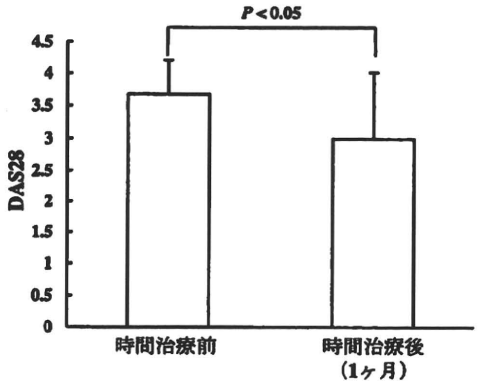

図5　RA患者を対象としたMTXの時間治療によるDAS28への影響
被験者は12例で、データは平均値±標準偏差で示した。

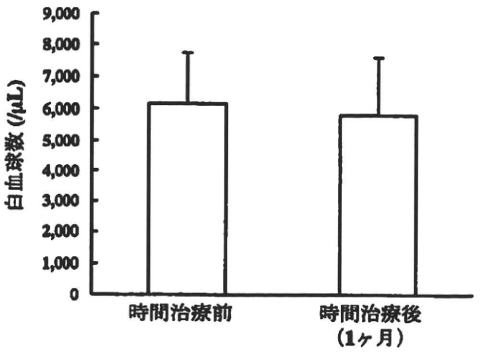

図7　RA患者を対象としたMTXの時間治療による白血球数への影響
被験者は12例で、データは平均値±標準偏差で示した。

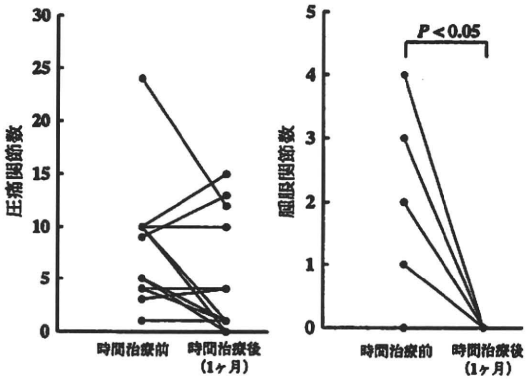

図6　RA患者を対象としたMTXの時間治療による圧痛関節数及び腫脹関節数への影響
被験者は12例である。

時点において平均血中濃度を用いてモーメント解析を行った結果、AUC_∞は5:00投薬群で23,622μg/mL・hr、17:00投薬群で32,305μg/mL・hrであり、5:00投薬群と比較し17:00投薬群のAUC_∞は約1.4倍高かった。

6. RA患者を対象としたMTXの時間治療による抗リウマチ効果への影響

時間治療開始1ヶ月後におけるDAS28の平均値は2.99であり、時間治療変更時のDAS28の平均値は3.67であった。時間治療を開始することで時間治療開始前と比較し、被験者のDAS28は有意に減少した（$P<0.05$; 図5）。多くの症例で、圧痛関節数は減少傾向にあり、腫脹関節数ではすべての被験者で腫脹が消失した（$P<0.05$; 図6）。

7. RA患者を対象としたMTXの時間治療による骨髄抑制への影響

時間治療開始1ヶ月後では、時間治療変更時と比較し骨髄抑制に有意な差異は認められなかった（図7）。

考　察

本研究では、RAモデル動物を用いて、炎症性サイトカイン等の概日リズム解析及びMTXの時間薬理学的検討、及びRA患者を対象とした時間治療に関する臨床研究を実施した。

RAモデル動物には、慢性的で破壊的な多発性関節炎を示すなどRA患者と多くの点で相同性が認められているCIAマウスと自然発症型リウマチモデル動物であるMRL/lprマウスを使用した。これらのRA動物におけるTNF-α濃度の概日リズムは、RAを発症することで、明期に高値、暗期に低値を示す明瞭な概日リズムが認められた。また、炎症の指標であるSAA濃度もRAを発症することで、明期に最高値を示す概日リズムが両モデル動物で現れた（一部未提示）。SAAは、C-reactive

protein（CRP）と同様に急性期の炎症反応を評価する指標でIL-1やIL-6、TNF-αなどの炎症性サイトカインによって惹起されることが知られている。したがって、RAの特徴である関節の痛みや朝のこわばりなどの炎症の概日リズムには、炎症性サイトカインの概日リズムが密接に関連していると考えられる。非常に興味深いことに、RA患者の炎症性サイトカインの概日リズムもまた、早朝に最高値となる概日リズムを示す[7]。一般的にRA患者は昼行性であり、RAモデル動物は夜行性である。このように活動リズムが異なる場合、グルココルチコイドをはじめとする多くの生体成分の概日リズムは、ヒトとげっ歯類で逆位相となる。そのため、病状が発現する時刻は、ヒトとげっ歯類では、約12時間異なることが多い。しかし、RA症状の概日リズムでは、非常に近似した位相をヒトとげっ歯類で示すことが明らかとなった。したがって、活動リズムに関与せず、ヒトとげっ歯類で共通した位相を有する生体成分が、RAにおける炎症などの概日リズム形成に重要な役割を有するのではないかと考えられる。今後、RAモデル動物を用いて、RA発症前から経時的にRA発症症状の進行に伴う病態及び生体成分の変化を時間生物学的な観点より評価することで、複雑なRA発症機序の解明が可能になると考えられる。

次に、炎症性サイトカインの概日リズムを考慮し、MTXの投薬タイミングの違いによる抗リウマチ効果への影響について検討した。CIAマウスは四肢の関節が腫脹する特徴を有するRAモデル動物であるため、四肢の関節炎をスコア化し評価した。MTX投与初期より投薬時刻の違いにより関節炎抑制効果に差異が現れ、最終投薬後7日目（Day 42）の関節炎スコアは、control群及び17:00投薬群と比較し5:00投薬群で有意に軽減できることが明らかとなった[9]。次に、血液学的にRA患者と非常に近似しているMRL/lprマウスでは、SAAやTNF-α濃度さらにはRA発症の指標となるIgG-RF濃度を測定した。その結果、TNF-α濃度やIgG-RF濃度は、control群と比較し1:00投薬群で有意に減少した。また、1:00投薬群のSAA濃度は、control群及び13:00投薬群と比較し有意に低値を示した[10]。以上より、MTXの投薬時刻の違いによって炎症反応に差異が現れることが明らかとなった。そして、治療効果が高かった時刻は、いずれのRAモデル動物においても1日の中でTNF-αが増加し始める時間帯にMTXを投薬した時間帯であった。したがって、抗リウマチ薬の効果の向上には、投薬タイミングを考慮することが重要であることが考えられる。

