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# Treatment of Antigen-induced Arthritis in Rabbits with Liposome-entrapped Methotrexate Injected Intra-articularly

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**Abstract**—Rabbits with a bilateral antigen-induced arthritis were injected intra-articularly in one joint with methotrexate as the free drug or entrapped in liposomes. Free methotrexate (1 mg) injected as a single dose at the time of antigen challenge, suppressed the development of joint swelling and the rise in skin surface temperature of treated joints by 20–30% compared with contralateral control arthritic joints. The beneficial effect of methotrexate occurred within 24 h of injection and was maintained for at least 56 days. However, methotrexate was ineffective in suppressing arthritis when injected 7 days after antigen challenge. Liposomal methotrexate suppressed the development of arthritis at a dose one-tenth that of the free drug and it was also effective in suppressing arthritis of 7 days duration, although significant beneficial effects of liposomal methotrexate were delayed for 10 to 14 days after injection. Neither free nor liposomal methotrexate was effective in suppressing an established arthritis, having no significant effect on joint swelling or skin surface temperature when injected 21 and 35 days after antigen challenge. At the end of the study, 8 or 9 weeks after induction of arthritis, the joints were examined morphologically and histologically. Free methotrexate generally had no significant effect on joint pathology. However, liposomal methotrexate suppressed the development of synovial hyperplasia, cellular infiltration and the erosion of cartilage and bone when injected at the time of antigen challenge or 7 days later, but affected none of these parameters in an established arthritis of 3 weeks duration.

Methotrexate has in recent years attracted increasing attention as a valuable second-line agent in the treatment of rheumatoid arthritis (Wilke et al 1987; McKendry & Cyr 1989). Methotrexate compares favourably with gold in decreasing the symptoms, the serological changes and the radiological progression of rheumatoid arthritis (Rau et al 1991). Methotrexate has also been administered intra-articularly (i.a.) to control the synovitis in arthritic joints. Franchi et al (1989) reported a beneficial response to i.a. methotrexate in a group of five patients, but the results with this route of methotrexate administration have generally been disappointing (see Wilke et al 1987). The poor response to i.a. therapy may be due to the rapid clearance of methotrexate from the joint, peak plasma levels of drug being detected 1–2 h after i.a. injection (Bird et al 1977; Wigginton et al 1980). We have shown that radiolabelled methotrexate injected into arthritic rabbit joints is retained in the joint much longer if the drug is entrapped in liposomes (Foong & Green 1988b). For example, 24 h after i.a. injection of the free drug, less than 0·6% was recovered from the injected joint, compared with 45% recovery of liposomal methotrexate, an 80-fold increase. The enhanced retention of liposomal methotrexate in arthritic joints was due, at least in part, to the phagocytic uptake of liposomes by cells of the diseased synovium. It was therefore anticipated that liposome-entrapment would enhance the efficacy of methotrexate injected into arthritic joints. Moreover, the greater retention of methotrexate at the injection site should reduce potential adverse systemic effects of i.a. methotrexate therapy.

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## Materials and Methods

### *Materials*

Cholesterol, dicetylphosphate, egg phosphatidylcholine type VE and ovalbumin were from Sigma. Other materials were obtained as follows: Freund's complete adjuvant (Difco, East Molesey, UK), methotrexate sodium (Lederle, Fareham, UK) and promethazine hydrochloride (Fisons, Loughborough, UK).

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 methotrexate was injected.

### *Liposome preparation*

Methotrexate 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). Free and liposome-entrapped methotrexate were separated by repeated washing and centrifuging at 40000 *g* ($r_{av}$ 8·2 cm) at 4°C for 20 min. The liposomes were suspended in phosphate-buffered saline (pH 7·4) and had a mean diameter of 1·07 μm as measured by photon correlation spectroscopy.

### *Antigen-induced arthritis*

Arthritis was induced in the knee joints 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 subcutaneously 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, intradermally), those animals

producing a positive skin reaction of at least 16 mm diameter 24 and 48 h later being considered adequately sensitized.

