J Pharmacol Sci **116**, 264 – 273 (2011)

Journal of Pharmacological Sciences

Full Paper

Dosing Time–Dependency of the Arthritis-Inhibiting Effect of Tacrolimus in Mice

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Received February 5, 2011; Accepted May 1, 2011

Abstract. Stiffness and cytokine in blood levels show 24-h rhythms in rheumatoid arthritis (RA) patients. We previously revealed that higher therapeutic effects were obtained in RA patients and RA model animals when the dosing time of methotrexate was chosen according to the 24-h rhythms to cytokine. In this study, we examined whether a dosing time–dependency of the therapeutic effect of tacrolimus (TAC) could be detected in collagen-induced arthritis (CIA) and MRL/lpr mice. To measure the levels of cytokines and serum amyloid A (SAA), blood was collected from CIA mice at different times. TAC was administered at two different dosing times based on these findings and its effects on arthritis and toxicity were examined. Plasma tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and SAA concentrations showed obvious 24-h rhythms with higher levels during the light phase and lower levels during the dark phase after RA crisis. The arthritis score and leukocyte counts were significantly lower in the group treated at 2 h after the light was turned on (HALO) than in the control and 14 HALO–treated groups. Our findings suggest that choosing an optimal dosing time could lead to the effective treatment of RA by TAC.

Keywords: tacrolimus, chronopharmacology, rheumatoid arthritis, circadian rhythm, cytokine

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder of unknown etiology and a chronic progressive disease (1, 2). Human blood and synovial fluid contain high concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which are inflammatory cytokines, and excess production of these cytokines plays a central role in the pathogenesis of RA (3 – 5). Morning stiffness is a characteristic feature of RA (6). It is generated in the period from midnight to early morning and is seldom recognized in the daytime (7 – 9). Although the mechanism behind the 24-h rhythm of the morning stiffness has not been fully elucidated, the inflammatory response may contribute to the rhythm. It was reported that plasma C-reactive protein (CRP) levels showed a 24-h rhythm that peaked in the early morning in RA patients, which matches the rhythms of pain and stiffness (10). The TNF-α and IL-6 secreted from activated monocytes and macrophages promote CRP expression in hepatocytes. In previous studies, clear 24-h rhythms in the blood concentrations of these cytokines with higher levels in the early morning were seen in RA patients (11, 12). Since the 24-h rhythms of CRP and cytokines are similar, it is considered that cytokine rhythms contribute to the rhythm of CRP.

Chronotherapy has decreased adverse effects and improved therapeutic effects in basic and clinical studies (13 – 15). RA chronotherapy has been studied using glucocorticoid and benoxaprofen (16 – 18). We also revealed that optimizing the dosing time of methotrexate, which is a disease-modifying antirheumatic drug that has been used to treat many RA patients, improved the symptoms of RA compared to the current standard dosing methods (19, 20).

Tacrolimus (TAC) has been extensively evaluated for

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Published online in J-STAGE on June 18, 2011 (in advance)
doi: 10.1254/jphs.11029FP

use in the transplantation field such as for liver transplantation. Its immunosuppressive action inhibits the activation of calcineurin, a phosphatase (21, 22). These are exerted by inhibiting the mRNA transcription of cytokine production such as IL-2 production, which is required for the activation of T cells (23). TAC was expected to be useful as an RA therapeutic drug because it inhibits the production of inflammatory cytokines (24, 25). It was reported that the adverse effects of TAC depend on dosing time in rats (26, 27). Therefore, we considered that choosing an optimal dosing time associated with the 24-h rhythm of RA symptoms affected by cytokines levels could lead to effective treatment of RA by TAC.

In the present study, to detect the 24-h rhythms of serum amyloid A (SAA) and cytokines, we measured their concentrations at six different times in collagen-induced arthritis (CIA) mice. TAC was administered at two different dosing times based on these findings and then its efficacy and toxicity were evaluated.

Materials and Methods

Animals

DBA/1J male mice (6-week-old), MRL/lpr male mice (9-week-old) and ICR male mice (5-week-old) were purchased from Charles River Japan, Inc. (Yokohama). The mice were housed under standardized light–dark cycle conditions (lights on and off at 7:00 and 19:00, respectively) 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 had been received from the Institutional Ethical Committee for Research on Animals.

Induction of arthritis induced by collagen (CIA)

Bovine type II collagen (CII), which was isolated and purified from bovine articular cartilage, was purchased from Chondrex, Inc. (Redmond, WA, USA). The seven-week-old DBA/1J mice were intradermally immunized at day 0 by the administration of 100 μg CII in Freund's complete adjuvant (FCA, Chondrex, Inc.). A booster injection of 200 μg CII in FCA was intradermally administered on day 14.

Preparation of tacrolimus

TAC, which was supplied by Astellas Pharma, Inc. (Tokyo), was dissolved in saline (final concentration: 0.4 mg/mL). TAC was intraperitoneally (i.p.) administered to the mice at 0.01 mL/g.

