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Inflammation Research

Manipulation of the induction of adjuvant arthritis in Sprague-Dawley rats

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Abstract. *Objective:* To investigate the roles of various variables in the induction of adjuvant-induced arthritis (AIA) in the outbred Sprague-Dawley (SD) rats, and further characterize its arthritic features by comprehensive examinations.
Methods: The roles of different preparative techniques, inoculation routes and doses of *Mycobacterium tuberculosis* (MT) suspension as well as the sex preference in the induction of AIA were comparatively studied using clinical assessment. The hind paws of animals were analyzed by radiological and histological examinations. The serum levels of cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were determined by ELISA.
Results: The particle size and dose of MT played a dominant role in the induction and severity of AIA. Male rats manifested markedly more severe arthritic signs than female rats. After subcutaneously inoculated with 500 μg MT, male rats developed pronounced arthritis with 100 % incidence and low variable clinical signs. Even using only 62.5 μg MT, AIA was efficiently induced in male rats and characterized by up-regulated expression profiles of IL-1β, IL-6 and TNF-α.
Conclusions: Since outbred SD rats are much cheaper and more readily available than Lewis rats, this well-developed SD rat AIA model is an efficient and cost-effective arthritis model available for screening novel anti-arthritic agents.

Key words: Adjuvant-induced arthritis – Sprague-Dawley rats – *Mycobacterium tuberculosis* – Pro-inflammatory cytokines

Introduction

Adjuvant-induced arthritis (AIA) was initially observed by accident when complete Freund's adjuvant (CFA) was used for immunization [1]. Since then, induced AIA has been used extensively as a model for studying pathogenic and pathological processes of rheumatoid arthritis (RA) and other arthritic diseases in humans, as well as for screening and testing novel anti-arthritic agents [2, 3]. However, use of the AIA model is hampered by the fact that there are wide variations in the incidence and severity of arthritis even using the inbred Lewis rats, which is currently the most commonly-used strain of animals for induction of AIA.

The classical method of inducing AIA is with ground, heat-killed *Mycobacterium tuberculosis* (MT) H37Ra suspended in incomplete Freund's adjuvant (IFA) commonly known as CFA. As an alternative for MT H37Ra, *M. butyricum* or a mixture of three strains of MT (C, DT, and PN) can be used [4]. Although various strains of rats were studied for establishment of AIA model, the Lewis rats are most frequently used for induction of AIA currently [4]. Sex preference has been reported in some rat strains [5, 6], but there is no significant sex restriction on the susceptibility to arthritis. Thus, both male and female rats are used indiscriminately in current laboratory studies. Nevertheless, wide variations in the frequency and severity of arthritic symptoms are revealed in different rat strains both in male and female animals. Swingle et al. [7] analyzed the severity and incidence of AIA in four strains of rats. In their experiments, both male and female Sprague-Dawley (SD), Holtzman, Charles River and Buffalo strains of rats were injected in the plantar surface of the right hind foot with 500 μg *M. butyricum*. Subsequently the Holtzman and Buffalo rats were found to develop the most and the least severe arthritis among the four strains, respectively. Only the Holtzman strain of rats showed a 100 % incidence of the secondary inflammatory and arthritic signs in the front paws and left hind paws. There was no marked difference in the magnitude of foot swelling between male and female rats of any particular strains. Rosenthale [8] reported that subplantar injection of MT suspension containing 250 μg MT in mineral oil in the right hind foot effectively induced much more severe and less variable AIA in inbred male Lewis rats, showing an incidence of 92 % affliction with secondary polyarthritis symptoms, while in the out-

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bred SD rats there was only a 60 % incidence. Accordingly, the study concluded that the outbred SD strain of rats were genetically less susceptible and more variable with respect to AIA. Banik et al. [9] recently reexamined the differences in susceptibility to AIA among male Lewis, Wistar and SD rats. The results of intradermal inoculation of *M. butyricum* suspended in mineral oil at the base of the tail of the animals showed that the inflammatory and arthritic signs in Lewis rats appeared earlier, more severely and more consistently than in Wistar and SD rats. With inoculation of *M. butyricum* at a dose of 600 µg/rat, 100 % incidence of arthritis was seen in Lewis rats. By contrast, only 64 % and 38 % incidences were observed in Wistar and SD rats, respectively, although the dose of *M. butyricum* was increased to 1,000 µg/rat for Wistar rats and 1,200 µg/rat for SD rats. In addition, the capacity of development of arthritic lesions in response to Freund's complete adjuvant was present in Dark Agouti (DA) rats but absent in Fisher 344 (F344) rats [10], while the Germfree F344 rats were found to develop rather more severe arthritis with 100 % incidence than the specific-pathogen-free (SPF) and conventional rats, probably due to the inhibitory effect of the bacterial flora on the development of the disease [11]. Unfortunately, no detailed descriptions of the manipulation techniques, and doses of MT influencing the induction and severity of AIA in different strains of rats were included in the above literatures. Thus, the roles and the underlying mechanisms of the arthritogenic factors, particularly the manipulation techniques and the doses of MT, in different rat strains in inducing AIA, have not yet been extensively investigated.

