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Prevention of Arthritic Inflammation Using an Oriental Herbal Combination BDX-1 Isolated from *Achyranthes bidentata* and *Atractylodes japonica*

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An oriental herbal combination (BDX-1) was isolated from *Achyranthes bidentata* and *Atractylodes japonica*. We previously tested the clinical effectiveness of BDX-1 in rheumatoid arthritis (RA) patients and found that it has a beneficial therapeutic effect. Here, we provide experimental evidence for the effectiveness of BDX-1 on RA in murine models. The oral administration of BDX-1 was found to markedly inhibit collagen-induced arthritis, adjuvant-induced arthritis, and zymosan-induced inflammation. It also inhibited carrageenan-induced acute edema and acetic acid-induced writhing response. In addition, the biological activity of BDX-1 was found to be strongly increased by fermentation. Our results suggest that BDX-1 could be useful for the treatment of rheumatoid arthritis.

Key words: BDX-1, *Atractylodes japonica*, *Achyranthes bidentata*, Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the inflammation of joints and accompanying synovial hyperplasia, which ultimately leads to joint destruction and deformity (Ono *et al.*, 2004). Current therapeutic approaches focus on controlling the symptoms and signs of this disease and ameliorating its damaging effects on articular cartilage. Disease modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, and steroids are used to control RA, and more recently TNF-α-neutralizing therapy in combination with methotrexate has been found to provide a sustained clinical benefit (Lipsky & Maini 2000; Lipsky *et al.*, 2000). However, the long-term efficacies of these treatments have not been proven, and adverse events have been reported, which limit their usefulness in terms of long term administration (Inoue *et al.*, 2004). Recently, oriental herbal medicines have been recognized to offer an alternative for the treatment of many inflammatory diseases including RA. One of the most promising advantages of oriental herbal medicines is that they are intrinsically safe and free of severe adverse effects after prolonged administration. Thus, we have screened and investigated many herbal medicines in the hope of finding anti-inflammatory agents, especially against RA.

Atractylodes japonica is listed in the Chinese, Korean, and Japanese pharmacopoeias and is traditionally used as a diuretic and stomachic (Jang *et al.*, 2004; Kitajima *et al.*, 2003; Min *et al.*, 2001; Satoh *et al.*, 1996). It has been reported that the Atractylodes family possesses anti-inflammatory activity by inhibiting the production of inflammatory mediators, such as nitric oxide and prostaglandins (Jang *et al.*, 2004). Polysaccharide isolated from *Achytanthes bidentata* is known to have antitumor activity by mediating immunostimulation (Meng *et al., 2005;* Mitaine-Offer *et al., 2001;* Tian *et al.*, 1995; Yu & Zhang 1995) and may inhibit asthma by enhancing eosinophil apoptosis (Li *et al.*, 2003). Moreover, its water extract is known to inhibit both pain and inflammation (Lu *et al.*, 1997). Like other traditional oriental medicines BDX-1 consists of a mix of medicinal plants. BDX-1 was prepared from *Atractylodes japonica* and *Achyranthes bidentata*,

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and then processed by malt fermentation. In this study, we assessed the efficacy of BDX-1 as a therapeutic agent for RA in rat and mouse models.

MATERIALS AND METHODS

Materials

All mice and rats used in the present study were purchased from Charles River Japan (Kanagawa, Japan) and maintained under SPF conditions until required. The KRIBB Animal Experimentation Ethics Committee approved all procedures used in this study. BDX-1 was provided by the BIORADIX Co. Ltd (Korea). The roots of *Atractylodes japonica* and *Achyranthes bidentata* were extracted with 30% ethanol for 24 h; a water extract also showed similar biological activity (data not shown). The supernatant was cooled at 4°C for 5 days, fermented with malt for 4~36 h, and the supernatant was lyophilized to produce a powdered extract (Lee *et al.*, 2001).

