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A comparison of the treatment of thyroidectomized rats with free thyroxine and thyroxine encapsulated in erythrocytes

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Summary

Erythrocytes loaded with thyroxine (T_4) have been tested as a slow release system in vivo in thyroidectomized rats. The encapsulated compound was more effective in restoring and maintaining basal metabolic rates after thyroidectomy than the free compound where the effect was short-lived. Administration of the encapsulated compound also allowed the animals to increase body mass over the experimental period compared with controls.

The use of erythrocytes as a circulating slow release system in vivo for drugs has attracted considerable research interest and has recently been reviewed (Ropars et al., 1987). In this communication the technique has been explored as a slow release system in vivo for thyroxine (T_4) in thyroidectomized rats. L-Thyroxine (Sigma Chemical Co., Poole, Dorset) was encapsulated into erythrocytes by the pre-swell technique that we have used in a previously published study (Pitt et al., 1983). By using this gentle loading method for drugs, erythrocytes, when returned to the circulation, are able to avoid removal by the reticuloendothelial system (RES). To test the use of erythrocytes as a slow release system in vivo, the following procedure was employed.

Blood was obtained by cardiac puncture from male Wistar strain rats (b.wt. 150–175 g) and after centrifugation at 600 *g* for 5 min the plasma and buffy coat (leukocytes) were carefully removed with a pipette. The pellet of packed erythrocytes was used for encapsulation. In a typical encapsulation, 1 ml of packed cells was transferred to a siliconized test tube and mixed gently with 4 vols. of Hank's reversed balanced salt solution (reversed HBSS) (KCl 10.18 g/l, KH_2PO_4 0.1 g/l, $NaHCO_3$ 1.273 g/l, NaCl 0.316 g/l, $Na_2HPO_4 \cdot 2H_2O$ 0.1 g/l and glucose 2.0 g/l). In this solution the sodium and potassium concentrations of normal HBSS are reversed to maintain intracellular levels of potassium during the encapsulation. The isotonicity of the reversed HBSS was adjusted to $\times 0.67$ normal. After mixing the swollen cells were centrifuged at 600 *g* for 5 min when the supernatant was removed. Meanwhile, a small quantity of haemolysed cells was prepared by diluting packed erythrocytes 1 : 1 v/v with water. A 200 μl portion of this haemolysate was carefully layered on top of the swollen cells obtained by centrifugation of the hypotonic suspension.

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Without disturbing this layer a further 200 μl layer of thyroxine (T_4) solution in aqueous ethanol (1 : 1 v/v) was layered on top of the haemolysate layer and the contents of the tube gently mixed by inversion. The purpose of the haemolysate layer was to provide an osmotic barrier against the hypotonic drug solution and also to provide a pool of cytosolic constituents to replace losses during the encapsulation procedure. Preliminary experiments had shown that the addition of 200 μl of T_4 solution (1 $mg \cdot ml^{-1}$) was sufficient to bring the swollen cells to the point of lysis. Further addition of T_4 solution caused complete lysis. However, immediately prior to the point of lysis T_4 was able to enter the cells through "pores" that open in the swollen membrane (Pitt et al., 1983). The cells were resealed by the addition of the calculated amount of hypertonic HBSS (10 $\times$ normal tonicity) to restore isotonicity to the erythrocytes. The resealed cells were recovered by centrifugation and washed twice with HBSS to remove non-encapsulated drug. For comparative purposes T_4, at the same concentration as used in the encapsulation, was incubated with 1 ml of packed erythrocytes suspended in normal isotonic HBSS for the same period as the encapsulation procedure (1 h). In experiments where it was required to distinguish the T_4-loaded cells from normal cells the erythrocytes were incubated for 1 h in HBSS saturated with fluorescein isothiocyanate (FITC). Excess FITC was removed by washing in normal HBSS until the washings were colourless. The labelled cells were distinguished from normal cells under UV light viewed through a Zeiss standard microscope fitted with a deuterium lamp. Cells were counted in a haemocytometer.

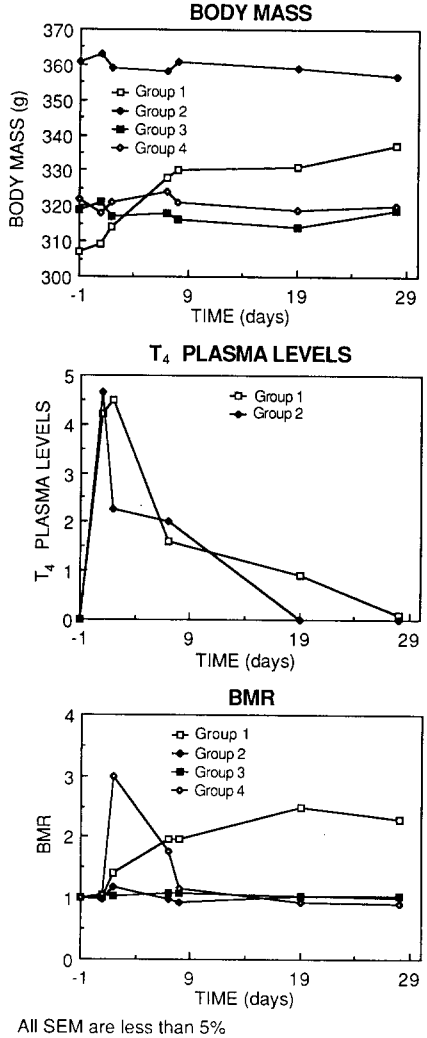
Rats selected for the experiments were thyroparathyroidectomized and to ensure complete inhibition of iodine organification were maintained weekly with 4 $mg \cdot kg^{-1}$ i.p. of the goitrogen methimazole (Solomon, 1971). The rats were placed on a diet of rat chow (15 g/day) supplemented with 0.1% m/v KCl to protect against electrolyte inbalance. Thyroidectomy was monitored by determining the BMR of each animal daily. This was commenced before the thyroidectomy to establish a baseline value for treatment and continued after treatment to asses the value of T_4 administration. An 8 inch vacuum desiccator was adapted as a respirometer with a bubble flow meter, a soda lime trap to remove carbon dioxide, and a water trap to allow replacement of air used in each experiment. The metabolic rate was calculated from the mean value of 5 readings of oxygen consumption. Each rat was allowed to settle for 10 min before readings were taken. The surface area of each rat was calculated from the formula:

