British Journal of Pharmacology (1999) 128, 234–240
http://www.stockton-press.co.uk/bjp

Interleukin-1β (IL-1β) inhibition: a possible mechanism for the anti-inflammatory potency of liposomally conjugated methotrexate formulations in arthritis

*,[1]A.S. Williams, [1]S.G. Jones, [1]R.M. Goodfellow, [1]N. Amos & [1]B.D. Williams

[1]Rheumatology Research Laboratory, University of Wales College of Medicine, Heath Park, Cardiff, CF4 4XN

1 Liposomes with conventional and long-circulation times were employed as carriers for the methotrexate derivative MTX-γ-DMPE (MTX-EPC and MTX-PEG respectively), their mechanism of action was investigated *in vitro* and *in vivo* and their therapeutic efficacy assessed using the rat collagen-induced arthritis (CIA) model.

2 At non-toxic dose, both MTX-EPC and MTX-PEG inhibited the lipopolysaccharide (LPS) induced release of IL-1β from activated rat peritoneal macrophages (rPMΦ) in a dose and time dependent manner. Free methotrexate (MTX) was not active in this respect. After a single intravenous injection (i.v.), and at equivalent doses, both free MTX (500 μg) and MTX-EPC inhibited the LPS induced rise in plasma IL-1β levels observed in MTX-PEG and saline treated rats.

3 When used to treat established CIA, MTX-EPC resulted in significantly lower clinical score (CS) (1.0 ± 0.42 ($P<0.001$)) and hind paw diameter (HPD) (6.5 ± 0.34 mm ($P<0.001$)) measurements than controls (3.0 ± 0.26; 7.33 ± 0.41 mm), after only two i.v. doses, and remained significantly lower for the entire experimental period. By day 24 both CS (2 ± 0.61 ($P<0.001$)) and HPD (6.97 ± 0.25 mm ($P<0.002$)) measurements had also become significantly lower in MTX-PEG treated rats than in saline treated controls (3.62 ± 0.17, 7.92 ± 0.38 mm) and remained lower until day 30. Joint inflammation in MTX treated rats was completely ameliorated by day 20 but the health and well being of the animals was compromised and the experiment terminated at this time-point.

4 Our results clearly demonstrate that both MTX-EPC and MTX-PEG liposomes have potential for development into therapeutic modalities for the treatment of inflammatory joint disease in man.

Keywords: Liposomes; methotrexate; collagen-induced arthritis; interleukin-1β

Abbreviations: CIA, collagen-induced arthritis; CHOL, cholesterol; CS, clinical score; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidyl choline; HPD, hind paw diameter; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MPS, mononuclear phagocyte system; MTX, methotrexate; MTX-γ-DMPE, methotrexate-γ-dimyristoylphosphatidylethanolamine; MTX-EPC, liposomes (100 nm) containing MTX-γ-DMPE and the phospholipids EPC : CHOL : PA in a molar ratio of 7 : 2 : 1; MTX-PEG, liposomes (100 nm) containing MTX-γ-DMPE and the phospholipids DSPC : CHOL : PEG in a molar ratio of 10 : 5 : 1; PA, phosphatidic acid; PEG, distearoylphosphatidylethanolamine conjugated to polyethyleneglycol; RA, rheumatoid arthritis; rPMΦ, rat peritoneal macrophages; SUV, Small unilamellar vesicles

Introduction

Oral MTX provides an effective treatment for rheumatoid arthritis (RA); adverse events occur frequently but are generally reversed by cessation of MTX therapy (Furst & Kremer, 1988; Sinnett *et al*., 1989; Fries *et al*., 1990). In an attempt to reduce these effects whilst maintaining MTX's anti-inflammatory efficacy alternative routes of administration and drug formulations have been investigated (Franchi *et al*., 1989; Wilke *et al*., 1987; Foong & Green, 1992). The results to date have generally been disappointing and have been attributed to poor formulation characteristics resulting in low drug concentrations being maintained within the inflamed joint.

Liposomes are microvesicles composed of continuous bilayers of phospholipid surrounding an aqueous phase. They have been adopted by numerous researchers as the vehicle of choice for drug (Asao *et al*., 1992; Janoff, 1992; Chopra *et al*., 1991) and vaccine (Gregoriadis, 1990; Alving, 1991) delivery and targeting. After parenteral administration, liposomes have been most successfully used in the treatment of human disease where the mononuclear phagocyte system (MPS) is the disease tissue target site. This natural targeting has been exploited in the treatment of Leishmaniasis and systemic fungal infections where liposome encapsulated agents are more effective and less toxic than the free drug (Alving, 1983; Lopez-Berestein *et al*., 1985).

Following systemic administration so-called conventional liposomes, of approximately 100 nm in size, containing the phospholipids; egg phosphatidyl choline (EPC), cholesterol (CHOL) and phosphatidic acid (PA) in a molar ratio of 7 : 2 : 1 accumulate in the actively inflamed joints of patients with RA and in the inflamed paw tissue of rats with adjuvant induced arthritis (O'Sullivan *et al*., 1988; Williams *et al*., 1986; 1987; Love *et al*., 1989; 1990). The utility of these liposomes for delivering encapsulated therapeutic agents to sites of inflammation may, however, be severely limited by their rapid removal from the blood by cells of the MPS resulting in circulation half-lives of approximately 15 min after intravenous administration. Recently liposomes with increased stability in the circulation and which avoid rapid uptake by the MPS have been introduced; typical formulations contain

*Author for correspondence.

the high phase transition temperature phospholipid distearoylphosphatidylcholine (DSPC), CHOL and distearoylphosphatidyl-ethanolamine conjugated to polyethyleneglycol (PEG) (Klibanov *et al*., 1990; Papahadjopoulos *et al*., 1991; Blume & Cevc, 1990). Since these so-called long circulating liposomes exhibit dramatically different pharmacokinetic and distribution profiles, with circulation half-lives extended to hours rather than minutes, new therapeutic opportunities have arisen, these include enhanced efficacy of delivery of antineoplastic agents to tumors.

