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Research Paper

Therapeutic index of methotrexate depends on circadian cycling of tumour necrosis factor-α in collagen-induced arthritic rats and mice

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Abstract

Objectives Rheumatoid arthritis is an autoimmune disorder of unknown aetiology. Morning stiffness, a characteristic feature of rheumatoid arthritis, shows a 24-h rhythm. Noticing this rhythm, we hypothesized the presence of a similar rhythm for a rheumatoid arthritis indicator, in addition to dosing-time dependency of the anti-rheumatic effect of methotrexate in arthritis induced by collagen in rats and mice, which reflect the symptomatology of rheumatoid arthritis patients.

Methods To measure tumour necrosis factor (TNF)-α concentration, blood was taken at different times (2, 6, 10, 14, 18 or 22 h after the light was turned on (HALO)) in collagen-induced arthritic mice. Methotrexate was administered at two different dosing times based on these findings to estimate arthritis.

Key findings The arthritis score was significantly lower in the 22 HALO-treated group than in the control and 10 HALO-treated groups in collagen-induced arthritic rats and mice. Plasma TNF-α concentrations showed obvious 24-h rhythms, with higher levels at light phase and lower levels at dark phase after rheumatoid arthritis crisis. Arthritis was relieved after administration of methotrexate during the dark phase in synchronization with the 24-h rhythm.

Conclusions Our findings suggest that choosing an optimal dosing time associated with the 24-h cycling of TNF-α could lead to effective treatment of rheumatoid arthritis by methotrexate.

Keywords methotrexate; rheumatoid arthritis; 24-h rhythm; tumour necrosis factor-α

Introduction

Chronotherapy is defined as the administration of medication in accordance with biological rhythms in order to optimize therapeutic outcomes or control adverse effects, and it has been reported that many drugs, such as anti-tumour drugs, antidepressants and analgesics, show rhythm-dependent differences in their effects and pharmacokinetics.[1–3] These effects arise from the 24-h rhythms found in elements of cellular physiology such as the cell cycle, receptors, hormones and enzymes.[4–6] Moreover, it has been reported that 24-h rhythms exist for asthma attacks and synthesis of cholesterol, and chronotherapy has been actively applied to the medicinal treatment of asthma and hyperlipidaemia.[7,8]

Rheumatoid arthritis is an autoimmune disorder of unknown aetiology and is a chronic progressive disease that reduces the quality of life.[9,10] Although many requirements must be met to establish the diagnosis, morning stiffness is a well-known characteristic feature of rheumatoid arthritis.[11] Morning stiffness shows a 24-h rhythm with a peak in the early morning.[12–14] The appearance of cytokines in the joint tissue, synovial fluid and serum of rheumatoid arthritis patients suggests that they may play a role in local and systemic inflammatory responses. It was reported that cytokine and melatonin concentrations in the blood also showed 24-h rhythms, with peaks in the middle of the night to the early morning that mirror the timing of morning stiffness.[15,16] The 24-h rhythm of cytokines and melatonin may therefore participate in the 24-hour rhythm of rheumatoid arthritis symptoms.

Chronotherapy of rheumatoid arthritis has been studied using glucocorticoid and benoxaprofen.[17,18] In recent years, it was reported that chronotherapy using modified-release prednisone was effective against rheumatoid arthritis compared with standard immediate-release prednisone.[19] Thus, the clinical application of chronotherapy is expected. The dosing

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time dependency of more popular disease-modifying antirheumatic drugs (DMARDs) has not been assessed, although they are administered to many rheumatoid arthritis patients. Methotrexate is a DMARD that is widely used in rheumatoid arthritis patients. Toxicity caused by methotrexate varies significantly depending on the dosing time.[20] Therefore, we considered that choosing an optimal dosing time associated with the 24-h rhythm of symptoms could lead to effective treatment of rheumatoid arthritis by methotrexate.

In this study, to detect 24-h rhythms for tumour necrosis factor (TNF)-α, we measured its serum concentrations at six different times in mice with arthritis induced by collagen (CIA mice). Methotrexate was administered at two different dosing times based on these findings to estimate arthritis. The dosing time dependency of methotrexate pharmacokinetics was determined after a single dose of methotrexate.

Materials and Methods

Animals

Lewis rats (7 weeks old) were purchased from Kyudo Co. Ltd (Kumamoto, Japan). DBA/1J mice (5 or 8 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Rats and mice were housed under standardized light–dark cycle conditions (lights on 0700–1900 h) at a room temperature of 24 ± 1°C and humidity of 60 ± 10% with free access to food and water. Experiments were performed after formal approval by the Institutional Ethical Committee for Research on Animals.

