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The prolongation of the survival times of mice implanted with TLX5 cells by treatment with methotrexate encapsulated in erythrocytes

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By using gentle loading techniques it is possible to encapsulate drugs into erythrocytes which when resealed will survive upon return to the circulation [1]. Possible applications of this technique to cancer therapy reviewed include the encapsulation of enzymes such as asparaginase to remove tumour-promoting amino acids or the encapsulation of lower molecular weight drugs which after encapsulation may diffuse slowly from the cell into the circulation [2]. A possible advantage of this type of slow-release system is that until the drug is released into the circulation it is not subjected to inactivation by metabolism. This may prolong the therapeutic efficacy of the drug over a much longer period than when the drug is administered systemically, allowing less frequent dosage.

In this communication we have encapsulated methotrexate (MTX) into erythrocytes and examined the effect of the encapsulated preparation on the survival time of CBA/CA mice implanted with TLX5 tumour cells. The rate of release of methotrexate from erythrocytes was also studied *in vivo* and *in vitro*.

MTX was encapsulated in CBA/CA male mouse erythrocytes by a technique based on pre-swelling erythrocytes in hypotonic solutions [3]. In this method blood was obtained by cardiac puncture from CBA/CA male mice by a heparinized syringe. The pooled blood from several animals was centrifuged at 600 *g* for 7 min. The plasma and white cells were discarded. A 1 ml portion of the packed erythrocytes was transferred to a plastic centrifuge tube and 4 ml of a modified K^+-reversed Hank's solution was added with gentle mixing by inversion (KCl 10.18 g/l, KH_2PO_4 0.1 g/l, $NaHCO_3$ 1.273 g/l, NaCl 0.316 g/l, $Na_2HPO_42H_2O$ 0.1 g/l and glucose 2.0 g/l). The Hank's solution had been diluted with water to adjust its final tonicity to 0.67 that of the cells. The cell suspension was centrifuged at 600 *g* for 5 min and the supernatant removed leaving a pellet of "pre-swollen" erythrocytes. A separate portion of packed erythrocytes was lysed by the addition of an equal volume of water and 0.2 ml of this lysate was layered on top of the cells. Meanwhile MTX was dissolved in 0.05 M $NaHCO_3$ to give a final concentration of 8 mg/ml. The MTX used contained 7 μCi of ^{3}H-MTX as a tracer (Amersham International plc, Amersham, U.K.). The purity of the radiolabelled material was confirmed by us as in excess of 96% when we examined the material by paper chromatography using *n*-butanol saturated with 2N ammonia as developing solvent followed by scanning the dried chromatogram using a 4II windowless gas flow radiochromatogram scanner (Tracerlab Instruments). Radiolabelled MTX was used as quickly as possible after receipt in order to avoid stability problems.

Two 0.1 ml portions of the MTX solution was layered on top of the swollen erythrocyte suspension and the tube contents were mixed by gentle inversion. The tube was centrifuged at 600 *g* for 5 min. At this stage the supernatant layer was observed to have darkened but was clearly differentiated from the layer of packed cells. Accordingly a further 0.05 ml of MTX solution was layered on to the supernatant and mixed with the cells by gently inverting the tube. The tube was centrifuged at 600 *g* for 5 min and the supernatant examined. It was observed that the supernatant layer had darkened further and that a small number of erythrocyte ghosts were present at the interface between the supernatant and the packed cells. These "ghosts" were probably due to the lysis of older cells which have greater osmotic fragility. Since the visual examination indicated that the cells were approaching the point of lysis [4] they were resealed by adding the calculated amount of 10 times hypertonic Hank's balanced salt solution (HBSS) to restore isotonicity to the cells. The cells were centrifuged at 600 *g* for 7 min and the supernatant discarded. The cells were washed twice with normal HBSS to remove excess MTX. As a control the same amount of MTX was incubated

with erythrocytes in isotonic HBSS for the same period of time as the encapsulation process (approx. 1 hr).

Cells containing encapsulated MTX which were used for *in vivo* studies were labelled with fluorescein [4]. The amount of MTX present in washed erythrocytes was determined by liquid scintillation counting (Beckman LS230 scintillation counter).

In order to test whether erythrocytes containing encapsulated MTX would survive *in vivo* 0.2 ml of a cell suspension in 0.9% w/v sodium chloride containing 70 μg MTX were injected into male CBA/CA (body wt 25 g) mice via a tail vein. In order to ensure a clean injection was made the animals were placed in a warm situation prior to injection and the tails were warmed immediately prior to injection. By placing the syringe needle directly in the vein it was possible to deliver the whole dose without contaminating the tail. If the tail was contaminated the animal was discarded. Two min after the injection a drop of blood was taken from the tip of the tail after discarding the first drop of blood and a differential count of fluorescein-labelled and normal cells was made in a haemocytometer under tungsten and ultra-violet light (Zeiss Universal microscope). Further microscope counts were made over the period of the experiment. The rate of release of MTX from cells returned to the circulation was determined by measuring the excretion of radiolabelled drug in the urine of CBA/CA mice that have been injected with a single i.v. dose of the encapsulated drug (70 μg in 0.2 ml packed cells). Controls were injected i.v. at the same time with 70 μg MTX.

