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European Journal of Pharmacology 435 (2002) 253–258

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Weekly pulse therapy of methotrexate improves survival compared with its daily administration in MRL/lpr mice

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Received 30 July 2001; received in revised form 21 November 2001; accepted 23 November 2001

Abstract

The purpose of this study was to determine whether weekly pulse therapy was superior to daily administration of methotrexate in MRL/lpr mice. Oral methotrexate was given to 6-week-old MRL/lpr mice at doses of 0.3, 1.0, or 3.0 mg/kg 5 days a week or at a dose of 15.0 mg/kg once a week until 35 weeks of age. The effects of methotrexate on physical, serological, and pathological findings were assessed. The survival rate and articular destruction on X-ray films were also evaluated. Both weekly pulse therapy and daily administration of methotrexate at the same weekly dose improved nephropathy and articular destruction of MRL/lpr mice when compared with control. However, weekly pulse therapy with methotrexate prolonged the survival of MRL/lpr mice when compared with the daily administration of the same weekly dose of methotrexate and control. Methotrexate did not suppress the increase in anti-DNA antibody and rheumatoid factor. Daily administration of methotrexate reduced the red and white blood cell counts, whereas weekly pulse therapy caused little reduction. In conclusion, weekly pulse therapy was superior to daily administration of methotrexate with respect to the survival rate, possibly due to a reduction in toxicity.

Keywords: Methotrexate; MRL/lpr mouse; Pulse therapy weekly; Survival; Myelosuppression

1. Introduction

Recently, low-dose methotrexate pulse therapy has become a standard strategy for the treatment of patients with rheumatoid arthritis (Ryan and Brooks, 1999). However, it has not been clarified whether weekly pulse therapy with methotrexate is superior to daily administration of the same total dose. Investigation of methotrexate pulse therapy has not been sufficiently performed in experimental models and the mechanism of the anti-inflammatory actions of methotrexate remains poorly known. We and other investigators have previously shown an anti-inflammatory effect of low-dose pulse methotrexate on soft tissue swelling in the hind paws of rats with adjuvant-induced arthritis as well as an inhibitory effect on articular destruction and bone loss (Kawai et al., 1997; Suzuki et al., 1997).

In the present study, we investigated the efficacy of weekly pulse therapy with methotrexate as compared with daily administration in MRL/lpr mice, which spontaneously develop an autoimmune disease that mimics human rheumatoid arthritis (Hang et al., 1982; O'Sullivan et al., 1985) and/or systemic lupus erythematosus (Andrews et al., 1978). These mice produce autoantibodies to DNA and rheumatoid factor that give rise to immune complex-mediated glomerulonephritis and eventual death from renal failure. The present study demonstrated a beneficial effect of weekly pulse therapy with methotrexate on the natural history of MRL/lpr mice.

2. Materials and methods

2.1. Animals

Male MRL/lpr mice were purchased from Nippon SLC, Hamamatsu, Japan. They were bred in our laboratory. Standard rodent chow (Japan Kurea, Tokyo, Japan) and water were freely available. All procedures complied with the regulations of the commission for animal protection and were approved by the Committee for Animal Studies.

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01555-2

2.2. Methods

Methotrexate was supplied by Wyeth Lederle Japan. It was suspended in a 0.5% solution of carboxymethyl cellulose (CMC) for administration. MRL/lpr mice were divided into four treated groups (10 animals/group) and one untreated control group. Methotrexate was given to three groups of mice orally five times a week at doses of 0.3, 1.0, or 3.0 mg/kg from 6 to 35 weeks of age. Other mice were administered methotrexate once a week at a dose of 15.0 mg/kg, which resulted in the same total dose as 3.0 mg/kg five times per week, and vehicle (0.5% CMC solution) was given on the other 4 days a week to these mice. The control group received a comparable volume of 0.5% CMC solution for 5 days. Then, the dosing regimens were comparable among five groups.

