency of therapeutic interventions. There is, however, a paucity of in vivo studies on these cellular carriers in man that are a prerequisite to clinical trials with therapeutic agents. Ensuring a near-physiological survival time of carrier erythrocytes in vivo is essential if they are to be successful as a sustained and targeted therapeutic delivery system.

Different methods have been used for therapeutic agent and macromolecule encapsulation including iso-osmotic lysis induced by high voltage electric fields and hypo-osmotic haemolysis either by direct dilution with a hypo-osmotic solution or by dialysis of the cells against a hypo-osmotic solution [1–3]. Of the erythrocyte ghosts prepared by these various methods, those prepared using hypo-osmotic dialysis retain to a greater extent the biochemical and physiological characteristics of the intact erythrocyte. In vivo survival studies of such dialysis erythrocyte ghosts in Beagle dogs revealed a half-life of 7 days compared with normal $^{51}$Cr-labelled erythrocytes which have a half-life of 18 ± 1 days [4]. Restoration of normal cellular ATP levels (and hence further improvement of biochemical and physiological parameters) by dialysing erythrocytes with the addition of glucose,

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magnesium chloride and adenosine in the rescaling buffer and using low centrifugation speeds during the washing steps increases cell survival in the dog to 18 days [5]. These energy-replete erythrocytes, both in the dog and human, show normal cellular morphology and retention of soluble cytoplasmic proteins and biochemical parameters, and are referred to as carrier erythrocytes to distinguish them from erythrocytes prepared by other methods [6]. Macromolecule entrapment into human carrier erythrocytes can be increased by extending the hypo-osmotic dialysis period. The lysed erythrocytes were rescaled by transferring the dialysis bags to containers holding 150 ml of PBS supplemented with 5 mmol/l adenosine, 5 mmol/l glucose and 5 mmol/l MgCl₂ and continuing rotation at 6 rev./min in the incubator, now at 37 °C, for 60 min. The energy-replete carrier erythrocytes were washed three times in 3 volumes of supplemented PBS with centrifugation at 100 g for 15 min and finally pooled.

METHODS

Volunteers

Nine healthy volunteers (four females and five males) aged 21 to 43 years (mean 25.6 ± 2.3 years) took part in the study. Ethics approval was granted by the local research ethics committee and informed consent was obtained from all subjects.

Blood preparation

Sterile materials and aseptic radiopharmacy facilities were used throughout. Forty millilitres of blood were collected and placed into two tubes containing 4 ml of anticoagulant citrate phosphate dextrose (n = 2) or 200 units of heparin (n = 7). The blood samples were centrifuged for 10 min at 1100 g; the supernatant plasma was removed and kept for later use and the buffy coat was discarded. The erythrocytes were washed twice in cold (4 °C) iso-osmotic PBS, pH 7.4 (2.68 mmol/l KCl, 1.47 mmol/l KH₂PO₄, 136.89 mmol/l NaCl, 8.10 mmol/l NaHPO₄), and centrifuged for 10 min at 1100 g.

Carrier erythrocyte preparation

Energy-replete carrier erythrocytes were prepared using a hypo-osmotic dialysis technique [4,9]. Washed and packed fresh erythrocytes (10.5 ml) were mixed with 4.5 ml of cold PBS. Five millilitres of this cell suspension were placed into each of three dialysis bags (molecular mass cut-off of 12000 Da, Medicell International Ltd, London, U.K.) sealed at both ends with clips. Each dialysis bag was placed in a container and supported firmly by wedging the dialysis clips against the container side. Dialysis was against 150 ml of hypo-osmotic phosphate buffer, pH 7.4 (5 mmol/l KH₂PO₄, 5 mmol/l K₂HPO₄), at 4 °C in a specially modified LabHeat refrigerated incubator (BoroLabs Ltd, Berks, U.K.) with rotation at 6 rev./min. Macromolecule entrapment can be increased by doubling the hypo-osmotic dialysis time to 180 min [7,8]. We therefore dialysed the erythrocytes for 90 (n = 6) or 180 (n = 3) min to ascertain that cell survival in vivo was not adversely affected by an extended hypo-osmotic dialysis period. The lysed erythrocytes were rescaled by transferring the dialysis bags to containers holding 150 ml of PBS supplemented with 5 mmol/l adenosine, 5 mmol/l glucose and 5 mmol/l MgCl₂ and continuing rotation at 6 rev./min in the incubator, now at 37 °C, for 60 min. The energy-replete carrier erythrocytes were washed three times in 3 volumes of supplemented PBS with centrifugation at 100 g for 15 min and finally pooled.