Induction of collagen-induced arthritis

In rats, bovine type II collagen (CII; Collagen Research Center, Tokyo, Japan) and Freund's incomplete adjuvant (Difco, Kansas, Mo, USA) were mixed, and the emulsion was prepared at a concentration of 1 mg/ml of CII. The rats were intradermally sensitized at ten sites (100 μl each site) at day 0 by administration of 1 mg CII. Seven and eleven days later, the rats received an intradermal booster injection of one-tenth of the volume used for sensitization.

In mice, CII isolated and purified from bovine articular cartilage was purchased from Chondrex, Inc. (Redmond, USA). Mice were intradermally immunized at day 0 by administration (100 μl) of 100 μg CII in Freund's complete adjuvant (FCA; Chondrex, Inc.). A booster injection (100 μl) of 200 μg CII in FCA was given intradermally on day 14.

Preparation of methotrexate

Methotrexate, supplied by Wyeth K.K. (Tokyo, Japan), was dissolved in 7% sodium bicarbonate. Methotrexate was prepared at a suitable concentration and perorally administered by gavage at 2 ml/kg in rats and intraperitoneally administered at 0.01 ml/g in mice.

Methotrexate dosing time dependent suppression of collagen-induced arthritis

In rats, methotrexate or sodium bicarbonate (7%) was administered one day after the first immunization. Methotrexate (0.1 mg/kg) was administered orally at 10 or 22 hours after the light was turned on (HALO) every day for 3 weeks in CIA rats (n = 10, respectively). Sodium bicarbonate (7%) was administered in the control group (n = 9). In mice, methotrexate or sodium bicarbonate (7%) was administered 21 days after the first immunization. Methotrexate (60 mg/kg) was injected intraperitoneally at 10 or 22 HALO every 7 days for 3 weeks in CIA mice (n = 9 or 10). Sodium bicarbonate (7%) was administered in the control group (n = 14).

In rats, arthritis score was recorded every day after the first immunization. We used a previously described arthritis scoring system[21] that evaluates individual joints and weights the arthritis severity by joint size, as follows: (a) for the interphalangeal joints, each of the 4 lateral digits in hind legs was scored as 0 or 1 (0 = no arthritis and 1 = arthritis present); and (b) for the ankle and midfoot joints, each was scored on a scale of 0–4 (0 = normal, 1 = minimal swelling, 2 = moderate swelling, 3 = severe swelling and 4 = severe swelling and non-weight bearing). The macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 32.

Mice were examined visually for the appearance of arthritis in peripheral joints and disease severity was graded on a scale using the modified method of Nandakumar *et al*.[22] as specified below. Mice were considered to have arthritis when significant changes in redness or swelling (or both) were noted in the digits or in other parts of the paws. Each inflamed toe counted as 1 point. Arthritis was graded on a scale of 0, 1, 2, 3, 4 and 5 for each wrist/ankle: 0 = no changes; 1 = slight erythema of limb; 2 = minimal swelling; 3 = moderate swelling and erythema of limb; 4 = marked swelling and erythema of the limb; 5 = maximal swelling and redness of the limb and, later, ankylosis. The macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 40.

Methotrexate dosing-time dependent leucopenia

To investigate leucocyte counts, 8-week-old DBA/1J mice were divided into the 10 and 22 HALO-treated groups. Blood samples were drawn by orbital sinus collection 4 day after a single injection of methotrexate or sodium bicarbonate (7%) in the 4 groups (n = 4–6). Leucocyte counts were measured immediately after blood drawing.

Twenty-four-hour rhythm in plasma TNF-α concentrations in collagen-induced arthritic mice

To measure TNF-α concentration, blood was taken at different times (2, 6, 10, 14, 18 or 22 HALO) in normal (n = 5 or 6) or CIA (n = 8 or 9) mice. Blood of CIA mice was drawn by orbital sinus collection on day 28 after immunization when arthritis showed in all mice. All blood samples were immediately centrifuged at 3000 rev/min for 15 min, after which the plasma was removed and frozen at −20°C until assay. To measure TNF-α concentrations, multianalyte profiling was performed using the Luminex-100 system (Luminex Corporation, Austin, USA). Acquired fluorescence data were analysed by the MasterPlex QT software (Ver. 1.2; MiraiBio, Inc., San Francisco, USA). Plasma concentrations of TNF-α were determined by Mouse Inflammatory Cytokine

4-Plex (Biosource, San Jose, USA). All analyses were performed according to the manufacturer's protocols.