The urine was collected from metabolism cages and the volume of urine excreted over 24 hr periods measured. Portions (0.2 ml) of the urine were diluted with 18 ml of a toluene : methanol mixture (7 : 3 v/v) containing 3 g/l of the scintillant 2,5-diphenyloxazolyl-benzene. The radioactivity in the samples was determined.

To measure the rate of release of MTX *in vitro* dialysis sacs 100 × 10 mm were made by sealing strips of standard dialysis tubing (Visking). Portions of either MTX encapsulated in 0.5 ml packed erythrocytes or free drug in 0.5 ml of HBSS were placed in these sacs which were immersed to a depth of 50 mm in HBSS (25 ml). McCartney bottles were used as containers and these were incubated at 37° during the experiment. Samples (1 ml) were withdrawn at 30 min intervals and the amount of radioactive MTX in solution determined. Both human and mouse cells were used in this experiment.

The therapeutic efficacy of encapsulated MTX against the TLX5 tumour cell line in CBA/CA mice was also determined. The mice were injected i.p. with 2×10^5 cells but the mice were not treated with MTX until 3 days after the initial injection. MTX was encapsulated into erythrocytes and on day 3 of the experiment encapsulated MTX (0.2 ml packed cells) was administered via a tail vein at a dose of 2.8 mg/kg. Controls were given free drug i.v. and others were left untreated. The survival time of the mice in the three groups was determined.

The maximum efficiency of encapsulation of MTX in erythrocytes was found to be 28.4 ± 4% (N = 5) (e.g. 568 ± 23 mg per ml packed cells) of the total amount of drug added to the cells. Controls where the drug was incubated in isotonic medium with the cells took up 0.4% of the added drug. Where lower doses were required for injection, cells were diluted with HBSS. The standard injection volume was 0.2 ml.

The survival time of the encapsulated preparation is shown in Fig. 1 where the preparation survived for 30 days *in vivo*. Immediately after returning the cells to the circulation a sample of blood was taken to determine the percentage of returned cells to resident cells in the circulation. This was accomplished by counting the number of returned cells (fluorescein-labelled) as a proportion of the total cells in a haemocytometer under an ultra-violet microscope. The initial value was 12% which declined to 11.5% after 24 hr showing a survival of 95% for the cells over that period. Even after 8 days half the returned cells were still circulating and some cells survived for 30 days *in vivo*. This experiment shows that the relatively mild conditions of the encapsulation procedure allowed the returned cells to escape rapid removal by the reticuloendothelial system. This result is similar to the survival times of returned cells containing MTX to rats when a half-life of 11 days was reported [4].

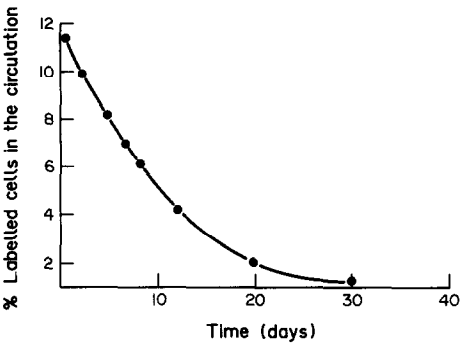

Fig. 1. The survival *in vivo* of CBA/CA mice erythrocytes containing encapsulated methotrexate. On return to the circulation the cells made up 12% of the total cells in circulation in the animals. Twenty four hours later this figure was 11.5% showing a loss of only 0.5% (less than 5% of the returned cells). The half-life of the returned cells was approximately 10 days but about 1% (10% of the returned cells) were still in circulation after 30 days. Experimental details of loading are given in the text.

The rate of release of the drug *in vivo* is shown in Table 1. When the drug was encapsulated only one-third of the dose administered was excreted in 24 hr compared with over a half of the dose where the drug was not encapsulated. Later samples confirmed that MTX, when administered in the encapsulated form, was retained in the body for longer periods than when MTX was administered in the free form. The experiment has similarities with a previous experiment we conducted with rats [4] where free and encapsulated MTX was administered i.v. to rats. The urine levels of MTX were determined in this experiment by the inhibition of dihydrofolic acid reductase. It was found that significant levels of MTX were being excreted on days 4 and 5 after the injection of encapsulated MTX but where the drug had been injected in the free form most of the drug had been excreted in the first three days.