The general condition of the mice was observed every day. Body weight and proteinuria were measured weekly. Superficial lymphadenopathy was assessed on the days of methotrexate administration. Proteinuria was measured using a colorimetric strip test (Wako, Osaka, Japan) (Free et al., 1957) and the result was graded from negative to 4+ using the manufacturer's instructions. Blood samples were taken from the retro-orbital venous plexus at intervals of 2 weeks and serum was stored at -20 °C until assayed for blood urea nitrogen, anti-dsDNA antibody, and rheumatoid factor. Blood urea nitrogen was measured by the urease indophenol method (Wako) (Searcy et al., 1967). Serum anti-dsDNA antibody was measured by enzyme-linked immunosorbent assay (ELISA) according to the method of Eaton et al. (1983). Native DNA was prepared by dissolving calf thymus DNA (Sigma, St. Louis, MO). The assay involved sequential treatment of microtiter plates with DNA, mouse serum (diluted 1:200), peroxidase-conjugated affinipure immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA), and the enzyme substrate. The enzymic reaction was stopped by addition of sodium hydroxide. Rheumatoid factor (IgM) was measured by ELISA using mouse IgG as the antigen (The Binding Site Institute of Research and Development, San Diego, CA), as described previously by Andrews et al. (1978). Peroxidase-labeled goat anti-mouse IgM was purchased from Jackson Immunoresearch. On completion of the anti-dsDNA antibody or rheumatoid factor (IgM) assay, the optical density was read at 405 nm with a multiple well plate reader and was used to calculate the serum antibody level. Serum samples from C57BL/6 mice were used as a control; the titers of anti-dsDNA antibody and rheumatoid factor in these animals were less than 0.25 and 0.1, respectively.

In another experiment that was performed separately, male MRL/lpr mice were orally administered methotrexate at a dose of 3.0 mg/kg daily for 5 days per week, or 15 mg/kg weekly and vehicle for the other 4 days, or vehicle only for 5 days from 6 to 20 weeks of age. We evaluated the influence of methotrexate on the bone marrow, the kidneys, and the progression of articular destruction by hind-paw radiography. Blood was obtained by cardiac puncture at 20 weeks of age, and the red blood cell count and white blood cell count were determined with an automatic blood cell counter (ERMA particle counter PC-607, Japan). To evaluate articular destruction, the lower extremities of these mice were resected and were immediately fixed in 10% formalin. The limbs were positioned over a cassette containing Kodak X-ray film and radiographs were obtained with a conventional microradiographic unit (SCMB-12, Softex, Tokyo, Japan) at 35 kV and 6 mA for 60 s. Each hind-paw radiograph was evaluated by the method of Clark et al. (1979) with some modifications. Briefly, six features (bone demineralization, bone erosion, periostitis, cartilage space, soft tissue, and alignment) were evaluated blindly by three researchers and were graded from 0 to 4 (with 0 indicating normal and 4 indicating severe changes). The sum of the score for each of the six features was defined as the articular score for each hind paw. The minimum and maximum possible scores were 0 and 24, respectively, and the mean score for each hind paw was calculated from the evaluations of the three researchers. Then the mean $\pm$ S.D. value for each group ($n = 10$) was calculated.

After the animals were killed, the kidneys were removed for histological studies. Kidney specimens were fixed in 10% formalin, embedded in paraffin, sectioned on a microtome, and stained with hematoxylin and eosin. The severity of glomerulonephritis was evaluated using the scoring system for histologic features of Wernick et al. (1993). Each specimen was assessed blindly for the six components of the activity index, comprising cellular proliferation, leukocyte infiltration, fibrinoid necrosis or karyorrhexis, cellular crescents, hyaline thrombi or wire loops, and mononuclear cell infiltration. Each component was scored as 0 (normal), 1, 2, or 3 (severe abnormality). When calculating the activity index, fibrinoid necrosis and cellular crescents were weighted by a factor of 2, so the maximum possible activity index was 24 points.

2.3. Statistical analysis

The probability of survival was estimated over time by the Kaplan–Meier method using SigmaStat software (SPSS, Chicago, IL). The significance of differences was estimated by the log-rank test. Multiple Student's *t*-tests with Bonferroni's correction were used for comparison of the mean values among the different groups of mice. A probability value of less than 0.05 was taken to indicate a significant difference.

