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Intra-articular therapy of experimental arthritis with a derivative of triamcinolone acetonide incorporated in liposomes

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Abstract—Triamcinolone acetonide-21-palmitate was synthesized and incorporated into liposomes for intra-articular treatment of an experimentally-induced arthritis in the knee joints of rabbits. The liposomal formulation was more efficient than free triamcinolone acetonide in solution in suppressing the arthritis. Using radioactive tracers, it was found that triamcinolone acetonide-21-palmitate incorporated into liposomes was retained in the articular cavity, together with the liposomal lipids, for a much longer period than free triamcinolone acetonide, and this correlated with its anti-inflammatory effect.

The treatment of rheumatoid arthritis with systemically administered glucocorticoids leads to a number of secondary effects. To avoid these effects, corticosteroids have been injected intra-articularly, since relatively high concentrations of drug can be attained in the joint cavity causing minimal systemic toxicity. The efficacy of intra-articular therapy is reduced by the rapid clearance of drugs from the joint cavity.

However, the administration of anti-inflammatory drugs encapsulated in liposomes prolongs the residence time of the drug in the joint cavity and has been shown to be successful in both man (De Silva et al 1979) and rabbit (Dingle et al 1978; Phillips et al 1979).

We report experiments in which triamcinolone acetonide palmitate incorporated into liposomes and injected intra-articularly in rabbits, exerted a substantial anti-inflammatory effect which correlated with its prolonged retention in the injected joint.

Materials and methods

Chemicals and laboratory materials. [1-^{14}C]Cholesteryl oleate (74 mCi mmol^{-1}) and [6,7-^{3}H(*N*)] triamcinolone acetonide (42·5 mCi mmol^{-1}) were purchased from Dupont, Dreieich, Germany. Dipalmitoylphosphatidylcholine and phosphatidic acid were obtained from Lipid Products, Redhill, Surrey, UK. Triamcinolone acetonide was from Sigma, Poole, Dorset, UK. Cholesterol, palmitoyl chloride and *N,N*-dimethylformamide were from Merck, Darmstadt, Germany, and pyridine was from Panreac, Barcelona, Spain. All other chemicals were of analytical grade.

Synthesis of triamcinolone acetonide-21-palmitate. This was essentially as described by Goundalkar & Mezei (1984). Briefly, 1 mmol triamcinolone acetonide was dissolved in 4 mL *N,N*-dimethylformamide followed by 2 mmol pyridine and 2 mmol palmitoyl chloride. This mixture was then incubated under magnetic stirring at room temperature (21°C) for 22 h and was then added to 1600 mL of 0·5 M sulphuric acid with vigorous stirring for 10 min. The product was purified by filtration in a chromatography column packed with silica-gel, using as eluant a mixture of toluene/ethyl acetate/acetic acid 90:10:1 (v/v/v). The purified product was recrystallized from a methanol solution and the structure of triamcinolone acetonide-21-palmitate verified by Fourier transform infra-red spectroscopy and ^{1}H nuclear magnetic resonance. The same procedure was used to synthesize the 21-palmitate derivative of radioactively-labelled triamcinolone acetonide.

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Preparation of liposomes. Lipids were dissolved in 2:1 chloroform/methanol (v/v), to give mixtures of dipalmitoylphosphatidylcholine/cholesterol/phosphatidic acid/triamcinolone acetonide-21-palmitate; molar ratio 8:3:1:0·9. The organic solvent was evaporated under a stream of nitrogen and the last traces eliminated under vacuum for at least 2 h. The dry lipid was then resuspended in sterile saline (0·9% NaCl) by heating at 60°C and vigorous stirring for 1 h to give a triamcinolone acetonide palmitate concentration of 2 mg mL^{-1}. In order to eliminate any cholesterol crystals, liposomes were filtered through polycarbonate filters (12 μm pore diam.). After filtration liposomes were centrifuged for 25 min at 22700 *g*. The pellet was then resuspended in sterile saline and the concentration of triamcinolone acetonide palmitate estimated by UV-spectroscopy at 239 nm in methanol solution ($\varepsilon = 18886$ cm^{-1} M^{-1}). The liposome suspension was finally diluted with saline to give the desired concentration of triamcinolone acetonide-21-palmitate.

Induction of experimental arthritis. Arthritis was induced in knee joints of New Zealand White rabbits (~2 kg) by injecting 0·5 mL of a 1% solution of carrageenan in saline intra-articularly. After three injections at weekly intervals this induces a sub-acute arthritis (Shoji et al 1986; Aaron et al 1987).

Assessment of inflammation. Inflammation was assessed by measuring the diameter of the knee joint using a Vernier caliper. To carry out histological studies, the joint capsule of the right limb was removed, fixed in 10% formalin, and processed through routine paraffin embedding and sectioning. After staining by haematoxylin and eosin, the sections were examined by light microscopy.

Treatment of experimental arthritis. Liposomes containing triamcinolone acetonide-21-palmitate (0·25 mg kg^{-1}) or free triamcinolone acetonide (0·25 mg kg^{-1}) were injected intra-articularly at weekly intervals for 3 weeks. In each case the first of the three doses was injected 1 week after the last injection of carrageenan used to induce arthritis.