SD rats are the most commonly used strain of rats in several laboratories around the world. SD rats are much cheaper and more readily available, in particular compared to the inbred Lewis rats; in addition, SD rats are outbred, such that the resulting AIA model possibly better mimics the genetic features of human RA than other experimental models of autoimmune arthritis. Hence, a SD rat AIA model would offer marked advantages for study. Moreover, an optimal intensity of disease in AIA model would have a significant impact on responses of different classes of agents. For this reason, efficient induction and modification of the disease severity of AIA produced by optimal manipulation techniques and arthritogenic conditions would be of great interest when novel anti-arthritic agents are being tested. We, therefore, initiated the present study to investigate the roles of various important variables such as different manipulation techniques for preparing MT suspension, the doses of MT in the suspension, as well as the inoculation routes and the sex preference in the induction and severity of AIA in the outbred SD rats. Finally, we characterized the features of this AIA model by using comprehensive analyses including clinical, serological, histopathological, and radiological parameters.

Materials and methods

Preparation of mycobacteria suspension

Besides the commercial CFA preparation (5.0 mg/ml MT H37Ra, Chondrex, Redmond, WA, USA), a ground MT suspension was freshly prepared according to the classical method [4]. Briefly, the heat-killed MT H37Ra (Difco, Detroit, MI, USA) was put into a roughened mortar and ground intensively until its color changed from gray to white, then mineral oil was added gradually during continued grinding until a paste was achieved. The grinding time was normally not less than 5 min. Moreover, as described previously [12], a homogenized suspension was prepared using a T8 ULTRA-TURRAX® homogenizer with S8N-5G Dispersing element (IKA-WERKE, Staufen, Germany) to mix the heat-killed MT in mineral oil at 25,000 rpm for 30 s, and a mixed suspension was prepared using a stirrer (Jenway, Dunmow, UK) to mix vigorously heat-killed MT in mineral oil for 60 s. The particle number-size distribution of MT in the suspension prepared by different manipulation techniques was determined by an optical method described previously (Axioskop 2 Microscope, Carl Zeiss Microimaging, Thornwood, NY, USA) [12].

Induction of AIA

SPF outbred male and female SD and male Lewis rats (Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong; Laboratory Animal Unit, the University of Hong Kong, Hong Kong, China), aged 6–7 weeks, were used. Rats were housed 4 per cage with food and water provided *ad libitum* throughout the experiments. The AIA model was induced on day 0 by a single injection of 0.1 ml of the MT suspension prepared by different manipulation techniques or the commercial CFA at the base of the tail of animals through intradermal or subcutaneous routes. All of the experimental protocols involving animals and their care were approved by the Committee on Use of Human & Animal Subjects in Teaching and Research of the Hong Kong Baptist University and were carried out according to the regulations of the National Institutes of Health of USA and the Department of Health of Hong Kong Special Administrative Region.

Clinical assessment of AIA

Rats were inspected daily for the onset of arthritis characterized by edema and erythema in the paws. Disease severity and progression were evaluated by arthritic scoring and measurements of both hind paw volumes and body weight on days 0, 9, 12, 18, 24 and 30 after the induction of arthritis. Lesions of four paws of each rat (i. e., the arthritic signs) were graded by two separate investigators from 0 to 4 according to the extent of both erythema and edema of the periarticular tissues; 16 was the potential maximum of the combined arthritic scores per animal [4]. The hind paw volumes were measured using a plethysmometer chamber (7140 UGO. Basile, Comerio, Italy), and expressed as the mean volume of both hind paws of rats. After the induction of arthritis (day 0), the increase in hind paw volume was calculated by subtracting the hind paw volume measured as the baseline on day 0. The body weight of the rats was monitored with a 0.1 g precision balance (Sartorius AG, Goettingen, Germany).

Haematological examination and measurement of serum cytokine levels

Blood samples were collected from the arteries of the tails of animals for laboratory tests on days 12, 18, 24, and 30 after the induction of arthritis. Erythrocyte sedimentation rate (ESR) was determined by a modified method based on ICSH (International Council for Standardization in Haematology) selected methods [13]. Briefly, a 120 µl sample of blood was taken and directly dropped into 30 µl of 0.109 mol/L sodium citrate, mixed well, and then transferred into a 1.0 mm × 100 mm capillary tube (VWR International, West Chester, PA, USA). The tubes were held obliquely at an angle of 45 °C, and the results were recorded after 15 min.

Levels of the pro-inflammatory cytokines interleukin-1 (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) in blood serum were measured using commercially available ELISA kits for IL-1β (Pierce Biotechnology, Rockford, IL, USA), IL-6 and TNF-α (BD Biosciences, San Diego, CA, USA) according to the manufacturers' recommendations.

Radiological and histological examinations

At the end of the experiments, rats were sacrificed by diethyl ether asphyxiation and the hind paws were radiographed on Fuji HR-Fast film, using a Giotto HT Mammography system (IMS, Bologna, Italy). Radiographs of each rat for evaluation of soft tissue swelling and bone erosion were scored blindly by two independent observers, on a scale of 0 (normal), 1 (mild changes), 2 (moderate changes) and 3 (severe changes) [14]. Total radiological scores were calculated from the sum of both hind paws, giving a maximum possible score of 6 for each radiological parameter per rat.

