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Liposomal Retention of a Modified Anti-Inflammatory Steroid

By IAN H. SHAW, C. GRAHAM KNIGHT and JOHN T. DINGLE
*Tissue Physiology Department, Strangeways Research Laboratory, Wort's Causeway,
Cambridge CB1 4RN, U.K.*

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In studies with synthetic lecithins, dipalmitoylphosphatidylcholine was found to be the preferred form for liposomal retention of cortisol esters at 37°C. Cortisol palmitate was retained longer than cortisol octanoate, whereas unesterified cortisol escaped readily from liposomes. Such a liposome composition may allow the controlled release of modified anti-inflammatory agents, particularly when used for intra-articular administration.

The treatment of rheumatoid arthritis by the systemic administration of glucocorticoids is often accompanied by serious side effects. Prolonged therapy with anti-inflammatory steroids can lead to an increased susceptibility to infection, myopathy, osteoporosis and adrenal insufficiency (Hess & Goldman, 1972). Such side effects often limit the use, or necessitate the withdrawal, of an otherwise effective therapeutic agent.

Direct intra-articular steroid administration in rheumatoid arthritis has been shown to bring about transient amelioration of the disease (Hollander, 1972). However, the effective intra-articular dose of cortisol is often in excess of 20 mg (Hollander, 1972) and there is evidence of steroid loss from the joint space within 24 h (Goetzel *et al.*, 1974). Localization of steroid within rheumatoid cells, mediated through a carrier, could eliminate many of these side effects.

Systemic administration of liposomes has been proposed as a pharmacological carrier in the treatment of inherited lysosomal enzyme deficiencies (Gregoriadis *et al.*, 1971), cancer (Gregoriadis *et al.*, 1974) and heavy-metal poisoning (Rahman *et al.*, 1974). We considered that the local administration of liposomes into a closed cavity bounded by phagocytic cells, as exemplified by the rheumatoid joint, would greatly enhance the probability of liposome–target-cell interaction. Thus in preliminary experiments it was shown (J. T. Dingle, J. L. Gordon, C. G. Knight, D. P. Page-Thomas, N. C. Phillips & I. H. Shaw, unpublished work) that intra-articular injection, into experimentally arthritic rabbits, of unsonicated liposomes containing an anti-inflammatory drug allows substantial uptake of the liposomes by phagocytic synovial lining cells, and minimizes the escape of the particles from the joint cavity. A liposomal carrier system therefore allows the use of very small quantities of a drug, with a consequent decrease in any systemic effect.

To determine the most suitable liposome composition for the entrapment and retention of anti-inflammatory steroids at physiological temperatures,

liposomes containing entrapped cortisol octanoate were prepared from dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine. The incorporation of cortisol derivatives and their subsequent release from dipalmitoyl phosphatidylcholine liposomes was studied.

Materials and Methods

Synthetic L- α -lecithins, dimyristoyl, dipalmitoyl and distearoyl phosphatidylcholine, and cortisol were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Phosphatidic acid (grade 1) was from Lipid Products, Redhill, Surrey, U.K. [1,2,6,7(n)- ^3H]Cortisol (95 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Octanoyl chloride and palmitoyl chloride were from Aldrich Chemical Co., Gillingham, Dorset GP8 4JL, U.K. Silica gel (Bio-Sil A, 100–200 mesh) was from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Silica-gel sheets (13179) for t.l.c. were from Kodak Ltd., Kirkby, Liverpool, U.K.

T.l.c. was performed in an Eastman Chromogram developing apparatus (13259) with chloroform/acetic acid (9:1, v/v) as solvent. Radioactivity was counted in a Packard Tri-Carb liquid-scintillation spectrometer, model 3324. Samples in chloroform, and aqueous samples (0.1 ml), were added to 10 ml of toluene/2-ethoxyethanol (7:3, v/v), containing 4 g of 2,5-diphenyloxazole/litre (Hall & Cocking, 1965). Counting efficiency was 19% for the aqueous samples.

Preparation of [^3H]cortisol octanoate

Cortisol (50 mg, 0.14 mmol) was dissolved in pyridine (0.7 ml) at 21°C. [^3H]Cortisol (0.3 mCi) in chloroform (0.3 ml) and octanoyl chloride (0.05 ml, 0.29 mmol) were added, and the mixture was stirred at 21°C for 16 h. The solvent was removed *in vacuo* at 40°C and the residue dissolved in chloroform (3 ml).

The chloroform was evaporated to give an oil, which was dissolved in chloroform (10ml). This solution was extracted in succession with 0.6M-NaCl (3×10ml), 0.48M-NaHCO₃ (3×10ml), 0.48M-citric acid containing 0.6M-NaCl (3×10ml) and 0.6M-NaCl (10ml). The chloroform solution was dried (MgSO₄) and evaporated to dryness *in vacuo* at 40°C to give a pale-yellow oil (88mg; expected yield 67mg). The radiochemical purity of the product was assessed by t.l.c. More than 95% of the radioactivity migrated with the solvent front and was presumed to be the ester product. Untreated [³H]cortisol had *R_F* 0.35. The specific radioactivity was 3.76Ci/mg. The [³H]cortisol octanoate was used without further purification. Unlabelled cortisol octanoate was prepared by the same method.