The retention of drug in the knee joint was followed by measuring ^{3}H and ^{14}C in the joint tissues as described by Foong & Green (1988).

Results

The injection of carrageenan resulted in an increase of the diameter of the knee joints of the rabbits, from 20·85 ± 0·16 mm in the group of untreated animals used as a control, to 22·21 ± 0·07 mm in the treated animals, three weeks after the first injection. Triamcinolone acetonide-21-palmitate associated with liposomes, administered one week after the last injection of carrageenan, caused a substantial reduction in inflammation

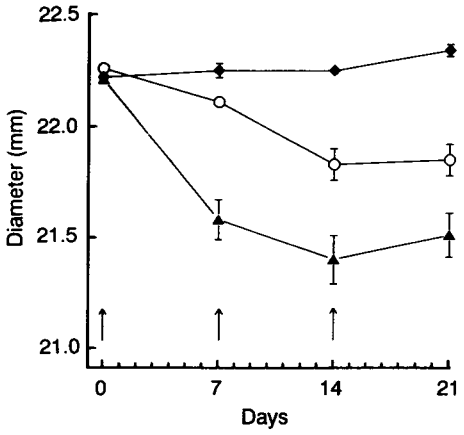

FIG. 1. Changes in diameter of arthritic knee joints in control rabbits (◆) and in animals following intra-articular injections (arrows) of free triamcinolone acetonide (○) or liposomal triamcinolone acetonide-21-palmitate (▲). Each point represents the mean value of four animals ± s.e.

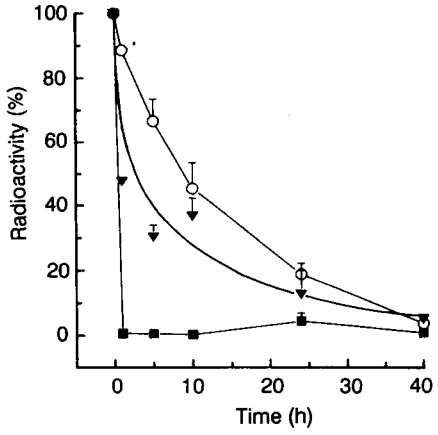

FIG. 3. Recovery of radioactivity at different times after intra-articular injection of free [6,7-^{3}H(*N*)]triamcinolone acetonide (■) and [6,7-^{3}H(*N*)]triamcinolone acetonide-21-palmitate incorporated in liposomes (○). The liposomal marker [1-^{14}C]cholesteryl oleate is represented by (▼). Each point represents the mean value of three different animals ± s.e.

when measured 7 days later (Fig. 1); free triamcinolone acetonide has a considerably lesser effect. The same trend continued after the second injection of corticosteroid, with a further reduction in knee joint diameter given by both agents, although this reduction was greater in the case of the drug associated with liposomes. The third dose of either drug did not result in further reduction of the diameter of the knee joint.

Histological examination of control arthritic joints injected with carrageenan showed synovial hyperplasia with abundant inflammatory foci (compare Fig. 2A with Fig. 2B) as reported by Lowther & Gillard (1976). Treatment with free triamcinolone acetonide 7 days after the last injection of carrageenan suppressed the inflammatory response although some hyperplasia was still present (Fig. 2C), with inflammatory cells in the joint cavity. However, Fig. 2D shows that in joints treated with triamcinolone acetonide palmitate associated with liposomes, very few inflammatory cells are present, although some hyperplasia can still be observed.

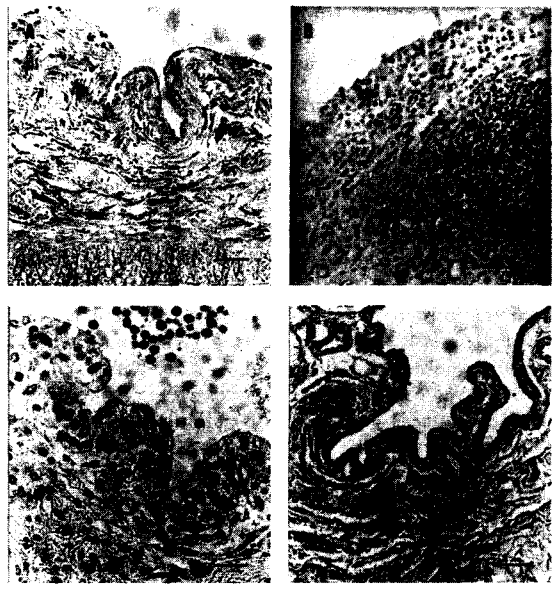

FIG. 2. Light microscopy examination of synovium of treated and untreated rabbits. Control healthy-animal is shown in A; synovium at day 42 after the beginning of the treatment with carrageenan is shown in B. C and D show the effect of the treatment of arthritic animals with free triamcinolone acetonide and triamcinolone acetonide-21-palmitate associated with liposomes, three weeks after the beginning of the treatment with anti-inflammatory drugs. The bars correspond to 25 μm in A, B and D, and 12·5 μm in C.