Chronopharmacokinetics of methotrexate

To investigate the pharmacokinetics, 8 week-old DBA/1J mice were divided into the 10 and 22 HALO-treated groups (n = 5 or 6). Blood samples were drawn by orbital sinus collection at 0.5, 1, 2, 4, 6 and 8 h after methotrexate (60 mg/kg) was intraperitoneally administered. The samples were immediately centrifuged at 3000 rev/min for 15 min. Plasma was stored at −20°C until analysis. Methotrexate concentrations in plasma were quantified by Abbott TDx II methotrexate assays.

Statistical analysis

The data were recorded as the mean ± standard deviation (SD), excluding the arthritis score. Arthritis score was shown as a mean and box plot. For each box plot, the median value is represented by the central horizontal line; the upper and lower quartiles (75 and 25 percentiles) are represented by the upper and lower borders of the box; the upper and lower extents of the vertical lines extending from the box represent the value of 90 and 10 percentiles. Intra-group post-hoc testing was done using the Mann–Whitney U-test with Bonferroni correction after Kruskal–Wallis test. Differences in leucocyte counts were compared by two-way analysis of variance. Differences in methotrexate concentrations between two groups were analysed by Student's t-test. Statistical moment analysis was performed by calculating pharmacokinetic parameters such as area under the plasma–time concentration curve (AUC) and mean residence time (MRT). We defined that the 24-h rhythmicity was statistically significant when both Cosinor analysis and one-way analysis of variance were significant. $P < 0.05$ was considered to be significant.

Results

Influence of dosing time on arthritis score during methotrexate administration in collagen-induced arthritic rats and mice

In rats, arthritis score was significantly lower in the 22 HALO-treated group than the control and 10 HALO-treated groups on day 22 ($P < 0.01$ and $P < 0.05$, respectively; Figure 1). On day 42, the 22 HALO group demonstrated significantly inhibited increase in arthritis score compared with the control and 10 HALO groups in mice ($P < 0.01$, respectively; Figure 2). The 10 HALO groups were not different from the control groups for the entire duration of observation in rats and mice.

Influence of dosing time on leucocyte counts after methotrexate administration in mice

There was no significant difference in leucocyte counts among the control and drug-treated groups (Figure 3).

Twenty-four-hour rhythm of plasma TNF-α concentrations in normal and collagen-induced arthritic mice

The plasma TNF-α concentrations in normal mice showed a significant 24-h rhythm with higher levels at the light phase and lower levels at the dark phase ($P < 0.01$, Figure 4). After immunization, TNF-α levels in CIA mice were 3.03- to 5.39-fold higher than those in normal mice at all sampling times. A significant 24-h rhythm was demonstrated for TNF-α concentrations in CIA mice ($P < 0.01$, Figure 4), and the levels were higher at the light phase and lower at the dark phase as observed in normal mice.