We have previously described the synthesis and characterization of a lipophilic derivative of MTX and the phospholipid dimyristoylphosphatidylethanolamine (MTX-γ-DMPE) (Williams *et al*., 1992). The analogue was formed with a view to increasing MTX loading and improving the drugs' retention within a liposomal delivery system. Thus, provided that liposomal characteristics are defined for optimal localization a drug such as MTX may be targeted to the site of inflammation.

In this paper we investigate the therapeutic potential of conventional and long circulating liposomal preparations containing MTX-γ-DMPE. Their effects were studied *in vitro*, upon LPS induced IL-1β synthesis, and *in vivo*, upon LPS induced plasma IL-1β levels and the treatment of established joint inflammation in the rat CIA model. These studies were undertaken with a view to developing a safe and effective delivery system for MTX to treat arthritis in man.

Methods

Synthesis of MTX-γ-DMPE

MTX-γ-DMPE was formed by the covalent attachment of the phospholipid dimyristoylphosphatidylethanolamine (DMPE) to the γ-carboxylic acid residue in the glutamyl portion of the MTX molecule by an amide linkage. The synthesis and characterization of MTX-γ-DMPE has been described previously (Williams *et al*., 1992).

Liposome preparation

Liposomes were prepared by hydrating dry phospholipid films with 0.9% saline. Small unilamellar vesicles (SUV's, 100 nm) were formed by probe sonication (MSE Soniprep 150) and were sized by photon correlation spectroscopy (Autosizer Lo-C and series 7032 multi-8 Correlator; Malvern Instruments Limited, Malvern, U.K.). Conventional SUV's contained EPC, CHOL and PA to a molar ratio of 7 : 2 : 1 (Lipid Products, Crabhill Lane, S. Nutfield, U.K.) and long-circulating SUV's contained DSPC (Genzyme, West Malling, U.K.), CHOL (Sigma Chemical Company, Poole, U.K.) and PEG (Genzyme, U.K.) in a molar ratio of 10 : 5 : 1. Predetermined quantities of MTX-γ-DMPE were added to the respective lipid combinations prior to hydration with 0.9% saline (MTX-EPC and MTX-PEG respectively).

Induction and collection of macrophages

The induction and collection of rat peritoneal macrophages has been described previously (Williams *et al*., 1994b,c). Briefly, activated macrophages were isolated from the peritoneal cavity 3 days post-injection with 4% brewers' thioglycollate medium. After purification the cell population contained $98 \pm 0.8\%$ macrophage (mean $\pm$ s.e.mean; $n = 6$), as determined by α-Naphthyl acetate esterase staining and their viability, as judged by trypan blue exclusion, was found to exceed 95%.

Macrophages (2×10^5 cells per well) were cultured (37°C, 5% CO_2/air) in 96-well flat bottomed microtitre plates containing minimum essential medium (Sigma, U.K.) supplemented with 10% foetal calf serum (Sigma, U.K.), L-glutamine (2 mM, Flow Laboratories, High Wycombe, U.K.) and penicillin/streptomycin (50 international units/ml and 50 μg/ml respectively; Flow Laboratories, High Wycombe, U.K.).

The concentration of freshly prepared test liposomes (MTX-EPC or MTX-PEG) was adjusted to 300 μg of MTX (as MTX-γ-DMPE) per 3 mg lipid in 1.0 ml and were diluted serially in macrophage culture medium immediately prior to use. Control liposomes (EPC-LIPO and PEG-LIPO) were diluted identically to contain an equivalent concentration of lipid. 100 μl of liposomes or free MTX (Sigma, U.K.) were added to the macrophages in quadruplicate and incubated for 0, 4 or 8 h before 20 μl of lipopolysaccharide (LPS, 0.1 mg/ml; from *Escherichia coli* Serotype 0111 : B4; Sigma, U.K.) was added to each well. The macrophages were incubated with each test agent and stimulated with LPS for 20 h, the resulting supernatants were reserved for IL-1β assay by ELISA.

Macrophage viability at the end of the incubation period was determined by ATP assay (Williams *et al*., 1994b,c). Cellular-associated ATP (%) was then calculated after comparison with untreated macrophage populations.

In vivo *LPS induced IL-1β synthesis*

Small groups of normal Lewis rats (150 g, $n = 3$) were given a single i.v. injection of either free MTX, MTX-EPC, MTX-PEG (at a dose equivalent to 500 μg MTX) or saline. After 24 h, the rats were given a single intra-peritoneal injection of LPS (100 μg/ml, lowest LPS dose to induce a detectable rise in plasma IL-1β). The rats were monitored for a further 5 h (time of peak plasma IL-1β levels) before they were sacrificed and blood samples collected into heparinized tubes. Baseline IL-1β plasma levels were obtained from blood samples taken from a superficial tail vein before MTX or liposome dosing and prior to LPS injection.

Determination of interleukin-1β

IL-1β levels were quantified by immunoradiometric assay following a method previously described (Bristow *et al*., 1991). Affinity-purified sheep anti-rat IL-1β was a kind gift from Dr S Poole at the Division of Endocrinology, National Institute for Biological Standards and Control, Blanche Lane, Hertfordshire.

Induction of collagen arthritis

Bovine type II collagen was extracted from bovine nasal septa by pepsin digestion and extensively purified by differential salt precipitation and dialysis against phosphate buffers (Morgan *et al*., 1992). Male inbred Lewis rats (130 g) were obtained from Bantin & Kingman (Hull, U.K.). The animals were allowed food and water *ad libitum* and were kept in the Biomedical Services Department for 1 week prior to their first immunization. The animals were housed in light–dark cycles of 12 hours. On two occasions, 7 days apart, rats were immunized by an intradermal injection on the back, near the base of the tail (in 4–5 sites), with 0.5 ml of an emulsion of the type II collagen (0.5 mg in 0.05 M acetic acid) and Freunds incomplete adjuvant (1 : 1 v v^{-1}). Day 0 being the day of the first immunization. On day 16 the rats were weighed and divided

into four groups which were matched for arthritis severity by clinical score (CS). The rats were treated daily with either free MTX ($n=5$; 500 μg), MTX-EPC ($n=10$; 500 μg MTX, as MTX-γ-DMPE per 16 mg lipid), MTX-PEG ($n=10$; 500 μg MTX, as MTX-γ-DMPE per 16 mg lipid) or saline ($n=10$) administered intravenously for 7 days.

