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Effect of Three Lipophilic Methotrexate Derivatives Upon Mediator Release by Lipopolysaccharide-stimulated Rat Peritoneal Macrophages

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Abstract—The ability of methotrexate and three lipophilic derivatives (methotrexate-γ-dimyristoylphosphatidylethanolamine (MγD), methotrexate-α-dimyristoylphosphatidylethanolamine (MαD) and methotrexate-α-γ-di-dimyristoylphosphatidylethanolamine (MαγD) to modulate mediator release by lipopolysaccharide-stimulated rat peritoneal macrophages was investigated. At nontoxic concentrations, approximately 10 nmol/10^5 cells, MαD and MγD produced 11·06 ± 1·0 and 75·6 ± 5·2%, respectively, inhibition of tumour necrosis factor (TNF) release (mean ± s.e.m., n = 4). At this same dose MαγD resulted in 68·8 ± 2·1% inhibition of TNF but cellular ATP levels were reduced by 80%. The inhibitory activity of all three derivatives was dose-dependent. Non-derivatized methotrexate at a concentration of 25 nmol/10^5 cells had no inhibitory effect upon TNF release (14·7 ± 0·8%, n = 3). Determination of prostaglandin E_2 (PGE_2) levels in the same samples demonstrated that all three conjugates were powerful inhibitors of prostaglandin release. At a quarter of the conjugate concentrations described above the monoamides MαD (3·1 nmol/10^5 cells) and MγD (2·5 nmol/10^5 cells) maintained their effects on PGE_2 production with 73 ± 2·3 and 71 ± 2·0% (n = 4) inhibition, respectively. At this lower concentration, however, the diamide MαγD (3·1 nmol/10^5 cells) was less effective in reducing the amount of PGE_2 released from the macrophages (29 ± 18%, n = 4). Maximal PGE_2 inhibition by each of the conjugates was attained at approximately 5 nmol/10^5 cells. Unconjugated methotrexate (range of 2·5–20 nmol/10^5 cells) did not inhibit the release of PGE_2 from lipopolysaccharide-stimulated macrophages.

Even though methotrexate effectively suppresses the joint inflammation associated with animal models of arthritis (Welles et al 1985; Ridge et al 1986; Suarez et al 1987; Hu et al 1988) and rheumatoid arthritis (Kremer & Lee 1986; Rose et al 1990; Shiroky et al 1991) its mode of action is not known. Controlled clinical studies, using weekly oral doses of 5–15 mg, have not only shown its efficacy but have highlighted the frequency and severity of the side-effects associated with its use (Furst & Kremer 1988; Sinnett et al 1989; Fries et al 1990).

In an attempt to limit the toxicity of methotrexate, alternative routes of administration and drug formulations have been investigated. Intra-articular administration of methotrexate failed to control the synovitis associated with rheumatoid arthritis since adequate concentrations of the drug could not be maintained within the joint (Wigginton et al 1980). Improved intra-articular retention in the inflamed synovium and a reduced clearance from the joints was shown in rabbits when methotrexate was incorporated within liposomes (Foong & Green 1988a). Rapid leakage of the entrapped drug nevertheless took place and free methotrexate was detected in plasma within a few minutes of intra-articular injection. Leakage may not, however, be a significant problem with liposomes in which the drug is incorporated within the lipid bilayers as a minor integral component. These considerations led us to synthesize three lipophilic amide derivatives of methotrexate by covalent linkage to dimyristoylphosphatidylethanolamine (DMPE). These derivatives were subsequently characterized as methotrexate-γ-dimyristoylphosphatidylethanolamine (MγD), methotrexate-α-dimyristoylphosphatidylethanolamine (MαD) and methotrexate-α-γ-di-dimyristoylphosphatidylethanolamine (MαγD) (Williams et al 1992a).

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Work previously undertaken in our laboratory showed that intravenously administered liposomes accumulate within the inflamed paw tissue of rats with adjuvant-induced arthritis and within the actively inflamed joints of patients with rheumatoid arthritis (Williams et al 1986, 1987; O'Sullivan et al 1988). Thus, when liposomal characteristics are defined for optimal localization, these lipophilic analogues of methotrexate could be targeted to the site of inflammation following entrapment, providing a stable and effective delivery system for methotrexate. It was, therefore, important to characterize the effects of these methotrexate analogues in a relevant in-vitro cell environment before studies with liposomes were commenced.

