Original Article

Combination effect of Bucillamine and Methotrexate on rat type II collagen-induced arthritis model

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Purpose: To expect disease-modified anti-rheumatic drugs (DMARDs) combination therapy on rheumatoid arthritis (RA), combination effect of bucillamine (Buc) and methotrexate (MTX) on type II collagen-induced arthritis model (CIA) was investigated. IgM production from B lymphocytes was examined to understand one of the mechanisms of combination effects.

Methods: Rat CIA was induced by bovine type II collagen (CII) sensitization. Buc 3 mg/kg was orally administrated once daily for 21 days from the day of sensitization. MTX 0.2 mg/kg was orally administrated on three times a week for three weeks as the mimic of the clinical dose. IgM production from B lymphocytes was examined with the stimulation of IL-2 and *Staphylococcus aureus*, and Buc or MTX were treated *in vitro*.

Results: Buc and MTX combination additively reduced the hind paw swelling and bone destruction in CIA. α1-acidic glycoprotein, one of the inflammation markers in serum, and anti-CII antibody titer were also decreased. Buc and MTX combination additively reduced *Staphylococcus aureus* and IL-2 induced IgM production from B lymphocytes *in vitro*.

Conclusion: This study provides the first evidence that Buc exerts additive effect with MTX in CIA rats. Regulating B cell function by Buc and MTX may contribute to their additive effects on rat CIA to some degree.

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Key words methotrexate (MTX), bucillamine (Buc), collagen-induced arthritis (CIA), combination therapy, rheumatoid arthritis (RA)

Introduction

Methotrexate (MTX), Bucillamine (Buc), and Salazosulfapyridine (SASP) have been used as major disease-modified anti-rheumatic drugs (DMARDs) for the treatment of rheumatoid arthritis (RA) in Japan. DMARDs are effective against RA, but therapy with DMARDs frequently needs to be changed due to insufficient effectiveness or loss of its efficacy, so-called escaping the efficacy. For many years, attempts have been made to use multiple DMARDs in combination with patients exhibiting poor responses to treatment with one DMARD. Although TNF-binding therapeutic biologic agents have been marketed and their effectiveness has recently come to be widely accepted, treatment

with a combination of conventional DMARDs is often selected even at present because of the high cost of biologics and the risk of serious adverse reactions to such biologics[1]. However, clinical evidence for the usefulness of combined DMARDs therapy has been scarcely reported. From the results of two large-scale clinical studies of combined MTX and SASP therapy in Europe[2,3], therapeutic advantage of this treatment compared to monotherapy could not be found. On the other hand, combination therapy of MTX and SASP therapy has recently proved more effective than monotherapy in RA[4]. These contradictions are both reported, thus, the combined therapy was selected based on the empirical judgment of individual clinicians. In addition, there is little evidence for the usefulness of Buc and MTX combination therapy. Under these circumstances, the members of the Ministry of Health, Labour, and Welfare (MHLW) Study Group, led by Ichikawa et al., conducted the first clinical study in Japan on combined DMARDs therapy, using a combination of MTX and Buc, to obtain findings regarding the usefulness of combined DMARDs therapy[5]. In that study, it was confirmed that treatment with MTX or Buc alone for two years using dosage regimen prevailing in Japan resulted in improvement in about 45% of all patients, according to the 20% improvement criteria of the American College of Rheumatology. The study also showed that, when assessed using the same criteria, about 80% of patients exhibited improvement in response to combined treatment with MTX and Buc. These results thus clearly demonstrated the usefulness of combined MTX and Buc therapy[5]. We speculate that the result of combination therapy by Japanese clinical study depends on a difference of action mechanism. Therefore, the present study was undertaken to reproduce the clinically observed additive effects of combined MTX and Buc therapy in a rat model of collagen-induced arthritis (CIA), a representative animal model used in studies of RA. It was also designed to examine the additive effects of Buc and MTX, focusing on bone destruction[6,7] and antibody production[8], which appear to be effects of Buc.

Materials and Methods

1)Induction of CIA in rats

An emulsion prepared from bovine type II collagen (CII) solution (Collagen Research Center) (final concentration 1 mg/mL) and Fround's incomplete adjuvant (DIFCO) was injected intracutaneously into the back of female Lewis rats (8 weeks old, body weight 150-170 g, SLC Japan) in a volume of 500 μL (first immunization). Seven days later, the same emulsion was injected intracutaneously into the root of tail of each rat in a volume of 100 μL (secondary immunization) to induce arthritis. All of the animal experiments were performed under the ethical code for experimental animals ruled by Nara Research and Development center in Santen Pharmaceutical Co., Ltd.