3. Results

3.1. Proteinuria

The mice were given orally methotrexate five times weekly or once a week from 6 to 35 weeks of age. Severe

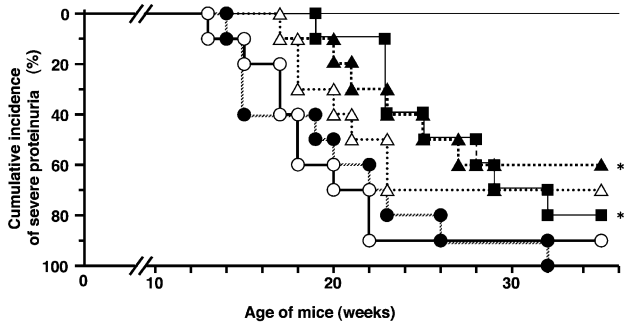

Fig. 1. Cumulative incidence of severe proteinuria after treatment with methotrexate. Methotrexate was administered to MRL/lpr mice orally five times a week at doses of 0 (control, ○), 0.3 (●), 1.0 (△), 3.0 (▲) mg/kg or once a week at a dose of 15 (■) mg/kg between the ages of 6 and 35 weeks. * indicates significant ($P<0.05$) difference vs. control by log-rank test of Kaplan–Meier life table method.

proteinuria was defined as 3+ proteinuria for at least 2 weeks. Fig. 1 shows that daily administration of methotrexate delayed the onset of severe proteinuria in a dose-dependent manner. The onset of severe proteinuria in the mice given 3.0 mg/kg methotrexate daily or 15 mg/kg weekly pulse therapy was significantly ($P<0.05$) delayed compared with that in the untreated control group by the log-rank test. No significant difference in the occurrence of proteinuria was observed between the groups given 3 mg/kg of methotrexate daily and 15 mg/kg weekly, which was the same total weekly dosage of methotrexate.

3.2. Lymphadenopathy

Superficial lymphadenopathy showed a delayed onset in methotrexate-treated mice compared with the untreated control group. However, the incidence of superficial lymphadenopathy did not differ among the various methotrexate-treated groups (data not shown).

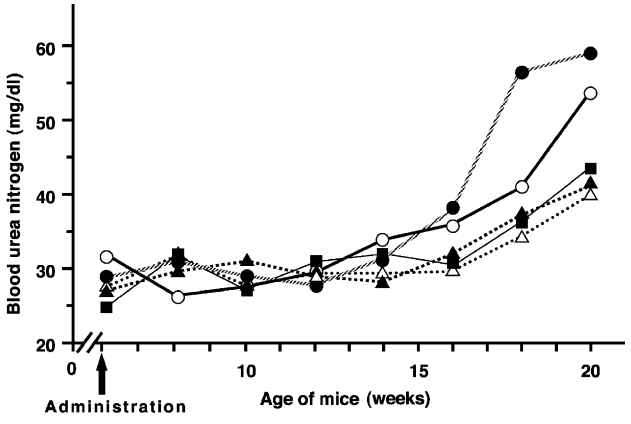

Fig. 2. Mean blood urea nitrogen levels after treatment with methotrexate in MRL/lpr mice. Groups receiving orally five times a week at doses of 1.0 (△), 3.0 (▲) mg/kg or once a week at a dose of 15 (■) mg/kg showed a smaller increase in blood urea nitrogen level, but no significant difference in the average levels was observed among the groups including the 3, 0.3 (●) mg/kg daily, and control (○) groups.

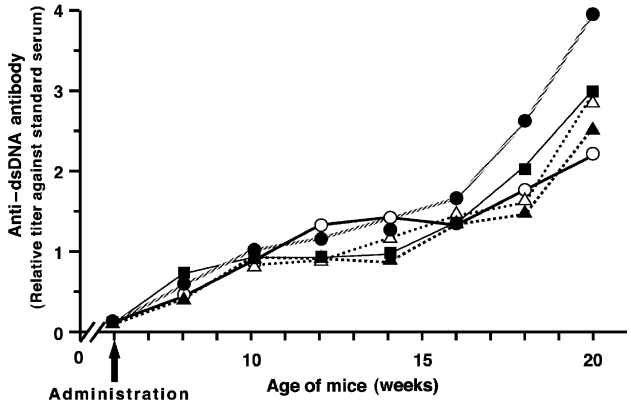

Fig. 3. Mean titers of anti-dsDNA antibody after treatment with methotrexate in MRL/lpr mice. Increases of titers of anti-dsDNA antibody in all groups [orally five times a week at doses of 0 (control, ○), 0.3 (●), 1.0 (△), 3.0 (▲) mg/kg and once a week at a dose of 15 (■) mg/kg] are shown. Methotrexate caused no significant effect on the titers of the antibody.