After the X-ray check, the hind paws were fixed in 10% PBS-buffered formalin. The fixed tissues of the ankle joints were then decalcified in formic acid, embedded in paraffin, longitudinally cut into 5 µm sections, and stained with hematoxylin and eosin (H&E). Grading of cellular infiltration (polymorphonuclear cells, macrophages or lymphocytes), edema, angiogenesis, joint capsular fibrosis, and joint space narrowing, synovial hyperplasia, pannus formation, cartilage and bone erosion of the ankle joints were examined blindly by two independent observers using a semiquantitative scale from 0 (normal), 1 (mild changes), 2 (moderate changes) and 3 (severe changes) [15, 16]. Histological scores were combined and expressed as the sum of both tibiotarsal joints to give a maximum score of 6 for each histological parameter per rat.

Statistical analysis

Data are expressed as the mean ±SEM. Student's *t*-test was used to calculate the difference between two groups. One-way analysis of variance with multiple comparisons using Student-Newman-Keuls test was used to analyze differences between the different groups. Statistical significance was accepted for $p < 0.05$.

3. Results

Susceptibility of SD and Lewis rats to AIA

Male SD and Lewis rats were intradermally inoculated with the ground MT suspension containing 500 µg MT. The onset of arthritis in all Lewis rats occurred around days 7 to 9, while arthritic signs became visible in all SD rats within 9–12 days. In both strains of rats, the arthritic signs characterized by erythema and/or edema first occurred in the hind paws and then in the front paws, mostly symmetrically. The inflammation at its peak extended from the ankles and wrists all the way through the digits. From day 12 onwards, a closely similar severity of disease was observed in two strains of rats (Figs. 1A and B).

Difference in arthritogenic ability of the commercial CFA and ground MT suspension

Male SD rats were intradermally injected with the commercial CFA or the ground MT suspension containing 500 µg MT. In the group receiving the ground MT suspension, all animals began to develop arthritic lesions in paws from days 9 to 12 after inoculation. The arthritic scores of disease on days 9, 12, 18 and 24 were 1.3 ± 0.3, 5.1 ± 0.4, 9.2 ± 0.8 and 11.4 ± 1.0, respectively; while those of rats treated with the commercial CFA on days 9, 12, 18 and 24 were 0.0 ± 0.0, 0.0 ± 0.0, 1.8 ± 0.7 and 2.6 ± 0.8, respectively. Very significant difference in arthritic score was obtained between two groups of rats (n = 10). In parallel, a similar time course of the hind paw volume was observed between two groups of rats (data not shown).

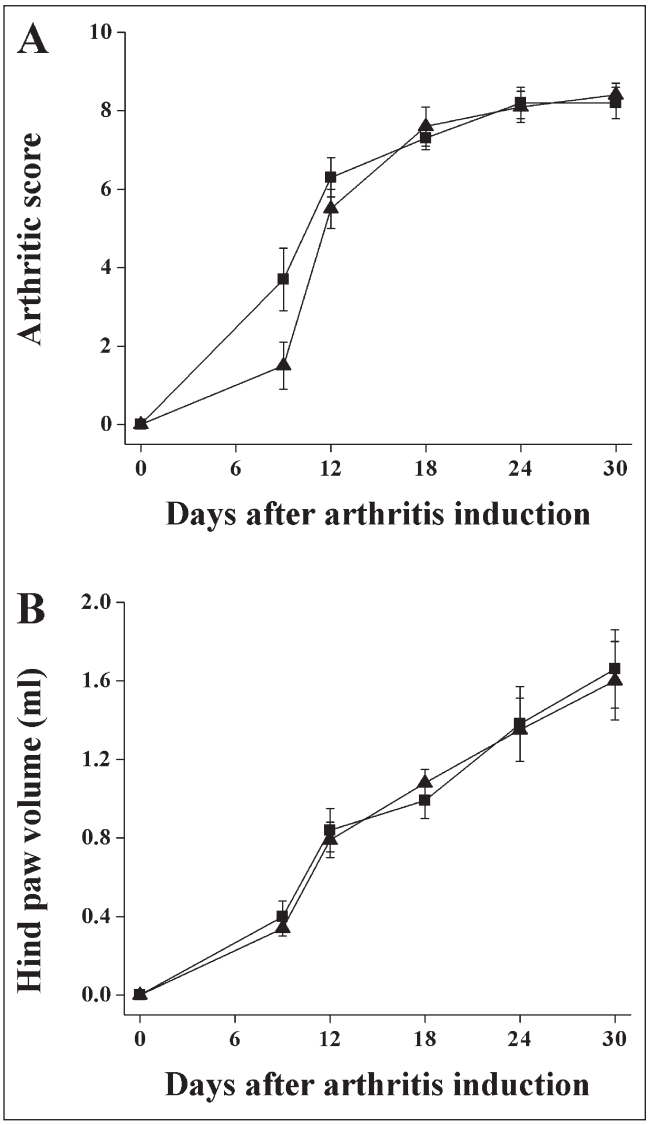

Fig. 1. Susceptibility of SD and Lewis rats to AIA. Male SD (▲) and Lewis rats (■) were intradermally inoculated with the ground MT suspension containing 500 µg MT. Following inoculation with MT, the inflammatory and arthritic lesions appeared in all Lewis rats around days 7 to 9 and in all SD rats within 9–12 days. From day 12 onward, both strains of rats experienced closely similar disease progression, showing similar clinical signs of arthritic score (A) and hind paw volume (B). Data are expressed as mean ±SEM (n = 8).