本邦では、MTXの投薬は1週間を1単位として1日目朝・夕、2日目朝を基本とする投薬がなされている。RA患者では、早朝にこわばりや炎症性サイトカインが増加する概日リズムがあることから[7]、動物実験の結果を含め総合的に判断すると、RA患者では夜間にのみMTXを投薬することで、より高い抗リウマチ効果が得られるのではないかと考えられた。そこで、本研究ではRA患者を対象に従来の治療法からMTXの投与量や投薬回数を変えずに服用時刻のみを寝る前に変更する時間治療を実施することで、治療効果が得られるか否か検討した。現在、20例のエントリーがあり、そのうち12名の被験者で時間治療を開始し1ヶ月が経過した。時間治療開始後1ヶ月間でDAS28は平均で0.68減少し、時間治療を開始することで有意にDAS28の低下が認められた。また、時間治療開始後わずか1ヶ月であるにもかかわらず、12例中4例で臨床的寛解に到達した。1ヶ月間の時間治療では、炎症反応の低下はそれほど認められないが、本研究で得られたデータとして特徴的なものとして、腫脹関節数がすべての被験者で消失したことが挙げられる。圧痛関節数も減少傾向にあり、関節痛の低減はRA患者のQOLを非常に向上できることからも、MTXの時間治療の有用性は高いと考えられる。また、寝る前に投薬する時間治療を導入することでMTX排泄遅延による副作用の増大が危惧されたが、骨髄抑制が現れた被験者はなく、安全にMTXの投薬が実施できている。以上より、MTXは生物学的製剤と比較し薬剤費が安

く、世界的にアンカードラッグとして最も使用されている抗リウマチ薬であるため、MTXの時間治療に関する本研究は、RA療法において非常に大きなインパクトを与えるものと期待できる。

本臨床研究は20症例のRA患者を対象に、時間治療による3ヶ月間の薬効評価試験を実施しており、途中経過ではあるが非常に良好な成果が得られている。また、現在エントリーしている被験者に対して12ヶ月間の長期試験を継続するための手続きを行っている。今後は、より科学性の高い評価を行うために、無作為化比較試験を実施し、RAにおけるMTXの時間治療の有用性を検証する。

本研究では、RAモデル動物を用いて、明期に高値を示す概日リズムがRA症状に存在することを明らかにした。また、これらの概日リズムを基盤に抗リウマチ薬MTXの投薬タイミングを設定することで、より高い抗リウマチ効果が得られることを明らかにした。そして、これらの基礎研究をベースに、本邦のMTXによる薬物療法を考察し、寝る前にのみMTXを投薬する時間治療法を提案し、臨床研究を実施している。現在、途中経過ではあるが、時間治療開始わずか1ヶ月であるにもかかわらず、有意なRAの症状改善が認められている。以上より、RA治療において、MTXの時間治療は有用な治療法の一つであると考えられる。

謝　辞

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Methotrexate Chronotherapy is Effective Against Rheumatoid Arthritis

Hideto To,[1] Hiromichi Yoshimatsu,[2] Mari Tomonari,[4] Hiroaki Ida,[5,6] Toshiyuki Tsurumoto,[7] Yasuhiro Tsuji,[8] Emi Sonemoto,[8] Noriko Shimasaki,[4] Satoru Koyanagi,[3] Hitoshi Sasaki,[4] Ichiro Ieiri,[2] Shun Higuchi,[2] Atsushi Kawakami,[6] Yukitaka Ueki,[8] and Katsumi Eguchi[6]

[1]Department of Medical Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Toyama, Japan, [2]Clinical Pharmacokinetics, [3]Pharmaceutics, Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, [4]Department of Hospital Pharmacy, Nagasaki University Hospital, Nagasaki, Japan, [5]Division of Respirology, Neurology, and Rheumatology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan, [6]Unit of Translational Medicine, Department of Immunology and Rheumatology, [7]Department of Macroscopic Anatomy, Graduate School of Biomedical Science, Nagasaki University, Nagasaki, Japan, [8]Sasebo Chuo Hospital, Center for Rheumatic disease, Sasebo, Japan

Methotrexate (MTX) is the most important drug for treating rheumatoid arthritis (RA). It has been stated that cytokines play an important role in the pathogenesis of RA, and that cytokine levels increase and show 24-h rhythms in RA patients. Previously, we found that arthritis was relieved after the administration of MTX at specific times in synchronization with the 24-h rhythm of tumor necrosis factor (TNF)-α in collagen-induced arthritis (CIA) animals. Based on our findings in an earlier study of the dosing time–dependent effects of MTX in MRL/lpr mice, which develop autoimmune disorders that share similarities with human RA, we examined here the utility of MTX chronotherapy in Japanese RA patients. In an initial animal modeling study, we collected blood from MRL/lpr mice at different times (2, 6, 10, 14, 18, or 22 hours after the light was turned on [HALO]), and we measured TNF-α mRNA expression in leukocytes. MTX was administered to the mice at two different dosing times (6 or 18 HALO), and various blood parameters were measured to estimate arthritis activity. TNF-α mRNA levels showed a clear 24-h rhythm with a peak at 22 HALO and a trough at 18 HALO after RA had developed. In these MRL/lpr mice, inflammation and TNF-α were markedly reduced when the MTX dosing time was matched to the time (18 HALO) when the TNF-α level began to increase. We then applied these findings to Japanese RA patients by switching them from the standard MTX three times/wk (day 1: after breakfast and supper; day 2: after breakfast schedule), to chronotherapy, in which the dose and number of doses/wk were not changed but MTX was administered once-a-day at bedtime. Disease Activity Score (DAS)28, modified health assessment questionnaire (MHAQ), and adverse effects were assessed. With MTX chronotherapy, DAS28, which is commonly used to quantitatively assess RA symptoms, was significantly improved at all follow-up clinical visit times compared with the baseline (vs. 1 mo: $p = .0197$, 2 mos: $p = .0107$, 3 mos: $p = .0087$). Significant symptom recovery was observed in 41.2% of patients, and 23.5% of patients achieved clinical remission during the 3 mos of follow-up. Functional capacity of RA patients, as indicated by the MHAQ, was markedly improved by chronotherapy. There were no severe adverse effects. Thus, we demonstrated (i) inflammation and plasma TNF-α concentrations were significantly reduced in MRL/lpr mice treated with MTX at 18 HALO, the time when TNF-α mRNA level began to increase; and (ii) MTX bedtime chronotherapy was safe, markedly reduced disease activity, and improved the functional capacity of RA patients. The findings on RA patients show that bedtime MTX chronotherapy can improve RA symptoms compared to the current standard dosing methods. (Author correspondence: hide-to@umin.net)

Keywords: Chronotherapy, Human trials, Methotrexate, Rheumatoid arthritis, Rodent models, Ig-GRF, Serum Amyloid A, TNF-α

INTRODUCTION

Methotrexate (MTX) is one of the most commonly used disease-modifying antirheumatic medications, and it induces a high American College of Rheumatology improvement response rate (Cohen et al., 2001), inhibits joint inflammation (Kremer et al., 1992; Weinblatt et al., 1992), and conveys marked survival benefit (Choi et al., 2002) in rheumatoid arthritis (RA) patients. However, MTX also causes adverse effects, such as myelosuppression and interstitial pneumonitis, because it is an

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Address correspondence to Hideto To, PhD, Department of Medical Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Tel.: +81-76-415-8811, Fax: +81-76-415-8811; E-mail: hide-to@umin.net

anticancer agent. Therefore, it is necessary to design a safe and effective dosing protocol for MTX treatment.

