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Retention and Distribution of Liposome-entrapped [^{3}H]Methotrexate Injected into Normal or Arthritic Rabbit Joints

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Abstract—Normal or arthritic rabbits were injected intra-articularly (i.a.) with free [^{3}H]methotrexate ([^{3}H]MTX) or liposomes containing [^{3}H]MTX with [^{14}C]cholesteryl oleate as a lipid marker. The distribution of ^{3}H and ^{14}C in the injected joint and other tissues was determined. Free [^{3}H]MTX was rapidly cleared from the joint, 79% being excreted in the urine within 24 h of injection. Liposome-entrapment retarded [^{3}H]MTX clearance from the joint ($P<0{\cdot}001$), 45·5% being recovered from the joint 24 h after injection. Uptake of liposomes by the inflamed synovium was lower than expected, 4% liposomal [^{3}H]MTX injected being associated with the synovium after 24 h. Nevertheless, this was 40-fold greater than when free [^{3}H]MTX was injected. Liposome entrapment should improve the efficacy and reduce the side effects of drugs injected directly into the joint cavity.

The rationale for treating arthritic joints with drugs injected intra-articularly (i.a.), is that high concentrations of drug can be attained in the joint cavity whilst causing minimal systemic toxicity. However, the efficacy of i.a. therapy is compromised by the rapid clearance of drugs from the joint, so that the beneficial effect is of short duration. Hunneyball (1986) has recently reviewed developments which improve drug retention in the joint. For example, the effects of corticosteroids are prolonged by using a water-insoluble microcrystalline suspension and the retention of ^{198}Au or ^{90}Y in the joint to induce radiosynovectomy is effected by using a colloidal system. Other approaches have involved the use of carriers such as drug-protein conjugates (Foong et al 1985), biodegradable polymers (Ratcliffe et al 1984; Davis et al 1985) or liposomes (Shaw et al 1979). The latter workers have shown that liposome-entrapment greatly enhances the anti-inflammatory effects of corticosteroids injected into arthritic joints, although the duration of effect was limited to 3 or 4 days.

Methotrexate (MTX) has been administered i.a. to control the synovitis in arthritic joints, but the results have generally been disappointing, possibly because adequate concentrations of drug could not be maintained in the joint (Bird et al 1977; Wigginton et al 1980). We have investigated the possibility of entrapping MTX in liposomes for direct injection into the joint cavity to suppress synovitis in arthritic joints. It was anticipated that liposome-entrapped MTX injected i.a. would be selectively taken up by phagocytic cells in the inflamed synovium, so exerting a prolonged inhibitory effect on synovial proliferation, whilst causing minimal systemic toxicity or damage to the articular cartilage.

We report here on the retention and distribution of liposomes containing [^{3}H]MTX with [^{14}C]cholesteryl oleate as a lipid marker injected into arthritic rabbit joints.

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Materials and Methods

Materials

[3′,5′,7-^{3}H]Methotrexate sodium (160 mCi mmol^{-1}) ([^{3}H]MTX and cholesteryl [1-^{14}C]oleate (58 mCi mmol^{-1}) were obtained from Amersham International. Specific activity of [^{3}H]MTX was adjusted to 4·7 mCi mmol^{-1} with methotrexate sodium (MTX) from Lederle. Cholesterol, dicetylphosphate, egg phosphatidylcholine type VE and ovalbumin were from Sigma. Other materials were obtained as follows: Freund's complete adjuvant (Difico), heparin (Evans), pentobarbitone sodium (May & Baker), promethazine hydrochloride (Fisons) and Soluene 100 (Packard Instruments).

Rabbits were inbred of the Old English strain. They weighed 1·4–1·6 kg at the time of antigen sensitization and 1·9–2·1 kg when [^{3}H]MTX was injected.