Experiment I: 24-h rhythm in the plasma SAA concentrations of CIA mice

Blood was collected at different times (2, 6, 10, 14, 18, or 22 h after the light was turned on (HALO) from CIA mice (n = 5) on day 24 after immunization or normal mice (n = 5 or 6). All blood samples were immediately centrifuged at 3,000 rpm for 15 min, after which the plasma was removed and frozen at −80°C until the assay. Plasma SAA was measured using a Mouse SAA ELISA KIT (SW type) (Shibayagi Co., Ltd., Shibukawa).

Experiment II: 24-h rhythms in plasma TNF-α, IL-6, and IL-1β concentrations in CIA mice

To measure the concentrations of TNF-α, IL-6, and IL-1β, blood was collected at different times (2, 6, 10, 14, 18, or 22 HALO) from CIA mice (n = 11 or 16) on days 24, 31, and 38 after immunization or normal mice (n = 5 or 6). All blood samples were immediately centrifuged at 3,000 rpm for 15 min, after which the plasma was removed and frozen at −80°C until the assay.

Experiment III: TAC dosing time–dependent suppression of CIA

TAC or saline was administered 17 days after the first immunization. TAC (4 mg/kg) or saline was i.p. injected into the CIA mice (n = 14) at 2 or 14 HALO every day for 3 weeks. Saline was administered in the control group (n = 24).

The mice were visually examined for the appearance of arthritis in their peripheral joints, and disease severity was graded on a scale according to the modified method of Nandakumar et al. (28) as specified below. The mice were considered to have arthritis when significant changes in redness and/or swelling were noted in their digits or in other parts of their paws. Each inflamed toe counted as 1 point. Arthritis was graded on a scale of 0 – 5 for each wrist/ankle: 0 = no changes, 1 = slight erythema of the limbs, 2 = minimal swelling, 3 = moderate swelling and erythema of the limbs, 4 = marked swelling and erythema of the limbs, 5 = maximal swelling and redness of the limbs and ankylosis. The macroscopic score was expressed as the cumulative value for all paws, with a maximum possible score of 40.

Experiment IV: TAC dosing time–dependent renal toxicity

TAC (4 mg/kg) or saline was i.p. injected at 2 or 14 HALO every day for 2 weeks in ICR mice. Saline was administered in the control group. To measure blood urea nitrogen (BUN) (n = 11, 12) and creatinine (Cr) (n = 4, 5), blood was taken at 2 or 14 HALO on days 14 after the initiation of administration. All blood samples were immediately centrifuged at 3,000 rpm for 15 min, after which the plasma was removed and frozen at −80°C until assay. The plasma concentrations of BUN and Cr were determined by the urease ultraviolet method and the al-

kalic picric acid method, respectively.

To assay *N*-acetyl-*β*-glucosaminidase (NAG) activity, the mice had their urine collected for a day from day 13 (n = 4, 5). The urine was frozen at −80°C until the assay. The NAG activity of urea was determined using the Shionogi NAG test (Shionogi & Co., Ltd., Osaka).

Experiment V: Chronopharmacokinetics of TAC

To investigate the pharmacokinetics of TAC, ICR mice were divided into the 10 and 22 HALO–treated groups (n = 6). Blood samples were obtained at 0.25, 0.5, 1, 2, 4, 8, and 12 h after TAC (4 mg/kg) had been i.p. administered. The samples were stored at −80°C until the analysis. The TAC concentrations in blood were quantified using the Abbott IMx® Tacrolimus-II assay system (Abbott Japan Co., Ltd., Tokyo).

Experiment VI: TAC dosing time–dependent cytokines in CIA mice

TAC or saline was administered 17 days after the first immunization. TAC (4 mg/kg, n = 8) or saline (control, n = 16) was i.p. injected at 2 or 14 HALO every day for 3 weeks in the CIA mice. The normal mice were not treated (n = 6). To measure the concentrations of TNF-*α* and IL-6, blood was taken at 6 HALO on days 24, 31, and 38, and the samples were immediately centrifuged at 3,000 rpm for 15 min. Plasma was stored at −80°C until it was analyzed.

Experiment VII: TAC dosing time–dependent leukocyte counts in MRL/lpr mice

Twelve-week-old MRL/lpr mice were i.p. given TAC (4 mg/kg) at 2 or 14 HALO every days for 2 weeks (n = 5). Saline was administered in the control group (n = 7). The blood samples were drawn by orbital sinus collection at 2 HALO on days 0, 7, and 14 after the initiation of administration, and then leukocyte counts were measured.

Cytokine assay

Multianalyte profiling was performed using the Luminex-100 system (Luminex Corporation, Austin, TX, USA). The acquired fluorescence data were analyzed using the MasterPlex™ QT software (Ver. 1.2; MiraiBio, Inc., San Francisco, CA, USA). The plasma concentrations of TNF-*α*, IL-6, and IL-1*β* were determined by the Mouse Inflammatory Cytokine 4-Plex kit (Biosource, San Jose, CA, USA). All analyses were performed according to the manufacturer's protocols.