Chronotherapy, the dosing of medications with reference to critical 24-h rhythms of disease activity and the pharmacokinetics and pharmacodynamics of medications, has been applied for the treatment of various medical conditions, for example, asthma, hyperlipidemia, and hypertension (D'Alonzo et al., 1995; Hermida et al., 2010; Haus, 2007; Saito et al., 1991; Smolensky et al., 2007). Moreover, it has been shown to decrease the adverse effects of anticancer drugs in basic and clinical studies (Kodama et al., 2009; Lévi et al., 1997; Tabuchi et al., 2005). In previous studies, MTX showed greater therapeutic effects against childhood leukemia when administered in the evening than in the morning (Rivard et al., 1993; Schmiegelow et al., 1997). MTX also was found to show dosing time-dependent toxicity in mice (Ohdo et al., 1997).

RA is an autoimmune disorder of unknown etiology, and morning stiffness is a well-known characteristic (Arnett et al., 1988). It was reported in recent years that chronotherapy using modified-release prednisone was more effective against RA than standard immediate-release prednisone (Buttgereit et al., 2008). However, chronotherapy involving MTX, which is the first-line disease-modifying antirheumatic drug (DMARD) treatment against RA, has not been examined. In recent years, it has been found that cytokines play an important role in the pathogenesis of RA (Chu et al., 1991) and that the levels of proinflammatory cytokines are increased in RA patients. Blood cytokines show 24-h rhythms in RA patients (Arvidson et al., 1997; Sulli et al., 2002), and these rhythms correspond to morning stiffness. We, therefore, considered that RA therapy associated with cytokine 24-h rhythms might be more effective than the RA therapy protocol currently used in clinical practice. It was previously reported that collagen-induced arthritis (CIA) mice, which show a similar pathology, immunology, and genetics to RA patients, also showed similarly augmented cytokine levels (Marinova-Mutafchieva et al., 1997; Mussener et al., 1997). We previously studied whether MTX exhibited a dosing time-dependent efficacy and toxicity using CIA model animals. We found a daily variation in plasma tumor necrosis factor (TNF)-α concentration in the CIA model after RA had developed and that arthritis was relieved after the administration of MTX when synchronized to the 24-h rhythm of TNF-α (To et al., 2009). Thus, we expected the clinical application of MTX chronotherapy to improve its antirheumatic effect. Here, we assessed the dosing time-dependent effects of MTX in MRL/lpr mice, which develop autoimmune disorders that share similarities with human RA (Abe et al., 1980; Koopman & Gay, 1988). Then, based on our findings of the animal studies and the 24-h TNF-α rhythms of RA patients, we changed the dosing schedules of RA patients from the standard MTX schedule, in which MTX is administered three times/wk (day 1: after breakfast and supper; day 2: after breakfast only), to a chronotherapy schedule, in which the dose and number of doses/wk were not changed, but MTX was administered once-a-day at bedtime, to examine whether a dosing-time dependency of the therapeutic effects of MTX treatment could be detected in the RA patients.

METHODS

Animals

Male MRL/MpJ-*Tnfrsf6*^{lpr}/Crlj (MRL/lpr) mice were purchased from Charles River Japan (Japan). They were housed 6–10/cage under standardized light-dark cycle conditions (lights-on and lights-off at 07:00 and 19:00 h, respectively) at a room temperature of 24°C±1°C (range) and relative humidity of 60%±10% (range) and were allowed free access to food and water.

Patients

Participants were enrolled at Sasebo Chuo Hospital in Japan from July to September 2009. All were outpatients, aged 20 yrs or older, had a diagnosis of adult-onset RA, a disease duration of ≥3 mos, and Disease Activity Score (DAS)28 of ≥3.2, and were not using biological drugs. The patients had to have received MTX for ≥3 mos before the study and to have received a stable dose for ≥2 mos before the screening. Exclusion criteria included treatment with unstable doses of DMARD within 3 mos of screening, prior treatment with tacrolimus or mizoribine, current use of ≥10.1 mg/day of prednisolone, and beginning prednisolone treatment within 1 mo of screening. Individuals with important concurrent medical diseases, who had undergone surgery within 6 mos, or pregnant were not excluded.

Ethics

All experiments were conducted ethically (Portaluppi et al., 2010). Animal experiments were performed after formal approval had been received from the Institutional Ethical Committee for Research on Animals of Nagasaki University, and human trials were conducted after all participants gave their written informed consent before the screening. The study protocol was approved by the ethics committees for each site and was registered with the UMIN Clinical Trials Registry under number UMIN000000928.

Preparation of MTX in MRL/lpr Mice

MTX was dissolved in 7% sodium bicarbonate, prepared at a suitable concentration, and intraperitoneally (i.p.) administered at 0.01 mL/g.

Twenty-Four-Hour Rhythm of TNF-α mRNA Expression in MRL/lpr Mice

Blood was taken from the hearts of 10- and 15-wk-old MRL/lpr mice (n = 5–7) at different times (2, 6, 10, 14, 18, or 22 hours after the light had been turned on [HALO]), and leukocytes were obtained. Total RNA was extracted from the leukocytes using TRIzol reagent

(Invitrogen), and cDNA was synthesized and amplified using an ExScriptTM RT Reagent Kit. Quantification of TNF-α mRNA was performed by real-time polymerase chain reaction (PCR) detection, using an ABI PRISM 7000 sequence detection system (Applied Biosystems) and SYBR Green detection of the amplification products. The copy numbers of the transcripts were measured against copy-number standard curves for the cloned target templates consisting of 10-fold serial dilution points. The amplification mixtures contained 10 μL of 2× SYBR Premix Ex TaqTM, 1 μL of cDNA synthesis mixture, 4 pmol each of the forward and reverse primers, and distilled water in a total volume of 20 μL. The *mTNF*-α primers used were as follows: forward: 5'-ACAAGGCTGCCCCGACTAC-3' and reverse: 5'-TTTCTCCTGGTATGAGATAGCAAATC-3'. All mRNA expression levels were normalized to the levels of β-actin.