The recovery of radioactivity from injected joints corresponding to [6,7-^{3}H(*N*)]triamcinolone acetonide-21-palmitate, the liposomal marker [1-^{14}C]cholesteryl oleate and the free parent drug are shown in Fig. 3. Cholesteryl oleate was used as a marker of liposomes, since this is a non-exchangeable lipid. Free triamcinolone acetonide was rapidly lost from the joint cavity whereas the palmitate derivative incorporated into liposomes and the cholesteryl oleate used as a marker of the liposomes were retained for much longer, providing evidence that the drug remains in the cavity, associated with the liposomes.

Discussion

The anti-inflammatory effect of triamcinolone acetonide in solution has been compared with that of its 21-palmitate derivative incorporated into liposomes. It was previously shown (Goundalkar & Mezei 1984) that the chemical modification of triamcinolone acetonide to give its palmitate derivative, dramatically improves the incorporation efficiency of the parent drug in liposomes. The palmitate derivative will be anchored in the membrane and hence its association to liposomes would be expected to be more stable.

Our results clearly indicate that triamcinolone acetonide-21-palmitate is considerably more effective than the free parent drug for the treatment of arthritis. This result is in agreement with other observations indicating that cortisol palmitate is also more effective than free cortisol (Shaw & Dingle 1980). The reason for this may be that liposome-associated corticosteroids remain in the articular cavity for a longer period than the free parent drug as demonstrated in Fig. 3. Our results show retentions very similar to those found by Shaw & Dingle (1980); 40% after 8 h of treatment, compared with 38% retention in our study (Fig. 3). Foong & Green (1988) found considerably longer retention, although they used a non-steroidal drug (methotrexate) and a lipid composition based on egg yolk lecithin-dicetylphosphate.

We conclude that triamcinolone acetonide palmitate included in liposomes provides a stable carrier system for the efficient treatment of chronic arthritis. This system will have the advantages of intra-articular injection together with an increased efficiency with respect to the use of the free corticoid.

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Conversion of flosequinan to the sulphone metabolite in subcellular fractions of human liver, in-vitro

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Abstract—Flosequinan was metabolized in a NADPH-dependent reaction, by *S*-oxidation to its principal metabolite, the sulphone. Most of the sulphone metabolite was formed within 20 min and reached a plateau by 60 min, following incubations of flosequinan (31, 61 or 122 μM) with the microsomal fraction. Flosequinan was metabolized mainly by the microsomal fraction. The *S*-oxidation of flosequinan in incubations containing mitochondrial and cytosolic fractions was 13 and 5%, respectively, of that detected in the microsomal fraction. The route of metabolism of flosequinan in-vitro correlates closely with that previously observed in-vivo. This model could be useful for studying the potential effects of other drugs on flosequinan metabolism in-vitro.

Flosequinan (7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone) is a new cardiovascular agent which has vasodilatory effects on both arterial and venous vascular beds (Cowley et al 1984). Flosequinan has been shown to be metabolized in man in-vivo by *S*-oxidation to its principal phase I metabolite, flosequinan sulphone (Fig. 1), which is also haemodynamically active (Wynne et al 1985). The aim of the present study was to evaluate the metabolism of flosequinan in-vitro in human liver microsomes and to compare this with in-vivo findings. The extent and route of metabolism of flosequinan in other subcellular fractions were also investigated.

Materials and methods

Chemicals and reagents. Flosequinan (BTS 49 465), the sulphone metabolite (BTS 53 554) and the internal standard for HPLC analysis (BTS 49 037, Fig. 1) were provided by Boots Pharmaceuticals, Nottingham, UK. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma Chemicals, UK. All other chemicals and reagents used were of HPLC or reagent grade.

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Tissue preparation and incubation. Human livers were obtained up to 4 h after death due to cerebral haemorrhage from six donors (four males, two females, age range 32–66 years) with normal liver function. Liver samples were stored at −80°C. Liver homogenates were prepared at 4°C with 4 vols of 0·1 M phosphate buffer, pH 7·4, using a glass–Teflon homogenizer. The mitochondrial fraction was prepared by centrifuging the homogenate at 4°C for 10 min at 700 *g*, followed by centrifugation of the resulting supernatant at 4°C for 15 min at 12 500 *g*, using a Sorval RC-5B (Du Pont) centrifuge. The pellet was washed three times by adding 5 vols of buffer, resuspending and centrifuging at 12 500 *g*. The microsomal and the cytosolic fractions were prepared from the same homogenates by differential centrifugation (Kamali et al 1988), using a Sorval ODT 65B (Du Pont) ultracentrifuge. The protein content of the microsomal fractions was determined according to an established method (Lowry et al 1951).

The time course for the metabolism of flosequinan in the microsomal fraction was assessed by incubating the drug at 37°C, in separate tubes in duplicate, in air at times: 0, 5, 10, 15, 20, 30, 40 and 60 min. The incubation mixture contained in 1 mL (final volume): phosphate buffer, pH 7·4 (6–9 mM), microsomal protein (1 mg mL^{-1}), NADPH (1 mM) and flosequinan (31, 61 or 122 μM). Microsomes were also incubated with flosequinan (122