Difference in severity of AIA induced by different inoculation routes

Male SD rats were inoculated either intradermally or subcutaneously with the ground MT suspension containing 500 µg MT. All animals began to develop arthritic lesions on days 9–12 after inoculation. From day 12 onwards, the animals treated by intradermal inoculation appeared to experience a little more severe arthritis than those treated subcutaneously, but there was no statistical significance in the clinical signs of arthritic score, hind paw volume, and body weight between the two groups (data no shown).

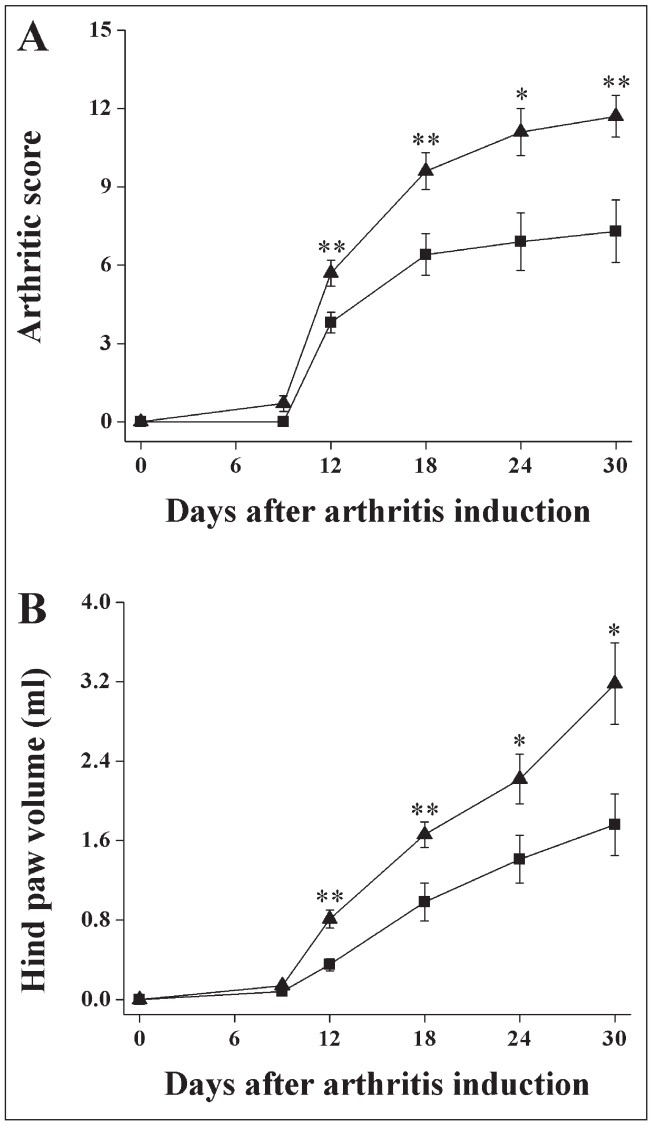

Fig. 2. Sex preferences in severity of AIA. Male (▲) and female (■) SD rats were subcutaneously inoculated with the ground MT suspension containing 500 µg MT. The incidence of arthritis reached 100 % in both male and female SD rats on day 12 after the inoculation of MT; while male rats manifested more severe arthritic signs than female rats, showing significant differences in arthritic score (A) and hind paw volume (B). Data are expressed as mean ±SEM (n = 10). * $p < 0.05$; ** $p < 0.01$.