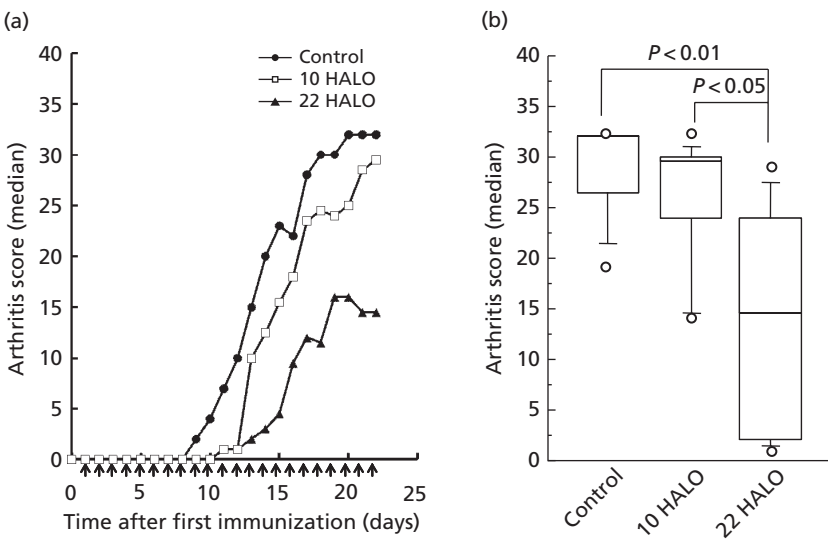

Figure 1 Influence of the dosing time of methotrexate on arthritis score in collagen-induced arthritic rats. Methotrexate (0.1 mg/kg) was administered orally at 10 or 22 hours after lights turned on (HALO) every day for three weeks in collagen-induced arthritic (CIA) rats (n = 10). Sodium bicarbonate (7%) was administered to the control group (n = 9). Methotrexate was administered one day after the first immunization. Arrows show methotrexate or sodium bicarbonate administration. (a) Each value represents the median. (b) The score on day 22 is indicated by box plots. On day 22, the 22 HALO-treated group showed a significantly decreased arthritis score when compared with the control and 10 HALO-groups ($P < 0.01$ and $P < 0.05$, respectively, Mann–Whitney U-test with Bonferroni correction).

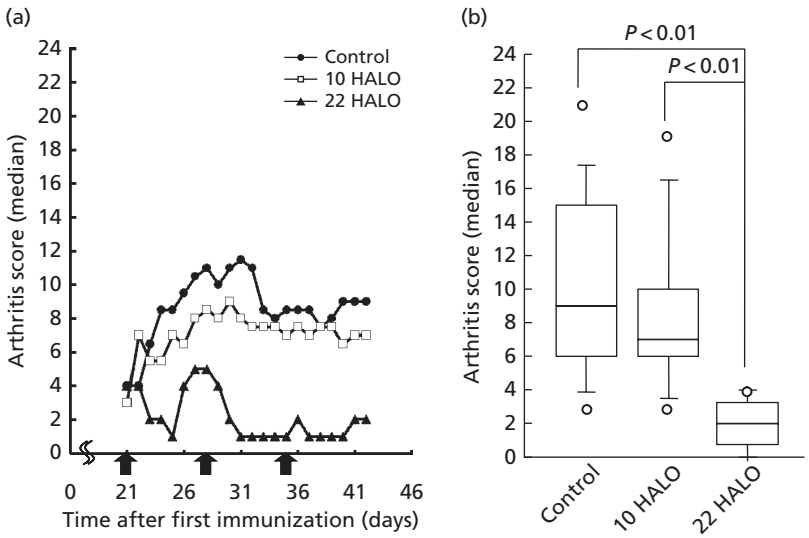

Figure 2 Influence of the dosing time of methotrexate on arthritis score in collagen-induced arthritic mice. Methotrexate (60 mg/kg) was administered at 10 or 22 hours after lights turned on (HALO) on days 21, 28 and 35 after the first immunization (n = 9 or 10). Sodium bicarbonate (7%) was administered to the control group (n = 14). Arrows show methotrexate or sodium bicarbonate administration. (a) Each value represents the median. (b) The score on day 42 is indicated by box plots. On day 42, the arthritis score was significantly lower in the 22 HALO group than the control and 10 HALO groups ($P < 0.01$, respectively, Mann–Whitney U-test with Bonferroni correction).

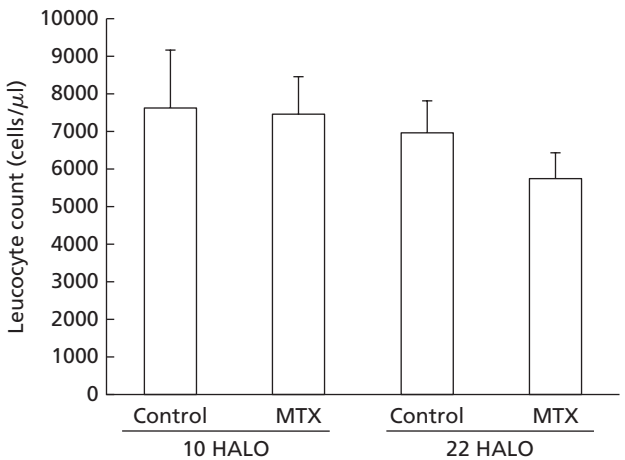

Figure 3 Influence of dosing schedule on myelosuppression after administration of methotrexate in mice. Methotrexate (MTX; 60 mg/kg, i.p.) was administered to the experimental group (n = 4–6); sodium bicarbonate (7%) was administered to the control group (n = 5 or 6). HALO, hours after lights turned on. Each value is the mean ± SD.