In view of the important role of macrophages in the pathogenesis of chronic inflammatory diseases the effects of methotrexate and three of its phospholipid derivatives, MαD, MγD and MαγD, upon two disparate cell functions namely tumour necrosis factor (TNF) and prostaglandin E_2 (PGE_2) production were examined in-vitro.

Materials and Methods

Synthesis of methotrexate-DMPE derivatives

The synthesis and purification of derivatized methotrexate has been described previously (Hashimoto et al 1985; Williams et al 1992a). The resulting conjugates MγD, MαD and MαγD were separated on analytical thin-layer plates (Silica gel 60 A, 20 × 20 cm, 0·2 mm thickness with fluores-

cent indicator 254 nm, Sigma Chemical Co., Poole, UK) eluted with a solvent system containing chloroform: methanol: water (70:30:5). The resulting fluorescent bands, which gave a positive test for phospholipid using the Stewart assay (Trudinger 1980; Stewart 1980), were individually scraped from the plates, suspended in 5·0 mL chloroform: methanol (1:1), passed through a sintered glass filter under vacuum and stored at −20°C.

Induction and collection of macrophages

Activated macrophages were isolated from male Wistar rat peritoneal cavities 3 days post-injection with 4% brewer's thioglycollate medium and harvested by a method previously described (Foong & Green 1988b). One hundred microlitre portions of cell suspension (2×10^6 cells mL^{-1}) were dispensed into microtitre plates. Adherent cells were purified after 2-h incubation at 37°C, 5% CO_2/air. The cell population contained 98 ± 0·8% (mean ± s.e.m., n = 4) macrophage, determined by α-naphthyl acetate esterase staining. Viability, as judged by trypan blue exclusion exceeded 95%.

MγD, MαD and MαγD were rotary evaporated to dryness and resuspended in sterile 0·9% NaCl immediately before use. The aggregates which formed, sized by photon correlation spectroscopy (Malvern, UK), were 294 ± 2·7, 752 ± 70 and 1299 ± 203 nm, respectively. One hundred microlitres of the respective conjugate dilutions (MγD, MαD and MαγD) were added to wells containing 100 μL culture medium. After 3·5-h incubation at 37°C, 5% CO_2/air, 20 μL lipopolysaccharide (0·1 mg mL^{-1}, Sigma Chemical Co., from *Escherichia coli* Serotype 0111:B4) was added to the cells. The stimulated macrophages were incubated for a further 14 h to ensure maximal mediator release; the resulting supernatants were then assayed for TNF and PGE_2. Methotrexate content of MαD, MγD and MαγD was determined spectrophotometrically after complete hydrolysis in 0·1 M NaOH at 37°C for 48 h.

Determination of macrophage-associated ATP

Each macrophage-containing well was washed twice with 100 μL phosphate-buffered saline (pH 7·4). ATP was removed from the cells by gentle agitation with 100 μL Bac-Extract (Steadman et al 1989). This solution was then added to 500 μL 25 mM HEPES/2 mM EDTA buffer (pH 7·5) to preserve ATP activity. An aliquot (100 μL) of this was then added to 50 μL luciferase-luciferin (Sigma Chemical Co., Poole, UK 8 mg mL^{-1}), mixed vigorously for 2 s and the maximum luminescence measured. Cellular-associated ATP (%) was then calculated after comparison with control macrophage populations.

Measurement of TNF

Test samples were incubated with monolayers of a plastic-adherent TNF-sensitive murine tumour cell line (L-929), generously supplied to us by N. Matthews (UWCM, Cardiff, UK). The cells were grown in RPMI medium with 5% foetal calf serum (Sigma Chemical Co., Poole, UK), glutamine (2 mM, Flow Laboratories) and penicillin/streptomycin (50 int. units mL^{-1} and 50 μg mL^{-1}, respectively, Flow Laboratories, High Wycombe, UK). Seventy five microlitre samples of cell suspension (3×10^5 mL^{-1}) were dispensed into the wells of a microtitre plate and incubated at 37°C, 5% CO_2, for 24 h before being challenged with test supernatants. Test samples were serially diluted then added to the L-929 cells which were incubated for an additional 16–24 h in the presence of emetine (1 μg mL^{-1}, Sigma Chemical Co., Poole, UK). Remaining adherent cells were fixed in 5% formaldehyde then stained with 1% crystal violet. After washing and drying, 100 μL acetic acid (33%) was added to each well. The absorbance at 540 nm was determined on an Elisa plate reader (Titertek Multiskan MCC/340).