Adjuvant-induced arthritis

Arthritis was induced by the subcutaneous (s.c.) injection of 0.5 mg of *Mycobacterium tuberculosis* (Difco Laboratory, Detroit, MI) in 50 mL of mineral oil into the left hind footpad of Sprague-Dawley rats (female and 6 weeks of age) on day 0. The volumes of right hind paws were measured by water displacement using a rat plethysmometer (MK-550, Muromachi Kikai Tokyo, Japan). Paw volumes of each mouse on day -1 were presented as 100%. BDX-1 was orally administered daily from day 0 to day 45 (Ishikawa *et al.*, 2005).

Collagen-induced arthritis

Bovine type II collagen was diluted in 0.05M acetic acid to a concentration of 2 mg/mL and emulsified with an equal volume of complete Freund's adjuvant (CFA, 2 mg/mL of *Mycobacterium tuberculosis*, Difco, Detroit, ML). Male DBA/1 mice (6 weeks) were immunized intradermally at the base of the tail with 100 μL of this emulsion. On day 21 postinjection, the animals were given an intraperitoneal (i.p.) booster injection of 100 μg of type II collagen dissolved in PBS. On days 28 and 49, the onset of arthritis was accelerated by administering a single i.p. injection of 40mg of lipopolysaccharide (LPS, Sigma, St. Louis). These mice were then treated or not with BDX-1 daily from the day following the LPS injection for 4 weeks. Mice were examined visually for the appearance of arthritis in joints, and severity scores (macroscopic score) were awarded as previously described (Han *et al.*, 2001; Ishikawa *et al.*, 2005). The clinical severity of arthritis was graded on a scale of 0-2 for each paw, in terms of redness and swelling, where 0 = no changes, 0.5 = significant, 1.0 = moderate, 1.5 = marked, 2.0 = maximal. The macroscopic score (mean +/- standard deviation) was defined as the cumulative value.

Zymosan-induced inflammation

Arthritis was induced by an s.c. injection of 50 mL of 3 mg/mL Zymosan A from *Saccharomyces cereviciae* (Sigma, St Louis, MO) into the hind footpad of C57BL/6 mice (female and 6 weeks of age). BDX-1 was orally administered from day 0 to day 6. On day 7, popliteal lymph nodes were isolated and weighed (Frasnelli *et al.*, 2005).

Carrageenan-induced paw edema

Paw edema was induced using an s.c. injection of 0.1 mL of 1% carrageenan into the rat left hind paw of C57BL/6 mice (female and 6 weeks of age). The volume of the right hind paw was measured by a water displacement using a plethysmometer. Paw volume was determined 2 h before and 3 h after carrageenan injection. BDX-1 was administered i.p. 1 h before carrageenan injection. Paw volumes of each mouse before BDX-1 injection were presented as 100% (di Meglio *et al.*, 2005).

Acetic acid-induced writhing response

BDX-1 was administered orally to C57BL/6 mice (female and 6 weeks of age) that had been fasted overnight, and 1h later mice were injected i.p. with 0.1 mL/10 g body weight of 0.7% acetic acid solution in saline. The number of writhing events was recorded over 15 min periods (Kou *et al.*, 2005).

Statistics

In vivo results represent 4~5 mice or rats per experiment, which were repeated at least four times. Some results are expressed as means +/- standard deviation. *P* values were calculated using the Student's *t*-test in Microsoft Excel.

RESULTS

Initially we examined the oral toxicity of BDX-1 in mice and rats, and found that it showed no observable toxicity at dosages up to 5 g/kg body weight (data not shown). In the following studies BDX-1 was orally administrated at <5 g/kg body weight.

Next, we examined the effect of BDX-1 on the development of RA using an adjuvant-induced arthritic model. Rats were injected s.c. with adjuvant in the left rear foot on day 0, and disease progression was monitored in right rear feet for 29 days. Control rats (n=5), which were administered saline vehicle only, showed severe inflammation in the right foot (Fig. 1A). Arthritic inflammation was observed in all right feet (n=5/5) on day 29 and paw