A bilateral arthritis was induced by injecting 5 mg ovalbumin in 0·5 mL saline into both knee joints through the supra-patella ligament using a 27 gauge needle. Thirty minutes before ovalbumin challenge rabbits were pretreated with promethazine (5 mg $kg^{-1}$, i.p.) to mitigate some of the effects of acute anaphylaxis.

*Assessment of arthritis*

The development of arthritis was monitored at regular intervals by measuring changes in knee joint diameter and skin surface temperature over the joint. Joint diameter was measured using a spring-loaded micrometer, the mean of 3 readings being taken with the joint flexed at an angle of 90°. Hair covering the knees was depilated and skin surface temperature over the suprapatella ligament was measured using a skin thermistor probe (AHI, DU35 Ellab Instruments) after the temperature had been allowed to equilibrate following a 2 min period of cooling with a fan. All measurements were carried out at a room temperature of $20 \pm 1$°C. Immediately before the injection of the challenging dose of antigen, the diameter of the knee joints was $18{\cdot}4 \pm 0{\cdot}2$ mm and the skin surface temperature $34{\cdot}1 \pm 0{\cdot}2$°C ($n = 30$). One week after injection of antigen, the joint diameter of untreated knees had increased to $23{\cdot}3 \pm 0{\cdot}4$ mm and the surface temperature to $37{\cdot}7 \pm 0{\cdot}2$°C ($n = 30$).

When the rabbits were killed, the knee joints were opened and their morphological appearance graded on a scale of 0 to 4 for each of the following parameters.

*Synovial fluid.* Very small volume, slight discoloration (1); small volume with discoloration (2); discoloration with some tissue debris (3); large volume with discoloration and tissue debris (4).

*Synovium.* Slight hyperplasia and vascularization (1); as (1) with occasional petechiae (2); hyperplasia with petechiae and some discoloration (3); hyperplasia with many petechiae and discoloration (4).

*Cartilage and bone.* Cartilage erosion (1); cartilage erosion with limited bone erosion of femoral condyles (2); extensive erosion of femoral condyles (3); erosion also affecting the intercondylar fossa and patella (4).

Histology of the joints was assessed after fixing soft tissue specimens in formal saline, embedding in paraffin wax and sectioning at 5 μm. Calcified tissue was decalcified in formic acid formalin decalcifying fluid (Gooding & Steward's fluid), several changes of solution being required to remove all of the calcium. Completion of decalcification was assessed using the method of Clayden (1952). The joints were then dehydrated and embedded in paraffin wax for sectioning. Sections were cut in the coronal plane at 5 μm, stained with haematoxylin and eosin, some being counterstained with alcian blue which stains the mucopolysaccharide of cartilage. The histology of the sections was assessed blind, being graded on a scale of 0 to 5 as follows: normal synovium with a few plasma cells and lymphocytes (1); moderate synovial hyperplasia with plasma cells and lymphocytes (2); increased hyperplasia and cellular infiltrate with vasculitis (3); cellular infiltration of whole synovium, pannus formation with abnormal chondrocyte distribution and cartilage erosion (4); dense cellular infiltrate with erosion of cartilage and bone (5).

*Statistics*

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

## Results

The irritant effects of free or liposome-entrapped methotrexate after i.a. injection into normal rabbit knee joints was studied by monitoring their effects on joint diameter and skin surface temperature. The injection of phosphate-buffered saline (1 mL) alone, or containing empty liposomes (egg phosphatidylcholine–cholesterol–dicetylphosphate, 3·3 μmol total lipid), caused a transient increase in joint diameter and surface temperature (Fig. 1). Morphological and histological examination of the joints 24 h after injection showed no evidence of inflammatory changes. The i.a. injection of empty liposomes at the time of antigen challenge did not affect the development or course of arthritis in the injected joint. Free methotrexate (1 mg) or liposome-entrapped methotrexate (0·1 mg) injected i.a. caused a significant increase in joint swelling and skin surface temperature which persisted for 12 h after injection of the free drug and for 72 h after injection of liposomal methotrexate. Joints injected