2)Drug treatment

Buc was administered once daily for 21 days, beginning on the day of first immunization. MTX was administered three times a week (as a clinical dosage, in the morning and the evening of the first days and in the morning of the second days of each week) beginning on the day of first immunization and according to a method similar to that used clinically (9 doses in total).

3)Measurement

The hind paw volume of rats with CIA was measured by the water replacement method, using a plethysmometer. Serum anti CII antibody titer was measured by ELISA using bovine CII (Collagen Research Center). The level of α1-acidic glycoprotein (α1-AG), an inflammatory maker protein, in rat serum was measured with a Pana Test A Series Rat α1-AG Kit (Panapharm Laboratories).

4)Effects on IgM production by B lymphocytes

A lymphocyte fraction was separated from each rat spleen by density-gradient centrifugation. Then, B lymphocytes were isolated with a Nylon fiber column. The isolated B lymphocytes were inoculated onto 96-well plates at a density of 2.0×10^5cells/well. Following treatment with *Staphylococcus aureus* (*SA*) (1/24000) plus 1 ng/mL of IL-2, an IgM production stimulator, and with Buc and MTX, the cells were incubated for 8 days. After incubation, IgM in the supernatant was quantified by ELISA[8]. SA was purchased as PANSORBIN® Cells from CALBIOCHEM, and IL-2 was purchased from R&D systems.

5)CT mean value

Images of the hind limb bones of CIA rats were taken into a computer using a micro-X ray CT device (TXMS-S; TSK Co., Ltd.) and the VGStudio MAX data analysis program (Volume Graphics) to re-establish three-dimensional images. After segmentation of the first tarsal bone, CT mean value, an indicator of bone destruction, was measured.

6)Statistical analysis

All values are expressed as the mean $\pm$ S.E.M. Statistical analyses were performed by EXSAS7.1.6 (SAS version8.2). Variance analysis for two groups was performed using F-test. Differences between the two groups were compared using Student's t-test for homoscedastic data or using Aspin-Welch t-test for others. Variance analysis for more than three groups was performed using Bartlett test. Differences between more than three groups were compared by Dunnett test for homoscedastic data or by Steel test for others. P values less than 0.05 were considered significant.

Results

The effects of MTX or Buc treatment and combined MTX

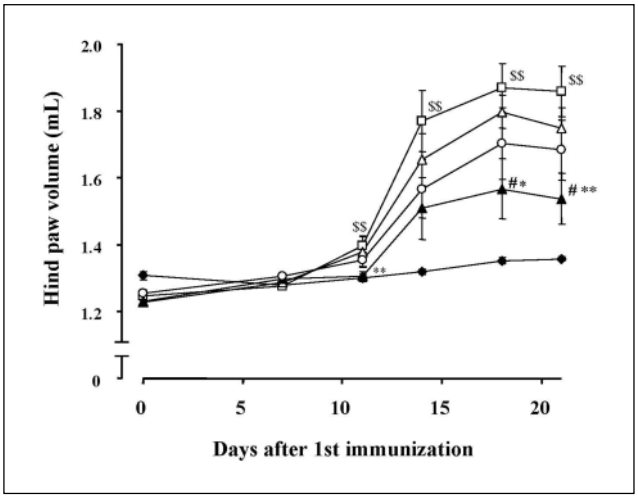

Fig.1 Combination effects of Buc and MTX on hind paw edema in CIA rats

Closed diamond: normal, open square: control, open triangle: 3mg/kg Buc, open circle: 0.2mg/kg MTX, closed triangle: Buc (3mg/kg) and MTX (0.2mg/kg).

$$: $p<0.01$, Aspin-welch t-test, vs normal, *: $p<0.05$, **: $p<0.01$, Student's t-test, vs control, #: $p<0.05$, Student's t-test, vs Buc3, N=8. Each point represents the mean ± S.E.M.