3.3. Body weight

The average body weight increased during the observation period in all groups, but in all treated groups, except that given 3.0 mg/kg daily, the average body weight was within 13% of the average for untreated controls. The average body weight of the 3.0 mg/kg daily group was at least 15% below that of the untreated controls.

3.4. Serum analysis

The mean blood urea nitrogen level was elevated after 16 weeks in all groups (Fig. 2). The three groups that were administered methotrexate at more than 1 mg/kg showed a smaller increase in blood urea nitrogen concentration, but no significant difference in the average level was observed at 20 weeks of age among these groups.

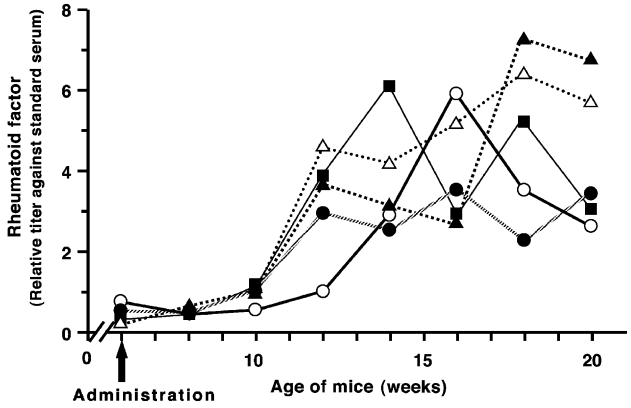

Fig. 4. Mean titers of rheumatoid factor after treatment with methotrexate in MRL/lpr mice. Increases in titers of rheumatoid factor in all groups [orally five times a week at doses of 0 (control, ○), 0.3 (●), 1.0 (△), 3.0 (▲) mg/kg body and once a week at a dose of 15 (■) mg/kg] are shown. Methotrexate caused no significant effect on the titers of rheumatoid factor.

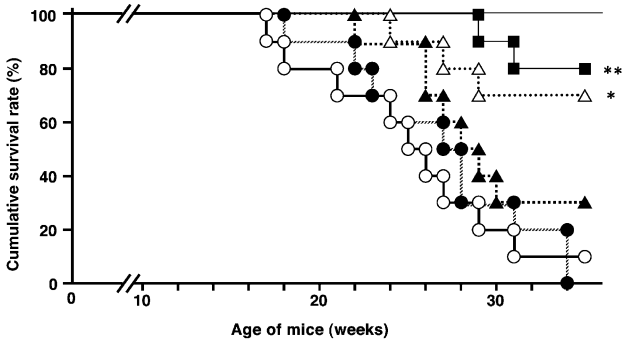

Fig. 5. Cumulative survival rate of MRL/lpr mice treated with methotrexate. Methotrexate was administered to MRL/lpr mice orally five times a week at doses of 0 (control, ○), 0.3 (●), 1.0 (△), or 3.0 (▲) mg/kg or once a week at a dose of 15.0 (■) mg/kg between the ages of 6 and 35 weeks. *indicates significant ($P<0.01$) difference vs. control. **indicates significant ($P<0.01$ and $P<0.05$) differences vs. control and daily 3.0 mg/kg groups, respectively. Significance was evaluated by log-rank test of Kaplan–Meier life table method.

To determine the effect of methotrexate on autoantibody production, we measured anti-dsDNA antibody and found an increase in its titer in all groups. Methotrexate caused no significant suppression of this antibody (Fig. 3). Rheumatoid factor was detectable in most mice after 15 weeks of age, and its titer was also not influenced by methotrexate treatment (Fig. 4).

3.5. Survival

Fig. 5 shows that survival was prolonged ($P<0.05$) in the group given 1.0 mg/kg of methotrexate daily when compared with the control group, but it was shortened in the group given 3.0 mg/kg daily. The survival rate of mice given weekly pulse therapy was significantly prolonged when compared with control group ($P<0.01$) and with the 3.0 mg/kg daily group ($P<0.05$). Differences in survival among the other groups were not significant.