Liposome preparation

[^{3}H]MTX (4·7 mCi mmol^{-1}) was entrapped in the aqueous phase of negatively charged multilamellar liposomes prepared by prolonged shaking (20 h) at 20 °C with lipids in the molar ratio, egg phosphatidylcholine–cholesterol–dicetylphosphate, 5:5:1 as previously described (White et al 1983). In addition [^{14}C]cholesteryl oleate was incorporated as a lipid marker contributing 0·2 mol % of the total lipid content. Free and liposome-entrapped [^{3}H]MTX were separated by repeated washing and centrifuging at 40 000 g (r_{av} 8·2 cm) at 4 °C for 20 min.

The liposomes had a mean diameter of 1·07 μm as measured by photon correlation spectroscopy. Liposomes routinely injected i.a. contained 1 mg MTX (10 μCi [^{3}H]MTX), 5 μCi [^{14}C]cholesteryl oleate and 99 ± 3 μmol lipid suspended in 1 mL phosphate buffered saline (pH 7·4).

Antigen-induced arthritis

An arthritis was induced in the knee joint of rabbits using a procedure similar to that described by Consden et al (1971). Ovalbumin 20 mg mL^{-1} in sterile 0·9% NaCl (saline) was

emulsified with an equal volume of Freund's complete adjuvant. Rabbits (1·4–1·6 kg) were sensitized by injecting s.c. a total of 1 mL emulsion at five sites between the scapulae. The sensitization procedure was repeated three weeks later. After a further 10 days the rabbits were skin tested with ovalbumin 10 μg i.d., those animals producing a positive skin reaction of at least 16 mm diameter 24 and 48 h later being considered adequately sensitized.

Arthritis was induced in one knee joint by injecting 5 mg ovalbumin in 0·5 mL saline into the joint cavity through the supra-patella ligament using a 27 gauge needle. 30 min before ovalbumin challenge rabbits were pretreated with promethazine 5 mg kg^{-1} i.p. to mitigate some of the effects of acute anaphylaxis. Arthritis was assessed by measuring changes in joint diameter and skin temperature over the joint. Three weeks after induction of arthritis, the diameter of control knee joints was $18·8 \pm 0·2$ mm and of contralateral arthritic joints $23·8 \pm 0·4$ mm (n = 20). Skin surface temperature of control joints was $34·5 \pm 0·3$ °C and of arthritic joints $38·0 \pm 0·5$ °C (n = 20).

Measurement of [^{3}H]MTX and [^{14}C]cholesteryl oleate distribution

Phosphate buffered saline (1 mL) containing [^{3}H]MTX free, or entrapped in liposomes with [^{14}C]cholesteryl oleate as lipid marker, was injected into normal knee joints or joints in which arthritis had been induced three weeks previously. The rabbits were housed in metabolism cages to enable radioactivity excreted in the urine to be monitored. Blood samples were collected at regular intervals from a marginal ear vein and the ^{3}H and ^{14}C content of 0·2 mL plasma aliquots determined. Total plasma volume of the rabbits was estimated from body weight by reference to tables (Spector 1956). The rabbits were killed 4 h, 24 h, 3 days or 7 days after injection of [^{3}H]MTX by injecting pentobarbitone sodium i.v. and the distribution of ^{3}H and ^{14}C in the joints and other tissues determined.

Urine was collected 4, 24, 48, 72 and 92 h after injection of [^{3}H]MTX. Rabbits were administered 20 mL water orally immediately after injection of [^{3}H]MTX and then every 24 h to increase the volume of urine excreted. When the rabbits were killed, urine in the bladder was pooled with that collected over the previous collection period.

Recovery of [^{3}H] and [^{14}C] from articular tissues

Synovial fluid was collected by injecting 0·6 mL heparinized saline (10 units mL^{-1}) into the knee joints immediately after the rabbits were killed. The joints were flexed and massaged before aspirating the fluid with gentle suction. Washing and aspiration of the joint cavity was repeated 10 times and the joint was then opened by cutting the patella ligament to expose the supra-patella pouch. The joint was again washed repeatedly with heparinized saline and the ^{3}H and ^{14}C content of the pooled washings measured.

The synovial membrane, underlying fat pads, patella, medial and lateral menisci were excised, washed in saline (4 °C), blotted dry, weighed and digested with Soluene 100 for 24 h at 40 °C. Radioactivity in the patella and menisci cartilage was measured in preference to the articular cartilage because of difficulty in obtaining representative samples of the latter.