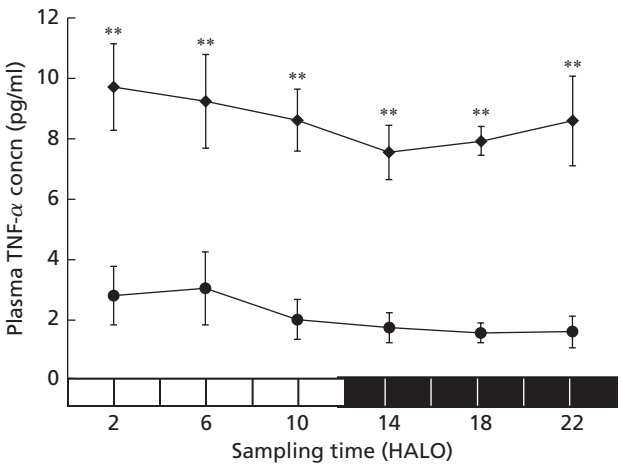

Figure 4 Twenty-four-hour rhythm of plasma tumour necrosis factor-α levels in normal and collagen-induced arthritic mice. Each value represents the mean ± SD of normal (n = 5 or 6) or CIA (n = 9) mice. HALO, hours after lights turned on. The plasma tumour necrosis factor (TNF)-α concentrations showed significant 24-h rhythms with higher levels at light phase and lower levels at dark phase in normal and collagen-induced arthritic (CIA) mice (normal: F from analysis of variance = 4.08, $P < 0.01$; P from cosinor < 0.01; day 28: F from analysis of variance = 3.93, $P < 0.01$; P from cosinor < 0.01). $^{**}P < 0.01$ compared with the normal group.

Influence of dosing time on pharmacokinetics after methotrexate administration in mice

Plasma methotrexate concentrations at 0.5 and 2 h after methotrexate injection in the 10 HALO group were significantly higher than in the 22 HALO group (0.5 h, $P < 0.05$; 2 h, $P < 0.01$) (Table 1). On the other hand, the 22 HALO group showed significantly higher plasma methotrexate levels than the 10 HALO group at 4, 6 and 8 h (4 h, $P < 0.01$; 6 h, $P < 0.01$; 8 h, $P < 0.05$) (Table 1).

Discussion

Collagen-induced arthritis (CIA) represents a true autoimmune reaction against major joint components with association of class II major histocompatibility complex genes and pannus formation. The CIA model is similar to rheumatoid arthritis in terms of pathology, immunology and genetics.[23,24] Using the

Table 1 Pharmacokinetic data after methotrexate (60 mg/kg, i.p.) administration to mice at 10 or 22 hours after lights turned on

	Methotrexate concentration (μg/ml)						AUC_∞ (μg h/ml)	MRT_∞ (h)
	0.5 h	1 h	2 h	4 h	6 h	8 h		
10 HALO	23.1 ± 4.07	8.64 ± 1.47	1.67 ± 0.52	0.037 ± 0.009	0.009 ± 0.006	0.020 ± 0.015	35.2	0.544
22 HALO	17.1 ± 1.92	6.37 ± 3.47	0.73 ± 0.37	0.113 ± 0.017	0.072 ± 0.023	0.039 ± 0.013	27.6	0.547
P value	< 0.05	NS	< 0.01	< 0.01	< 0.01	< 0.05		

Each value is the mean ± S.D. of 5 or 6 mice. AUC, area under plasma–time concentration curve; HALO, hours after lights turned on; MRT, mean residence time; NS, not statistically significant.