$$\frac{\text{Mass of rat (g)} \times 10\ m^2}{1000}$$

This, with the measurement of oxygen consumption corrected for STP, allowed the basal metabolic rate (*BMR*) to be calculated in kcal/day for each rat. Replacement therapy was commenced when the values for the rats were identical on two subsequent determinations. The body weight was also recorded for each rat throughout the experiment. Baseline levels were established for each rat and 5 rats were used in each experimental group. Encapsulated T_4 was administered to rats i.p. This route was chosen since DeLoach and Drolesky (1986) have reported that 80% of cells administered by this route were rapidly taken into the circulation. In preliminary experiments using FITC-labelled cells we obtained an identical result. Thyroxine levels in cells and tail vein blood samples were determined by radioimmunoassay with a commercial kit supplied by Amersham International. Levels were determined on blood samples prior to T_4 administration so that each animal acted as its own control. Thyroxine levels were monitored until the end of the experiment (day 29).

Four experimental groups were examined: *Group 1* were treated with encapsulated T_4, (1 ml packed cells haematocrit value 72, i.p.); *Group 2* with washed cells (1 ml packed cells, haematocrit value 72, i.p.) after incubation in isotonic T_4 solutions; *Group 3* with sham-encapsulated (i.e. cells subjected to the encapsulation procedure but where T_4 was omitted), packed cells, haematocrit value 72, i.p.); and *Group 4*, where free T_4 was injected (200 μg in 200 μl saline i.p.). In the encapsulation it was found that when 200 μg of T_4 was added to

1 ml of packed cells 145 μg was encapsulated. Where cells were only incubated with the drug 3 μg was measured for each ml of packed cells. Using FITC-labelled cells it was found that as in previous experiments (Pitt et al., 1983) the cells were able to survive when returned to the circulation. The FITC-labelled cells were found to survive in vivo up to day 29 when the experiments were terminated.



All SEM are less than 5%

Fig. 1. The graphs show the changes in mass, plasma T_4 levels and *BMR* expressed as a ratio of the baseline level adjusted for each group to the arbitrary value 1. Baseline values were determined 24 h before the commencement of treatments (day 1). Only two groups are plotted for T_4 levels since groups which did not receive T_4 in their treatments did not give detectable levels of T4 on analysis. Results are the mean $\pm$ S.E.M. for 5 animals in each group. Group 1 (encapsulated T_4); Group 2 (incubated T_4); Group 3 (sham encapsulation); Group 4 (free T_4).

Thyroxine levels in blood in all 4 experimental groups before the administration of T_4 were not detectable by RIA. The results in Fig. 1 show that only the group that received encapsulated T_4 put on weight over the experimental period. Plasma T_4 levels were only measurable in Group 1 (encapsulated) and Group 4 (free). The other groups gave values below the level of detection in the kit. The group that received free T_4 peaked rapidly with a maximum value after 24 h but the levels had halved at 48 h with no detectable T_4 on day 19.

The encapsulated T_4 took 48 h to reach peak values but T_4, although in decline, was still detectable at the end of the experiment (day 28). The effect on the *BMR* is also shown in Fig. 1. The baseline values for each group were arbitrarily fixed at 1.0 in the plot (day 1). The actual rates compared to 100% values before thyroidectomy were: Group 1 (encapsulation group) $56 \pm 4\%$; Group 2 (incubated cells) $65 \pm 7\%$; Group 3 (sham encapsulation) $62 \pm 5\%$; and Group 4 (free T4) $70 \pm 10\%$. As shown in Fig. 1, the group that received free T_4 showed a rapid rise in *BMR* but after 7 days the values were back to pretreatment levels. In contrast the group that received encapsulated T_4 showed a steady recovery of values to peak at day 19 with only a slight decline by day 29 when the experiment was terminated. A point of interest here was that the group treated with free T_4 were given a dose of 200 $\mu g \cdot kg^{-1}$ compared with 145 $\mu g \cdot kg^{-1}$. The *BMR* in the group treated with encapsulated drug is high on day 29 although the plasma T_4 level had fallen considerably. It seems likely that although the plasma T_4 levels had declined, considerable amounts of the drug were still present in other tissues accounting for the high *BMR* which was in the same order as the *BMR* before thyroidectomy.

In conclusion, the results show that the replacement of T_4 encapsulated in erythrocytes was more effective than the administration of free T_4. Therefore, erythrocytes may possibly be useful in hormone replacement therapy. A possible mode of action was by a slow release mechanism where the

drug leaked from the cells whilst in circulation. Another possible route is from the macrophages of the RES when cells are removed. Our previous work suggests that the former explanation is the more likely (Pitt et al., 1983).

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