Recovery of ^{3}H and ^{14}C from extra-articular tissues

The liver, spleen, kidneys and lungs were removed, washed in saline (4 °C), blotted dry and weighed. Each tissue was homogenized, 200 mg aliquots of the homogenate digested with Soluene 100 for 24 h at 40 °C and the ^{3}H and ^{14}C content measured. Radioactivity of tissue aliquots was measured by liquid scintillation counting and expressed as a percentage of the amount injected. In some cases the specific activity of tissues (d min^{-1} g^{-1}) was compared.

Statistics

Results are expressed as mean ± s.e. mean, statistical significance being determined using an unpaired Student's *t*-test.

Results

The efficiency of the recovery procedure used to determine the retention of [^{3}H]MTX in the joints was assessed by injecting [^{3}H]MTX (10 μCi) i.a., immediately killing the rabbits and measuring the [^{3}H] content of the articular tissues. $87·3 \pm 2·7$% of [^{3}H]MTX injected was recovered, most being in the synovial fluid (Table 1).

Retention and distribution in articular tissues

The clearance of free [^{3}H]MTX from normal or arthritic knee joints was rapid, in both cases only approx 3·7% of ^{3}H injected being recovered from the joint 4 h after injection (Table 1). However, a greater percentage of the ^{3}H recovered was associated with the synovium of arthritic joints than with normal joints ($P < 0·05$).

Liposome-entrapment greatly enhanced the retention of [^{3}H]MTX in injected joints. Thus, 24 h after injection of free [^{3}H]MTX, less than 0·6% was recovered from the joint, compared with $45·5 \pm 3·0$% when liposomal [^{3}H]MTX was injected, an 81-fold increase (Table 1). At this time approx 91% of ^{3}H and ^{14}C recovered from the joints was in the synovial fluid, $6·1 \pm 2$% being associated with inflammatory cells in the synovial fluid, predominantly polymorphonuclear leucocytes and lymphocytes. Liposome-entrapment increased ^{3}H associated with the synovium. Thus, 24 h after injection of liposomal [^{3}H]MTX, $4·0 \pm 0·3$% ^{3}H was associated with the synovium, compared with only $0·1 \pm 0·02$% when free [^{3}H]MTX was injected. There appeared to be no significant difference ($P < 0·05$) in the articular distribution of liposomal [^{3}H]MTX and [^{14}C]cholesteryl oleate.

To inhibit synovial proliferation and minimize cartilage damage, i.a. therapy for arthritis should result in much greater drug uptake by the synovium than by the cartilage. 24 h after injection of free [^{3}H]MTX, specific activity of ^{3}H in the synovial membrane was 2·1-fold ($P < 0·05$) greater than in the patella and menisci cartilage. By contrast, 24 h after injection of [^{3}H]MTX liposomes, the difference in specific activity was 14·8-fold ($P < 0·05$) and 7 days after injection had increased to 33-fold ($P < 0·001$). Thus, liposome entrapment selectively enhanced [^{3}H]MTX association with the synovium.

Although liposome-entrapment of [^{3}H]MTX favourably altered its retention and distribution in the joint, association of the drug with the synovium was relatively low, 3 days after injection approx 83% of [^{3}H]MTX recovered from the joint being in the synovial fluid. To ascertain whether the low

Table 1. Recovery of ^3H and ^{14}C from normal or arthritic joints after i.a. injection of [^3H]MTX or liposomes containing [^3H]MTX with [^{14}C]cholesteryl oleate. Values are percentage of injected ^3H or ^{14}C recovered (mean ± s.e. mean, n = 4 to 6).