Arthritis assessments

Assessment of joint inflammation was carried out blindly by an independent observer with no knowledge of the treatment protocol. The severity of the arthritis in each hind paw was quantified, on alternate days, by a CS and hind paw diameter (HPD) measurement. CS was applied to each hind paw in integers from 0 to 5 (0 = normal, 1 = swollen ankle joint, 2 = ankle and up to two other joints swollen, 3 = ankle and up to six other joints swollen, 4 = ankle and more than six other joints involved and 5 = all joints swollen, red and stiff). HPD was measured using a digital micrometer. All rats were humanely killed on day 30 by a Schedule 1 method (Animals (Scientific Procedures) Act 1986).

Pharmacokinetic studies

The clearance of both MTX-EPC and MTX-PEG from the circulation after intravenous administration was quantified. The organ/tissue accumulation of the respective liposomal preparations was also determined in rats with established arthritis using previously described dual labelling methodology (Williams *et al*., 1995; 1996). Briefly, two groups of animals ($n=3$) were injected intravenously with either MTX-EPC or MTX-PEG labelled with ^{3}H-cholesterol. These animals were simultaneously injected with ^{99m}Tc radiolabelled erythrocytes (blood pool marker). Blood samples (50 μl) were collected over a 24 h period and the clearance of the liposomes from the circulation calculated as a percentage of the injected dose remaining in the circulation at specified times. In separate experiments, when 80% liposome clearance from the circulation was achieved, the total specific accumulation of the respective liposome preparations was calculated, as a percentage of the injected dose per gram of tissue.

Statistical analysis of data

The one-way analysis of variance (ANOVA) was used to determine whether CS or HPD (day 16 readings were used as baseline values) were significantly different between MTX, MTX-EPC or MTX-PEG and saline treated control rats. The Student's *t*-test was used to identify which groups were different.

Results

At concentrations equivalent to 15 μg of MTX and 0.15 mg of lipid macrophage cellular ATP levels exceeded 95%, at the end of the incubation period, for MTX-EPC, MTX-PEG, EPC-LIPO, PEG-LIPO and MTX treated cells respectively. At these non-toxic concentrations, both MTX-EPC and MTX-PEG respectively inhibited the LPS induced release of IL-1 ($77\pm2.3\%$; $79\pm4.0\%$; mean $\pm$ s.e.mean % inhibition) from rPMΦ (Figure 1). At concentrations equivalent to 7.5 μg of MTX and 0.07 mg of lipid per well, IL-1β inhibition was significantly greater in MTX-EPC treated rPMΦ ($54\pm3.7\%$, $P<0.01$) than in MTX-PEG ($32\pm4\%$) treated cells. A liposome pre-incubation period of 8 h was required for MTX-PEG, prior to LPS stimulation, before the dose dependent inhibition of IL-1β release from rPMΦ was observed. In contrast, MTX-EPC (7.5 μg per 0.7 mg lipid) added simultaneously with LPS to rPMΦ in culture inhibited IL-1β release to the same degree as cells pre-incubated with MTX-PEG for 8 h prior to LPS stimulation, at an equivalent dose of MTX. The inhibition of IL-1β release was dose and time dependent with MTX-EPC being more potent than MTX-PEG in this respect. Neither free MTX nor empty control liposomal preparations (EPC-LIPO; PEG-LIPO; data not shown) inhibited IL-1β release from LPS stimulated rPMΦ.

In order to assess whether these observations were reproducible *in vivo*, normal rats were given a single i.v. injection of either MTX, MTX-EPC or MTX-PEG, at a dose equivalent to 500 μg of free drug (Figure 2). After 24 h, the LPS induced rise in plasma IL-1 levels was compared between the treatment groups and saline treated controls. There was no significant difference (ANOVA) in plasma IL-1β levels when treatment and control groups were compared either before the treatment protocols were initiated or before they were stimulated with LPS. There was no significant difference in plasma IL-1β levels (mean $\pm$ s.e.mean) pre- and post-LPS stimulation in either MTX (111 ± 43, 115 ± 44 pg ml^{-1}) or MTX-EPC (79 ± 11, 104 ± 25 pg ml^{-1}) treated animals respectively. However, plasma IL-1β levels (mean $\pm$ s.e.mean) were significantly different pre- and post-LPS stimulation rising from 37 ± 12 pg ml^{-1} and 57.48 ± 12 pg ml^{-1} to 148 ± 10 pg ml^{-1} and 208 ± 48 pg ml^{-1} in MTX-PEG ($P<0.002$) and saline ($P<0.03$) treated animals respectively.

The time dependent clearance of MTX-EPC and MTX-PEG from the circulation following intravenous administration is shown in Figure 3. The time for 80% of the injected dose to clear from the circulation was significantly longer with MTX-PEG (24 h; ($P<0.001$)) than with MTX-EPC (1.5 h).