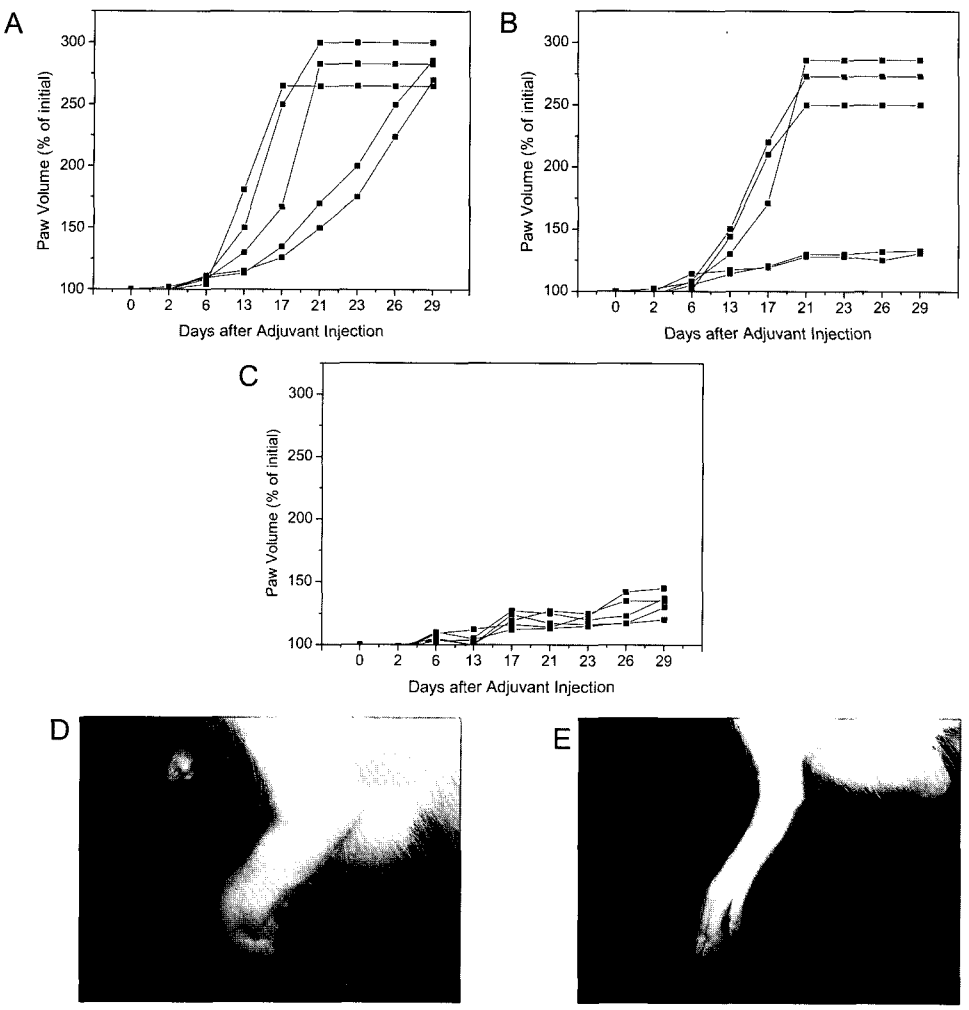

Fig. 1. Effect of BDX-1 on adjuvant-induced arthritis. Arthritis was induced by injecting s.c. adjuvant *Mycobacterium tuberculosis* into the left hind footpad of SD rats (n=5, Day 0). The volumes of right hind paws were measured by water displacement using a rat plethysmometer. Paw volumes of each mouse on day -1 were presented as 100%. Rats were orally administered with saline (A), unfermented (B) or fermented BDX-1 (C) at 500 mg/kg body weight daily from day 0 to day 29. Representative photographs of hind paws from BDX-1-untreated (D) and fermented BDX-1-treated rat (E) are shown.

volume increased by 180% compared with initial paw volume. After preparing non-fermented herbal extract, we administrated this agent orally at 500 mg/kg body weight from day 0 to day 29. As shown in Fig. 1B, the oral administration of non-fermented BDX-1 slightly inhibited the pathogenesis of the arthritis. Two out of five animals showed mild inflammation on day 29 with a mean paw volume increase by 114% versus the initial volume. However, fermented BDX-1 markedly suppressed arthritic inflammation, and all five rats showed only mild inflammation on day 29 with a mean paw volume increase by 33% (Fig. 1C). Representative images of right hind feet from BDX-untreated (Fig. 1D) or BDX-1-treated rats (Fig. 1E) are shown, and illustrate the effect of BDX-1 on arthritic inflammation. We used fermented BDX-1 in the following experiments.