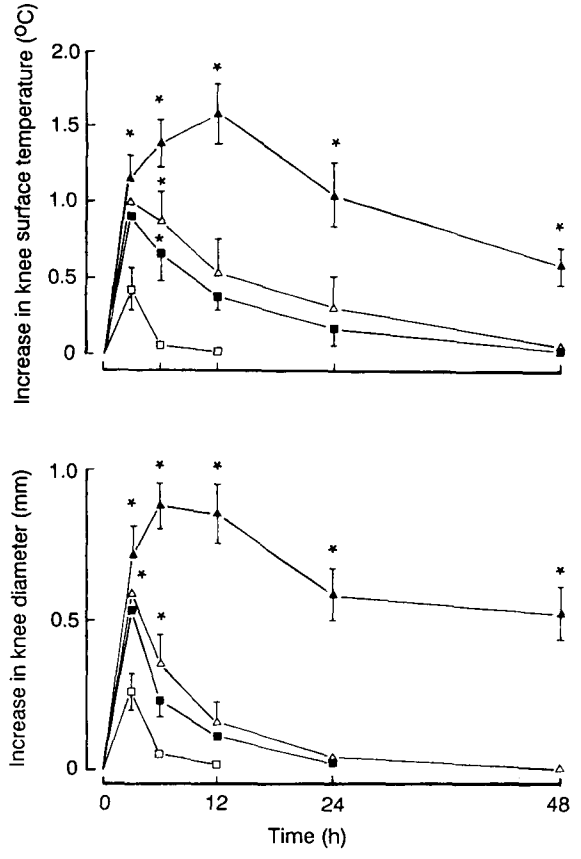


FIG. 1. Effect of empty liposomes (■), free methotrexate 1 mg (△), liposomal methotrexate 0·1 mg (▲) or phosphate-buffered saline 1 mL (□) injected into normal knee joints on joint diameter and surface temperature. Values indicate mean ± s.e.m., $n = 4$. *$P < 0{\cdot}05$ compared with control joint.

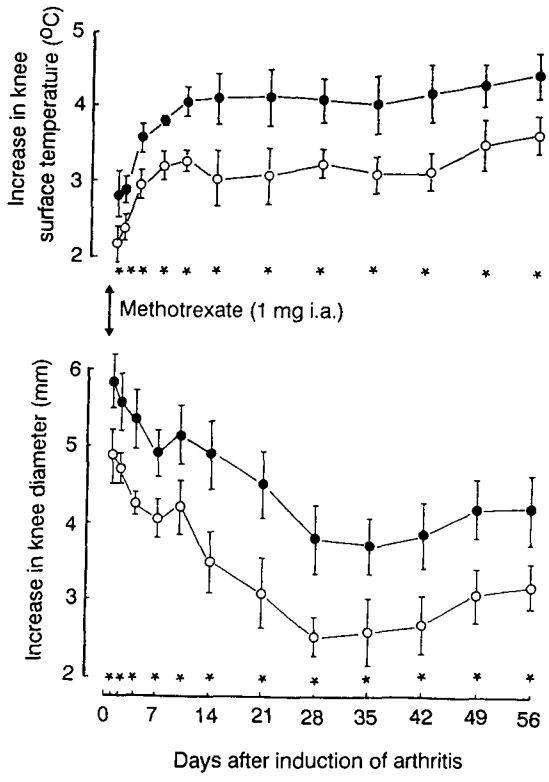


FIG. 2. Effect of free methotrexate 1 mg i.a. (○) on joint diameter and surface temperature of arthritic joints when injected at the time of antigen challenge. Saline (●) was injected into the contralateral joint. Values indicate mean ± s.e.m., n = 5. *$P < 0.05$ compared with control arthritic joint.

with free or liposomal methotrexate showed moderate synovial hyperplasia and polymorphonuclear leucocyte infiltration when the joints were opened and examined 24 h after injection, these effects being greatest in joints injected with liposomal methotrexate. Inflammatory changes were still apparent 3 days after injection of liposomal methotrexate and the tissues were heavily discoloured by retained drug.