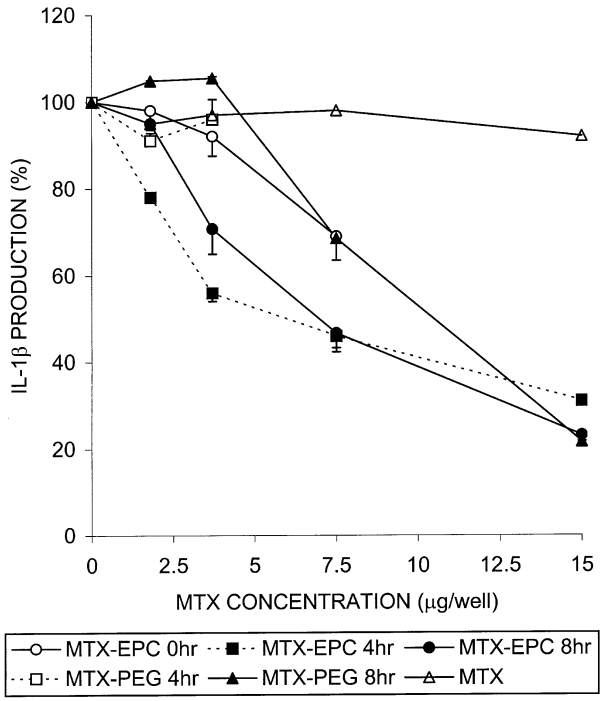

Figure 1 The time and dose dependent inhibition of IL-1β release from LPS stimulated, thioglycollate elicited, rat peritoneal macrophages after incubation with either free MTX, MTX-EPC or MTX-PEG *in vitro*.

The specific accumulation of each liposome preparation after 80% clearance from the circulation is shown in Table 1.

The effects of MTX-EPC and MTX-PEG upon the progression of rat collagen induced arthritis are shown in Figures 4a,b. Cumulative data from two separate experiments are represented in these figures. On day 16, 78% of the rats which had been immunized on day 0, and day 7 exhibited moderate hind paw inflammation with (mean ± s.e.mean) CS of 2.61 ± 0.19 and HPD of 7.09 ± 0.39 mm. Liposome treatment was initiated on day 16. From day 16 to 22 neither CS nor HPD measurements in MTX-PEG treated rats were significantly different from saline treated controls. After only two intravenous doses MTX-EPC resulted in significantly

Table 1 Specific tissue/organ accumulation of MTX-EPC and MTX-PEG after intravenous administration to arthritic rats

	MTX-EPC	*MTX-PEG*
Liver	0.7 ± 0.12	0.13 ± 0.11*
Spleen	1.41 ± 0.21	1.31 ± 0.21
Arthritic joint	0.10 ± 0.02	0.33 ± 0.07**

Animals were sacrificed after 80% of the liposome dose had cleared from the circulation at 1.5 h and 24 h respectively for MTX-EPC and MTX-PEG. Mean accumulation as the percentage of the injected dose per gram of the tissue ± s.e.-mean ($n = 3$); $*P < 0.005$, $**P < 0.01$ for each pair of data.

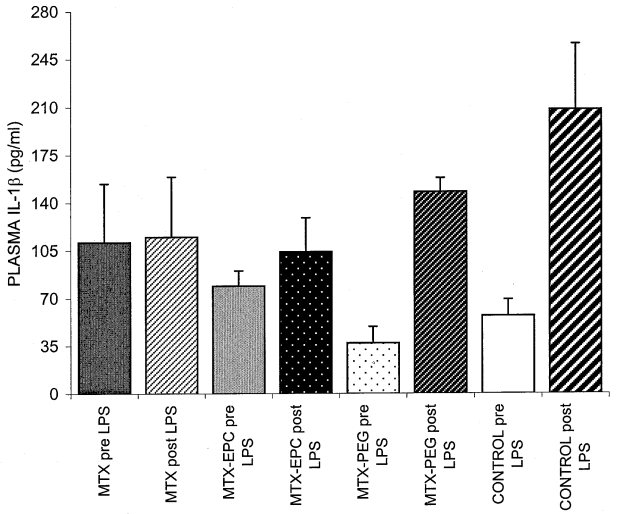

Figure 2 *In vivo*, LPS induced plasma IL-1β levels in normal rats pre-treated with, either free MTX, MTX-EPC, MTX-PEG or saline.

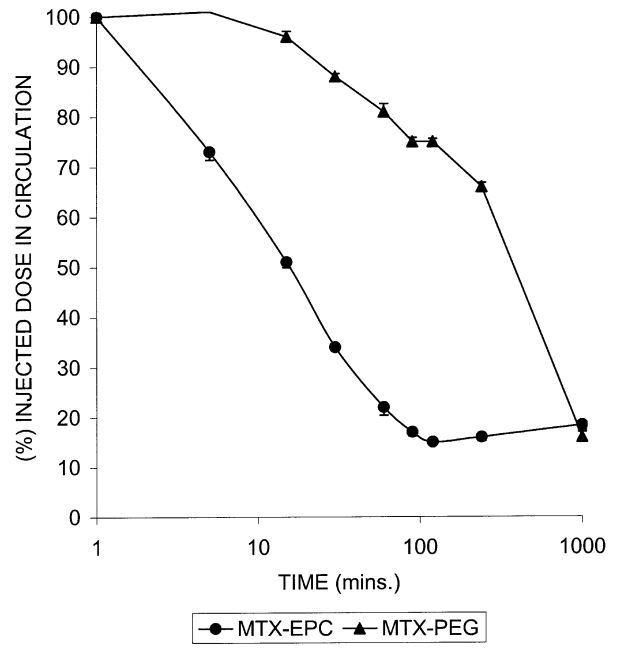

Figure 3 Time dependent clearance of MTX-EPC and MTX-PEG from the circulation following intravenous administration.