In order to calculate the Median Effective Dose (ED_{50}), we administered BDX-1 orally at different doses from 30 to 3,000 mg/kg body weight and determined this to be 113 mg/kg body weight (Fig. 2). By repeated experimentation, the average ED_{50} of BDX-1 was found to be 167 mg/kg body weight, ranging from 80 to 261 mg/kg body weight. During the course of this experiment, no changes in body weight or other macroscopic appearance were observed in BDX-1-treated rats.

Collagen-induced arthritic inflammation was induced in DBA-1 mice (n=4) by collagen, CFA, and LPS. As shown in Fig. 3A, arthritic inflammation was noticed from day 50, and the oral administration of 500 mg/kg body weight of BDX-1 markedly inhibited the pathogenesis of arthritis, based on the macroscopic arthritic score (Fig. 3B). The average clinical score of BDX-1-treated mice at the end of experiment was 3.8, which represented a 47% reduction in score compared with untreated control mice. The onset

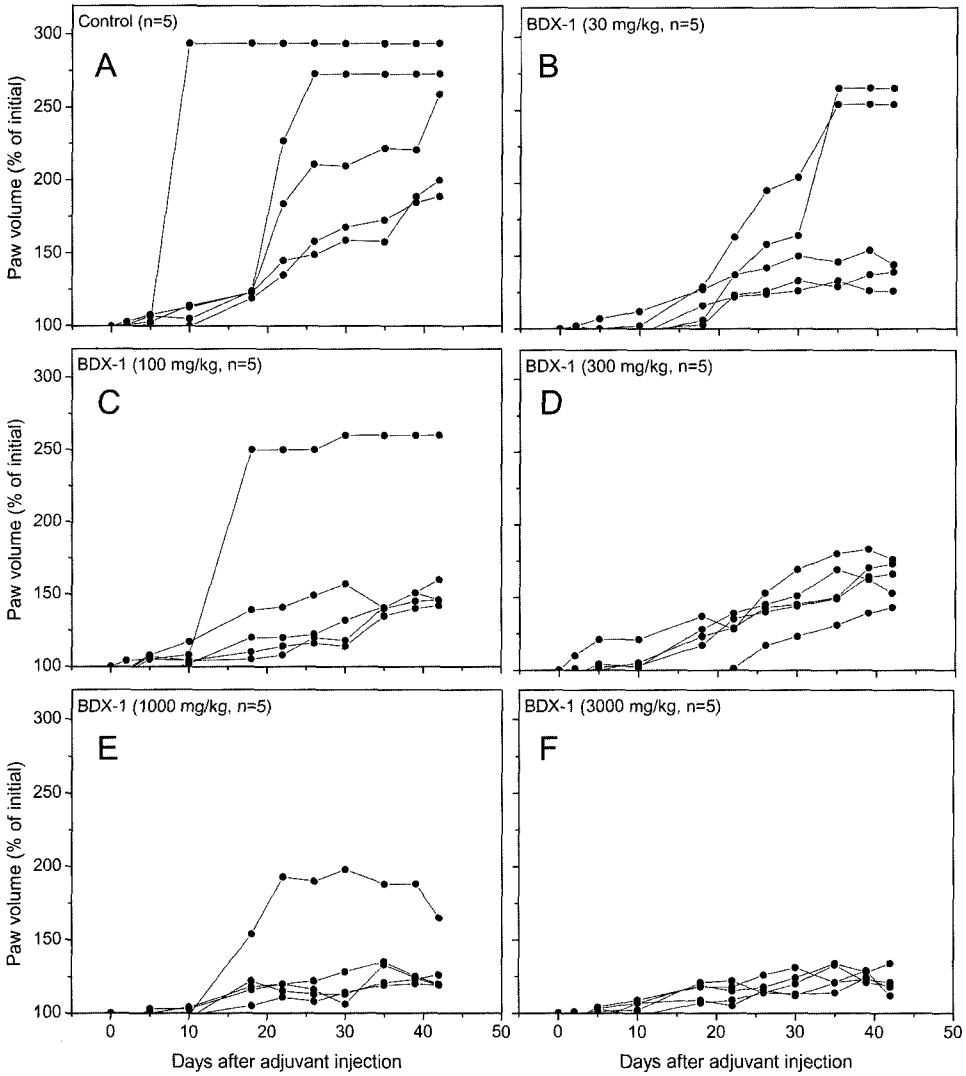

Fig. 2. Dose-response graph for the effect of BDX-1 on adjuvant-induced arthritis. Arthritis was induced by the s.c. injection of adjuvant *Mycobacterium tuberculosis* into the left hind footpad of SD rats (n=5, Day 0). The volumes of right hind paws were measured by water displacement using a rat plethysmometer. Paw volumes of each mouse on day -1 were presented as 100%. Rats were orally administered with saline (A) or fermented BDX-1 at 30 (B), 100 (C), 300 (D), 1000 (E), or 3000 mg/kg body weight (F) daily from day 0 to day 42.