Statistical analyses

All data were recorded as the mean ± standard deviation (S.D.), excluding the arthritis score. Differences between two groups were analyzed by the Student's *t*-test. Groups were compared by one-way analysis of variance (ANOVA), two-way ANOVA, or repeated ANOVA; and differences between groups were determined using Scheffe's test. The arthritis score is shown as the median. The arthritis score was compared among the various dosing groups using the Kruskal-Wallis test, and the Mann-Whitney U test with Bonferroni correction for non-parametric data was used as a post-hoc test. A probability level of less than 0.05 was considered to be significant. The 24-h rhythmicity was defined to be statistically significant when both Cosinor analysis and one-way ANOVA were significant.

Results

Daily variation in plasma SAA concentrations in normal and CIA mice

After immunization, the SAA levels in the CIA mice were significantly higher than those in the normal mice at all sampling times ($P < 0.01$, Fig. 1). The SAA concentrations showed obvious daily variations with higher levels in the early morning in both the normal and CIA mice (normal group: F from ANOVA = 3.11, $P < 0.05$; CIA group on day 24: F from ANOVA = 2.96, $P < 0.05$; Fig. 1).

Twenty-four hour rhythms of plasma cytokine concentrations in normal and CIA mice

The plasma TNF-*α* concentrations in the normal and CIA mice showed significant 24-h rhythms with higher

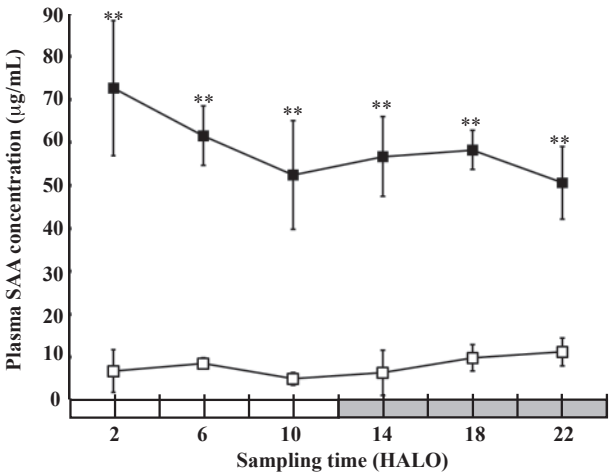

Fig. 1. Daily variation in plasma SAA levels in the normal (open squares) and CIA mice (closed squares). Each value represents the mean ± S.D. of 4 or 5 normal mice and 5 CIA mice. $^{**}P < 0.01$, compared with the normal group at the corresponding sampling time (Student's *t*-test). There were clear daily variations in SAA levels in both the normal and CIA mice ($P < 0.05$, respectively; ANOVA).

levels in the light phase and lower levels in the dark phase (normal group: F from ANOVA = 4.08, $P < 0.01$, P from Cosinor < 0.01; CIA group on day 24: F from ANOVA = 15.64, $P < 0.01$, P from Cosinor < 0.01; CIA group on day 31: F from ANOVA = 14.15, $P < 0.01$, P from Cosinor < 0.01; CIA group on day 38: F from ANOVA = 11.97, $P < 0.01$, P from Cosinor < 0.01, Fig. 2: A – D). After immunization, the TNF-α levels in the CIA mice on days 24, 31, and 38 were significantly higher than those in normal mice at all sampling times ($P < 0.05$ and $P < 0.01$, respectively).

Figure 2, E – H, shows the IL-6 levels in the normal and CIA groups. There was no significant 24-h rhythm in IL-6 levels in the normal group. The plasma IL-6 concentrations in the CIA mice on days 24 and 31 after the first immunization showed obvious 24-h rhythms with higher levels in the light phase and lower levels in the dark phase (day 24: F from ANOVA = 2.27, $P = 0.056$, P from Cosinor < 0.01; day 31: F from ANOVA = 5.73, $P < 0.01$, P from Cosinor < 0.01). After immunization, the IL-6 levels in the CIA mice on days 24, 31, and 38 were increased compared with those in the normal mice at many sampling times ($P < 0.05$ and $P < 0.01$, respectively). IL-1β was not detected in the plasma of the normal or CIA mice.

Influence of dosing time on arthritis score during TAC administration in CIA mice

Arthritis was observed in all groups on day 24 after the first immunization, and the arthritis score increased day by day. On day 38, the median arthritis score was 17 in the control group, 4.5 in the 2 HALO–treated group, and 11.5 in the 14 HALO–treated group. The arthritis score was significantly lower in the 2 HALO–treated group than in the control and 14 HALO–treated groups ($P < 0.05$ and $P < 0.01$, respectively; Fig. 3).

Influence of dosing time on renal toxicity during TAC administration in CIA mice

Figure 4 shows plasma BUN and Cr levels and urinary NAG activity, which are markers of renal toxicity. The BUN concentration was significantly lower at 14 HALO than at 2 HALO in the normal group ($P < 0.01$), and the 2 HALO–treated group a showed significantly lower BUN value than the normal group ($P < 0.01$). However, none of the TAC-treated groups displayed exacerbated renal function compared with the normal group.