*Treatment with free methotrexate*

Methotrexate (1 mg) injected i.a. immediately after antigen challenge suppressed the increase in joint diameter and skin surface temperature by 20–30% compared with the contralateral control arthritic joint (Fig. 2). The beneficial effects were evident within 24 h of injection and persisted for at least 56 days, when the rabbits were killed to allow internal examination of the joints. Morphological examination of the opened joints showed that methotrexate-treated joints had significantly ($P < 0.05$) lower scores than contralateral control joints for inflammatory changes in the synovium and synovial fluid, but erosion of cartilage and bone was not significantly reduced (Table 1). The histology of the treated joints was not significantly different from control joints, both groups showing marked synovial hyperplasia, dense lymphocytic infiltration and pannus formation with erosion of cartilage and bone (Table 1).

Free methotrexate (1 mg) injected i.a., 7 days or 21 and 35 days after antigen challenge had no significant effect ($P > 0.05$) on the established arthritis as judged by the measurement of joint diameter and skin surface temperature, or by morphological and histological assessment (Table 1).

Table 1. Morphological and histological assessment of arthritis in control arthritic joints and arthritic joints injected with free or liposomal methotrexate.

| Treatment and time of injection after arthritis induced | | Time of death after arthritis induced | Macroscopic assessment: Synovial fluid | Synovium | Cartilage and bone erosion | Histological assessment |
|---|---|---|---|---|---|---|
| Free methotrexate | | 56 days | | | | |
| 1 mg | (5) | Control | 3·6 ± 0·2 | 3·7 ± 0·2 | 3·7 ± 0·2 | 4·4 ± 0·2 |
| Day 0 | | Treated | 3·2 ± 0·1* | 3·0 ± 0·2* | 3·4 ± 0·2 | 4·0 ± 0·3 |
| | | 56 days | | | | |
| 1 mg | (5) | Control | 3·7 ± 0·2 | 3·7 ± 0·3 | 3·8 ± 0·2 | 4·7 ± 0·2 |
| Day 7 | | Treated | 4·0 ± 0·0 | 3·7 ± 0·2 | 3·7 ± 0·3 | 4·7 ± 0·2 |
| | | 63 days | | | | |
| 1 mg | (3) | Control | 3·8 ± 0·2 | 3·7 ± 0·3 | 3·5 ± 0·0 | 4·7 ± 0·2 |
| Day 21 and 35 | | Treated | 4·0 ± 0·0 | 3·4 ± 0·2 | 3·7 ± 0·2 | 4·5 ± 0·2 |
| Liposomal methotrexate | | 56 days | | | | |
| 0·1 mg | (6) | Control | 3·6 ± 0·2 | 3·8 ± 0·2 | 3·3 ± 0·4 | 4·6 ± 0·4 |
| Day 0 | | Treated | 2·9 ± 0·4* | 3·0 ± 0·2* | 2·6 ± 0·2* | 3·8 ± 0·2* |
| | | 56 days | | | | |
| 0·1 mg | (5) | Control | 3·7 ± 0·3 | 3·5 ± 0·3 | 3·7 ± 0·3 | 4·7 ± 0·3 |
| Day 7 | | Treated | 2·9 ± 0·3* | 2·7 ± 0·3* | 2·6 ± 0·3* | 4·0 ± 0·2* |
| | | 63 days | | | | |
| 0·1 mg | (3) | Control | 3·3 ± 0·2 | 3·5 ± 0·3 | 3·7 ± 0·2 | 4·7 ± 0·2 |
| Day 21 and 35 | | Treated | 3·0 ± 0·3 | 3·8 ± 0·2 | 3·0 ± 0·5 | 4·7 ± 0·2 |
| | | 63 days | | | | |
| 1 mg | (3) | Control | 3·3 ± 0·3 | 3·8 ± 0·2 | 3·7 ± 0·5 | 4·6 ± 0·2 |
| Day 21 and 35 | | Teated | 3·0 ± 0·0 | 3·7 ± 0·2 | 3·0 ± 0·2 | 4·5 ± 0·3 |

A bilateral arthritis was induced in rabbits and free or liposomal methotrexate injected into one joint at various times after induction. When the rabbits were killed, the morphological (graded 0 to 4) and histological (graded 0 to 5) appearance of the joints was scored at 0·5 intervals. Values indicate means ± s.e.m. Figures in parentheses indicate the number of rabbits in each group. *$P < 0.05$ when compared with contralateral control arthritic joints.