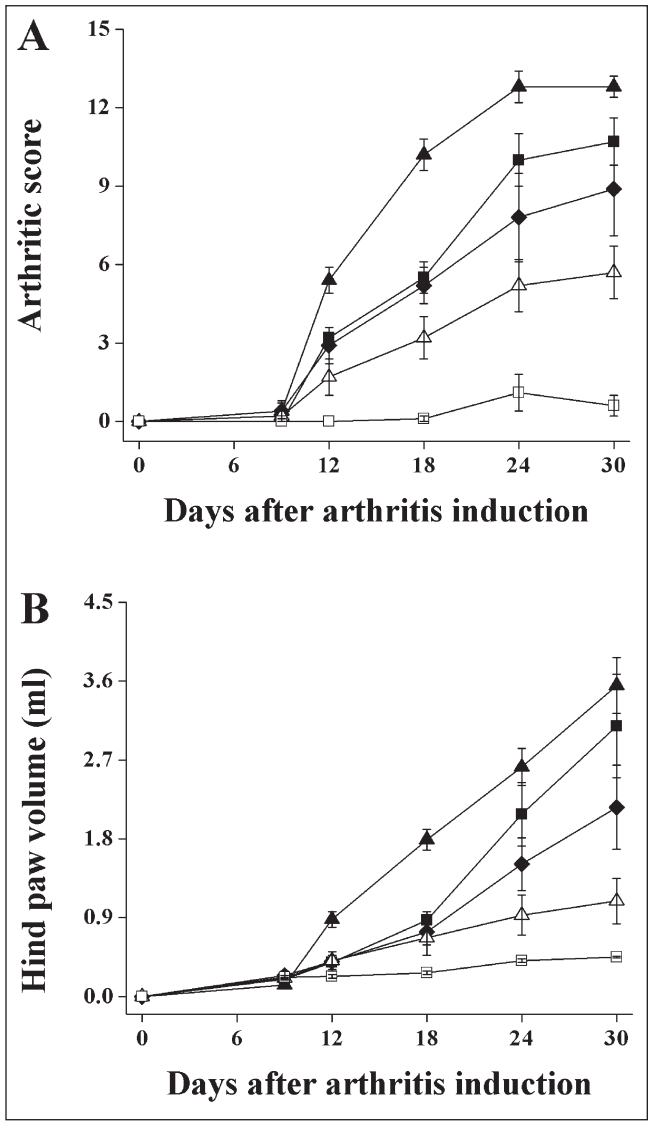

Fig. 3. Influence of the doses of MT in the suspension on incidence and severity of AIA. Male SD rats were subcutaneously inoculated with different doses of the ground MT suspension containing 500 µg (▲), 250 µg (■), 125 µg (◊), 62.5 µg (Δ) and 31.25 µg MT (□), respectively. All doses of MT except 31.25 µg induced 100 % incidence of arthritis, and the dose of MT significantly affected the intensity of disease represented by arthritic score (A) and hind paw volume (B) in a dose-dependent manner. Data are expressed as mean ±SEM (n = 7–9).

Sex preference in incidence and severity of AIA

Male and female SD rats were injected subcutaneously with the ground MT suspension containing 500 µg MT. The secondary inflammatory and arthritic lesions characterized by erythema and/or edema in the paws appeared around days 9 to 12 after inoculation in all animals, both male and female rats; however, the male SD rats developed much more severe arthritis, and significant differences in the arthritic score and hind paw volume were observed between male and female rats from day 12 onwards (Figs. 2A and B).

Influence of the doses of MT in the suspension on incidence and severity of AIA

Male SD rats were injected subcutaneously with different doses of the ground MT suspension, i. e., containing 500, 250, 125, 62.5 or 31.25 µg MT. The clinical signs of arthritis first became visible in the animals starting around day 9 after inoculation. From day 12 onwards, the dose of MT in the suspension markedly affected the severity of AIA (Figs. 3A and B) and counteracted the body weight of animals (data not shown) in a dose-dependent manner. Except with the dose of 31.25 µg, all other doses of MT induced 100 % incidence of AIA.

Table 1. The particle number-size distribution in the ground, homogenized and mixed MT suspensions

Size range (μm)	Ground MT suspension	Homogenized MT suspension	Mixed MT suspension
<10	82.1 %	71.6 %	61.2 %
10–20	10.8 %	18.3 %	13.6 %
>20	7.1 %	10.1 %	25.2 %
Maximum	149.0 μm	264.0 μm	348.0 μm

Influence of MT particle size on arthritogenic ability of the MT suspension

The particle number-size distribution in different MT suspensions prepared by grinding, homogenizing and mixing techniques was measured. As noted in Table 1, in the ground, homogenized and mixed MT suspensions at 5.0 mg/ml of MT concentration, more than 80 %, 70 % and 60 % of MT particles were less than 10 μm in diameter, respectively.

Correspondingly, the arthritogenicity of different MT suspensions was significantly associated with the particle sizes of MT. When male SD rats were subcutaneously injected with MT suspensions containing 500 μg MT prepared by the grinding, homogenizing or mixing techniques, it is seen in Figures 4A and B that the ground MT suspension had the highest arthritogenic ability producing markedly more severe arthritis than that produced by the homogenized or mixed MT suspension.

Rats inoculated with the homogenized MT suspension developed a relatively moderate level of disease, while the incidence and severity of arthritis induced by the mixed MT suspension revealed significantly lower arthritic lesions in comparison to that induced by the ground or the homogenized MT suspension (Figs. 4A and B).

Correlations of clinical arthritic signs and the levels of ESR and serum cytokines in AIA rats

To characterize the AIA model in SD strain of rats by ESR and serum IL-1β, IL-6 and TNF-α levels during disease progression of AIA, the male SD rats were injected subcutaneously at the base of the tail with 0.1 ml of the ground MT suspension containing 62.5 μg MT. The control rats were injected with the saline. On days 12 to 18 after inoculation, most of the animals (90 %) developed AIA, showing significant increase in the hind paw volume and arthritic score (data available upon request). Moreover, the clinical signs of arthritis were significantly associated with the elevated ESR levels. In Figure 5A, it shows that on days 12, 18, 24 and 30 after inoculation, the ESR level was significantly higher in MT-treated animals than in control rats, peaking on day 18 and then falling sharply, indicating a relatively early but lasting stimulation of acute-phase protein production.