CIA model, we estimated the preventive effect and therapeutic effect against the development of arthritis when methotrexate was administered for 21 days before or after the onset of arthritis. When CIA rats received 0.1 mg/kg methotrexate orally at 10 or 22 HALO, starting on day 1 after the first immunization, the arthritis score in the 22 HALO group showed significantly lower values compared with the control and 10 HALO groups. Inhibition of the increase in arthritis depended on dosing time in the preventive treatment with methotrexate. In the clinical situation, it is difficult to perform preventive treatment for rheumatoid arthritis although early diagnosis has been advancing year after year. Thus, we examined whether the dosing time dependency of therapeutic effect was shown in the CIA model after the onset of arthritis. The effect of methotrexate in CIA rats after the onset of arthritis was reported by Cuzzocrea *et al*.[25] We studied the anti-rheumatic effect after the onset of arthritis according to this report, using clinical doses. However, there were no significant differences between the control and methotrexate-treated groups (data not shown). Because the arthritis score in CIA rats increased rapidly after the last sensitization, low-dose methotrexate using clinical doses may not show an anti-rheumatic effect. On the other hand, the arthritis score in CIA mice gradually increased compared with CIA rats after the last sensitization. In a preliminary study, we had clarified that the score in the methotrexate (60 mg/kg) group treated at 22 HALO was lower than that in the control group in CIA mice after the onset of arthritis. Therefore, therapeutic effect was estimated in CIA mice. The 22 HALO-treated group showed a significantly reduced arthritis score when compared with the control and 10 HALO-treated groups, and the arthritis score decreased to almost normal levels in many 22 HALO mice despite lack of score reduction for the 10 HALO group. Leucopenia showed no significant dosing time dependence for methotrexate (60 mg/kg) injection in mice. In a past study, there were adverse effects when 400 mg/kg of methotrexate was administered in mice.[20] However, adverse effects were not observed in this study because only 60 mg/kg of methotrexate was administered in mice. These findings suggest that both the preventive effect and therapeutic effect are evidently high in groups treated with methotrexate at 22 HALO compared with those treated at 10 HALO.

To clarify the mechanism underlying dosing time dependency, we investigated the influence of dosing time on the pharmacokinetics of methotrexate. Methotrexate is excreted largely in urine. Both renal blood flow and glomerular filtration rate have been found to follow a 24-h rhythm, with a peak during the active period of animals.[26,27] The AUC in the 10 HALO group, which showed no decrease in arthritis score, was 1.28-fold higher than that in the 22 HALO group, which had a significantly reduced score. The AUC may be high in the 10 HALO group compared with the 22 HALO group since methotrexate was administered during the inactive period. In this study, a relationship was not shown between the concentration of methotrexate and its efficacy. Thus, the 24-h rhythm in the sensitivity of the body seems to play an important role in the daily variation of anti-rheumatic effect rather than pharmacokinetics of methotrexate.

In recent years, it has been stated that cytokines are an important factor in the pathogenesis of rheumatoid arthritis,[28] and that levels of pro-inflammatory cytokines increase in rheumatoid arthritis patients. Blood cytokines show 24-h rhythms in rheumatoid arthritis patients,[15,16] and the rhythms correspond to morning stiffness. We considered that rheumatoid arthritis therapy that is associated with cytokine 24-h rhythms might be more effective than the therapy used commonly in clinical practice. It was previously reported that CIA mice showed an augmentation of cytokine levels similar to that seen in rheumatoid arthritis patients.[29,30] Thus, to clarify whether there is a 24-h rhythm for the plasma concentration of TNF-α, an important causal factor of rheumatoid arthritis, we measured its concentration in normal and CIA mice. In normal mice, the plasma TNF-α concentration showed a significant 24-h rhythm with higher levels during the light phase and lower levels in the dark phase. Although the TNF-α concentration increased after immunization, an obvious 24-h rhythm with a peak at light phase and a trough at dark phase was maintained in CIA mice. The development of arthritis was significantly inhibited in groups treated with methotrexate at 22HALO when the plasma TNF-α level began to increase. MRL/lpr mice are another rheumatoid arthritis model known to develop autoimmune disorders sharing similarities with human rheumatoid arthritis and systemic lupus erythematosus.[31,32] Thus, we examined the 24-h rhythm of TNF-α and the dosing time dependency of methotrexate effects in MRL/lpr mice. In these results, the daily variation of arthritis inhibition caused by methotrexate corresponded to the 24-h rhythm of TNF-α levels in the same manner as in the CIA model (data not shown). From these results, it is thought that methotrexate has a significant dosing-time dependent anti-inflammatory action, and this effect may be due to the 24-h rhythm of TNF-α levels rather than pharmacokinetics.

Conclusions

This study showed that daily variation occurred in the plasma TNF-α concentration in the CIA model after rheumatoid

arthritis developed, and that arthritis was relieved after administration of methotrexate at specific times in synchronization with the 24-h rhythm of TNF-α. Choosing an optimal dosing time associated with the 24-h rhythm of TNF-α is therefore expected to lead to effective therapy of rheumatoid arthritis with methotrexate. However, the dose and dosing schedule of methotrexate in the rheumatoid arthritis model animal did not always correspond to that in the rheumatoid arthritis patient because a high dose of methotrexate was administered in the model animal to produce sufficient anti-rheumatic effect in this study. Therefore, we are studying the usefulness of chronotherapy of methotrexate in Japanese rheumatoid arthritis patients.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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