Influence of dosing time on the pharmacokinetics of TAC after its administration in mice

The TAC concentrations at 0.25 and 4 h after TAC

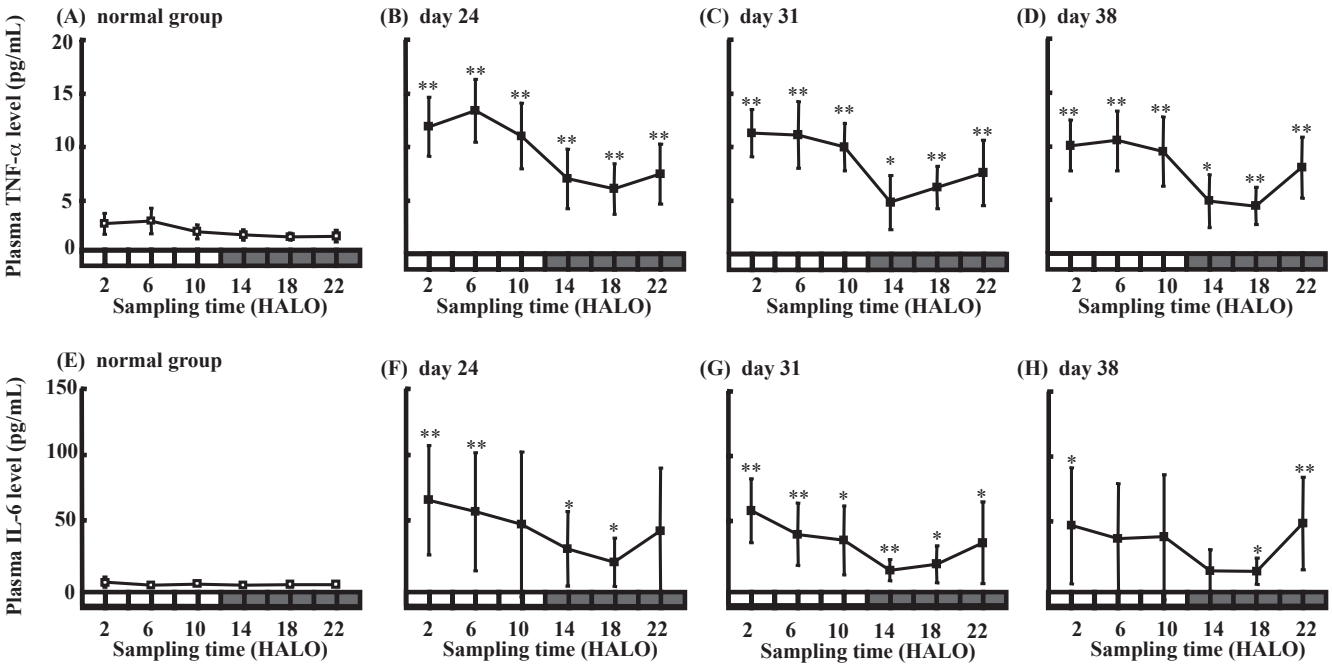

Fig. 2. Daily variation in plasma TNF-α (A, B, C, D) and IL-6 (E, F, G, H) levels in the normal (A, E: open squares) and the CIA groups (closed squares) on day 24 (B, F), day 31 (C, G), and day 38 (D, H). Each value represents the mean ± S.D. of 5 or 6 normal mice and 11 – 16 CIA mice. *$P < 0.05$ and **$P < 0.01$, compared with the normal group at the corresponding sampling time (Student's t-test). There were significant 24-h rhythms in TNF-α levels in CIA mice ($P < 0.01$, respectively; ANOVA and Cosinor analysis).

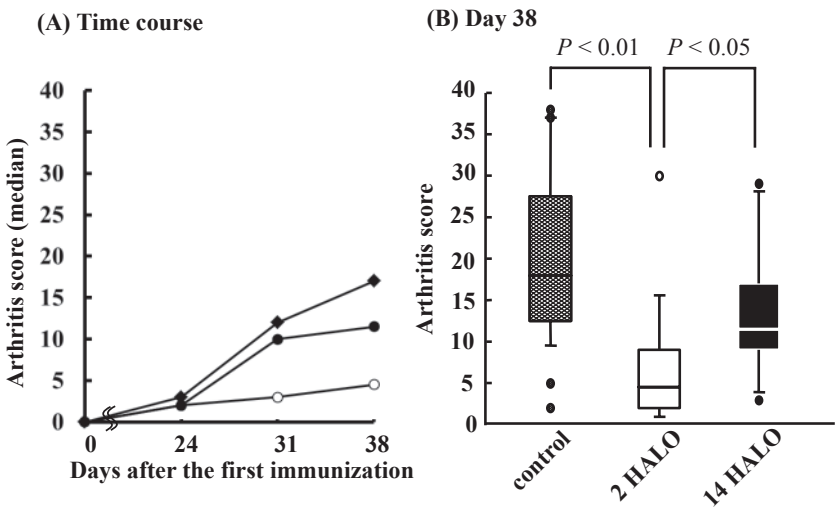

Fig. 3. Influence of TAC dosing-time on the arthritis score in mice. TAC (4 mg/kg) was i.p. administered once a day at 2 (open circle) or 14 (closed circle) HALO for 3 weeks from day 17 after the first immunization (n = 14, respectively). Saline was administered in the control group (closed rhomb, n = 24). The scores observed on day 38 are indicated by box-plots. For each box-plot, the central line represents the median value; the upper and lower borders of the box represent the 75th and 25th percentile, respectively; and the upper and lower extents of the vertical lines extending from the box represent the 90th and 10th percentile, respectively. The 2 HALO–treated group showed significantly lower arthritis score compared with the control and 14 HALO–treated groups ($P < 0.01$ and $P < 0.05$, respectively; Mann-Whitney U test with Bonferroni correction).