The correlations of the clinical signs and the kinetics of pro-inflammatory cytokines in serum during disease progression of AIA were also investigated. The results showed that the serum levels of IL-1β, IL-6 and TNF-α in the AIA rats were significantly up-regulated in association

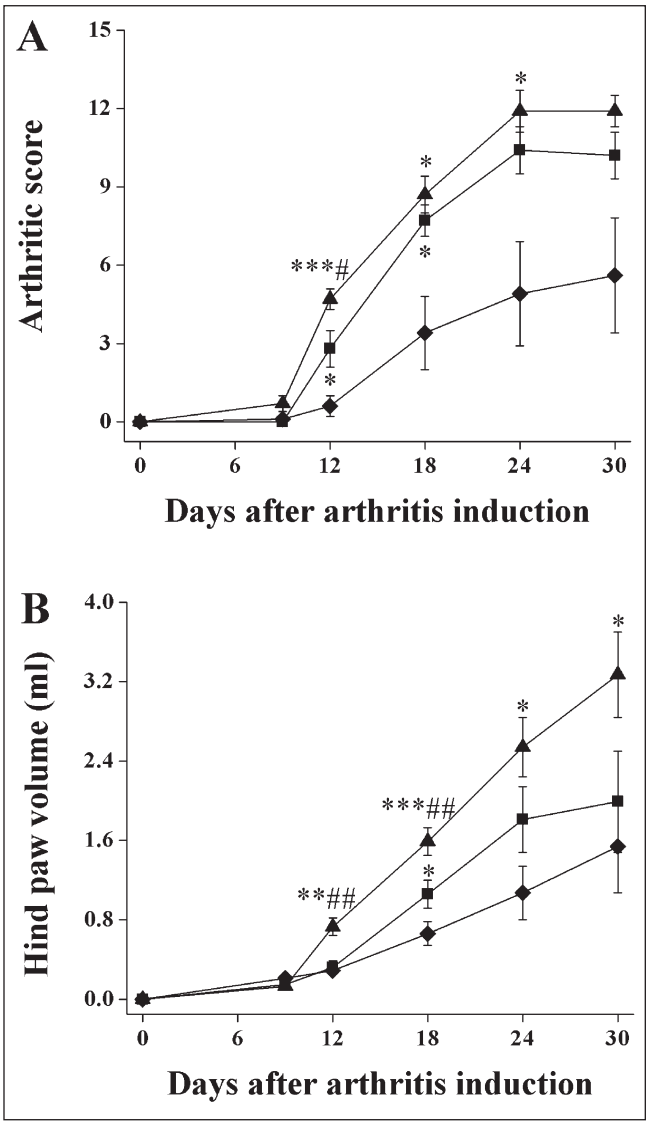

Fig. 4. Effects of the particle size of MT on arthritogenic ability of MT suspension. Male SD rats were subcutaneously inoculated with different MT suspensions containing 500 μg MT prepared by grinding (▲), homogenizing (■) or mixing techniques (◆), respectively. The ground, homogenized, and mixed MT preparations induced relatively severe, moderate, and slight arthritic signs, respectively. Marked differences in arthritic score (A) and hind paw volume (B) were observed between these groups of rats beginning from day 12. Data are expressed as means ±SEM (n = 9–10). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus the mixed MT suspension. # $p < 0.05$; ## $p < 0.01$ versus the homogenized MT suspension.

with the development of disease from days 12 to 30 after inoculation. In detail, the serum IL-1β level in AIA rats was markedly elevated from days 12 to 30 after arthritis induction in comparison with that of the control rats, especially in the earlier phase of the disease with a peak on day 18 (Fig. 5B). AIA rats showed also a significant increase of serum IL-6 level on day 18 after inoculation but markedly declined thereafter (Fig. 5C). This kinetic change of the serum IL-1β and IL-6 levels was well in line with the ESR characteristics during the disease course of AIA. Moreover, during disease progression of AIA, the serum levels of TNF-α on days 12