Table 1. Percentage of the initial dose of methotrexate excreted in the urine

Day	Free methotrexate	Encapsulated methotrexate
1	54.5 ± 7.9	33.8 ± 2.1
2	15.1 ± 1.6	18.1 ± 4.4
3	8.3 ± 1.1	10.8 ± 1.3
4	Not detected	9.1 ± 2.2

Results given as mean of 3 determinations ± SEM from urines pooled from 6 mice.

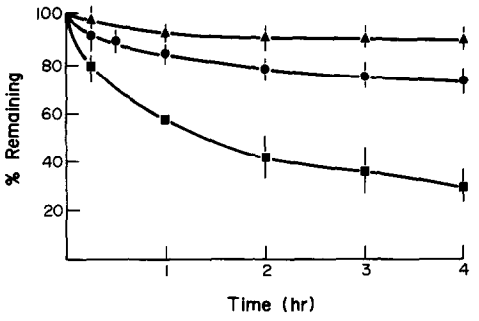

Fig. 2. The *in vitro* release of methotrexate after encapsulation in either mouse or human erythrocytes. ■ free methotrexate; ● methotrexate encapsulated in mouse erythrocytes; ▲ methotrexate encapsulated in human erythrocytes. Results are mean values ± SEM from 5 determinations. Experimental details are given in the text.

The *in vitro* studies showed that whereas free methotrexate rapidly diffused out of the dialysis sacs according to Ficks Law very little MTX leaked out of the erythrocytes over the experimental period (4 hr) (Fig. 2). When plasma was incorporated into the medium the results were not significantly different. Both human and mouse cells were similar in that mouse cells lost 13.6% of their encapsulated drug over 2 hr compared to 9.6% for human cells. Tyler and Ryman [5] found that rat and human cells lost about 10% of encapsulated MTX in an experiment similar to ours. However, their encapsulation efficiency was only 3.2% compared with our 28%.

The efficiency of the encapsulated preparation was shown in the survival experiments where encapsulated MTX produced a 70% increase in survival time over the untreated controls (Table 2). In contrast mice treated with the same amount of free drug produced only a 33% increase in survival time. This doubling of the survival time of the animals would appear to be due to circulating MTX levels remaining higher in the circulation when the encapsulated preparation was administered. The release of MTX from the cells may either be due to direct release of the cells by diffusion or indirect release when the cells are taken up by the reticuloendothelial system (RES) (e.g. Kupffer cells). The excellent survival times of the cells suggest that the uptake by RES cells was slow. It appears likely that appreciable amounts of MTX were released directly from circulating erythrocytes. One advantage of MTX being encapsulated in erythrocytes is that the MTX will be protected from inactivation until it is released from the erythrocytes. The cells will therefore supply, by a slow release system, active MTX to sustain a circulating pool.

Table 2. Antitumour activity of methotrexate against the TLX5 lymphoma tumour line in the CBA/CA mouse

Treatment	Mean survival time (days ± SD)
Controls (No treatment)	9.9 ± 0.4
Free drug	12.6 ± 0.5
Encapsulated drug	16.3 ± 1.2 ($P < 0.001$)

Each mouse was given 2×10^5 tumour cells i.p. Treatment with either free or encapsulated MTX was commenced three days after the tumour cells were administered. Six animals were used in each experimental group. The P value (<0.001) for the encapsulated treatment was significant against both the controls and free-drug treated groups (Student's *t*-test).

In the experiments described, MTX encapsulated in erythrocytes was returned to the circulation shortly after encapsulation. Other experiments have shown that cells with encapsulated drugs can be stored at 4° for 2 weeks without loss of viability *in vivo* (unpublished results). Attempts to freeze-dry cells were unsuccessful.

In conclusion, the encapsulation of MTX in intact erythrocytes has produced a therapeutic advantage over the free drug when used to treat the TLX5 tumour in mice. Our loading technique has been reported to have similar advantages in the use of asparginase in the treatment of the 6C3HED tumour in mice [6] and in the treatment of adjuvant arthritis in the rat with corticosteroids [7]. It has been reported [8] that MTX encapsulated in human erythrocytes increases the osmotic fragility of cells. In our experiments half the cells introduced into the mouse were still surviving at eight days and in the rat [4] at eleven days. We did not investigate the osmotic fragility of cells in the present investigation but clearly cells encapsulating MTX showed good survival times in both rats and mice. Either the cells possessed good osmotic fragility or the relationship between osmotic fragility and *in vivo* survival does not hold with rats and mice. It would be of interest to investigate for species differences in erythrocytes, in their resistance to MTX.

Recently encapsulation procedures have produced survival times approaching that of normal erythrocytes [9] together with the development of equipment allowing large-scale encapsulations using dialysis [10] and continuous-flow hollow-fiber dialysis [11] producing high encapsulation efficiencies. All this is encouraging for the practical exploitation of erythrocytes as drug carriers. A serious limitation at present is their relatively short storage time *in vitro*.

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