Preparation of [³H]cortisol palmitate

Cortisol (120mg, 0.33mmol) was dissolved in pyridine (2ml) at 21°C. [³H]Cortisol (0.7mCi) in chloroform (0.7ml) and palmitoyl chloride (0.2ml, 0.66mmol) were added and the mixture was stirred at 21°C for 15h. The solvent was removed *in vacuo* at 30°C and the residual oil was dissolved in chloroform (2ml). The chloroform was removed *in vacuo* at 30°C and the residue was redissolved in chloroform (1ml). This solution was applied to a column (1cm×60cm) of silica gel and eluted with chloroform (40ml/h). Fractions (8ml) were collected, and a portion (10μl) of each fraction was taken for scintillation counting. Fractions representing the first radioactive peak eluted from the column were combined and evaporated *in vacuo* at 30°C to give a colourless oil (13.5mg). On t.l.c., 95% of the radioactivity migrated with the solvent front. This material was presumed to be the ester product and was used without further purification. The specific radioactivity was 2.88μCi/mg.

Liposome preparation

Dimyristoyl, dipalmitoyl or distearoyl phosphatidylcholine and [³H]cortisol octanoate (1.5μCi) were dissolved in chloroform (1.25ml) containing phosphatidic acid (1mg) in lecithin/steroid ratios (w/w) of 8.5:0.5, 8.0:1.0, 7.0:2.0, 6.0:3.0 and 5.0:4.0 respectively. The total weight of phospholipid and steroid in each preparation was 10mg.

The chloroform was removed from the lipid mixture by rotary evaporation *in vacuo* under N₂, at a temperature 20°C above the phase-transition temperature of the constituent lecithin (Shimshick & McConnell, 1973); thus the bath temperature was 43°C for dimyristoyl phosphatidylcholine, 61°C for dipalmitoyl phosphatidylcholine and 73°C for distearoyl phosphatidylcholine. The dried lipid film was dispersed in 5mm-sodium phosphate, pH7.35, containing 0.15M-sodium chloride (2ml), and

vigorously shaken at the corresponding temperature. After standing at 21°C for 1h the liposome suspension was washed three times in the same buffer (1ml) by centrifugation at 50000g for 10min at 21°C, and then resuspended in buffer (1ml).

[³H]Cortisol (3mg, 1.43μCi) or [³H]cortisol palmitate (3mg, 1.25μCi) was entrapped within dipalmitoyl phosphatidylcholine (6mg) liposomes as described above.

Assessment of steroid retention

The liposome suspension (1ml) was diluted in 5mm-sodium phosphate, pH7.35, containing 0.15M-sodium chloride (9ml), and incubated at 37°C after duplicate samples (0.1ml) were taken at zero time for scintillation counting. At 24h intervals the suspension was centrifuged at 50000g for 10min at 21°C, the liposomes were resuspended in fresh buffer (10ml) and further samples taken for counting of radioactivity as above.

To assess the variability of liposomal retention of steroid ester, dipalmitoyl phosphatidylcholine/[³H]cortisol octanoate (6:3, w/w) liposomes (4ml) were prepared, and portions (0.5ml) were suspended in buffer (4.5ml) and incubated at 37°C for 24h. After centrifugation and resuspension, portions (0.1ml) were taken for scintillation counting.

Results and Discussion

The lecithins used in this study have saturated fatty acid chains of defined lengths differing from each other by two carbon atoms and therefore have different phase-transition temperatures. We have used defined lecithins because of the enhanced degree of structural organization conferred on the liposomal bilayers resulting in a clearly defined physical state at physiological temperatures (Shimshick & McConnell, 1973; Rand *et al.*, 1975).

Liposomes were prepared by using 1mg of phosphatidic acid and 9mg of lecithin. Steroid-containing liposomes were prepared by replacing some of the lecithin with cortisol esters. When 30% of the lipid was cortisol octanoate, there was maximum incorporation (1.63–1.82mg/ml of liposome suspension) irrespective of the lecithin used, whereas increasing the steroid content to 40% resulted in a marked decrease in uptake. Liposomes prepared by using up to 30% of the steroid incorporated between 61 and 64% cortisol octanoate when the structural lecithin was dimyristoyl phosphatidylcholine, between 44 and 59% for dipalmitoyl phosphatidylcholine and between 54 and 63% for distearoyl phosphatidylcholine.