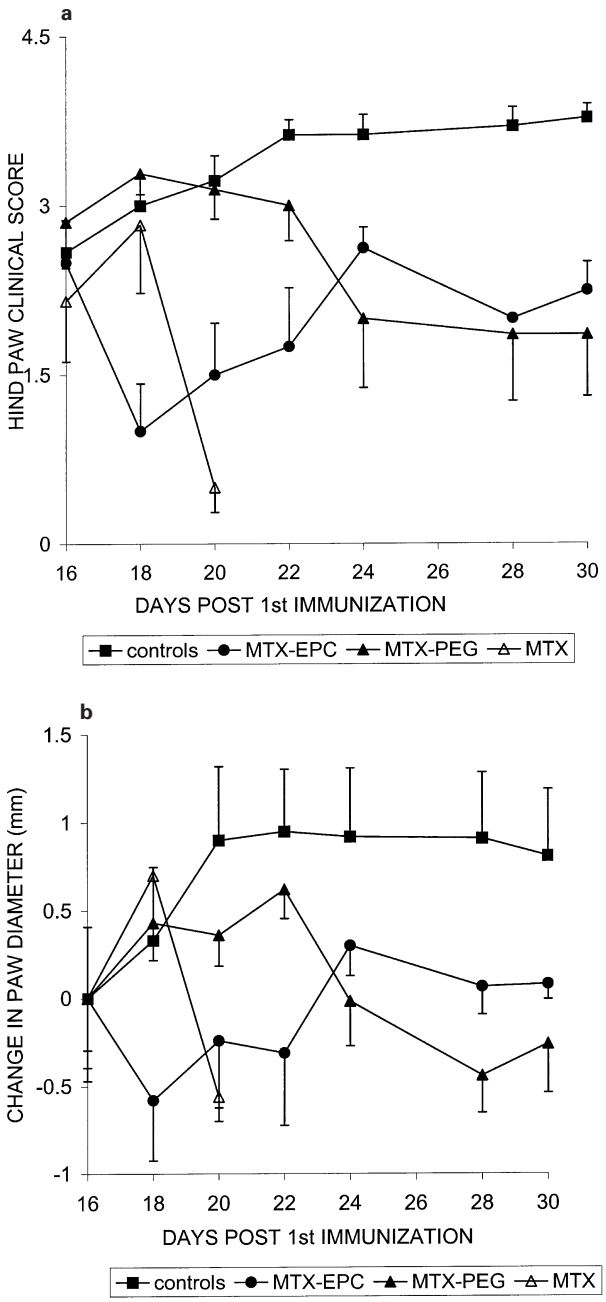

Figure 4 The effect of free MTX, MTX-EPC, MTX-PEG and saline treatment upon the progression of joint inflammation, as assessed by clinical score (a) and hind paw diameter (b), in rats with collagen-induced arthritis.

lower CS (1.0 ± 0.42 ($P < 0.001$)) and HPD (6.5 ± 0.34 mm ($P < 0.001$)) measurements than controls (3.0 ± 0.26; 7.33 ± 0.41 mm) and remained significantly lower for the entire experimental period. By day 22 hind paw inflammation as assessed by CS only (mean $\pm$ s.e.mean) in MTX-PEG (3.0 ± 0.3 ($P < 0.05$)) treated rats was significantly lower than in control rats (3.62 ± 0.13). However, by day 24 both CS (2 ± 0.61 ($P < 0.001$)) and HPD (6.97 ± 0.25 mm ($P < 0.002$)) measurements were significantly lower in MTX-PEG treated rats than in saline treated controls (3.62 ± 0.17, 7.92 ± 0.38 mm) and remained lower until day 30 when the experiment ended.

Joint inflammation in MTX treated rats was completely ameliorated in each experimental animal by day 20, however, the rats had diarrhoea, vasculitic areas were visible on their fore-paws and their weight (mean $\pm$ s.e.mean; 144 ± 2.5 g ($P < 0.01$)) had dropped significantly compared with control rats (161 ± 3.0 g). Under these circumstances this experiment was terminated, the animals were sacrificed and blood samples taken by cardiac puncture for haematological assessment (Table 2). The experiment was repeated, excluding the MTX treated group, and was allowed to run to day 30. Day 22 blood counts in MTX-EPC treated rats were not significantly different from controls, however, white blood cell ($1.0 \pm 0.2 \times 10^9\ l^{-1}$ ($P < 0.001$)) and platelet ($49 \pm 49 \times 10^9\ l^{-1}$ ($P < 0.0002$)) counts were significantly lower in MTX treated rats than in control or MTX-EPC treated rats.

Discussion

Our present study demonstrates that, both MTX-EPC and MTX-PEG are potent inhibitors of LPS induced IL-1β release from rPMΦ and that at their highest, non-toxic incubation concentration there was no significant difference in inhibitory activity. *In vitro* IL-1β inhibition was dependent on both incubation time and liposome dose. Although both MTX-EPC and MTX-PEG inhibited IL-1β release in a dose dependent fashion, MTX-EPC had 2 fold greater anti-inflammatory potency than MTX-PEG. MTX-EPC concentrations of 7.5 μg well^{-1} had equivalent inhibitory potency to MTX-PEG at 15 μg well^{-1}. Free MTX did not inhibit mediator release under the experimental conditions described. Our preliminary experiments showed that the *in vitro* uptake of radio-labelled SUV's containing EPC : CHOL : PA (7 : 2 : 1) by activated rat macrophages was 3 fold greater than liposomes containing DSPC:CHOL:PEG (10 : 5 : 1) after 20 h incubation (data not shown). Consequently, the greater inhibitory potency of MTX-EPC compared with MTX-PEG, is probably a reflection of the preferential uptake of MTX-EPC than MTX-PEG by macrophages.

Table 2 Haematology of blood isolated from rats with collagen-induced arthritis treated with either free MTX or liposomal conjugated methotrexate

Haematological parameter	*Treatment protocol*			
	Control	*MTX*	*MTX-EPC*	*MTX-PEG*
Red blood cells ($\times 10^{12}\ l^{-1}$)	8.7 ± 0.2	3.2 ± 3.2	7.6 ± 0.3	7.7 ± 0.1
White blood cells ($\times 10^9\ l^{-1}$)	15 ± 1.8	1.0 ± 0.2*	9 ± 0.3	10.8 ± 0.9
Platelets ($\times 10^9\ l^{-1}$)	1014 ± 57	49 ± 49**	777 ± 58	487 ± 16***

There was no significant difference in red cell count when MTX, MTX-EPC, MTX-PEG and controls were compared (ANOVA). The white blood cell (ANOVA; $P < 0.003$) and platelet (ANOVA; $P < 0.01$) counts were significantly different when the groups were compared. *$P < 0.001$; **$P < 0.0002$ and ***$P < 0.001$ (t-test, when compared with controls).