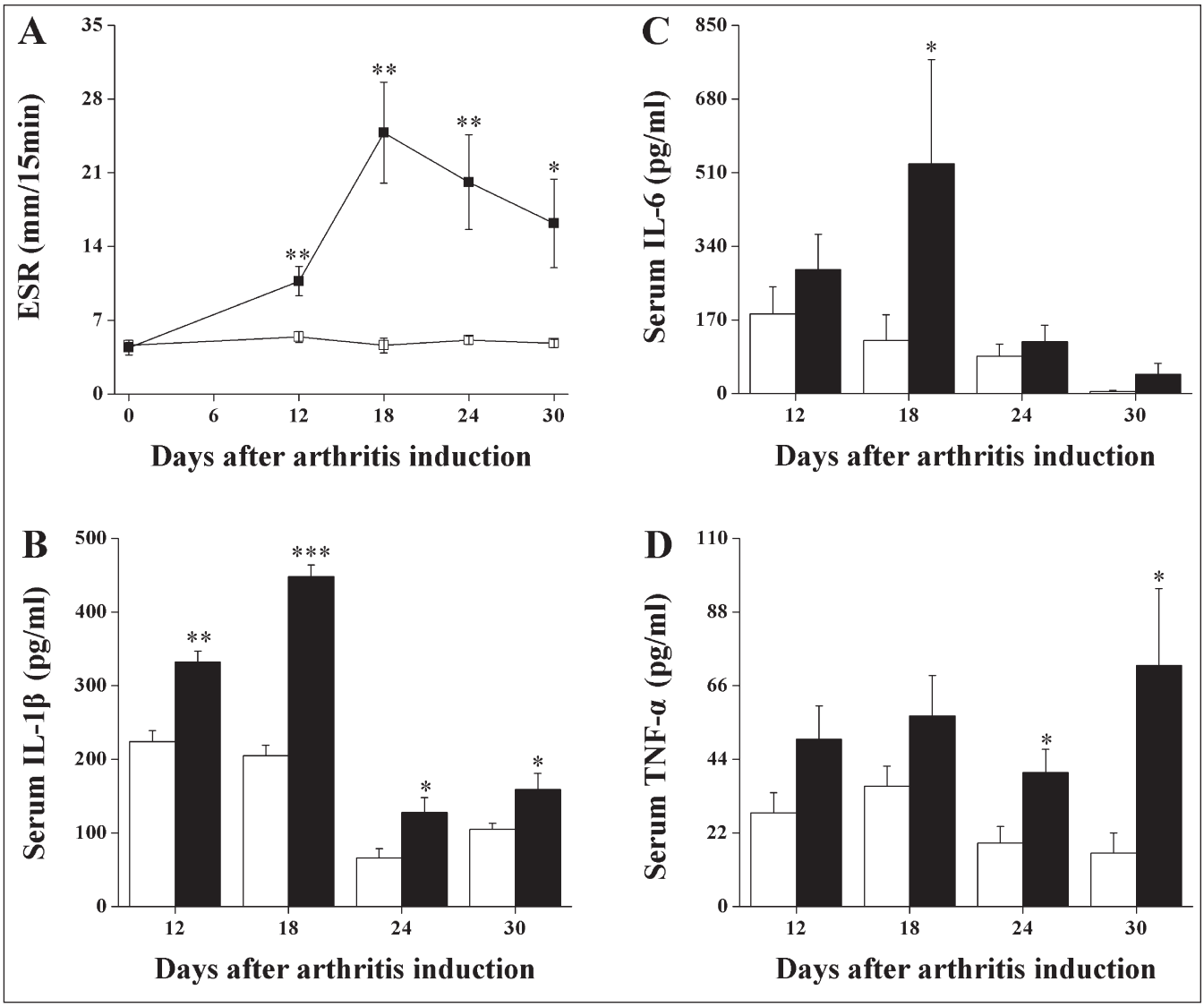

Fig. 5. ESR (A) and serum levels of the pro-inflammatory cytokines IL-1β (B), IL-6 (C) and TNF-α (D) in the SD control (□) and AIA rats (■) inoculated with the ground MT suspension containing 62.5 μg MT during a 30-day disease course. Compared to control rats, significantly elevated ESR levels in AIA rats (A) were observed from days 12 to 30 with a peak on day 18. Serum IL-1β levels (B) remained markedly higher in AIA rats from days 12 to 30, with abundant production occurring in the early phase of the disease. The serum concentration of IL-6 (C) markedly peaks on day 18 and thereafter decreased sharply. Serum TNF-α production (D) in AIA rats were significantly increased on days 24 and 30. Data are expressed as mean ±SEM (n = 10). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus control.

and 18 after inoculation were in some degrees increased, but a significant elevation appeared only in the late phase of the disease, i. e., on days 24 and 30 (Fig. 5D).

Radiological examination of the hind paws of AIA and control rats

In connection with the clinical signs of AIA (Fig. 6C) and control rats (Fig. 6A), radiological examination of hind paws revealed severe soft tissue swelling and bone erosion in the arthritic joints of AIA rats (Fig. 6D) as compared to the normal joints of control rats (Fig. 6B), suggesting active arthritis with joint swelling and destruction in AIA animals. Statistically significant differences were also obtained in the radiological score for soft tissue swelling and bone erosion between the AIA and control rats (Fig. 6E).