Labelling of carrier erythrocytes with sodium [⁵¹Cr]chromate

Carrier erythrocytes were labelled using a standard ⁵¹Cr erythrocyte-labelling technique [10]; the washed and packed cells were gently mixed with 0.75 MBq of sodium [⁵¹Cr]chromate (Amersham International, Bucks, U.K.) and allowed to stand at room temperature for 30 min. Unbound chromium was removed with either 100 mg of ascorbic acid (100 mg/ml, Evans Medical, Leatherhead, U.K.), which reduces the chromate ion to the non-permeable chromic ion, followed by a single wash (n = 7), or by three washes (n = 2) in supplemented PBS. After resuspension in an equal volume of autologous plasma, the carrier erythrocytes were injected slowly over a period of 5 min into the volunteer. Autologous erythrocytes were used throughout these studies.

Assessment of carrier erythrocyte survival

In vivo survival was assessed by monitoring the disappearance of ⁵¹Cr label from the circulation; 10-ml blood samples were taken from a vein in the contralateral arm 15, 30, 60, 120 and 180 min after injection, then twice in the first week and weekly until activity was not noticeably above background. To check for intravascular haemolysis, plasma was measured for ⁵¹Cr activity injected. Urinary excretion of label was assessed in seven volunteers by making 24-h urine collections for the first 72 h after injection; ⁵¹Cr activity in the urine was expressed as a percentage of the total ⁵¹Cr activity injected. ⁵¹Cr activity in the liver, spleen and heart was measured at various times between 0 and 6 days after carrier erythrocyte injection by surface counting using a gamma counter (John Caunt Scientific, Oxon, U.K.) in seven volunteers. The position of the organs was marked on the skin surface to ensure correct positioning of the gamma counter on subsequent days. The liver and spleen were
Figure 1  Survival in six subjects of carrier erythrocytes prepared using a hypo-osmotic dialysis period of 90 min; MCL, mean cell life

scanned because they are sites of erythrocyte sequestration and destruction, and the heart provided a measure of circulating labelled cells. Duplicate measurements were made for each organ; the count rates were then averaged and corrected for background counts and natural decay. The initial mean count rate over the heart was designated 1000, and this normalizing factor was then applied to all other count rates recorded.

Analysis of carrier erythrocyte survival data

Percentage raw cell survival was calculated by expressing c.p.m./ml packed cells as a percentage of the calculated zero time value, after correction for natural decay. The data were then plotted against time on a semi-logarithmic scale and a best-fit straight line drawn. $T_{1/2}$ carrier erythrocyte survival was taken as the time for the concentration of $^{51}$Cr in the circulating blood to fall to 50% of its initial value.

For the determination of mean cell life, c.p.m./ml packed cells (corrected for both natural decay and chromium elution from the erythrocytes) was expressed as a percentage of the corrected zero time value. Cell survival data were plotted against time on a linear scale and the mean cell life span derived from the intercept obtained by extrapolating the line to zero activity.

Because of an initial splenic sequestration, followed by a release of carrier erythrocytes (see Results), we were not able to calculate the value for zero time circulating label by standard methods. The zero time circulating label value was therefore determined by disregarding measure-
ments made up to 3 h after injection and any measurement which fluctuated between 1 and 6 days. The data (corrected for natural decay, but not elution) between 1 and up to 16 days were plotted on a linear scale, and a straight line drawn. The line was extrapolated back to the ordinate and the point of intersection was taken as the count rate which corresponds to the zero time value, representing 100% survival. The percentage lost on reinjection (determined by measuring $^{51}$Cr in the urine for the first 24 h and correcting for $^{51}$Cr elution from the cells) ranged between 0.3 and 3.9 (1.8 ± 1.3, mean ± S.D. of seven subjects).

RESULTS

The percentage of cells surviving the dialysis procedure was 93.0 (mean of six subjects, range 90.2–96.3) and 92.9 (mean of three subjects, range 90.7–94.6) for 90 and 180 min of hypo-osmotic dialysis respectively.

Figures 1 and 2 show the cell survival plots for carrier erythrocytes subjected to 90 and 180 min of hypo-osmotic dialysis respectively. One hundred percent survival represents the zero time circulating label (see above for determination). With both hypo-osmotic dialysis times there was a fall in circulating labelled cells, reaching the lowest levels between 3 and 19 h after injection; 70.3% and 88.3% of the injected carrier erythrocytes remained in the circulation respectively when hypo-osmotic dialysis periods of 90 and 180 min were used. These cell recoveries are the means of the lowest values measured and are depicted in Figure 3. Cell recovery then increased, reaching maximal levels of 94% and 99% for 90 and 180 min of hypo-osmotic dialysis respectively, between 24 and 144 h. These values are the means of the highest cell recoveries recorded (Figure 3).