MTX Dosing Time–Dependent Change in Serum Amyloid A (SAA), Immunoglobulin G–Rheumatoid Factor (IgG-RF), and TNF-α Concentrations in MRL/lpr Mice

Based on the 24-h rhythm of TNF-α mRNA level (see Figure 1), MTX (10 mg/kg) was administered to 10-wk-old MRL/lpr mice (n = 8) three times/wk for 2 wks (total of 60 mg/kg of MTX) at 6 or 18 HALO when the TNF-α level begins to decrease or increase. Sodium bicarbonate (7%) was administered to the control group (n = 14 or 15). On day 14, blood was taken at 2 HALO, and the samples were immediately centrifuged at 3,000 rpm for 15 min. Plasma was stored at –80°C until analyzed.

Measurement of SAA, IgG-RF, and TNF-α Levels in Blood

SAA and IgG-RF were measured using a Mouse SAA ELISA (enzyme-linked immunosorbent assay) kit (SW type) and a Mouse IgG Rheumatoid Factor ELISA kit (Shibayagi, Japan). Multianalyte profiling was performed using the Luminex-100 system (Luminex). The acquired fluorescence data were analyzed using MasterPlex QT software (Ver. 1.2; MiraiBio). Plasma TNF-α concentrations were determined using the Mouse Inflammatory Cytokine 4-Plex kit (Biosource). All analyses were performed according to the manufacturers' protocols.

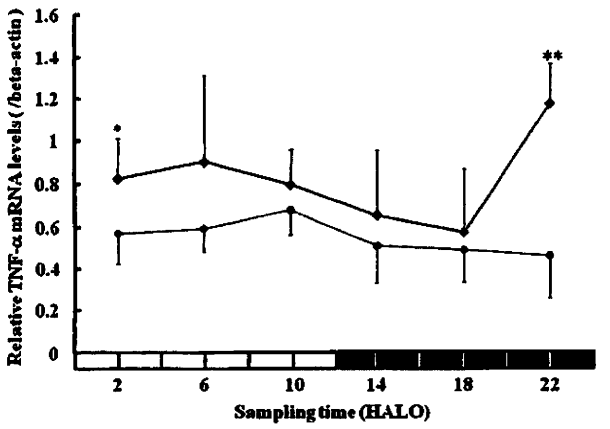

FIGURE 1. Twenty-four-hour rhythms of TNF-α mRNA in 10- (•) and 15- (♦) wk-old MRL/lpr mice. The relative expression levels of TNF-α mRNA compared to those of β-actin are shown. Each value represents the mean and SD of five to seven mice. $^{*}p < .05$ and $^{**}p < .01$ compared to the 10-wk-old group. TNF-α mRNA levels in leukocytes showed a clear 24-h rhythm with higher levels at 22 HALO and lower levels at 18 HALO in the 15-wk-old group (F from ANOVA = 3.55, $p < .05$; p from cosinor = .06).

Clinical Trial

The clinical trial was a prospective, single-arm study to assess the effects and safety of MTX chronotherapy in RA patients. In the chronotherapy, the dose and number of doses/wk were not changed from the original MTX dosing schedule of each patient, but MTX was orally administered once-a-day, always at bedtime. Patients were scheduled for routine follow-up clinic visits in wks 4, 8, and 12 of chronotherapy, and efficacy and safety-related assessments were performed. The primary endpoints were changes in the DAS28 and leukocyte count from the baseline (wk 0) to 3 mos of chronotherapy. The secondary endpoints included tender and swollen joint counts, the patients' assessment of their overall disease activity, modified health assessment questionnaire (MHAQ), and the C-reactive protein (CRP), SAA, matrix metalloproteinase (MMP)-3, RF, TNF-α, and interleukin (IL)-6 levels in serum.

DAS28 is a composite score based on tender and swollen joint counts (28 joints), the patient's global assessment of their disease activity (100 mm Visual Analog Scale [VAS]: 0 = no activity, 100 = extreme activity). DAS28 values were calculated as follows:

$$\text{DAS28 (CRP)} = 0.56 \times \sqrt{(\text{TJC28})} + 0.28 \times \sqrt{(\text{SJC28})} + 0.014 \times \text{GH} + 0.36 \times \ln(\text{CRP} + 1) + 0.96,$$

where TJC = tender joint count, SJC = swollen joint count, GH = general health, and CRP = C-reactive protein in mg/dL. A score of ≥5.1 indicates high disease activity, a score > 3.2 and ≤5.1 indicates moderate disease activity, a score < 3.2 indicates low disease activity, and a score < 2.6 indicates disease remission (van Gestel et al., 1996). The European league against rheumatism (EULAR) response states were classified as follows: good responders were patients displaying an improvement of > 1.2 and a present score of ≤3.2; moderate responders were patients displaying an improvement of > 0.6 and ≤1.2 and a present score of ≤5.1, or an improvement of > 1.2 and a present score of > 3.2; and nonresponders were any patients displaying an improvement of ≤0.6, or patients displaying an improvement of > 0.6 and ≤1.2 and a present score of > 5.1 (Fransen & van Riel, 2005).

Myelosuppression was evaluated according to the Common Toxicity Criteria for Adverse Events version 3.0 (CTCAE 3.0). Leukocyte counts were graded as: 1 (3500–3000 cells/mm^3), 2 (2999–2000 cells/mm^3), 3 (1999–1000 cells/mm^3), or 4 (< 1000 cells/mm^3).

MHAQ assesses the ability of patients to perform daily activities using eight questions. The final MHAQ score is the mean score of the eight questions and ranges from 0 to 3, with higher levels reflecting greater disability (Pincus et al., 1983).

CRP concentrations were determined at Sasebo Chuo Hospital, and SAA, MMP-3, and RF levels were analyzed by a central laboratory. TNF-α and IL-6 levels were determined using the Bio-Plex kit. All analyses were performed according to the manufacturers' protocols.

Statistical Analysis

The data are presented as the mean±standard deviation (SD), except for the data regarding the TNF-α mRNA, TNF-α, SAA, and IgG-RF levels of the mice and the DAS28 and DAS28-related parameters of the RA patients. Groups were compared by one-way analysis of variance (ANOVA) and repeated-measures ANOVA, and differences between groups were determined using Scheffe's or Fisher's protected least significant difference test. We defined 24-h rhythmicity as significant when both cosinor analysis and one-way ANOVA were significant. Cosinor analysis is a natural way of modeling cyclic behavior, and is performed by a single 24-h cosine curve fitting using software (Time Series Analysis Seriel Cosinor 6.3).