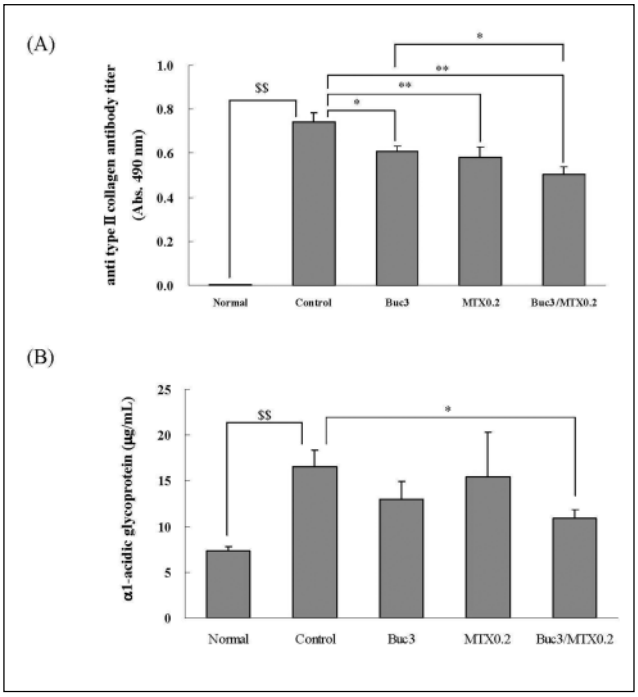

Fig.2 Combination effects of Buc and MTX on serum type II collagen antibody titer (A), serum α1-acidic glycoprotein level (B) in CIA rats

$$: $p<0.01$, Aspin-Welch t-test, *: $p<0.05$, **: $p<0.01$, Student's t-test, N=7-8.

Each bar represents the mean ± S.E.M.

plus Buc treatment on CIA-induced hind paw edema are shown in Figure 1 (N=8). The dose used in this study was 3 mg/kg for Buc and 0.2 mg/kg for MTX. These dose levels were effective but lower than optimal dose levels determined in the dose-response study of the effect on hind paw edema in CIA rats. In the previous dose-response study, Buc induced significant suppression of hind paw edema at a dose level of 10 mg/kg, while MTX exhibited maximal suppression at a dose level of 0.4 mg/kg. In addition, Buc at 1mg/kg, MTX at 0.1mg/kg, or 3mg/kg Buc plus 0.1mg/kg MTX did not affect (data not shown). The results indicate significant and additive suppression of CIA-induced hind paw edema following combined MTX plus Buc, compared to treatment with Buc or MTX alone. Moreover, combined MTX plus Buc significantly suppressed the onset of the hind paw swelling on day 11.

Figure 2 shows serum anti-typeII collagen (CII) antibody titers (A), serum α1-acidic glycoprotein (AG) levels (B) for each group on the 22nd day after first immunization. Following induction of CIA, serum anti-CII antibody titer and α1-AG, as a marker of inflammation level, increased significantly. The elevation of these parameters was more markedly suppressed by combined MTX plus Buc treatment than by treatment with MTX or Buc alone.

Figure 3 shows three-dimensional images obtained with a micro-X ray device in each group on the 22nd day after first immunization. In addition, as a shown in Figure 4, CT mean value for the first tarsal bone in the control group marked decreased. The decrease in CT mean value means the loss of bone mineral content, that is, the bone destruction. On the other hand, the decrease of CT mean value in CIA rats was also more markedly suppressed by MTX plus Buc combination than by Buc or MTX alone (Fig.4).

Following marked effects of combined MTX and Buc treatment on elevation of anti-CII antibody titer in rats with CIA, we evaluated the effects of combined MTX plus Buc treatment on antibody formation by B lymphocytes in vitro. Figure 5 shows the effects of combined MTX plus Buc treatment and MTX or Buc treatment on IgM antibody released into the supernatant of culture on stimulation of B lymphocytes with *Staphylococcus aureus* (SA) and IL-2. Treatment with Buc alone suppressed IgM antibody formation by B lymphocytes in a dose-dependent manner (A), as did that by MTX alone (B). Suppressive effects of sub-optimal concentration of MTX on IgM production by B