The specificity of the assay was confirmed by pre-incubating TNF-containing supernatants (150 μL) with a neutralizing rabbit anti-mouse TNF_α polyclonal antibody (at 1:150 dilution, Genzyme, Cambridge, UK) for 30 min at 37°C before performing the L-929 assay. At this antibody dilution L-929 cell death was inhibited by 98%.

Determination of PGE_2

The PGE_2 content of the supernatants was assessed by a double antibody radioimmunoassay method using specific anti-PGE_2 (BioClin, product AB-3050) polyclonal rabbit antiserum (Kaever et al 1988; Topley et al 1989). The comparative cross-reactivities of this antiserum with other prostanoids were: PGE_2 100%; PGE_1 10·0%; $PGF_{1\alpha}$ 3·3%; PGB_1 3·0%; PGD_2 0·01%; PGA_1 0·01%; PGA_2 0·01%; $PGF_{2\alpha}$ 0·01%; 13, 14-dihydro-6-keto-$PGF_{1\alpha}$ 0·01%; thromboxane B_2 0·01%; and 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ 0·01%.

Results

Rat peritoneal macrophage viability, after incubation with either free methotrexate, MαD, MγD or MαγD, was determined by assessment of total cellular ATP from the respective cell cultures. Macrophage viability was unaffected by incubation with underivatized methotrexate at concentrations up to 16 nmol/10^5 cells. At their highest incubation concentrations MαD (24 nmol/10^5 cells) and MγD (30 nmol/10^5 cells) were toxic to the macrophage with viability at 26·3 ± 5·1 and 9·1 ± 0·7%, respectively. The MαγD preparation was more toxic, with macrophages viability being 12·6 ± 2·2% at the lower dose of 12 nmol/10^5 cells (Fig. 1). At half these concentrations, macrophage viability remained low for MαγD (22 ± 2·2%) but increased above 75% for each of the monoamide derivatives, MαD and MγD. Of the conjugates MγD was least toxic to the macrophages.

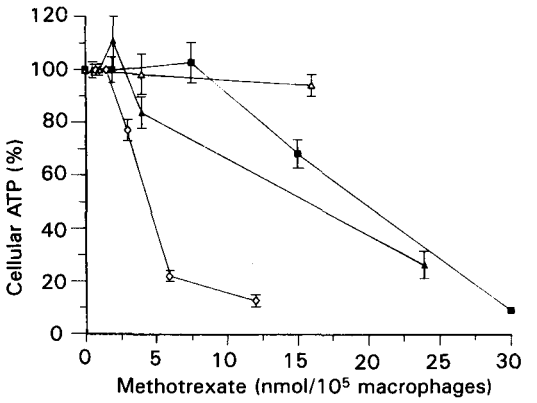

FIG. 1. The effect of free methotrexate (△) and three of its phospholipid analogues (MγD ■, MαD ▲, MαγD ◇) upon cellular ATP levels in rat peritoneal macrophages. Results are expressed as mean ± s.e.m. % cellular ATP, which are determined after comparison with control macrophages incubated with culture medium alone.

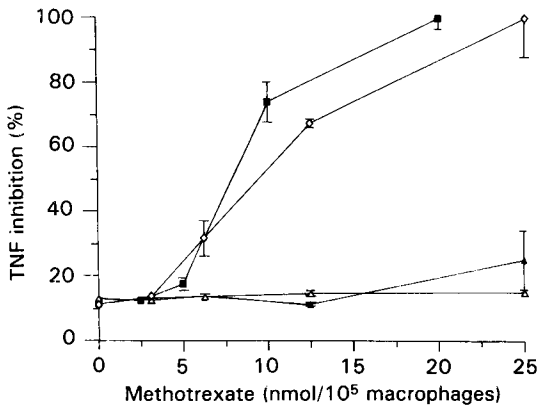

FIG. 2. Inhibition of TNF release from lipopolysaccharide-stimulated macrophages treated with methotrexate (△), MαD (▲), MγD (■) or MαγD (◇). TNF was measured by a cytotoxic cell assay. Results are expressed as % TNF inhibition (mean ± s.e.m.) determined after comparison with supernatants isolated from macrophages incubated with culture medium alone. Four supernatants were assayed per test methotrexate and methotrexate analogue concentration. TNF production by the macrophages was assessed by stimulating the macrophages incubated in culture medium with lipopolysaccharide.