The MHAQ results are shown as box plots. 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; and the upper and lower extents of the vertical lines extending from the boxes represent the values of the 90th and 10th percentiles, respectively. Leukopenia is shown as each patient's score. Among-group comparisons of MHAQ, RA-related parameters, and leukopenia were made using the Friedman test. Intragroup post hoc testing was performed using the Wilcoxon signed-rank test with Bonferroni's correction after the Friedman test. A probability level of less than .05 was considered to be significant.

RESULTS

Twenty-Four-Hour Rhythms of TNF-α mRNA Expression in Leukocytes of MRL/lpr Mice

TNF-α mRNA levels were detected after total RNA had been extracted from the leukocytes of MRL/lpr mice. There was no significant 24-h rhythm in TNF-α mRNA levels at 10 wks by ANOVA and cosinor analysis. However, at 15 wks, the TNF-α mRNA levels showed a clear 24-h rhythm with higher levels seen at 22 HALO and lower levels observed at 18 HALO (F from ANOVA = 3.55, $p < .05$; p from cosinor = .06; Figure 1).

Influence of MTX Dosing Time on SAA, IgG-RF, and TNF-α Levels in MRL/lpr Mice

MTX was administered three times/wk at 6 or 18 HALO based on the 24-h rhythm of the TNF-α mRNA level (Figure 1). The plasma TNF-α levels did not differ between the control and 6 HALO groups, although they were significantly lower in the 18 HALO group than in the control group ($p < .05$; Figure 2). The SAA concentration in the 18 HALO group was significantly lower than that in the control and 6 HALO groups (vs. control: $p < .01$; vs. 6 HALO: $p < .05$; Figure 2). The groups treated with MTX showed a markedly decreased IgG-RF index compared with the control group (vs. 18 HALO: $p < .05$; Figure 2).

Baseline Characteristics of the Japanese RA Patients

Twenty-two rheumatoid arthritis patients between 41 and 78 yrs of age were enrolled and (77%) received MTX chronotherapy for the entire 3 mos of the study. Their demographic and baseline characteristics are described in Table 1. One of the 17 patients had a DAS28 of >5.1, indicating high disease activity, and the other

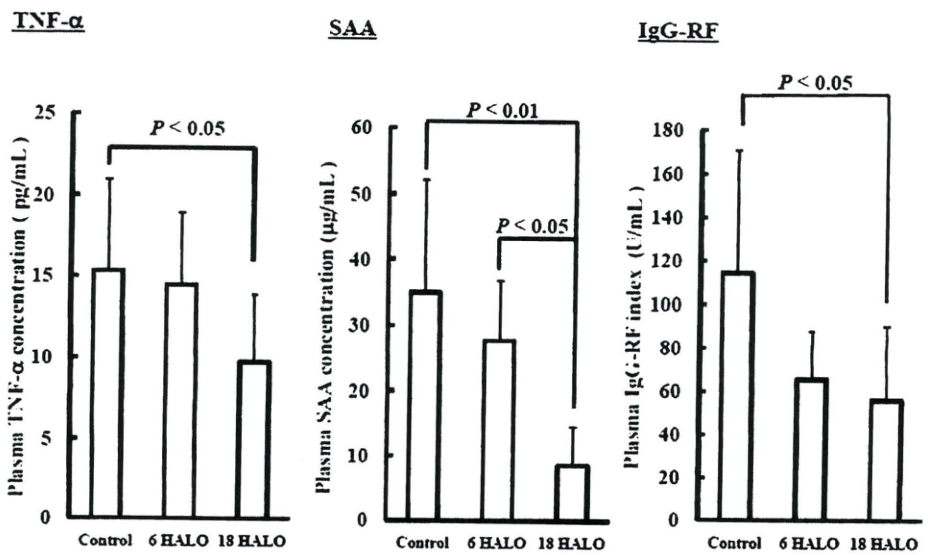

FIGURE 2. Influence of MTX dosing time on the plasma SAA concentration, IgG-RF index, and TNF-α concentration in MRL/lpr mice. MTX (10 mg/kg) was administered three times/wk for 2 wks (total of 60 mg/kg of MTX) at 6 and 18 HALO. Each value represents the mean and SD of 8 to 15 mice. The TNF-α level was significantly lower in the 18 HALO group than in the control group ($p < .05$). The SAA and IgG-RF levels were significantly decreased in the 18 HALO group compared with the control group ($p < .01$ and $p < .05$).

TABLE 1 Demographic and baseline characteristics of the Japanese RA patients

Number of patients	17
Age (yrs)	61 (41-78)[a]
Sex	5/12[b]
Body weight (kg)	50.2 (29.8-72.0)[a]
Duration of RA (yrs)	11.8 (2-33)[a]
MTX dose (mg/wk)	6 (4-8)[c]
Disease Activity Score 28	3.83 (3.25-5.30)[a]
Stage	
I	
II	0
III	4
IV	5
	8
Class	
I	1
II	16

[a]Mean (minimum-maximum).
[b]Male/female.
[c]Median (minimum-maximum).

patients had a DAS28 of between 3.2 and 5.1, indicating moderate disease activity.

Influence of MTX Chronotherapy on DAS28, MHAQ, and Adverse Effects in Japanese RA Patients

Table 2 shows the four factors involved in the calculation of the DAS28. The tender joint count decreased slightly after chronotherapy. On the other hand, the swollen joint count decreased markedly from 1.18 at baseline (0 mos) to 0.29 at 3 mos. The CRP level continued to improve throughout the 3-mo study period, and improved by 64.2% after the chronotherapy compared with baseline. VAS, which represents the patient's global assessment of his/her own disease activity, hardly changed despite the patients recognizing the change in the MTX administration method.

The mean DAS28, which is the most commonly used indicator for quantitatively estimating RA symptoms, was 3.65 at 2 mos before chronotherapy and 3.83 at the start of chronotherapy ($p = .279$), and the condition of the RA patients was maintained between moderate and high disease activity. DAS28 was significantly improved after MTX chronotherapy throughout the three clinic visits compared with the baseline (vs. 1 mo: $p = .0197$; 2 mos: $p = .0107$; 3 mos: $p = .0087$ of follow-up; Figure 3A). On the basis of the EULAR definitions of the treatment response according to DAS28 at 3 mos, seven (41.2%) patients achieved a moderate response, and four (23.5%) patients attained clinical remission (DAS28 < 2.6).

MHAQ, an indicator of functional capacity of RA patients, was markedly decreased by 3 mos of chronotherapy ($p = .0529$; Figure. 3B).

In this study, there were no severe adverse effects.

Influence of MTX Chronotherapy on RA-Related Factors in Japanese RA Patients

Secondary efficacy variables are shown in Table 3. The SAA concentrations gradually decreased over the 3 mos of the chronotherapy, and had improved by 60.6% after the chronotherapy compared with baseline. The other RA-related factors did not change throughout the study.