*Treatment with liposome-entrapped methotrexate*

Injection of liposomal methotrexate (0·1 mg) into joints at the time of antigen challenge suppressed the increase in joint swelling and skin surface temperature by approximately 30% (Fig. 3). This beneficial effect of liposomal methotrexate developed more slowly than when 1 mg of the free drug was injected, a significant suppressive effect on the development of arthritis not occurring until 14 days after injection. Gross morphological examination of the opened joints 56 days after induction of arthritis showed a significant reduction ($P < 0{\cdot}05$) in all of the parameters of arthritis assessed (Table 1). The beneficial effect of i.a. liposomal methotrexate was also reflected in the histological appearance of the treated joints, there being a significant reduction ($P < 0{\cdot}05$) in inflammatory changes and erosion of cartilage compared with contralateral control arthritic joints (Table 1). Increasing the amount of liposomal methotrexate injected from 0·1 to 1 mg only enhanced the suppression of arthritis slightly. Thus joint swelling was reduced by a maximum of 35%, a significant reduction ($P < 0{\cdot}05$) in joint diameter and skin surface temperature occurring within 10 days of injection.

Liposomal methotrexate (0·1 mg) injected into joints with an established arthritis of 7 days duration was also effective in reducing joint swelling and skin surface temperature (Fig. 4). A significant reduction ($P < 0{\cdot}05$) in skin temperature occurred within 7 days of injection, and in joint diameter 14 days after injection, the beneficial effect of liposomal methotrexate being maintained for at least 49 days when the rabbits were killed. There was also a significant reduction ($P < 0{\cdot}05$) in severity of arthritis assessed by gross morphological and histological examination of the joints (Table 1). However, injection of liposomal methotrexate (0·1 or 1 mg) into arthritic joints 21 and 35 days after induction of arthritis appeared to have no beneficial effect on the course of arthritis as measured by joint swelling and skin surface temperature. Similarly, there was no significant difference ($P > 0{\cdot}05$) between treated and control joints in their gross morphology and histology 9 weeks after induction of arthritis (Table 1).

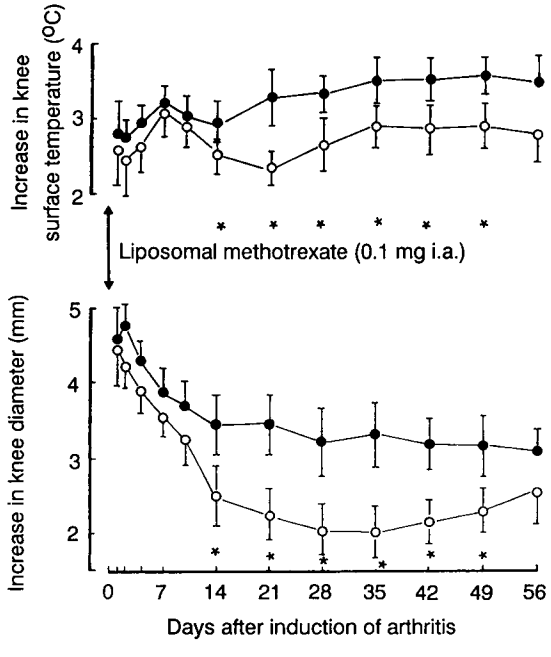


FIG. 3. Effect of liposome-entrapped methotrexate 0·1 mg i.a. (○) on joint diameter and surface temperature of arthritic joints when injected at the time of antigen challenge. Saline (●) was injected into the contralateral arthritic joint. Values indicate mean ± s.e.m., n = 6. *$P < 0{\cdot}05$ compared with control arthritic joint.