Surface counting demonstrated an initial loss of counts from the heart at both hypo-osmotic dialysis times. This coincided with an increase in splenic counts in three subjects injected with carrier erythrocytes exposed to 90 min of hypo-osmotic dialysis and in all cases of 180-min hypo-osmotic dialysis (Figures 4 and 5). These results show that the loss of labelled carrier erythrocytes from the circulation was due to sequestration by the spleen. There was no evidence of erythrocyte sequestration by the liver at this stage. The subsequent increase in heart counts and decrease in splenic counts (in all cases) is consistent with the release of a proportion of the sequestered carrier erythrocytes back into the circulation, and accounts for the observed increase in circulating label (Figures 4 and 5).

The mean daily urinary excretion of label was $1.7 ± 0.6\%$ (mean ± S.D. of four subjects) and $2.3 ± 0.7\%$ (mean ± S.D. of three subjects) for 90 min and 180 min of hypo-osmotic dialysis respectively. There were no significant differences in excretion between the two hypo-osmotic dialysis times ($P = 0.26$) and there was no detectable label in the plasma.

The mean cell half-life was $26.2 ± 3.7$ days (mean ± S.D. of six subjects) and $29.7 ± 7.5$ days (mean ± S.D. of three subjects) for carrier erythrocytes subjected to 90 and 180 min of hypo-osmotic dialysis respectively. There
Figure 3  Initial carrier erythrocyte recovery in nine subjects
Shown are the lowest levels of circulating labelled cells recorded at the time indicated in hours (open bar); and the subsequent increase to the highest levels of circulating labelled cells recorded at the time indicated (filled bar).

Figure 4  Normalized counts in heart (■), spleen (▲) and liver (●) in four subjects injected with carrier erythrocytes prepared using a hypo-osmotic dialysis period of 90 min

were no significant differences in mean cell half-life between the two hypo-osmotic dialysis times ($P = 0.36$). The mean cell life was $106.5 \pm 18.8$ days (mean ± S.D. of six subjects) and $113.7 \pm 27.7$ days (mean ± S.D. of three subjects) for carrier erythrocytes subjected to 90 and 180 min of hypo-osmotic dialysis respectively. There were no significant differences in mean cell life between the two hypo-osmotic dialysis times ($P = 0.66$).
DISCUSSION

Unloaded carrier erythrocytes prepared using both 90 min and 180 min of hypo-osmotic dialysis had in vivo mean cell half-lives within the normal range of 19 to 29 days for human erythrocytes. The mean cell life for normal erythrocytes in this laboratory is 110 days, ranging from 89 to 131 days; carrier erythrocytes prepared using both hypo-osmotic dialysis times had a mean cell life similar to normal erythrocytes. The fact that there was no label in the plasma and that the urinary excretions were within the normal $^{51}$Cr elution limits of 1.0 to 3.2% per day demonstrates that the label was cell associated, and that there was minimal intravascular haemolysis of the labelled cells. Previous studies in vivo in humans include those of Eichler et al. [11] where the survival of hypo-osmotic dialysis erythrocyte ghosts was determined by measurement of entrapped gentamicin. After a rapid loss for the first 4.5 h after injection, the gentamicin concentration decreased with a half-life of 22 days. Erythrocytes loaded with inositol hexaphosphate using an osmotic pulse technique and labelled with $^{51}$Cr had a 24-h post-injection mean cell life of 95 to 100 days [12]. Inositol hexaphosphate- and l-asparaginase-loaded erythrocytes prepared by a continuous-flow procedure had mean cell half-lives of 25 and 28 days respectively, 24 h after injection [13,14].

It is perhaps surprising that the longer hypo-osmotic dialysis time had no detrimental effect on cell survival since a prolonged exposure to hypo-osmotic conditions might be expected to have an adverse effect on cell viability. Increasing the dialysis time probably irretrievably damages the more fragile (and older) cells, which are then removed during the washing process. This is supported by the high in vivo survival observed between 24 and 144 h post injection and by studies with murine carrier erythrocytes reported by Chiarantini and DeLoach [15]. Extended hypo-osmotic dialysis periods are an important consideration where drug/enzyme entrapment is time dependent [7,8] and the efficiency of therapeutic agent entrapment is increased. It would, however, be necessary to determine for how long the hypo-osmotic dialysis period could be further extended without adversely affecting the biochemical and physical characteristics of the carrier erythrocytes.