Cortisol octanoate was released from all liposome formulations incubated at 37°C (Table 1). The rate of loss of encapsulated cortisol octanoate at 37°C was related to the fatty acid chain length of the liposomal

Table 1. Percentage retention of [³H]cortisol octanoate encapsulated within liposomes incubated at 37°C

Liposomes were formulated from 1 mg of phosphatidic acid and various combinations of dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine with [³H]cortisol octanoate as described in the text. The liposome suspension was incubated at 37°C, washed by centrifugation every 24 h, and resuspended in fresh buffer before sampling.

Time (days)	Lecithin/steroid ratio (w/w) ...	Retention of [³ H]cortisol octanoate (%)				
		8.5:0.5	8.0:1.0	7.0:2.0	6.0:3.0	5.0:4.0
1	Lecithin					
	Dimyristoyl phosphatidylcholine	86.8	76.9	80.3	70.7	93.6
	Dipalmitoyl phosphatidylcholine	87.7	73.1	79.6	74.8	98.7
2	Distearoyl phosphatidylcholine	94.5	87.1	81.4	81.4	72.8
	Dimyristoyl phosphatidylcholine	30.2	24.3	26.2	12.8	62.9
	Dipalmitoyl phosphatidylcholine	55.4	60.0	51.5	50.6	75.8
3	Distearoyl phosphatidylcholine	86.5	77.7	69.8	60.9	63.2
	Dimyristoyl phosphatidylcholine	3.9	2.3	6.6	2.1	35.5
	Dipalmitoyl phosphatidylcholine	39.5	29.6	32.7	34.1	48.0
	Distearoyl phosphatidylcholine	42.2	58.9	29.0	38.2	47.8

Fig. 1. Retention of [³H]cortisol, [³H]cortisol octanoate and [³H]cortisol palmitate in dipalmitoyl phosphatidylcholine liposomes at 37°C

Liposomes were prepared from 6mg of dipalmitoyl phosphatidylcholine, 1 mg of phosphatidic acid and 3 mg of [³H]cortisol (■), [³H]cortisol octanoate (▲) or [³H]cortisol palmitate (●) as described in the text. The initial incorporation of radioactivity was measured. At 24 h intervals the liposome suspension was washed by centrifugation and resuspended in fresh 5 mM-sodium phosphate, pH 7.35, containing 0.15 M-sodium chloride.

& McConnell, 1973); consequently the bilayers are disorganized and less able to retain the hydrophobic component of the cortisol octanoate. After 24 h, the variability of cortisol octanoate retention was $87 \pm 2\%$ (S.E.M., $n = 8$).

In a series of experiments involving liposomes prepared from egg-yolk phosphatidylcholine (phase-transition temperature -10°C) we established the importance of bilayer stability and entrapped drug form (Segal *et al.*, 1975) at temperatures above that of the phase-transition temperature for constituent lecithin. Despite the addition of cholesterol, the 21-succinate, 21-phosphate and 21-acetate derivatives of cortisol, and cortisol, which possess mixed hydrophobic and hydrophilic properties, all diffused out of the liposomes within 24 h (I. H. Shaw, C. G. Knight & J. T. Dingle, unpublished work).

Increasing the chain length of the substituent group on C-21 of cortisol caused a marked enhancement of steroid retention at 37°C in dipalmitoyl phosphatidylcholine liposomes prepared with 30% of the steroid (Fig. 1). Initial liposomal incorporation for the free alcohol was 1.21 mg, for cortisol octanoate 1.76 mg, and for cortisol palmitate 1.80 mg. After 3 days' incubation at 37°C, 0.15 mg of cortisol (12%), 0.71 mg of cortisol octanoate (40%) and 1.27 mg of cortisol palmitate (71%) remained entrapped within the liposomes.

The enhanced retention of cortisol palmitate in dipalmitoyl phosphatidylcholine liposomes, in comparison with cortisol octanoate, may reflect a greater degree of compatibility between the ester and the constituent palmitoyl fatty acid chains of the dipalmitoyl phosphatidylcholine, providing a more stable environment for the hydrophobic chain. The

lecithin. Cortisol octanoate was released from dimyristoyl phosphatidylcholine liposomes at a much greater rate than from the otherwise similar dipalmitoyl phosphatidylcholine or distearoyl phosphatidylcholine liposomes. At 37°C, dimyristoyl phosphatidylcholine liposomes exist in a fluid state (Shimshick

need for a compatible hydrophobic 'anchor' is clearly illustrated by the rapid loss of the free alcohol from dipalmitoyl phosphatidylcholine liposomes.

It is concluded that cortisol palmitate/dipalmitoyl phosphatidylcholine liposomes provide a stable carrier system for the investigation of liposomes as drug carriers in local therapy of rheumatoid arthritis. The variable rates of release of the various esters may allow the use of a mixed population of liposomes such that the active steroid may be differentially released so as to prolong the therapeutic effect. However, further experimental work is needed to show whether differential release can be achieved *in vivo*.

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