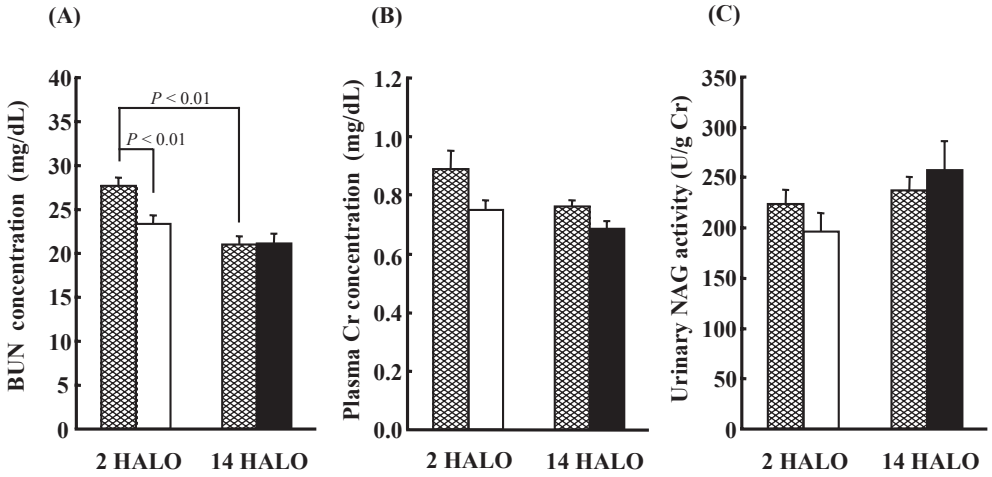

Fig. 4. Influence of TAC dosing-time on BUN concentration (A), plasma Cr concentration (B), and urinary NAG activity (C) after TAC administration (4 mg/kg, i.p.) at 2 (open column) or 14 (closed column) HALO for 2 weeks. Saline was administered in the normal group (dotted column). Each value represents the mean ± S.D. (n = 4 – 12). Groups were compared by two-way ANOVA, and differences between groups were determined using Scheffe's test.

injection in the 14 HALO group were significantly higher than those in the 2 HALO group (0.25 h: $P < 0.01$, 4 h: $P < 0$ .05; Fig. 5).

Influence of TAC dosing time on TNF-α and IL-6 levels during TAC administration in CIA mice

The TNF-α concentration was significantly increased in the control groups on days 24, 31, and 38 after immunization compared with the normal group ($P < 0.01$, respectively; Fig. 6: A – C). Although the TAC-treated groups showed significant inhibition of the increases in TNF-α levels compared with the control groups on days 24, 31, and 38 ($P < 0.01$, respectively), there were no significant differences in TNF-α levels between the two TAC-treated groups.

On days 24 and 31, the control groups showed higher IL-6 concentrations than the normal group ($P < 0.05$ and $P < 0.01$, Fig. 6: D and E). The IL-6 concentrations in the 2 and 14 HALO groups were significantly lower than those in the control group on day 31 ($P < 0.01$, respectively; Fig. 6: E). There were no significant differences in IL-6 levels between the two TAC-treated groups on days 24, 31, or 38.

Influence of TAC dosing time on inhibition of increasing leukocytes in MRL/lpr mice

The leukocyte counts of the control group were measured on days 0, 7, and 14 after the initiation of administration, and the leukocytes significantly increased by aging ($P = 0.01$, Fig. 7). When TAC was given to 12-week-old MRL/lpr mice (day 0) for 2 weeks, the leukocyte counts in the control and 14 HALO groups increased 1.4- and 1.6-fold, respectively, on day 14 compared with those on day 0. On the other hand, the 2 HALO group maintained a normal level of leukocyte counts on day 14. The group treated at 2 HALO showed