Treatment	Time of injection	Synovial fluid	Synovium	Patella & menisci	Total recovered from joint
		Recovery of ^3H			
Free [^3H]MTX injected into normal joints	0 h	87·22 ± 2·66	< 0·01	0·02 ± 0·00	87·26 ± 2·67
	4 h	3·45 ± 0·43	0·10 ± 0·03	0·16 ± 0·02	3·72 ± 0·45
Free [^3H]MTX injected into arthritic joints	4 h	3·18 ± 0·55	0·39 ± 0·05	0·16 ± 0·02	3·74 ± 0·53
	24 h	0·44 ± 0·01	0·10 ± 0·02	0·02 ± 0·01	0·56 ± 0·04
Liposomes (99 μmol phospholipid) containing [^3H]MTX & [^{14}C] cholesteryl oleate injected into arthritic joints	4 h	46·45 ± 4·60	2·04 ± 0·20	0·16 ± 0·03	48·65 ± 4·85
	24 h	41·38 ± 2·93	4·00 ± 0·31	0·11 ± 0·02	45·49 ± 2·96
	3 days	25·7 ± 2·43	5·19 ± 0·42	0·20 ± 0·02	31·14 ± 2·58
	7 days	4·66 ± 0·90	5·54 ± 0·34	0·11 ± 0·02	10·31 ± 0·98
		Recovery of ^{14}C			
Liposomes (99 μmol phospholipid) containing [^3H]MTX & [^{14}C] cholesteryl oleate injected into arthritic joints	4 h	48·32 ± 0·48	2·22 ± 0·22	0·14 ± 0·01	50·68 ± 0·58
	24 h	50·10 ± 2·65	4·72 ± 0·45	0·13 ± 0·01	54·95 ± 2·72
	3 days	19·37 ± 2·02	5·00 ± 0·16	0·14 ± 0·02	24·51 ± 2·37
	7 days	2·99 ± 0·74	5·64 ± 0·32	0·13 ± 0·03	8·76 ± 1·01
Liposomes (4·95 μmol phospholipid) containing [^{14}C]cholesteryl oleate injected into arthritic joints	4 h	34·87 ± 1·87	3·43 ± 0·15	0·05 ± 0·01	38·35 ± 1·94
	24 h	24·45 ± 1·60	6·91 ± 0·34	0·07 ± 0·01	32·43 ± 1·97

Columns "Synovial fluid" to "Total recovered from joint" are under the heading: Percentage of ^3H or ^{14}C recovered.

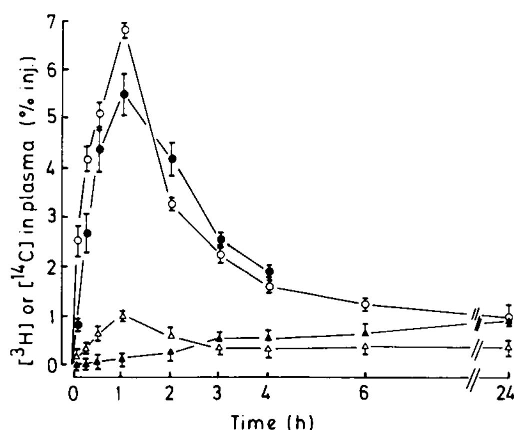

Fig. 1. ^3H or ^{14}C in blood plasma after i.a. injection of free [^3H]MTX or liposomes containing [^3H]MTX and [^{14}C]cholesteryl oleate. Free [^3H]MTX injected into normal (●) or arthritic joints (○). Liposomes containing [^3H]MTX (△) and [^{14}C]cholesteryl oleate (▲) injected into arthritic joints. (mean ± s.e.m., n = 4–6)

association of liposomal [^3H]MTX with the synovium was due to the large amount of lipid injected saturating the mechanisms involved in liposome uptake, the amount of liposomal lipid injected was reduced 20-fold. When 4·95 μmols instead of 99 μmol phospholipid were injected, the percentage of ^3H injected associated with the synovium 24 h later was approx 1·5 fold greater, whereas the percentage associated with the patella and menisci cartilage was approx 50% lower ($P < 0·01$) (Table 1).