3.6. Peripheral blood cell count

To investigate whether myelosuppression induced by methotrexate had an influence on survival, mice were given methotrexate orally 5 days per week at 3.0 mg/kg or once weekly at 15 mg/kg from 6 to 20 weeks of age, while the vehicle alone (0.5% CMC solution) was administered to the control group. The white blood cell count showed a tendency to be reduced by daily administration of methotrexate when compared with that in the 15 mg/kg weekly group and in the control group; however, there was no significant difference among these groups because of wide variation (Panel A, Fig. 6). The red blood cell count was significantly ($P<0.05$) reduced by daily administration of methotrexate when compared with that of the 15 mg/kg weekly group and that of the control group (Panel B, Fig. 6). Weekly pulse therapy with methotrexate had no influence on the white blood cell count or the red blood cell count.

3.7. Radiographic changes

Table 1 shows the effect of methotrexate on articular destruction in the hind paws of MRL/lpr mice. Methotrexate inhibited the progression of articular destruction compared

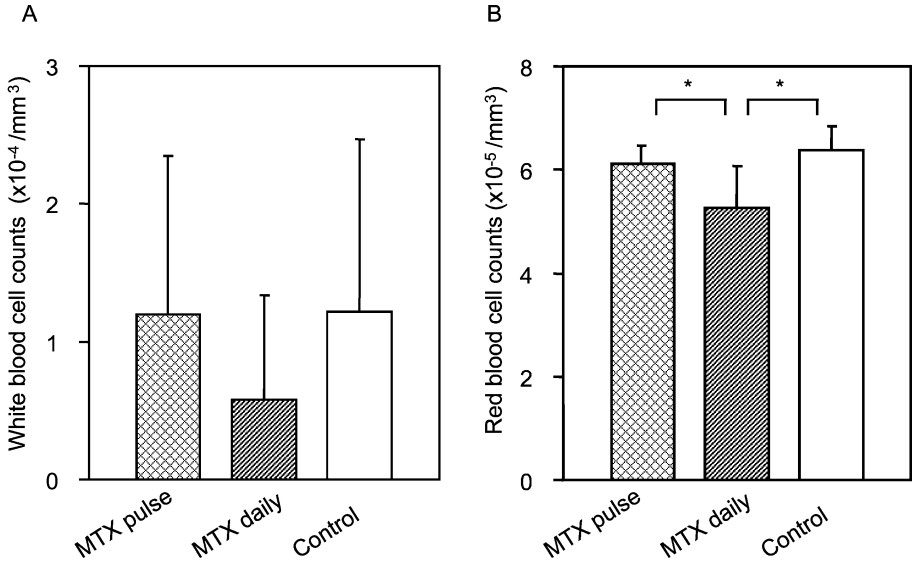

Fig. 6. Peripheral blood counts at 20 weeks of age in MRL/lpr mice treated with methotrexate. Panel A shows mean + S.D. values of peripheral white blood cell counts in MRL/lpr mice receiving oral methotrexate five times a week with dose of 3.0 mg/kg (MTX daily), weekly pulse therapy with 15.0 mg/kg (MTX pulse), and vehicle (Control). The white blood cell counts tended to be lower in the MTX daily group, but no significant difference among groups was detected. Panel B shows mean + S.D. values of peripheral red blood cell counts in MRL/lpr mice treated with the MTX daily, MTX pulse, and Control. The red blood cell counts in the MTX daily group were significantly (* $P<0.05$) reduced when compared with those of the MTX pulse and Control groups by Student's *t*-test with Bonferroni's correction.

Table 1
Effect of methotrexate (MTX) on articular destruction in MRL/lpr mice

	MTX pulse	MTX daily	Control
Bone mineralization	2.6 ± 0.4	1.9 ± 0.3[a]	2.5 ± 0.5
Erosion	1.6 ± 0.7	1.5 ± 1.0[b]	2.6 ± 0.7
Periostitis	1.7 ± 0.7[b]	1.7 ± 0.7[b]	2.6 ± 0.4
Cartilage space	2.0 ± 0.7[b]	2.4 ± 0.5[b]	3.2 ± 0.6
Soft tissue	1.7 ± 0.6	2.0 ± 0.5	1.8 ± 0.7
Alignment	1.4 ± 0.5	1.4 ± 0.5	1.6 ± 0.7
Arthritis score	10.9 ± 1.9[b]	10.8 ± 2.2[b]	14.2 ± 1.7

Values are the means ± S.D. ($n = 10$). MTX daily = five times a week with a dose of 3.0 mg/kg of methotrexate. MTX pulse = weekly pulse therapy with 15.0 mg/kg of methotrexate.
[a] $P < 0.05$ vs. MTX pulse and Control.
[b] $P < 0.05$ vs. Control (Student's t-test with Bonferroni's correction).

with that in the control group, as shown by a reduction of the scores for several items and a decrease of the arthritis score. There was no significant difference in the arthritis score between weekly pulse therapy and daily administration of methotrexate.