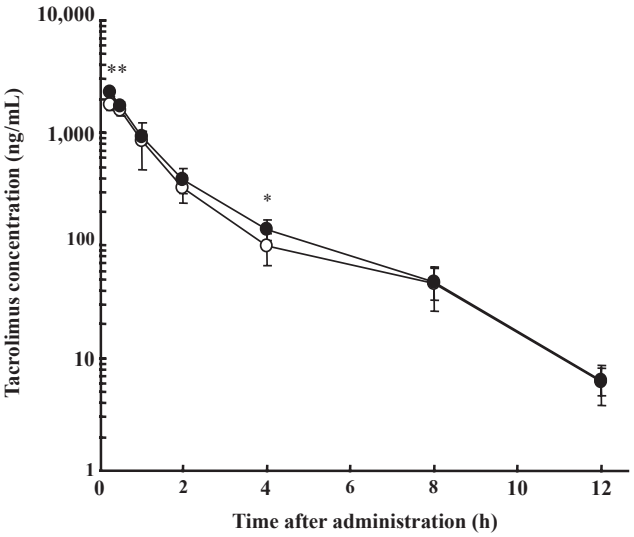

Fig. 5. Influence of dosing-time on the blood TAC concentration after a single dose had been administrered (4 mg/kg, i.p.) at 2 (open circle) or 14 (closed circle) HALO. Each value represents the mean ± S.D. (n = 6). *$P < 0.05$ and **$P < 0.01$, compared with the 2 HALO–treated group at the corresponding sampling time (Student's *t*-test).

a significant inhibition of the increase of leukocyte counts compared with that at 14 HALO ($P < 0.05$, Fig. 7).

Discussion

CIA represents a true autoimmune reaction against major joint components and is associated with class II major histocompatibility complex genes and pannus formation. The CIA model is similar to RA in terms of pathology, immunology, and genetics (29, 30). It is difficult to monitor stiffness in RA model animals; however, the 24-h rhythm of CRP was found to correspond to the morning stiffness in RA patients (10). Using the CIA model, we estimated the plasma SAA concentration, which is an acute-phase protein and a sensitive marker of acute inflammatory states, because the CRP level cannot be detected in mice. After RA onset, the SAA level was increased and showed obvious daily variations with higher levels at 2 HALO in CIA mice. MRL/lpr mice are another RA model, which are known to develop autoimmune disorders that share similarities with human RA

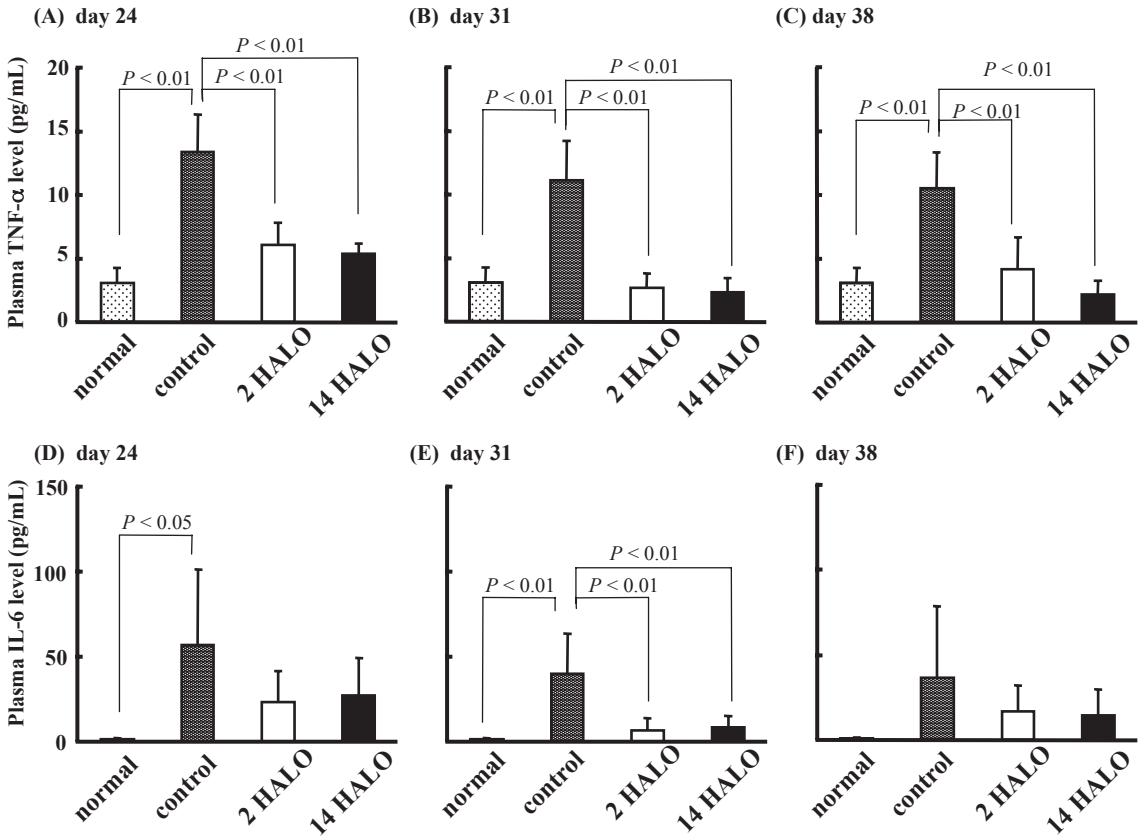

Fig. 6. Influence of TAC dosing-time on the plasma TNF-α level (A, B, C) and plasma IL-6 level (D, E, F) in CIA mice. TAC (4 mg/kg) was i.p. administered once a day at 2 or 14 HALO for 3 weeks from day 17 after the first immunization. The plasma TNF-α and IL-6 levels at 6 HALO were measured on day 24 (A, D), day 31 (B, E), and day 38 (C, F). Each value represents the mean ± S.D. of 5 or 6 normal mice and 7 – 16 CIA mice. The plasma TNF-α levels in the TAC-treated groups were significantly lower than that in the control group ($P < 0.01$, respectively; Scheffe's test).