of collagen-induced arthritis in Fig. 3A was slightly delayed compared to other reports (Han *et al.*, 2001). This may be due to differences in the experimental set-up (e.g., the mouse conditions, collagens, and LPS used). However, since our study incorporated effective internal controls to facilitate comparisons between BDX-treated and -untreated mice, the therapeutic activity of BDX-1 on collagen-induced arthritis is evident.

In addition, BDX-1 showed strong anti-inflammatory activity in the case of zymosan-induced inflammation. Zymosan was injected into the both foot pads of C57BL/6 mice, and BDX-1 administered orally at 100 or 300 mg/kg body weight reduced inflammation by 39 and 59%, respectively, compare to BDX-1-untreated mice (Fig. 4).

In order to examine the effect of BDX-1 on acute edema, carrageenan was injected into hind foot pads 1 h after an i.p. BDX-1 administration and foot pad swelling was examined 3 h after the carrageenan injection. The results obtained showed that the i.p. administration of BDX-1 strongly inhibited carrageenan-induced edema (Fig. 5); however, the oral administration of BDX-1 only slightly affected this edema (data not shown). This may have been due a protracted active component absorption time for the oral treatment. Moreover, this was supported by the observation that the effective dose of BDX-1 in i.p. injection studies was about 10 fold lower compared with that in oral injection studies. Meanwhile, rats given BDX-1 i.p. did not show any obvious behavioral changes, e.g., excitation, sedation, muscle relaxation.