DISCUSSION

Pain and stiffness show 24-h rhythms with a peak in the early morning in many RA patients (Bellamy et al., 1991, 2002; Kowanko et al., 1982), and the 24-h rhythm of pain and stiffness may play a role in local and systemic inflammatory responses. Herold and Günther (1987) reported that plasma CRP levels, an indicator of inflammatory responses, showed a 24-h rhythm with a peak in the early morning and a trough in the evening in RA patients, which matches the rhythm of pain and stiffness. Proinflammatory cytokines, such as TNF-α and interleukin (IL)-6, are secreted from activated monocytes, and macrophages promote CRP levels in hepatocytes. There were clear 24-h rhythms in the blood concentrations of these cytokines, with higher levels seen in the early morning in RA patients (Crofford et al., 1997; Perry et al., 2009). Since the 24-h rhythms of CRP and cytokines are similar, it is considered that cytokine rhythms contribute to the rhythm of CRP. In our preliminary study using CIA model mice, a significant 24-h rhythm was demonstrated for the concentration of SAA, a marker of the inflammatory response induced by cytokines, and SAA levels were higher at 2 HALO and lower at 22 HALO in mice (data not shown). The plasma TNF-α concentration also showed a 24-h rhythm with a peak at 2 HALO (To et al., 2009). In the current study, we examined TNF-α

TABLE 2 Changes in Japanese RA patients between before and after treatment with MTX chronotherapy

	Time (Mos) before and after MTX chronotherapy					
	-2	0	1	2	3	*p* value
Tender joint count	9.77 ± 8.71	10.35 ± 7.55	8.77 ± 8.07	8.77 ± 8.20	8.88 ± 7.78	*N.S.*
Swollen joint count	0.65 ± 1.58	1.18 ± 1.51	0.18 ± 0.73	0.41 ± 1.46	0.29 ± 0.99	*N.S.*
CRP (mg/dL)	0.60 ± 0.94	0.67 ± 1.29	0.67 ± 1.11	0.54 ± 0.94	0.43 ± 0.71	*N.S.*
VAS (mm)	41.18 ± 23.69	35.47 ± 25.65	32.94 ± 23.26	32.06 ± 21.94	32.94 ± 22.01	*N.S.*

Data are presented as the mean ± SD.

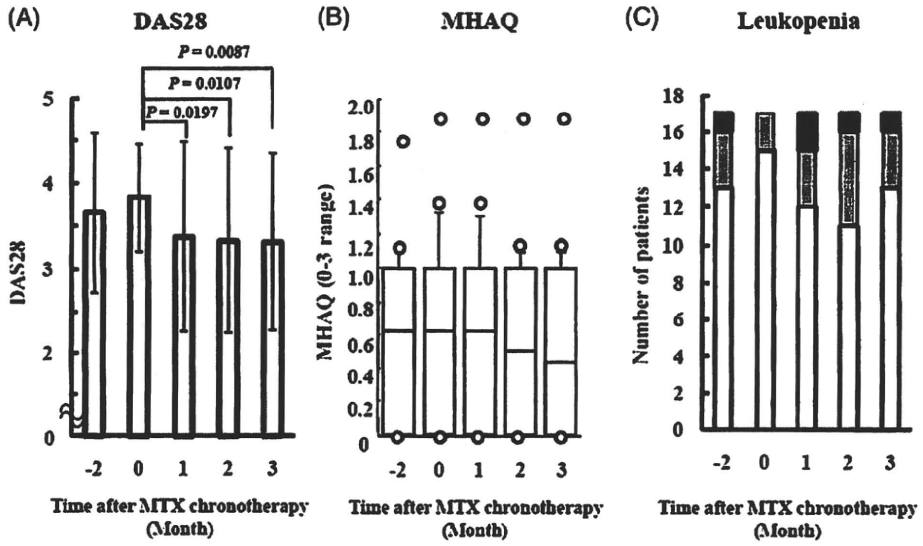

FIGURE 3. Efficacy and safety during 3 months of MTX treatment in Japanese RA patients. Values are presented as the mean and SD for DAS28 (A) and as a box plot of MHAQ (B) of the 17 patients. (C) Leukopenia □: Grade 0, ▨: Grade 1, ■: Grade 2. The mean DAS28 was 3.65, 3.83, 3.37, 3.33, and 3.31 at –2, 0, 1, 2, and 3 mos, respectively, and DAS28 was significantly improved at 3 mos compared with the baseline (vs. 1 mo: $p = .0197$; 2 mos: $p = .0107$; 3 mos: $p = .0087$).

TABLE 3 Changes in Japanese RA patients after 3 months of treatment with MTX chronotherapy

	Time after MTX chronotherapy (mos)				
	0	1	2	3	*p*-value
SAA (μg/mL)	13.2 [7.35–24.2]	7.0 [4.6–27.9]	9.5 [4.4–29.1]	8.0 [4.0–31.1]	*N.S.*
MMP-3 (ng/mL)	81.3 [50.1–151.0]	89.4 [50.5–162.3]	79.9 [54.9–139.3]	83.8 [51.1–163.8]	*N.S.*
RF (IU/mL)	57.0 [3.5–161.3]	58.0 [2.0–127.0]	54.0 [1.3–202.8]	52.0 [2.3–192.5]	*N.S.*
TNF-α(pg/mL)	6.44 [2.72–19.8]	6.44 [4.56–61.2]	4.56 [1.13–86.4]	6.44 [3.18–41.0]	*N.S.*
IL-6 (pg/mL)	7.37 [3.55–33.1]	16.0 [3.47–43.6]	8.40 [4.35–41.1]	9.09 [6.10–29.1]	*N.S.*

Data are presented as median values [25 percentile–75 percentile].

mRNA expression in MRL/lpr mice. A clear 24-h rhythm, involving higher levels during the late dark phase, was observed for TNF-α mRNA expression. Plasma SAA and TNF-α concentrations also showed 24-h rhythms with peaks at 2 HALO in MRL/lpr mice (data not shown). Therefore, changes in TNF-α concentration may affect the 24-h rhythm of SAA.

In a previous study, our findings with CIA model mice suggested that choosing an optimal dosing time that is associated with the 24-h cycle of TNF-α would lead to the more effective treatment of RA with MTX (To et al., 2009). In this study, in which we used MRL/lpr mice, the 18 HALO group showed significantly reduced plasma SAA levels compared to the control and 6 HALO groups and displayed significantly reduced TNF-α levels compared to the control group. MTX inhibits the production of cytokines by suppressing lymphocyte proliferation (Williams et al., 2001) and TNF-α transcriptional activity (Becker et al., 1998). However, neither the 6 nor 18 HALO group showed a significant decrease in their leukocyte counts compared with the control group (data not shown). Thus, MTX may reduce plasma TNF-α level by suppressing transcriptional activity rather than suppressing lymphocyte proliferation. In this study, the dosing time-dependent change in the SAA level corresponded to an identical change in TNF-α level. It is likely that the level of SAA is also reduced due to a decrease in TNF-α concentration after MTX administration at 18 HALO. In addition, arthritis and inflammation were reduced in the dark phase (activity span of mice), when plasma TNF-α concentration began to increase, in both the CIA model and MRL/lpr mice. These findings reveal that the therapeutic effects of MTX treatment can be improved by administering MTX when the TNF-α level begins to increase in the blood.