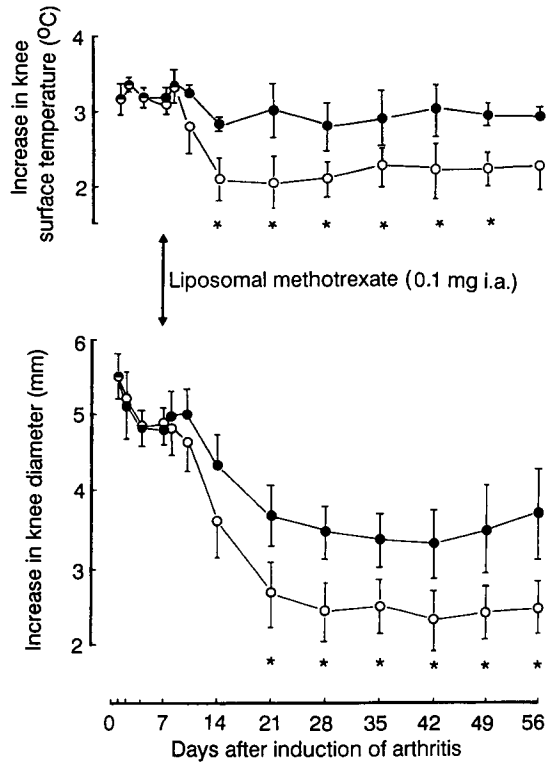


FIG. 4. Effect of liposome-entrapped methotrexate 0·1 mg i.a. (○) on joint diameter and surface temperature of arthritic joints when injected 7 days after antigen challenge. Saline (●) was injected into the contralateral arthritic joint. Values indicate mean ± s.e.m., n = 5. *$P < 0{\cdot}05$ compared with control arthritic joint.

## Discussion

Antigen-induced arthritis in rabbits provides one of the best models of rheumatoid arthritis available. The joint histopathology of antigen-induced arthritis closely resembles rheumatoid arthritis (Glynn 1968; Zvaifler 1973), and its responsiveness to anti-rheumatic and anti-inflammatory drugs is similar to that of the clinical disease. Non-steroidal anti-inflammatory drugs and corticosteroids have generally been found to reduce joint swelling and temperature, but to have little effect on the progress of antigen-induced arthritis (Davis 1971; Blackham & Radziwonik 1977; Goldlust & Rich 1981; Hunneyball 1981, 1984). The arthritis is not responsive to short-term treatment with penicillamine, but is suppressed by treatment over 100–200 days (Blackham & Radziwonik 1977; Hunneyball et al 1979). Both gold therapy and high doses of chloroquine are reported to be moderately effective in antigen-induced arthritis, suppressing changes in joint histopathology but not joint swelling (Blackham & Radziwonik 1977; Goldlust & Rich 1981). The arthritis is also reversibly suppressed by azathioprine and by cyclophosphamide (Brouilhet et al 1975; Blackham 1978; Goldlust & Rich 1981).

The rationale for treating arthritic joints with drugs injected 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. Dingle (1976) first proposed the use of liposome-entrapped drugs for i.a. injection, and subsequently showed that the retention of liposome-entrapped corticosteroids in the injected joint was greatly enhanced, so that smaller doses of drug were required to elicit a beneficial effect (Phillips et al 1979; Shaw et al 1979). These workers also showed that the cytotoxic agent daunorubicin injected i.a. was effective in suppressing joint swelling and skin surface temperature in arthritic rabbits, the effect being enhanced when the drug was entrapped in liposomes (Page Thomas & Phillips 1979). In the current study, methotrexate was selected as a suitable cytotoxic agent for liposome-entrapment and i.a. injection, partly because it has been shown to have a beneficial effect in the systemic therapy of rheumatoid arthritis, but also because its toxicity can be limited by folic acid rescue (see Wilke & Mackenzie 1986). This has important implications for i.a. therapy. Because methotrexate is cell-cycle specific, its effects being limited to cells in the S-phase, Wigginton et al (1980) suggested that for i.a. injected methotrexate to be effective it would need to be present in the joint in inhibitory concentrations for periods exceeding the cell cycle generation time of the rheumatoid pannus. They proposed that this could be achieved by repeated injections of the drug, systemic toxicity due to methotrexate leaking from the joint being prevented by the oral administration of calcium folinate. In preliminary studies in rheumatoid arthritic patients in which inhibitory concentrations of methotrexate were maintained in the joint for up to 48 h, they failed to produce a favourable therapeutic response. However, the feasibility of such a treatment regimen would obviously be improved by the use of liposomal methotrexate, since the requirement for multiple injections would be obviated.