Macrophages play a central role in chronic synovial inflammation, after activation they are capable of synthesizing mediators, such as the eicosanoid PGE_2 and the cytokines TNFα and IL-1β. In turn, these secretory products induce the production of a variety of enzymes which initiate cartilage and bone destruction (Bresnihan, 1992; Hopkins, 1991; Malone *et al*., 1984; Shore *et al*., 1986).

Several cytokines including TNFα, IL-1β, interferon (INFγ) (Williams *et al*., 1993) and transforming growth factor (TGFβ) have been implicated in the pathogenesis of CIA. Both TNFα and TGFβ accelerated the onset of rat CIA after intra-articular injection into the ankle joints (Cooper *et al*., 1992) and human recombinant IL-1β accelerated the onset and progression of CIA in mice and rats after subcutaneous administration (Hom *et al*., 1991; Hom *et al*., 1988). Thus, inhibition of these cytokines would be expected to exert anti-inflammatory effects in this animal model. This has been demonstrated by the administration of neutralizing anti-TNFα or anti-IL-1β antibodies which ameliorated joint disease in murine CIA (Van den Berg *et al*., 1994, Williams *et al*., 1992; 1994d; Geiger *et al*., 1993).

If the results of our *in vitro* cytokine studies were reproduced *in vivo* then we would speculate that both MTX-EPC and MTX-PEG would have anti-inflammatory activity in experimental models of arthritis, such as, rat collagen-induced arthritis.

Our present study clearly demonstrates that both free MTX and MTX-EPC completely inhibit the LPS induced rise in plasma IL-1β noted in rats treated with either MTX-PEG or saline. These data confirm an anti-inflammatory mechanism by which liposomally conjugated MTX could exert therapeutic efficacy in experimental models of arthritis.

The results of our *in vivo* treatment protocols demonstrate that both MTX-EPC and MTX-PEG have potent anti-inflammatory activity when applied as therapy in established rat CIA. Although MTX was efficacious it was also toxic, which necessitated cessation of therapy. Consequently, a free MTX treatment group was not followed to day 30.

We first demonstrated that liposomes containing MTX-γ-DMPE provided a safe and effective delivery system for the treatment of joint inflammation in the rat adjuvant-induced arthritis model. In these studies rats were dosed daily with MTX-EPC (100 μg MTX-γ-DMPE) by intravenous injection for 7 days, a fifth of the dose used in our present study, and although disease progression was inhibited it was not ameliorated in any of the treated rats (Williams *et al*., 1994a). At equivalent MTX or lipid doses respectively, inflammatory parameters in MTX, EPC-LIPO and untreated control rats were not significantly different to one another but were significantly higher than those reported in MTX-EPC treated animals. Furthermore, haematological data collected at the termination of this experiment demonstrated that free MTX (100 μg daily for 7 days) treatment produced a significant decrease in reticulocyte, red blood cell and white blood cell counts when compared with MTX-EPC, EPC-LIPO or untreated controls. In light of these data, and due to home office constraints, we were unable to continue with dose ranging studies in either the adjuvant- or the collagen-induced arthritis models. In our present study, rats with CIA were given seven daily doses of MTX-EPC (500 μg MTX-γ-DMPE) by intravenous injection. On day 16, when MTX-EPC treatment was initiated, 8/10 hind paws were moderately affected by arthritis. After 2 days disease was completely

ameliorated in three of these eight paws and of the five remaining arthritic paws two had CS's of 1 (mild arthritis). For the first time we have been able to demonstrate that MTX-EPC ameliorates joint inflammation in established rat arthritis. CIA in MTX-EPC treated rats then appears to progresses to day 22, albeit at a significantly lower level than in either MTX-PEG or saline treated animals, with 7/10 paws affected with arthritis by end of the experimental period. This may be explained by our previous observations in the rat adjuvant-induced arthritis model where MTX-EPC exerted significant anti-inflammatory effect on established joint inflammation but it did not affect the progression of the disease when administered prophylactically (Williams *et al*., 1995). Adjuvant-induced arthritis, like CIA, represents a T-lymphocyte driven, chronic, erosive polyarthritis with similar clinical features to RA. Our present data supports the observations made in rat adjuvant arthritis and infers that macrophages rather than T-lymphocytes are the cellular target for liposomally conjugated MTX in CIA.

On day 24 MTX-PEG begins to exert an anti-inflammatory effect on rat CIA, 7 days after MTX-EPC. The effective delivery of drugs using liposomes is dependent upon the quantitative retention of the drugs by the carrier, the rate of clearance from the circulation and their uptake by the target tissue. These criteria have been shown to be intimately connected with liposome composition. We previously showed that MTX-EPC inhibited tumor necrosis factor (TNFα) and prostaglandin (PGE_2) release from lipopolysaccharide (LPS) stimulated rat peritoneal macrophages (rPMΦ) (Williams *et al*., 1994c). In these experiments the macrophages were pre-incubated with the conjugate or MTX-EPC for 3.5 h prior to stimulation with LPS; almost complete mediator inhibition was achieved without cellular toxicity. Under these experimental conditions MTX-PEG did not inhibit mediator release. In our present study we found that a minimum pre-incubation period of 8 h prior to LPS stimulation was necessary to observe any mediator inhibition with MTX-PEG. This differential effect is probably a reflection of the time dependent uptake of MTX-PEG and MTX-EPC by the target cell, the macrophage. This could explain why MTX-EPC exerts its beneficial effects in CIA prior to MTX-PEG.

The insertion of MTX-γ-DMPE into these liposomal formulations has enabled us to examine whether avoidance of recognition of long circulating liposomal preparations by the MPS, with the associated possibility of enhanced accumulation in inflammatory tissue, could increase the therapeutic efficacy of MTX-γ-DMPE. As previously discussed, the effective delivery of drugs using liposomes is dependent upon the quantitative retention of the drugs by the carrier, the rate of clearance from the circulation and their uptake by the target tissue. Although the circulation time of MTX-γ-DMPE, following intravenous administration was extended in MTX-PEG compared with MTX-EPC, the uptake of the active constituent of the liposomes (MTX-γ-DMPE) by the target cell (the macrophage) may have been compromised during the early treatment phase.