At approximately 10 nmol/10^5 cells MγD inhibited TNF release from the lipopolysaccharide-stimulated macrophages by 74 ± 6·2%. Under identical incubation conditions MαD demonstrated no inhibition of the cytokines release (11·3 ± 0·4%) (Fig. 2). TNF inhibition by MαγD was due to cell cytotoxicity at concentrations of 5 nmol/10^5 cells and above. Furthermore, neither methotrexate nor DMPE (20 nmol/10^5 cells) exerted any inhibitory effect upon TNF release from lipopolysaccharide-stimulated macrophages and did not reduce cell viability (data not shown). None of the methotrexate-DMPE conjugates, DMPE or methotrexate showed any cytotoxic effects towards the effector L-929 cells (data not shown).

Upon lipopolysaccharide stimulation PGE_2 levels in supernatants isolated from untreated control macrophages

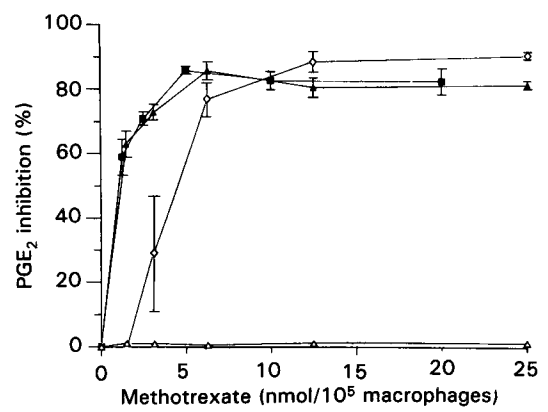

FIG. 3. The effect of free methotrexate (△) and phospholipid-conjugated methotrexate (MγD ■, MαD ▲, MαγD ◇) upon PGE_2 secretion by activated rat peritoneal macrophages stimulated with lipopolysaccharide. Results are expressed as % PGE_2 inhibition (mean ± s.e.m.) determined after comparison with control cells incubated with culture medium and stimulated with lipopolysaccharide.

increased from approximately 2 to 25 ng mL^{-1}. Pre-incubation of the macrophages with methotrexate did not inhibit PGE_2 release from lipopolysaccharide-stimulated macrophages, at any dose tested. In contrast, incubation with MγD, MαD or MαγD resulted in a dose-dependent inhibition of PGE_2 release (Fig. 3). At low doses, the monoamides MαD (3·1 nmol/10^5 cells) and MγD (2·5 nmol/10^5 cells) were more potent than the diamide MαγD (3·1 nmol/10^5 cells) resulting in 73 ± 2·4, 71 ± 2·0 and 29 ± 18% PGE_2 inhibition, respectively.

Discussion

This study was designed to investigate the effects of free methotrexate and three of its lipophilic derivatives on TNF and PGE_2 production by lipopolysaccharide-stimulated macrophages. Thioglycollate-elicited macrophages were used since it has been shown that they produce about 100-fold more TNF following lipopolysaccharide challenge than do the resident peritoneal macrophages (Stein & Gordon 1991). We have demonstrated that the diamide derivative of methotrexate, MαγD, is more toxic to macrophages than either free methotrexate, MαD or MγD. At MαγD concentrations of 5 nmol/10^5 cells, macrophage viability was reduced by 80%; thus, mediator inhibition by MαγD at this concentration and above was due to cell death. Mediator inhibition by MαD and MγD below 10 nmol/10^5 cells was not, however, related to a decrease in cell viability. At this concentration MγD inhibited TNF release from lipopolysaccharide stimulated macrophages by approximately 80%, whereas neither methotrexate nor MαD had any effect on the mediators release. These data alone suggest that free methotrexate and MαD do not enter the cell or that MγD, methotrexate and MαD exert differential effects upon TNF production in macrophages.