Intra-erythrocytic ATP levels are closely linked with the physical properties of erythrocytes. Declining ATP levels induce changes in erythrocyte shape and result in a diminished cell deformability. A loss of membrane deformability reduces the ability of erythrocytes to pass through the restricted areas of the microcirculation and the sinus regions of the reticulo-endothelial system, and enhances their sequestration by macrophages. The spleen is the most discriminating organ for the detection and removal of damaged erythrocytes [16].

Sequestration of some of the carrier erythrocytes followed by release back into the circulation was an unexpected finding that has not been reported previously. Although the release of sequestered cells from the spleen has been observed with the reticulocyte, the immature erythrocyte [17,18], in humans the spleen does not serve as a reservoir for mature erythrocytes, and after infusion
of $^{51}$Cr-labelled erythrocytes, activity in the spleen increases exponentially to reach equilibrium within a few minutes [19]. It seems probable that the released carrier erythrocytes were initially retained by the spleen for repair and were then released back into the circulation. Sequestered cell release was almost total because by 24 to 144 h, cell recovery was 94% and 99% for 90 and 180 min of hypo-osmotic dialysis respectively. The greater magnitude of (temporary) splenic sequestration observed in subjects 4 and 7 (see Figures 4 and 5) may be due to individual variations in spleen size and function and handling of (partially) damaged erythrocytes. In a preliminary pilot study, in which these carrier erythrocytes were loaded with Pegademase, used for enzyme replacement therapy for adenosine deaminase deficiency, there was a 90% cell recovery at 72 h [20]. Other studies in man have reported 24-h recoveries of 51%, 65% and 75% for erythrocytes loaded with gentamicin, inositol hexaphosphate and L-asparaginase respectively [11,13, 14]. These reduced recovery characteristics may be due to the effect of loading erythrocytes with these particular macromolecules or, more likely, due to the different methods of preparation used. All studies investigating the use of carrier erythrocyte-entrapped therapeutic agent should include a determination of cell survival to ascertain that the loaded material is not detrimental to carrier erythrocyte viability.

There have been few reported human clinical applications of erythrocytes as cellular carriers of therapeutic agents and the majority have used erythrocyte ghosts prepared without consideration of their morphological or biochemical characteristics or survival in vivo. Desferrioxamine-loaded erythrocyte ghosts prepared using a direct dilution hypo-osmotic procedure were investigated clinically for the treatment of iron overload as a result of repeated blood transfusions. There was an increased chelating efficiency of the drug and a biphasic survival with $t_{1/2}$ values of 12 to 42 min and 4.5 to 12.6 h [21]. The survival of hypo-osmotic dialysis erythrocyte ghosts loaded with glucocerebrosidase and administered intravenously to a patient with Gaucher’s disease was dependent upon the haematocrit at which the enzyme was entrapped. Cells loaded at a haematocrit of 95% had a half-life of 10 days, whereas those loaded at a haematocrit of 77% had a biphasic survival with half-lives of 14 h and 5 days [22]. However, a more recent study evaluating the use of L-asparaginase-loaded dialysis, energy-supplemented erythrocytes for the treatment of lymphoblastic leukaemia and non-Hodgkin’s lymphoma, demonstrated a sustained enzyme activity and a greater immune tolerance to the enzyme compared with intravenously administered L-asparaginase [23]. We are currently undertaking trials in an adult patient with adenosine deaminase deficiency of the metabolic effects of carrier erythrocyte-entrapped native adenosine deaminase therapy and the use of this therapy to replace intramuscularly administered polyethylene glycol bound adenosine deaminase (Pegademase) [24].

Autologous carrier erythrocytes were used in the present studies with healthy volunteers for ethical reasons to avoid the use of foreign blood products. Autologous cells are used in patient studies where possible but, based upon our studies in vivo in dogs [4,5], there are no reasons to negate the use of homologous carrier erythrocytes in therapy of patients, particularly where the patients have haemolytic disorders and more fragile cells. The use of a banked blood (transfusion) should possibly be avoided because the storage conditions used reduce erythrocyte viability (B. E. Bax, R. A. Chalmers and M. D. Bain, unpublished work).

The in vivo survival results reported here demonstrate the suitability of energy-replete carrier erythrocytes for sustained circulating levels of, and as a sustained delivery system for, therapeutic drugs and enzymes in the human. The results permit direct extrapolation into clinical trials.

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