In conclusion, a conventional liposome preparation with a short circulation time such as MTX-EPC delivers MTX-γ-DMPE more efficiently to macrophages within the inflamed joint and has greater anti-inflammatory potency than a long circulating liposome preparation such as MTX-PEG, in the short term. In the long term however, liposomal preparation such as MTX-PEG, with an extended circulation time following intravenous administration, also exert potent anti-inflammatory effects in rat arthritis. This anti-inflammatory potency may be mediated through inhibition of IL-1β synthesis. Both MTX-EPC and MTX-PEG liposomes have potential for development into therapeutic modalities for the treatment of inflammatory joint disease in man.

A.S. Williams would like to thank the Wales Office of Research and Development for their financial support during the course of this study.

References

ALVING, C.R. (1983). Delivery of encapsulated drugs to macrophages. *Pharmacol. Ther.,* **22,** 407 – 424.

ALVING, C.R. (1991). Liposomes as carriers of antigens and adjuvants. *J. Immunol. Methods.,* **140,** 1 – 13.

ASAO, T., SHIBATA, H.R., BATIST, G. & BRODT, P. (1992). Eradication of hepatic metastases of carcinoma H-59 by combination chemotherapy with liposomal muramyl tripeptide, 5-fluorouracil and leucovorin. *Cancer Res.,* **52,** 6254 – 6257.

BLUME, G. & CEVC, G. (1990). Liposomes for the sustained drug release *in vivo*. *Biochim. et Biophys. Acta.,* **1029,** 91 – 97.

BRESNIHAN, B. (1992). The synovial lining cells in chronic arthritis. *Br. J. Rheumatol.,* **31,** 433 – 436.

BRISTOW, A.F., MOSLEY, K. & POOLE, S. (1991). Interleukin-1β production in vivo and in vitro in rats and mice measured using specific immunoradiometric assays. *J. Mol. Endocrinol.,* **7,** 1 – 7.

CHOPRA, R., BLAIR, S., STRANG, J., CERVI, P., PATERSON, K.G. & GOLDSTONE, A.H. (1991). Liposomal amphoteracin B (AmBisome) in the treatment of fungal infections in neutropenic patients. *J. Antimicrob. Chemother.,* **28,** B93 – B104.

COOPER, W.O., FAVA, R.A., GATES, C.A., CREMER, M.A. & TOWNES, A.S. (1992). Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumor necrosis factor or transforming growth factor-beta. *Clin. Exp. Immunol.,* **89,** 244 – 250.

FOONG, W.C. & GREEN, K.L. (1992). Treatment of antigen induced arthritis in rabbits with liposome-entrapped methotrexate injected intra-articularly. *J. Pharm. Pharmacol.,* **45,** 204 – 209.

FRANCHI, F., SEMINARA, P., CODACCI-PISANE, G., ARONNE, T., AVELLA, A. & BONOMO, L. (1989). Intra-articular methotrexate in the therapy of rheumatoid arthritis. *Recent Prog. Med.,* **5,** 261 – 262.

FRIES, J., SINGH, G., LENERT, L. & FURST, D.E. (1990). Aspirin, hydroxychloroquine and hepatic enzyme abnormalities with methotrexate in rheumatoid arthritis. *Arth. and Rheum.,* **33,** 1611 – 1615.

FURST, D.E. & KREMER, J.M. (1988). Methotrexate in rheumatoid arthritis. *Arth. and Rheum.,* **31,** 305 – 314.

GEIGER, T., TOWBIN, H., COSENTI-VARGAS, A., ZINGEL, O., ARNOLD, J., RORDORF, C., GLATT, M. & VOSBECK, K. (1993). Neutralization of interleukin-1β activity *in vivo* with a monoclonal antibody alleviates collagen-induced arthritis in DBA/1 mice and prevents the associated acute-phase response. *Clin. Exp. Rheumatol.,* **11,** 515 – 522.

GREGORIADIS, G. (1990). Immunological adjuvants: a role for liposomes. *Immunol. Today,* **11,** 89 – 97.

HOM, J.T., BENDELE, A.M. & CARLSON, D.G. (1988). *In vivo* administration with IL-1 accelerates the development of collagen-induced arthritis in mice. *J. Immunol.,* **141,** 834 – 841.

HOM, J.T., GLISZCZYNSKI, V.L., COLE, H.W. & BENDELE, A.M. (1991). Interleukin-1 mediated acceleration of type II collagen-induced arthritis: Effects of anti-inflammatory or anti-arthritis drugs. *Agents and Actions,* **33,** 300 – 309.

HOPKINS, S.J. (1991). Cytokines and eicosanoids in rheumatic diseases. *Ann. Rheum. Dis.,* **49,** 207 – 211.

JANOFF, A.S. (1992). Lipids, liposomes and rationale drug design. *Lab. Invest.,* **66,** 655–658.

KLIBANOV, A.L., MARUYAMA, K., TORCHILIN, V.P. & HUANG, L. (1990). Amphipathic polyethyleneglycols prolong the circulation time of liposomes. *FEBS Lett.,* **268,** 235–237.

LOPEZ-BERESTEIN, G., FAINSTEIN, V., HOPFER, R.L., JULIANO, R.L. & BODEY, G.P. (1985). Liposomal Amphoteracin B for the treatment of systemic fungal infections in patients with cancer. *J. Infect. Dis.,* **4,** 704–710.

LOVE, W.G., AMOS, N., KELLAWAY, I.W. & WILLIAMS, B.D. (1989). Specific accumulation of technetium-99 m radiolabelled negative liposomes in the inflamed paws of rats with adjuvant induced arthritis. *Ann. Rheum. Diseases,* **48,** 143–148.