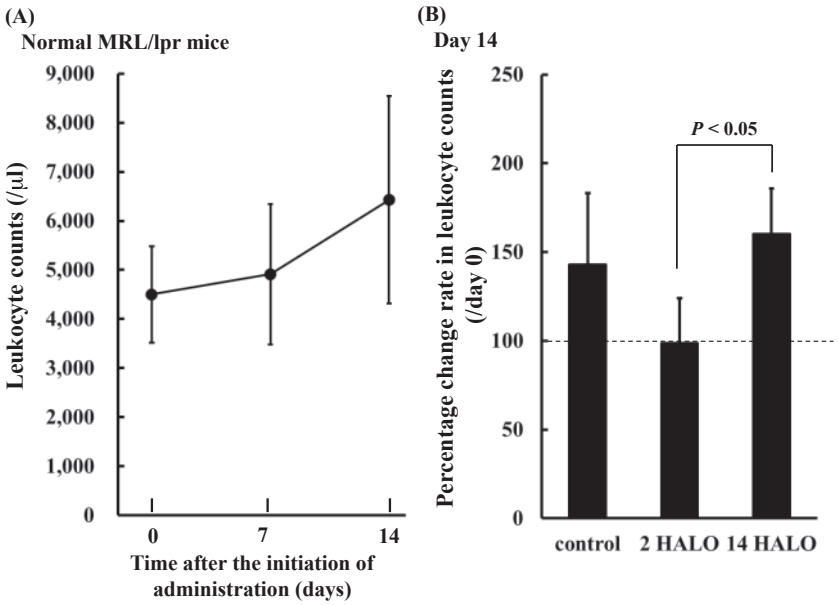

Fig. 7. Influence of TAC dosing-time on leukocyte counts in MRL/lpr mice. TAC (4 mg/kg) was i.p. administered once a day at 2 or 14 HALO for 2 weeks. Each value represents the mean ± S.D. of 5 – 7 MRL/lpr mice. A) Change in leukocyte counts in the control group. B) Change rate in leukocyte counts 14 days after TAC administration. The 2 HALO–treated group significantly suppressed the increasing leukocyte counts compared with the 14 HALO–treated group ($P < 0.05$, Scheffe's test).

and systemic lupus erythematosus (31, 32). An obvious 24-h rhythm in the plasma SAA concentration with higher levels at 2 HALO was observed in MRL/lpr mice that had developed RA (data not shown). It is considered that some body components that show 24-h rhythms in both RA model animals after RA onset are responsible for the cyclical nature of inflammatory.

SAA is synthesized in the liver upon stimulation by cytokines such as TNF-α and IL-6 (33, 34). We estimated the TNF-α, IL-6, and IL-1β concentrations in plasma at different times in the present study. IL-1β was undetectable in plasma in almost all mice both before and after immunization as was reported in past studies using CIA model mice (35, 36). On the other hand, the TNF-α and IL-6 concentrations were significantly higher than those in normal mice at all sampling times. Interestingly, increases in TNF-α and IL-6 concentrations induced by immunization were about twofold higher during the light phase than the dark phase, and the plasma TNF-α and IL-6 concentrations showed significant 24-h rhythms with higher levels in the light phase and lower levels in the dark phase. In the MRL/lpr mice, the plasma TNF-α level showed a 24-h rhythm with a peak at 2 HALO (data not shown). The synchronization of the 24-h rhythms of the inflammatory response and cytokine levels was also observed in RA patients (10, 37). It is thought that the inflammatory response contributes to morning stiffness in RA because pain and stiffness develop in the early morning when the CRP level is higher. Therefore, we consider that the 24-h rhythms of inflammatory cytokines play important roles in the expression of RA symptoms and that these rhythms are important for to diagnosing RA.

In the present study, we revealed that 24-h rhythms in the inflammatory response and cytokines levels were observed in CIA mice after RA onset that were similar to those seen in RA patients. We consider that CIA mice are an appropriate model for studying RA chronotherapy. Chronotherapy involves the optimization of dosing schedules after taking the 24-h rhythms of various kinds of elements in the body and the pharmacokinetics of drugs into consideration. Since the 24-h rhythms of cytokines peaked during the light phase and were lowest during the dark phase, TAC was administered at 2 or 14 HALO. Although the increase in arthritis score was hardly inhibited in the 14 HALO–treated group, the 2 HALO–treated group showed marked arthritis suppression compared with the control group. The optimum dosing time of TAC was similar to that of methotrexate found in our previous study (19), and both drugs showed a higher inhibitory effect on arthritis during the early morning when cytokine levels increased. Therefore, it was suggested that choosing an optimal dosing time according to the 24-h cycling of inflammatory cytokines could lead to augmentation of the RA therapeutic effect of TAC.