Finally, the oral administration of BDX-1 showed strong

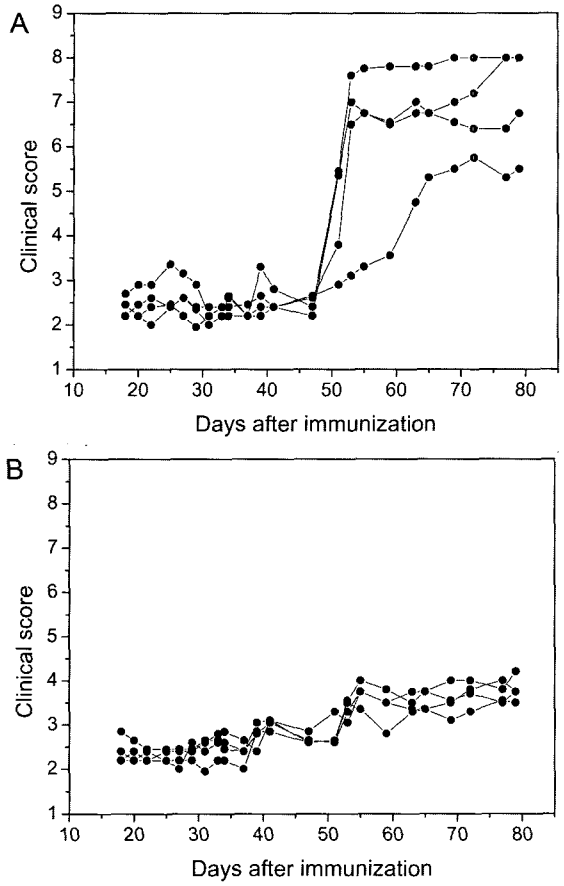

Fig. 3. Effect of BDX-1 on collagen-induced arthritis. Control DBA/1 mice (n=4) were treated with saline, which was also used to dissolve fermented BDX-1 (A). DBA/1 mice (n=4) were then treated orally with 500 mg/kg body weight of BDX-1 from day 0 to day 80 (B). Mice were examined visually for the appearance of arthritis in the joints. The clinical severity of arthritis was graded on a scale of 0-2 for each paw, in terms of redness and swelling, where 0 = no changes, 0.5 = significant, 1.0 = moderate, 1.5 = marked, 2.0 = maximal. The macroscopic score (mean +/- standard deviation) was defined as the cumulative value for all paws.

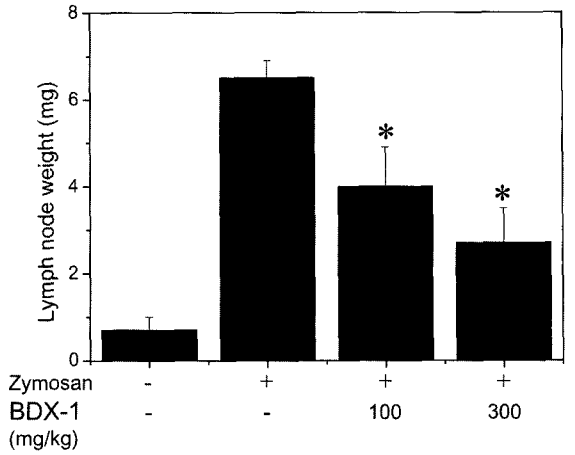

Fig. 4. Effect of BDX-1 on zymosan-induced arthritis. Arthritis was induced by the s.c. injection of Zymosan A into hind footpads of C57BL/6 mice (n=4). Fermented BDX-1 was administered orally from day 0 to day 6. On day 7, popliteal lymph nodes were isolated and weighed. Significance was determined using the Student's t-test versus BDX-1-untreated-treated controls (* $p<0.01$).

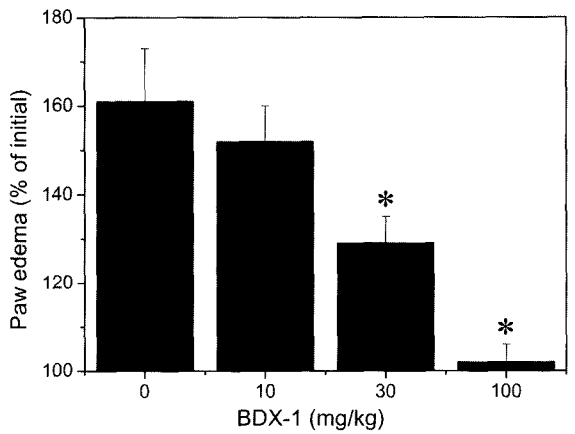

Fig. 5. Effect of BDX-1 on carrageenan-induced edema. Paw edema was induced by the s.c. injection of carrageenan into mouse left hind paws of C57BL/6 mice (n=4). Right hind paw volumes were measured by water displacement using a plethysmometer. Paw volumes were measured 2 h before and 3 h after carrageenan injection. Fermented BDX-1 was administered i.p. 1 h before carrageenan injection. Paw volumes of each mouse before BDX-1 administration were presented as 100%. Significance was determined using the Student's t-test versus BDX-1-untreated-treated controls (* $p<0.01$).

analgesic activity by the acetic acid-induced writhing test (Fig. 6), as did aspirin administration (500 mg/kg body weight).