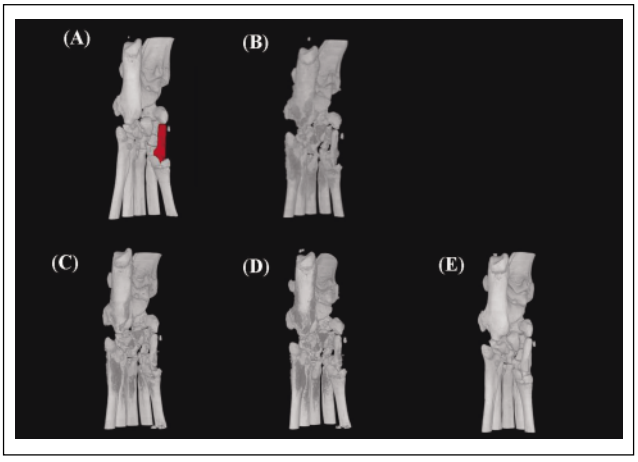

Fig.3 Representative three-dimensional images of the first tarsal bone of CIA rats with obtained with a micro-X-ray CT device

First tarsal bone is colored with red in normal group (A).
(A) normal, (B) control, (C) 3mg/kg Buc, (D) 0.2 mg/kg MTX
(E)Buc (3 mg/kg) and MTX (0.2 mg/kg).

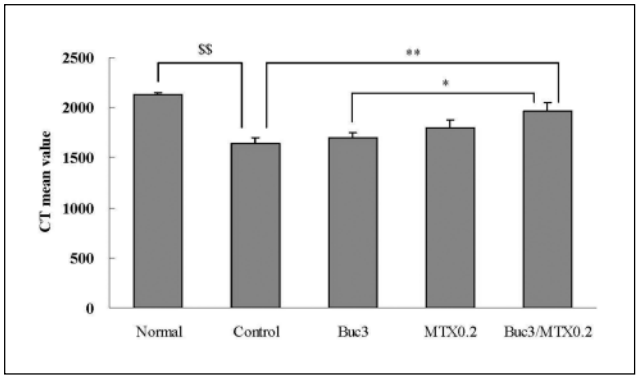

Fig.4 Combination effects of Buc and MTX on CT mean value of the first tarsal bone of CIA rats

$$: $p<0.01$, Aspin-Welch t-test, *: $p<0.05$, **: $p<0.01$, Student's t-test, N=8. Each bar represents the mean ± S.E.M.