From our studies using RA model animals and the 24-h rhythms of RA patients, we anticipated that higher therapeutic effects compared with those of the current, conventional, treatment schedule could be achieved with chronotherapy in which MTX was administered before bedtime and the dose and number of doses/wk remained the same. Bedtime MTX chronotherapy improved the DAS28 in 14 of 17 patients (82.4%), and the

mean DAS28 value was significantly decreased by 0.460 at 1 mo, 0.506 at 2 mos, and 0.521 at 3 mos after the start of chronotherapy. In particular, despite the dose and number of doses remaining the same, 23.5% of patients achieved clinical remission, and a significant therapeutic effect was observed in 41.2% of patients. Interestingly, four (80%) male and three (25%) female participants achieved a moderate response, with men showing a greater therapeutic effect compared with the women. Although the number of patients was not sufficient in this study, sex differences may lead to variations in the efficacy of the chronotherapy regimen.

DAS28 is calculated from the following four parameters: tender and swollen joint counts, patient's global assessment of his/her disease activity, and CRP concentration. The tender joint count and CRP level changed little throughout this study. On the other hand, the swollen joint count was markedly decreased in all patients after 3 mos of chronotherapy. The patients' global assessments of their disease activity were susceptible to bias because each patient evaluated their own degree of illness. In this study, the patients understood that the method of MTX administration had changed; however, the patients' global assessments of their disease activity did not show significant changes, even though a placebo effect of the chronotherapy was anticipated. Therefore, it was considered that the placebo effect did not contribute to the observed significant decrease in DAS28. It was revealed that MTX chronotherapy improved the functional capacity of RA patients, although we could not clarify the factors responsible for the improvement in MHAQ from the data obtained in this study. Almost all patients had mild leukopenia, although the incidence of leukopenia higher than Grade 1 increased from 11.8% to 23.5% throughout the study. Moreover, there were no severe adverse effects in the 17 patients who completed the trial. From these results, it was demonstrated that MTX chronotherapy is safe and markedly improved disease activity and the functional capacity of RA patients.

Daily variations in the pharmacokinetics of MTX may be involved in the higher therapeutic effect in RA patients brought about by administering MTX at night in the present study. Although the pharmacokinetics of MTX were not determined in this study, it was reported in previous studies that there were no dosing time-dependent changes in MTX pharmacokinetics in patients with cancer (Balis et al., 1989; Robinson et al., 1989). Moreover, no difference was noted in MTX pharmacokinetics according to the clock hour of injection when MTX was administered intramuscularly at 10:00 or 18:00 h to RA patients (Carpentier et al., 1998). In our previous study, CIA mice administered MTX in the rest phase showed a larger area under the plasma concentration-time curve of MTX than mice administered MTX in the active phase (To et al., 2009). However, the daily variations in the pharmacokinetics of MTX were not related to the dosing-time dependency of MTX efficacy. From the results of our studies and the 24-h cycles of cytokines in RA patients (Crofford et al., 1997; Perry et al., 2009), it is thought that MTX has a significant dosing time-dependent anti-inflammatory action and that this effect may be due to the 24-h rhythms of cytokine levels rather than the pharmacokinetics of MTX.

CONCLUSIONS

The present study, using MRL/lpr mice, revealed that administering MTX at specific times in accordance with the 24-h rhythm of TNF-α leads to decreased inflammatory responses to RA. It was demonstrated in RA patients that disease activity and the patients' functional capacity were improved while safety was maintained when MTX was given once-a-day at bedtime based on our results of the animal study and the 24-h rhythm of TNF-α in RA patients. Choosing an optimal dosing time that is associated with the 24-h rhythms of RA symptoms is, therefore, expected to lead to more effective MTX therapy for RA. We consider that MTX chronotherapy, which showed strong therapeutic effects compared with the current standard treatment in this study, has potential as an RA therapy. To confirm our findings, we are now performing a double-blind, randomized, controlled trial.

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〈記録〉第31回 日本臨床薬理学会年会 2010年12月1～3日 京都
シンポジウム25：時間疾患の現状

4．関節リウマチにおける病態の概日リズムと時間薬物療法

藤 秀人*

1．はじめに

関節リウマチ（RA）は関節滑膜を病変の主座とする進行性の全身性炎症性疾患であるが，発症機序は十分に明らかにされていない．これまでRAの薬物治療は痛みや炎症を抑えるために非ステロイド性抗炎症薬（NSAID）や副腎皮質ホルモン薬，RAの免疫異常を修飾することができるメトトレキサート（MTX）などの疾患修飾性抗リウマチ薬（DMRAD）が用いられてきた．また近年では，抗ヒトtumor necrosis factor-α（TNF-α）モノクローナル抗体製剤であるインフリキシマブなどの生物学的製剤の開発により，RAの炎症反応に関与する炎症性サイトカインの作用を効果的に抑制することで，従来の抗リウマチ薬と比較して高い有効性を示すようになった．しかし，生物学的製剤に対する抗体産生や高い薬剤費など多くの問題も山積している．

科学技術の発達によってさまざまなRAの診断法や薬物治療法が開発されており，RA薬物治療は今まさにパラダイムシフトの真っただ中にある．そのため，RA病態の特徴や既存の抗リウマチ薬の特性を理解し，より効果的な治療法を再度検討する良い機会であり，我々はRAの生体リズムという観点からRAの薬物療法について研究を行っている．

2．RAにおける病態の概日リズム

RA患者には他の疾患の患者と異なり，特徴的な症状として朝のこわばりが存在する．朝のこわばりは，深夜から早朝にかけて関節の痛みなどとともに現れ，日中にはほとんど消失する症状であり[1]，多くのRA患者に発現する．これらの原因の詳細は十分に明らかになっていないものの，炎症反応が一部関与していると考えられている．炎症反応の指標であるC-reactive protein（CRP）は，RA患者で関節の痛みやこわばりに沿って早朝に高値，夕方に低値を示す概日リズムを有することが報告されている[2]．CRPは，炎症性サイトカインによって肝細胞で産生されるが，これらの炎症性サイトカインはRAにおける炎症反応や病態形成に重要な役割を担っていることが知られている．したがって，CRPの概日リズムには，炎症性サイトカインの概日リズムが関与している可能性が考えられる．RA患者のinterleukin-6（IL-6）やTNF-αなどの炎症性サイトカインの血中濃度は，早朝に最高値を示すという概日リズムが認められる[3]．また，健常人や自己免疫疾患である結合組織症の患者ではIL-6の概日リズムは認められないため，このような概日リズムはRA患者特有の現象であると考えられる[3]．以上より，こわばりの発症機序に少なからず炎症性サイトカインの日周リズムが関与しているのではないかと考えられている．現在，このような日周リズム形成の制御因子解析が進んでおり，今後の研究の進展に期待したい．