Extra-articular distribution

Peak plasma concentrations of [^3H]MTX occurred 1 h after i.a. injection of either free or liposome-entrapped [^3H]MTX (Fig. 1). The highest plasma levels were attained when arthritic joints were injected with free [^3H]MTX, 1 h after injection, 6·8 ± 0·3% ^3H injected being present in the plasma, compared with 5·5 ± 0·9% when normal joints were injected. Plasma levels of ^3H attained after i.a. injection of liposomal [^3H]MTX were approx 7-fold less than levels produced by free [^3H]MTX. However, after liposome injection plasma levels of ^3H increased more rapidly than ^{14}C, suggesting that some entrapped [^3H]MTX leaked from the liposomes soon after injection.

[^3H]MTX which leaked from the joints was rapidly excreted in the urine. Thus, when free [^3H]MTX was injected, 35·2 ± 1·7% and 79·3 ± 2·2% was excreted in the urine within 4 and 24 h, respectively. By contrast, when liposome-entrapped [^3H]MTX was injected, 20·4 ± 3·6% was excreted over 24 h, with only 0·25 ± 0·12% ^{14}C detected in the urine at this time (Fig. 2). The liver and kidneys contained appreciable amounts of ^3H, levels in these organs being maintained longest in rabbits injected with liposomes, presumably due to the slower but sustained release of [^3H]MTX from the injected joint (Table 2).

Discussion

The rapid clearance of free [^3H]MTX from rabbit knee joints was similar to that reported for the clearance of MTX from

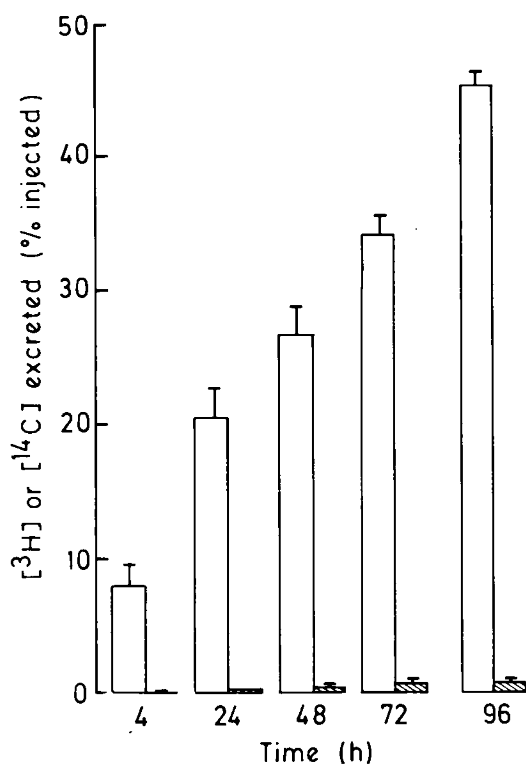

FIG. 2. Urinary excretion of ^{3}H (open columns) and ^{14}C (hatched columns) after i.a. injection of liposomes containing [^{3}H]MTX and [^{14}C]cholesteryl oleate (mean ± s.e.m., n = 4–6).

human arthritic joints, where peak plasma concentrations of MTX occurred 1 to 2 h after injection (Bird et al 1977). Liposome-entrapment reduced the clearance of [^{3}H]MTX from rabbit joints, plasma levels of ^{3}H 1 h after injection being 7-fold less than when free [^{3}H]MTX was injected. However, plasma levels of ^{3}H increased more rapidly than those of ^{14}C, suggesting that some entrapped [^{3}H]MTX leaked from the liposomes within a few minutes of injection. This is in accord with results we obtained on liposome stability in synovial fluid in-vitro, approx 10% of entrapped [^{3}H]MTX leaking from liposomes within the first hour of incubation (White et al 1983).