A main adverse effect of TAC is nephrotoxicity. To estimate renal toxicity induced by TAC administration, we measured plasma BUN and Cr levels and urinary NAG activity. However, no renal toxicity was seen in the TAC-treated groups. In a preliminary study, there was no significant increase in the BUN concentration in the tacrolimus groups compared with the nomal group even though we had i.p. administered TAC (6 mg/kg) once a

day for 4 weeks (data not shown). Therefore, it was thought that no dosing time–dependent renal toxicity was generated by the TAC dose that had inhibitory effects on arthritis.

To find the reason why the inhibitory effect of arthritis was affected by dosing time, we measured the whole blood TAC concentration. The mean area under the plasma concentration time curve (AUC) of TAC was 2,963 ng/mL per hour in the 2 HALO group and 3,126 ng/mL per hour in the 14 HALO group. There was no definite difference in TAC concentration between the two groups. In past reports, no difference in dosing time–dependent pharmacokinetics was found, which agrees with the present study (38). On the other hand, many reports have demonstrated that the blood concentration of TAC shows daily variations in which its C_{max} is markedly increased in the active phase compared with the inactive phase (27, 39, 40). The main difference between these reports and our study was the route of administration. Although daily variations in the pharmacokinetics of TAC were observed after it had been administered perorally, there was no dosing time–dependent difference in the plasma concentration when TAC was given i.p. Usually, TAC is orally administered during RA therapy. Further studies may be necessary to clarify the relationship between the daily variations in antirheumatic effects of TAC and its pharmacokinetics. However, the pharmacokinetics of TAC did not participate in the dosing time–dependency of the arthritis-inhibitory effect seen in this study. Therefore, the 24-h rhythms of another factor involved in RA may have been important for selecting the optimal dosing time of TAC.

It is known that TAC decreases the inflammatory cytokine levels in the blood (35). Since the dosing time–dependent effect of TAC matched the 24-h rhythm of cytokine levels in plasma, we studied the influence of TAC dosing time on the plasma TNF-α and IL-6 concentrations. Although the plasma TNF-α and IL-6 concentrations in the 2 and 14 HALO–treated groups were significantly lower those in the control group, there were no differences in plasma cytokine levels between the two groups. These results did not correspond to the dosing time–dependency of the inhibitory effect of TAC on arthritis. Cytokines are hard to detect in plasma despite the fact that high levels of cytokines can be measured in tissue in CIA and adjuvant-induced arthritis animals (35, 36).

Moreover, TNF-α and IL-6 are detected at high concentrations in the blood and synovial fluid of RA patients, and it was thought that the levels in blood correlate with disease activity and that the TNF-α and IL-6 in synovial fluid participate in inflammation and the destruction of cartilage and bone joints (41). In this study, we estimated the inhibitory effect of TAC on arthritis. It may be thought that the change in cytokines levels in plasma caused by the administration of TAC does not reflect the daily variation in arthritis score. The DBA/1J mice used in this study are relatively small, making it difficult to isolate sufficient amounts of synovial tissue to measure the cytokines. Detecting cytokines in synovial membranes and synovia in other RA model animals would clarify the mechanism of the dosing time–dependency of the inhibitory effects of TAC on arthritis, which we hope to do in a future study.

Leukocytes are an inflammatory marker because they induce inflammation, and the leukocyte counts increase in most RA patients (42, 43). Ueki et al. reported that RA symptoms markedly improved when the increased leukocytes in RA patients were eliminated by leukocytapheresis (44). Thus, it is considered that inhibiting the increased leukocyte counts, which induce inflammatory response, is one of the options available for the treatment of RA. MRL/lpr mice develop autoimmune disorders that share similarities with human RA (45, 46) and have serious RA symptoms as they get older. In this study, the leukocyte counts in MRL/lpr mice increased by aging. Moreover, IgG-rheumatoid factor (IgG-RF) level also increased in MRL/lpr mice following aging. When TAC was given at 2 or 14 HALO in 12-week-old MRL/lpr mice, the increase in leukocyte counts was observed in the 14 HALO group 14 days after the initial dose. However, the 2 HALO group showed an inhibition of the increase and maintained the normal leukocyte level. Deterioration of RA symptoms was induced by various factors including inflammatory cytokines produced from leukocytes. Therefore, suppressing the augmentation of leukocyte counts in the 2 HALO group may contribute to inhibition of the local inflammatory response and arthritis.

In summary, we revealed that TAC showed dosing time–dependent antirheumatic effects in this study. The inhibition of leukocyte counts, which are increased by RA, may be one of causes in the dosing time–dependency of the arthritis-inhibiting effect of TAC. Although further investigations are necessary to elucidate the mechanism in detail, selecting optimal dosing-time may help to increase the antirheumatic effects of RA therapy involving TAC.

Acknowledgments

We are indebted to Astellas Pharma, Inc. (Tokyo) for supplying the tacrolimus used in this study. This study was supported by a Grant-in-Aid for Young Scientists (B) (H.T., 17790126) from the Ministry of Education, Culture, Sports, Science, and Technology Japan and the Ministry of Health, Labour, and Welfare of Japan. This project was partially supported by a Grant from the Takeda Science Foundation (H.T.).

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