3.8. Renal activity index

The effect of methotrexate on glomerulonephritis was assessed using kidney specimens. Daily methotrexate administration significantly decreased the renal activity index (11.6 ± 2.9) compared with that in the control group (15.1 ± 1.3, $P < 0.05$). Weekly pulse therapy with methotrexate decreased the index (13.1 ± 1.8), but the difference was not significant compared with that in the other groups.

4. Discussion

Our present results clearly showed that high doses of methotrexate were effective in suppressing proteinuria and the histological features of nephropathy in MRL/lpr mice. Methotrexate had little influence on the appearance of lymphadenopathy and the production of autoantibodies, such as anti ds-DNA antibody or rheumatoid factor. We have already reported that low-dose pulse therapy with methotrexate inhibits articular destruction in rats with adjuvant arthritis (Kawai et al., 1997). We found similar results in MRL/lpr mice in this study, with no difference in the reduction of articular destruction between weekly pulse therapy and daily administration. These results suggest that methotrexate might improve nephropathy and articular destruction without having much influence on the immune system. Biochemical studies have demonstrated that inhibition of AICAR (5-amino-4-carboxamide ribonucleotide) transformylase by methotrexate results in increased release of adenosine by fibroblasts, endothelial cells, and other cells (Allegra et al., 1985; Cronstein et al., 1991). It is known that the anti-inflammatory effect of methotrexate is mediated by adenosine and its A_2-receptor. The adenosine A_2-receptor is expressed on various subsets of lymphocytes depending on their activation status, and adenosine has been shown to increase the cyclic AMP level and to inhibit several lymphocyte responses (Cronstein and Hirschhorn, 1990). Therefore, methotrexate might improve nephropathy and articular destruction via increased release of adenosine in our experimental model.

We evaluated the efficacy of methotrexate as weekly pulse therapy or as a 5-day/week treatment in MRL/lpr mice, using untreated animals as controls. There were no significant differences in serological and urinary findings, severity of glomerulonephritis, and articular destruction between a dose of 3.0 mg/kg on 5 days weekly and 15 mg/kg once weekly, both of which delivered the same total dose per week. We found that daily administration of methotrexate at the highest dose did not improve the survival rate. In contrast, weekly pulse therapy with methotrexate improved the survival rate. Mihara et al. (1992) studied that the effect of methotrexate on the development of autoimmune kidney disease in MRL/lpr mice after oral administration of the drug three times a week. They found that methotrexate delayed the appearance of proteinuria, prolonged survival, and inhibited the elevation of blood urea nitrogen, but they did not evaluate weekly pulse therapy, which is the standard regimen in patients with rheumatoid arthritis.

Daily administration of methotrexate suppressed bone marrow function in our study, while the weekly pulse therapy did not cause myelosuppression despite the mice receiving the same total dose per week. Therefore, this side effect may have contributed to the shorter life span of the mice on daily therapy. The terminal phase half-life of methotrexate in mice is reported as 12 h (Borsa et al., 1969), which is comparable with that (9 h) in patients with rheumatoid arthritis (Edno et al., 1996). Gutirrez-Urena et al. (1996) reported that 1.4% of patients with rheumatoid arthritis exposed to methotrexate developed pancytopenia, according to data from long-term prospective studies. Several patients have died from this complication of methotrexate despite medical intervention. Our findings indicated that weekly pulse administration of methotrexate was superior to daily administration in MRL/lpr mice because of an increase in the survival rate, possibly due to a reduction in the toxicity of methotrexate.

Acknowledgements

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japanese Government, and the Medical Research Laboratories of Wyeth Lederle Japan. We thank Ms. Sonoko Sakurai for secretarial assistance.

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