Plasma levels of ^{3}H after i.a. injection of [^{3}H]MTX inversely reflected drug retention in the joint. Thus, 24 h after injection of liposome-entrapped [^{3}H]MTX, 45·5% was recovered from the joint compared with less than 0·6% when free drug was injected. At this time only 4% of liposomal [^{3}H]MTX injected was associated with the synovium, and although this was 40-fold greater than when free drug was injected, it was much lower than has been reported by other workers for liposome-entrapped material. For example, Bard et al (1983) reported that injection of liposome-entrapped chelated ^{51}Cr into rabbit knee joints resulted in over 90% of the radioactivity being associated with the synovium 24 h later. Similarly, Shaw et al (1979) injected arthritic rabbits with liposome-entrapped corticosteroids and, depending upon the duration of the arthritis, found 10 to 60% of drug associated with the synovium, uptake being greatest in joints with arthritis of 2 days duration. In the current study the arthritis was of 21 days duration and the low uptake of liposomal [^{3}H]MTX by the synovium may have been due in part to the chronicity of the arthritis. Both Shaw et al (1979) and Bard et al (1983) induced arthritis by the i.a. injection of a complex of hyaluronate and poly-D-lysine. In our experience, arthritis induced by injection of the complex is less severe and less persistent than antigen-induced arthritis, perhaps accounting in part for the reported differences in liposome uptake by the synovium. Also, in the present study the large amounts of lipid injected may have saturated the phagocytic processes involved in liposome uptake by the synovium. Thus, in the initial study 99 μmol of liposomal lipid was injected, but when this was reduced 20-fold the percentage of the ^{14}C-lipid marker associated with the synovium increased approx 50%. By contrast, Bard et al (1983) injected only 2 μmol lipid, which could account for the high liposomal uptake (> 90%) by the synovium which they observed. Another factor responsible for the low uptake of

Table 2. Recovery of ^{3}H and ^{14}C from extra-articular tissues after i.a. injection of [^{3}H]MTX or liposomes containing [^{3}H]MTX with [^{14}C]cholesteryl oleate. Values are percentage of injected ^{3}H or ^{14}C recovered (mean ± s.e. mean, n = 6).

Treatment	Time of Injection	Percentage of ^{3}H or ^{14}C recovered			
		Liver	Spleen	Kidney	Lung
		Recovery of ^{3}H			
Free [^{3}H]MTX injected into arthritic joints	4 h	3·48 ± 0·40	0·03 ± 0·01	1·75 ± 0·08	0·03 ± 0·01
	24 h	0·41 ± 0·20	<0·01	0·14 ± 0·04	<0·01
Liposomes containing [^{3}H]MTX & [^{14}C] cholesteryl oleate injected into arthritic joints	4 h	1·89 ± 0·28	0·03 ± 0·02	1·70 ± 0·48	0·03 ± 0·01
	24 h	2·09 ± 0·25	0·04 ± 0·01	0·19 ± 0·06	0·18 ± 0·03
		Recovery of ^{14}C			
Liposomes containing [^{3}H]MTX & [^{14}C] cholesteryl oleate injected into arthritic joints	4 h	1·14 ± 0·40	0·01 ± 0·01	0·08 ± 0·01	0·08 ± 0·01
	24 h	0·83 ± 0·42	<0·01	0·07 ± 0·03	0·21 ± 0·09

liposomes by the synovium could have been their high cholesterol content. Liposomes with a high cholesterol content were used because they were shown to be stable in the presence of synovial fluid in-vitro (White et al 1983). However, a high cholesterol content has been shown to depress liposome uptake by macrophages in-vitro (Foong & Green 1988) and by the liver and spleen in-vivo (Patel et al 1983). Furthermore, the degradation of liposomes taken up by macrophages appears to be inhibited by a high cholesterol content (Johnson 1975).

MTX was selected as a suitable cytotoxic agent for this study because it has been shown to be beneficial in the systemic therapy of arthritis and it has the potential advantage that toxicity can be limited by folic acid rescue (see Wilke & Mackenzie 1986). In the current study, although the uptake of liposome-entrapped MTX by the synovium was less than had been expected, it is of note that association of [^{3}H]MTX with the synovium 24 h after injection was 40-fold greater than when free [^{3}H]MTX was injected, while levels in the patella and menisci cartilage were not significantly increased. Thus, liposome-entrapment should favourably enhance the inhibitory effect of MTX on synovial proliferation, whilst having minimal effects on the cartilage. Liposome-entrapped cytotoxic drugs may be preferable to radioisotopes for treating synovitis, since the latter, even when selectively taken up by the synovium, can irradiate and damage adjacent articular cartilage (Bard et al 1984).

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