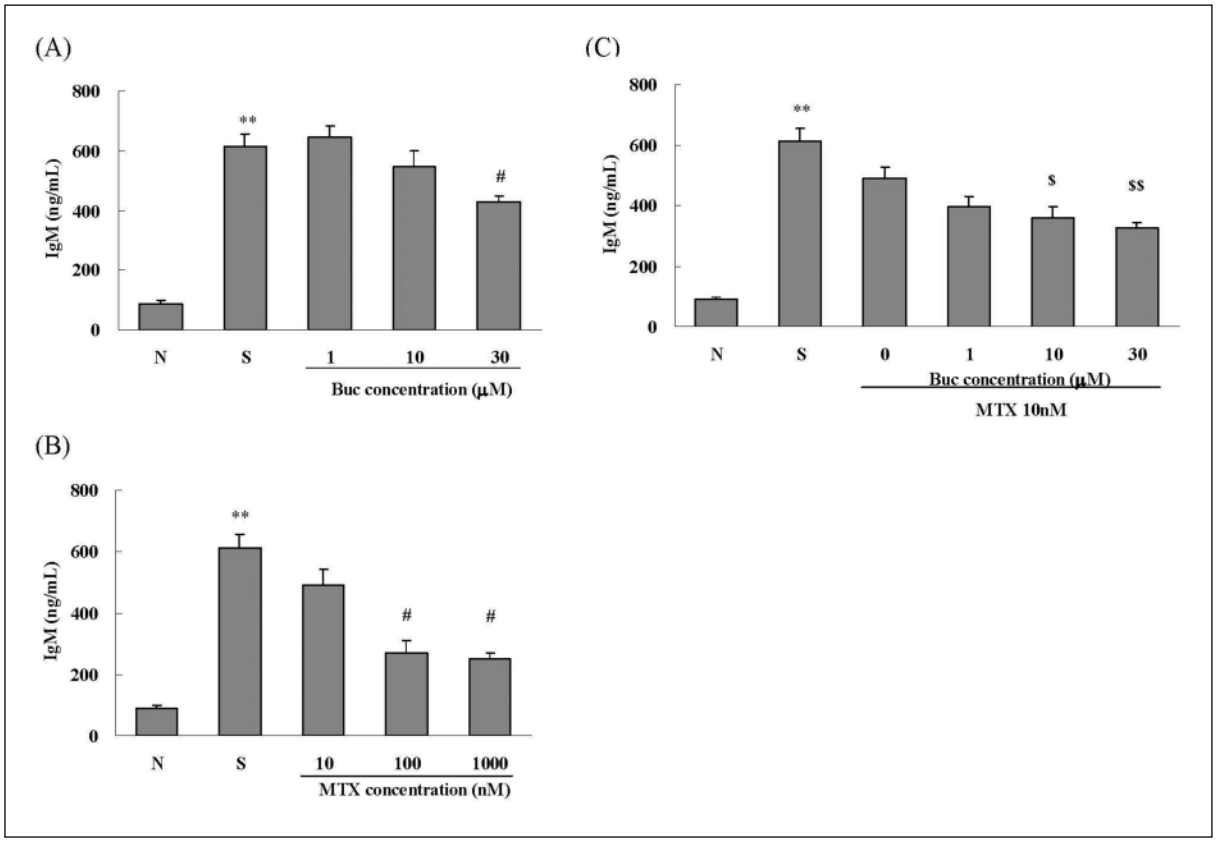

Fig.5 Combination effects of Buc and MTX on IgM formation by *Staphylococcus aureus* and IL-2-stimulated B lymphocytes

(A) Effects of Buc on IgM production, (B) Effects of MTX on IgM production, (C) Effects of combined MTX (10 nM) and Buc on IgM production.

N=normal, S=stimulation, **: $p<0.01$, Student's t-test, vs Normal, #: $p<0.05$, Steel test, vs S, $: $p<0.05$, $$: $p<0.01$, Dunnett test, vs Buc0. N=6 Each bar represents the mean ± S.E.M.

lymphocytes were enhanced by adding of Buc dose-dependently (C). Same results were obtained by adding of MTX to sub-optimal concentration of Buc (data not shown).

Discussion

In the present study, combination treatment with MTX and Buc suppressed the inflammation of joints, elevation of serum anti-CII antibody titer, markers of inflammation, and bone destruction more strongly than the treatment with MTX or Buc alone in rats of CIA. In addition, the combination treatment with MTX and Buc exerted the suppression effect of production of IgM by B lymphocytes in vitro more strongly than the monotherapy. Thus, in the present study of an animal model as in treatment of RA with DMARDs, efficacy in alleviating signs of arthritis is stronger with combined MTX and Buc than with use of MTX or Buc alone.

It is believed that the progression of RA is closely related to the activity of T lymphocytes, B lymphocytes, osteoclasts, and synoviocytes. Since RA was markedly alleviated in response to B lymphocyte depletion therapy using anti-CD20 antibody[9] and abnormal differentiation of osteoclasts was locally observed in pannus where advanced bone destruction had occurred[6], it has been considered very likely that B lymphocytes and osteoclasts are closely involved in the progression of RA. MTX has been reported not only to suppress cell division through inhibition of folic acid metabolism and to have anti-inflammatory effects mediated by A2 receptors through induction of adenosine pooling[10], but also to have direct effects on the differentiation of osteoclasts[7]. Buc has also been reported to act on RA by suppressing autoantibody production in B lymphocytes[8] and suppressing bone destruction by osteoclasts[7,11] although the mechanisms of these effects remain unclear in detail. Regarding the effects of MTX and Buc in suppressing the differentiation of osteoclasts, the study performed *in vitro* by Suematsu et al.[7] demonstrated that the points of action of these two drugs differed, and that suppressive effect was reinforced by combined use of these drugs. The suppressive effects of MTX on B lymphocytes appears to be due primarily to inhibition of cell division through inhibition of folic acid metabolism, though no report has been published concerning direct suppressive effects of Buc on cell division and Buc appears to directly suppress the function of B lymphocytes. It therefore appears quite likely that the points of action on B lymphocytes of these two drugs also differ. This conclusion was supported by the findings of the evaluation of effects of combined use of MTX and Buc on B lymphocytes in the present study.

Taken together, these findings suggest that MTX and Buc combination treatment can be expected to exert additive effects in alleviating the signs and symptoms of RA, since these two drugs suppress B lymphocyte activity and osteoclast differentiation through different mechanisms of action.

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