3．これまでのRA治療薬における時間薬物療法

RAにおけるこわばり等の概日リズムの存在は古くからよく知られていたが，このような周期に合わせた薬物療法（時間薬物療法）はほとんど行われていない．Arvidsonらは，合成副腎皮質ホルモン薬であるプレドニゾロンを2：00か7：30に1日1回投薬し，時間治療前後で治療成績を評価した．その結果，2：00投薬群では朝のこわばりなどにおいて7：30投薬群と比較して顕著に軽減できることを明らかにした[4]．しかし，残念なことに現在のRAの薬物治療においてこの時間治療は広く汎用されるには至っていない．その理由としては，有効性は認められるがプレドニゾロンを2:00に服用することが，臨床的には非現実的であったことなどが考えられる．

しかし，2008年にButtgereitらによってRAに対する時間治療の新たな取り組みがLancetに報告された[5]．本試験はプレドニゾンの徐放化製剤による試みであり，深夜服用が回避され寝る前22：00の服用が新製剤によって可能となった．従来の薬剤を用い朝投薬されるグループと新徐放性製剤を用いて寝る前に投薬されるグループの2群に分けられ，二重盲検比較試験が実施された．その結果，新徐放性製剤群が既存薬剤群と比較して有意に朝のこわばりを軽減できることが明らかとなった．

以上のように，RAにおける炎症抑制効果については，ステロイドの時間薬物療法の有用性が証明されている．

4．MTXにおける時間薬物療法の試み

これまでに炎症抑制については，ステロイドによる時間薬物療法の検討がなされているが，現在のRA治療の主要ターゲットとなっている免疫異常の改善を目的としたDMARDや生物学的製剤を対象とした時間薬物療法に関する研究報告は皆無に等しい．そこで，我々は，世界で最

* 富山大学大学院医学薬学研究部（薬学）医療薬学研究室
〒930-0194 富山市杉谷2630

も用いられているDMARDのMTXを用いて，RAモデル動物およびRA患者を対象に時間薬物療法の有用性を検証した．

四肢の関節にRA様の関節炎を発現するII型コラーゲン感作（CIA）マウスを対象に，未感作および感作後における血漿中TNF-α濃度を測定した結果，CIA群では，未感作群と比較しいずれの測定時点でもTNF-α濃度が有意に上昇し，9：00に最高値，21：00に最低値を示す有意な日周リズムが発現した（Fig. 1）．次に，この日周リズムを考慮して，TNF-α濃度が増加し始める5：00もしくは低下している17：00にMTXを投与して，投薬時刻の違いによる関節炎抑制効果を評価した．その結果，5：00投薬群において，control群および17：00投薬群と比較して有意に関節炎の増悪を抑制できることが明らかとなった[6]．

以上のRAモデル動物による実験結果とRA患者の炎症性サイトカインの日周リズムから，MTXの投薬タイミングを推計すると，MTXを夜間（就寝前）に投薬することで，より効果的な治療成果が得られることが予想された．そこで，RA患者を対象にMTXの時間薬物療法を行うことで，従来の治療方法と比較し治療効果の向上および副作用の軽減が可能か否かについて評価した．

Fig. 2には，MTXの時間薬物療法によって得られた代表的な症例の治療成績を示した．RAの臨床指標の1つであるDAS28は，時間治療開始前と比較し，時間治療開始後早期より低下し，高い治療効果が認められた．さらに，本試験全体では，17例の被験者（男性5例，女性12例）が3カ月間の時間治療を経験し，DAS28（平均値）は，時間治療開始前と比較し時間治療開始後3カ月で有意に減少し，23.5%の被験者が臨床的寛解に到達した．さらに，41.2%の被験者でEULAR改善基準の中等度反応が認められた．また，試験期間中重篤な副作用は認められなかった．

5．まとめ

RAには，疾患特異的な日周リズムを有しており，これらの制御因子の解明によって，将来より効果的な医薬品の開発につながると期待される．さらに，我々は，動物実験および臨床研究にて，MTXの時間薬物療法の有用性を明らかにした．現在，無作為割付二重盲検比較試験を実施しており，より科学的なエビデンスが構築できることを期待している．以上，今後，いくつもの検討課題は残るものの，比較的安価であるMTXを用いた時間薬物療法は，より効率的なRA薬物療法の提案が可能となり，医療費の削減にもおおいに寄与できると考えられる．

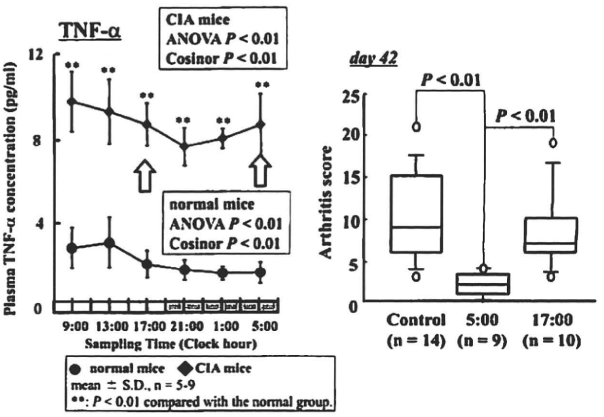

Fig. 1　RAモデル動物における血中TNF-αの日周リズムおよびMTXの投薬時刻の違いによる関節炎抑制効果への影響

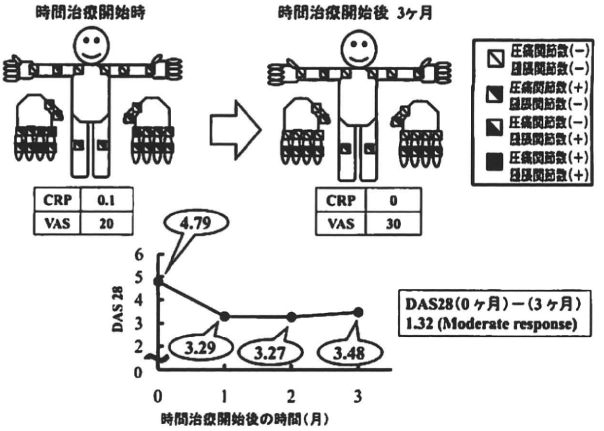

Fig. 2　MTXの時間薬物療法の一例

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