Interestingly, at the relatively nontoxic level of 10 nmol/10^5 cells, MγD and MαD exhibit identical PGE_2 inhibitory profiles but MαD does not affect TNF secretion, a finding which suggests that this lipophilic derivative of methotrexate exerts a differential and specific inhibitory effect upon PGE_2 release from macrophages stimulated with lipopolysaccharide. It has previously been documented that in-vitro TNF_α production by activated, lipopolysaccharide-stimulated rat peritoneal macrophages is suppressed by PGE_2 levels exceeding 10 ng mL^{-1} (Renz et al 1988). This direct suppressive effect does not appear to be occurring in our experimental system, since incubation of the macrophages with each methotrexate-DMPE derivative (10 nmol/10^5 cells) resulted in almost complete suppression of PGE_2. At this same concentration TNF production by these cells was only inhibited by MγD but was unaffected by methotrexate or MαD. The inhibition of TNF and PGE_2 release in our experimental system appear to be independent events, since under conditions where prostaglandin release is almost totally suppressed TNF secretion can either be co-suppressed or remain unaffected.

Methotrexate enters proliferating cells via a folate transport system and, after being polyglutamated, exerts its cytotoxic action primarily through inhibition of dihydrofo-

late reductase (DHFR) (Fan et al 1991). Methotrexate contains two carboxyl groups, α-carboxyl ($pK_a = 3{\cdot}36$) and γ-carboxyl ($pK_a = 4{\cdot}70$), in the glutamyl portion of the molecule. They have previously been utilized as coupling sites for fluorescein isothiocyanate to study the interaction of methotrexate with the target enzyme DHFR (Rosowsky & Forsch 1982; Rosowsky et al 1986). The γ-fluorescent derivative had the highest affinity for the methotrexate binding site and was a more potent inhibitor of bacterial and mammalian folate transport systems than the α-derivative (Fan et al 1991). Indeed an intact α-carboxyl group was generally considered essential for efficient transport of methotrexate into cells and tight binding to DHFR (Rosowsky et al 1986, 1988). This differential binding to DHFR would account for differences between the MαD and MγD derivatives. A different inhibitory profile and sensitivity to inhibition for PGE_2 and TNF production also suggests that these methotrexate-DMPE conjugates exert their inhibitory effects on different pathways.

The role of TNF_α in contributing to the development of arthritis has recently been studied in-vivo in the collagen-induced arthritis model DBA/1 mouse. Treatment of these mice with anti-TNF antibodies significantly reduced the clinical and histopathological severity of the arthritis (Williams et al 1991, 1992b). Further support for the role of TNF_α in rheumatoid arthritis has come from a recent study on transgenic mice expressing the human TNF_α gene (Keffer et al 1991). Such mice developed a polyarthritis, whose pathogenesis was totally inhibited by administering monoclonal anti-human TNF_α antibodies at birth. TNF_α has been identified in the synovial membrane and particularly at the cartilage-pannus junction of patients with rheumatoid arthritis (Chu et al 1990). This mediator may, therefore, represent an important therapeutic target in rheumatoid arthritis and if its action could be blocked locally within the joint, it is possible that the disease process would be greatly ameliorated.

Results obtained from studies performed on animal models of arthritis also implicate PGE_2 as a major mediator of both local tissue destruction and systemic immunoregulatory changes associated with rheumatoid arthritis (Malone et al 1984; Shore et al 1986; Ahern et al 1988; Johnson et al 1988; Rose et al 1990). In-vitro studies, however, reported that methotrexate did not inhibit prostaglandin release at concentrations equivalent to the highest methotrexate concentrations used in our study (Shiroky et al 1991). This latter observation is consistent with the results obtained in our laboratory where free methotrexate had no effect on PGE_2 release. In contrast, the present data clearly demonstrates that two phospholipid analogues of methotrexate (MαD and MγD) inhibit PGE_2 release at a concentration as low as $2{\cdot}5$ nmol/10^5 cells. These data suggest that the methotrexate-DMPE derivatives enter the macrophage and specifically interfere with arachidonic acid metabolism.

We have shown that, in-vitro, MγD is a potent inhibitor of two important mediator systems implicated in the pathogenesis of rheumatoid arthritis and that this lipophilic analogue was more effective than free methotrexate in this respect. Therefore, MγD could conceivably ameliorate the pathogenesis of rheumatoid arthritis provided these observations were reproduced in-vivo.

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