LOVE, W.G., AMOS, N., KELLAWAY, I.W. & WILLIAMS, B.D. (1990). Specific accumulation of cholesterol-rich liposomes in the inflammatory tissue of rats with adjuvant-induced arthritis. *Ann. Rheum. Diseases.,* **49,** 611–614.

MALONE, D.G., WAHL, S.M., TSOKOS, M., CATTELL, H., DECKER, J.L. & WILDER, R.L. (1984). Immune function in severe, active rheumatoid arthritis. A relationship between peripheral blood mononuclear cell proliferation to soluble antigens and synovial tissue immunohistologic characterisation. *J. Clin. Invest.,* **74,** 1173–1185.

MORGAN, K., REEVE-STEPHENSON, J.O, DENTON, A.J. & FREEMONT, A.J. (1992). Type II collagen induced arthritis: comparison of histological changes in arthritis-susceptible and arthritis-resistant rats. *Clin. Exp. Rheumatol.,* **10,** 109–116.

O'SULLIVAN, M.M., POWELL, N., FRENCH, A.P., WILLIAMS, K.E., MORGAN, J.R. & WILLIAMS, B.D. (1988). Inflammatory joint disease: a comparison of liposome scanning, bone scanning and radiography. *Ann. Rheum. Diseases,* **47,** 485–491.

PAPAHADJOPOULOS, D., ALLEN, T.M., GABIZON, A., MAYHEW, E., MATTHAY, K., HUANG, S.K., LEE, K.D., WOODLE, M.C., LASIC, D.D, REDEMANN, C. & MARTIN, F.J. (1991). Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci: U.S.A.,* **88,** 11460–11464.

SHORE, A., JAGLAL, S. & KEYSTONE, E.C. (1986). Enhanced interleukin-1 generation by monocytes *in vitro* is temporarily linked to an early event in the onset of exacerbation of rheumatoid arthritis. *Clin. Exp. Immunol.,* **65,** 293–302.

SINNETT, M.J., GROFF, G.D., RADDATZ, D.A., FRANCK, W.A. & BERTINO, J.S. (1989). Methotrexate pharmacokinetics in patients with rheumatoid arthritis. *J. Rheumatol.,* **16,** 745–749.

VAN DEN BERG, W.B., JOOSTEN, L.A.B., HELSEN, M. & VAN DE LOO, F.A.J. (1994). Amelioration of established murine collagen-induced arthritis with anti-IL-1 treatment. *Clin. Exp. Immunol.,* **95,** 237–243.

WILKE, W.S., BIRO, J.A. & SEGAL, A.M. (1987). Methotrexate in the treatment of rheumatoid arthritis and connective tissue diseases. *Clev. Clin. J. Med.,* **54,** 327–328.

WILLIAMS, A.S., CAMILLERI, J.P., AMOS, N. & WILLIAMS, B.D. (1995). Differential effects of methotrexate and liposomally conjugated methotrexate in rat adjuvant induced arthritis. *Clin. Exp. Immunol.,* **102,** 560–565.

WILLIAMS, A.S., CAMILLERI, J.P., GOODFELLOW, R.M. & WILLIAMS, B.D. (1996). A single intra-articular injection of liposomally conjugated methotrexate suppresses joint inflammation in rat antigen-induced arthritis. *Br. J. Rheumatol.,* **35,** 719–724.

WILLIAMS, A.S., CAMILLERI, J.P. & WILLIAMS, B.D. (1994a). Suppression of adjuvant-induced arthritis by liposomally conjugated methotrexate in the rat. *Br. J. Rheumatol.,* **33,** 530–533.

WILLIAMS, A.S., LOVE, W.G. & WILLIAMS, B.D. (1992). Synthesis of methotrexate-dimyristoylphosphatidylethanolamine analogs and characterisation of methotrexate release *in vitro*. *Int. J. Pharm.,* **85,** 189–197.

WILLIAMS, A.S., TOPLEY, N., AMOS, N. & WILLIAMS, B.D. (1994b). Effect of three lipophilic methotrexate derivatives upon mediator release by lipopolysaccharide-stimulated rat peritoneal macrophages. *J. Pharm. Pharmacol.,* **46,** 291–295.

WILLIAMS, A.S., TOPLEY, N. & WILLIAMS, B.D. (1994c). Effect of liposomally encapsulated MTX-DMPE conjugates upon TNFα and PGE_2 release by lipopolysaccharide stimulated rat peritoneal macrophages. *Biochim. et Biophys. Acta,* **1225,** 217–222.

WILLIAMS, B.D., O'SULLIVAN, M.M., SAGGU, G.S., WILLIAMS, K.E., WILLIAMS, L.A. & MORGAN, J.R. (1986). Imaging in rheumatoid arthritis using liposomes labelled with technetium. *Br. Med. J.,* **293,** 1143–1144.

WILLIAMS, B.D., O'SULLIVAN, M.M., SAGGU, G.S., WILLIAMS, K.E., WILLIAMS, L.A. & MORGAN, J.R. (1987). Synovial accumulation of technetium labelled liposomes in rheumatoid arthritis. *Ann. Rheum. Diseases,* **46,** 314–318.

WILLIAMS, R.O., FELDMANN, M. & MAINI, R. (1992). Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA.,* **89,** 9784–9788.

WILLIAMS, R.O., MASON, L.J., FELDMANN, M. & MAINI, R. (1994d). Synergy between anti-CD4 and anti-tumor necrosis factor in the amelioration of established collagen-induced arthritis. *Proc. Natl. Acad Sci. USA.,* **91,** 2762–2766.

WILLIAMS, R.O., WILLIAMS, D.G., FELDMANN, M. & MAINI, R.N. (1993). Increased limb involvement in murine collagen-induced arthritis following treatment with anti-interferon-gamma. *Clin. Exp. Immunol.,* **92,** 323–327.

(Received May 10, 1999
Revised June 11, 1999
Accepted June 16, 1999)