DISCUSSION

Although the etiology of RA is unknown, it is known that humoral and cellular immune responses are involved in the pathogenesis of RA. In fact, arthritis can be transferred to a naïve recipient by transplanting antibody-containing serum or activated lymphocytes (Inoue *et al.*, 2004; Kinne *et al.*, 1997). In particular, the production of cytokines by T cells and macrophages is critically required for the development of arthritic inflammation (Pohlers *et al.*, 2004). In addition, considerable evidence suggests that RA is a Th1-mediated inflammatory disease, which is associated with the productions of IL-2 and IFN-γ, which can stimulate macrophages to produce TNF-α and IL-1β. TNF-α contributes to inflammatory cell infiltration by up-regulating adhesion molecules on the endothelium, whereas IL-1β induces the production of prostaglandins and collagenases by joint-resident chondrocytes, fibroblasts, and synoviocytes. Indeed, the administration of cytokines, such as IL-2, IFN-γ, TNF-α, and IL-1β has been shown to exacerbate arthritis (Cooper *et al.*, 1988; Mauritz *et al.*, 1988; Williams *et al.*, 1992). In contrast, Th2-derived

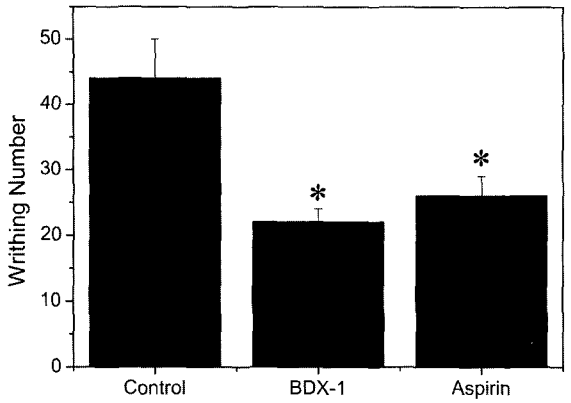

Fig. 6. Effect of BDX-1 on acetic acid-induced writhing response. Mice (n=4) were orally administered with fermented BDX-1 (500 mg/kg body weight). One hour after BDX-1 treatment, mice were injected i.p. with acetic acid solution. The number of writhing events was recorded over 15 min after acetic acid injection. Significance was determined using the Student's t-test versus BDX-1-untreated-treated controls (* $p<0.01$).

cytokines, such as IL-4 and IL-10, may exert minor, or even inhibitory effects on RA.

In the present study, we investigated the anti-arthritic effect of BDX-1 in murine models involving collagen- or adjuvant-induced arthritis. In addition, the anti-inflammatory and analgesic effects of BDX-1 were also evaluated *in vivo* in zymosan- or carrageenan-induced inflammation and acetic acid-induced writhing test. The results obtained showed that BDX-1 inhibited arthritic inflammation in all experimental models used in the present study. Although the mechanism by which BDX-1 suppresses the arthritic inflammation is unknown, we suggest that the modulation of cytokine production by Th1 and Th2 cells and by macrophages is mainly related with the anti-arthritic activity of BDX-1 (Lee *et al.*, 2001). This agent appears to inhibit IFN-γ production by Th1 cells, and TNF-α production by macrophages, which suggests that BDX-1 inhibits inflammatory cytokine production by Th1 cells and macrophages to affect its anti-arthritic effect. However, BDX-1 appeared to increase IL-4 and IL-10 production by Th2 cells in collagen-induced arthritic mice. It is known that Th2 cytokine induces IgG1 isotype switching, whereas Th1 cytokine stimulates IgG2a isotype switching (Iwai *et al.*, 2002; Mukherjee *et al.*, 2003; Shadidi *et al.*, 2003; Yamaki *et al.*, 2003). In fact, changes in Th1/Th2 cytokine profiles by BDX-1 were paralleled by a reduction of IgG2a and the induction of IgG1 in the blood of collagen-induced arthritic mice. These results strongly suggest that BDX-1 prevents arthritis by modulating cytokine networks.

We conclude that the present results suggest that BDX-1 has the potential to be used as a therapeutic agent for the treatment of RA. Further studies will be required to determine how fermentation increases the biological activity of BDX-1, and to isolate and characterize its active compound.

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