In the current study, free methotrexate injected i.a. at the time of antigen challenge had little if any effect on joint pathology, but caused a modest 20–30% reduction in joint swelling and temperature. The onset of the beneficial effect was rapid and it persisted for the duration of the study. However, free methotrexate was ineffective in suppressing any of the parameters of inflammation in an established arthritis of 7 days or longer duration. This finding of lack of efficacy in an established arthritis is in accord with studies on antigen-induced arthritis in mice, where systemic administration of methotrexate (1·5 mg $kg^{-1}$ daily) was found to suppress arthritis only if therapy was initiated at the time of antigen challenge, but to be ineffective if delayed for 14 days (Crossley et al 1987).

Liposome-entrapped methotrexate injected i.a. was approximately 10-fold more potent than the free drug in suppressing the development of arthritis when injected at the time of antigen challenge. Liposomal methotrexate was also effective in suppressing arthritis of 7 days duration, but not an established arthritis of 21 days. Increasing the dose of liposomal methotrexate 10-fold to 1 mg did not enhance its beneficial effects. This was disappointing, since we have shown liposome-entrapment to greatly prolong the retention of methotrexate in arthritic joints, up to 80-fold more methotrexate being recovered from the joints 24 h after injection of the liposomal drug (Foong & Green 1988b). However, not all of the liposomal methotrexate injected would be immediately available to exert an effect, which could account for the beneficial effect of liposomal methotrexate on joint swelling and temperature being delayed in onset. Thus, uptake of liposomal methotrexate by the diseased synovium in rabbits with an established antigen-induced arthritis has been shown to be only approximately 4% of the amount injected in the first 24 h (Foong & Green 1988b). The low availability of methotrexate might have been partly due to the liposomes having a high cholesterol content. The liposomes were formulated with a high cholesterol content because they had high stability to drug leakage in the presence of synovial fluid (White et al 1983), but a high cholesterol content will also reduce endocytic uptake of liposomes (Foong & Green 1988a). In retrospect, liposomes with a lower cholesterol content might have been more readily taken up by the diseased synovium and hence more effective in suppressing synovitis. Also, in this study, relatively large amounts of liposomal lipid were injected (up to 33 μmol), which would tend to saturate the limited endocytic capacity of the synovium (Foong & Green 1988a). Recent developments enabling cytotoxic drugs to be entrapped in liposomes more efficiently (Mayer et al 1986, 1990) should enable the amount of liposomal lipid injected to be greatly reduced. The endocytic uptake of liposomes by the rheumatoid synovium might also be enhanced by attention to the choice of liposomal lipids and to liposome size. The initial irritant effect of liposomal methotrexate on injected joints might be suppressed by the co-administration of an anti-inflammatory corticosteroid. Franchi et al (1989) achieved a favourable response in rheumatoid arthritis when i.a. methotrexate was co-administered with orgotein, an anti-inflammatory protein which inhibits superoxide dismutase.

Providing that the liposome formulation can be optimized, prospects for the successful clinical use of i.a. liposomal methotrexate are encouraging. Undoubtedly, if disease modifying anti-rheumatic drugs are to exert a local effect in arthritic joints, formulating them in liposomes for i.a. administration should improve their utility and reduce systemic toxicity.

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