Histolopathological examination of the ankle joints of AIA and control rats

Histopathological examination of the ankle joints of control rats revealed a clear space between the bones and thin synovial membrane (Fig. 7A), while the AIA rats showed marked joint swelling with cellular infiltration, synovial hyperplasia and joint space narrowing. Severe pannus formation, cartilage destruction, and bone erosion were also observed in AIA rats (Fig. 7B). Further histopathological scoring shown in Figure 7C demonstrate that the AIA rats were found to

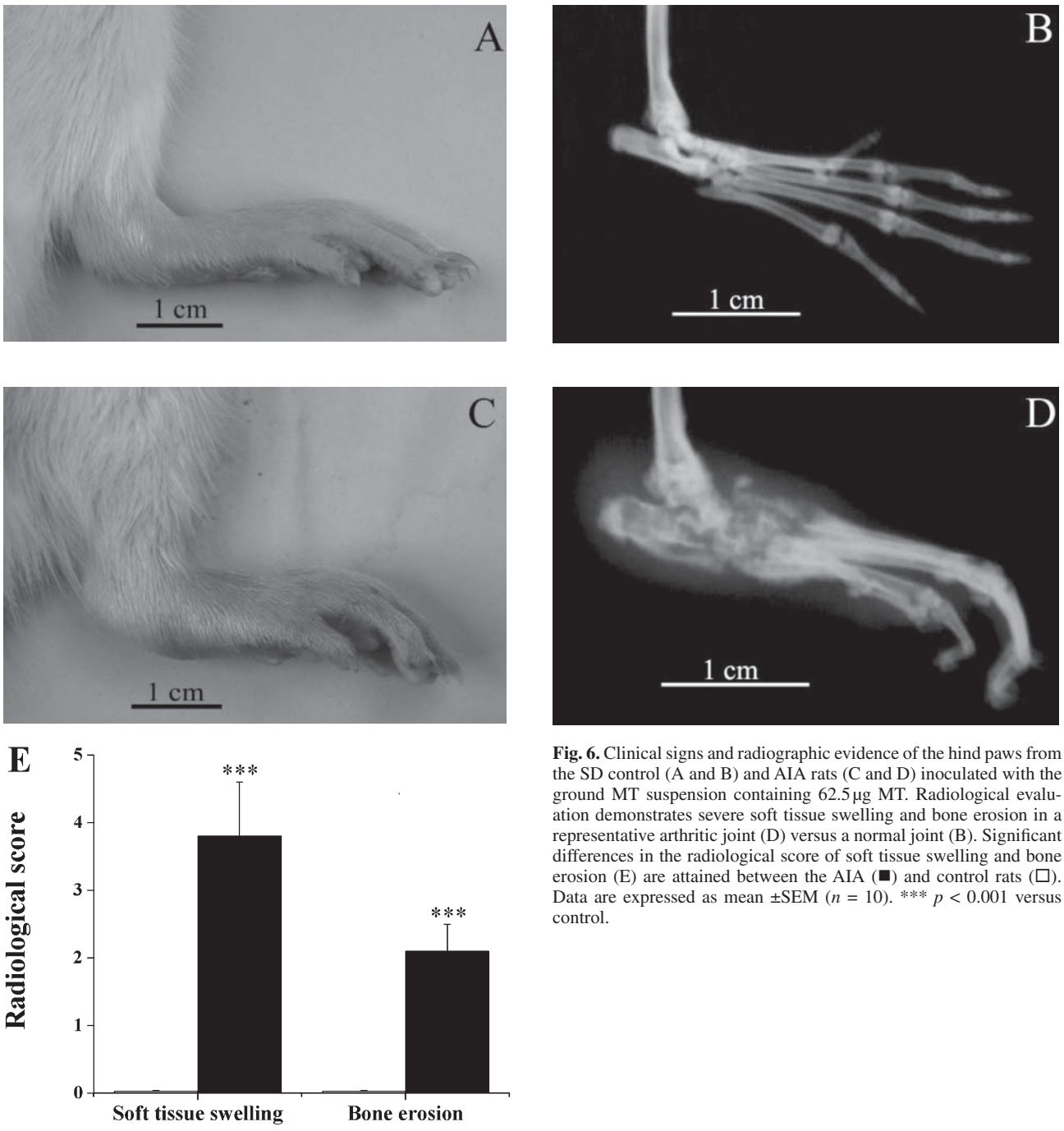

Fig. 6. Clinical signs and radiographic evidence of the hind paws from the SD control (A and B) and AIA rats (C and D) inoculated with the ground MT suspension containing 62.5 µg MT. Radiological evaluation demonstrates severe soft tissue swelling and bone erosion in a representative arthritic joint (D) versus a normal joint (B). Significant differences in the radiological score of soft tissue swelling and bone erosion (E) are attained between the AIA (■) and control rats (□). Data are expressed as mean ±SEM (n = 10). *** $p < 0.001$ versus control.

reveal significant edema, cellular infiltration, angiogenesis, synovial hyperplasia, pannus formation, joint capsular fibrosis and space narrowing, cartilage and bone erosion.

Discussion

AIA is the most frequently-used arthritis model for testing and developing anti-arthritic agents. However, it bears several unfavorable characteristics. In particular, wide variations in the incidence and severity of the arthritic signs, and the narrow number of susceptible rat strains available, restrict its extensive use.

Although heterogenic SD rats are the moderate-responder strain to AIA [7], we demonstrate, in the present study, that following intradermal or subcutaneous inoculation with the ground MT suspension containing 500 µg MT at the base of the tail, the outbred SD rats developed severe and low variable arthritis at a 100 % incidence. The results are readily reproducible. In parallel, using the same induction protocol, the same incidence and highly similar severity of AIA were observed in the inbred Lewis rats (Fig. 1). Rosenthale

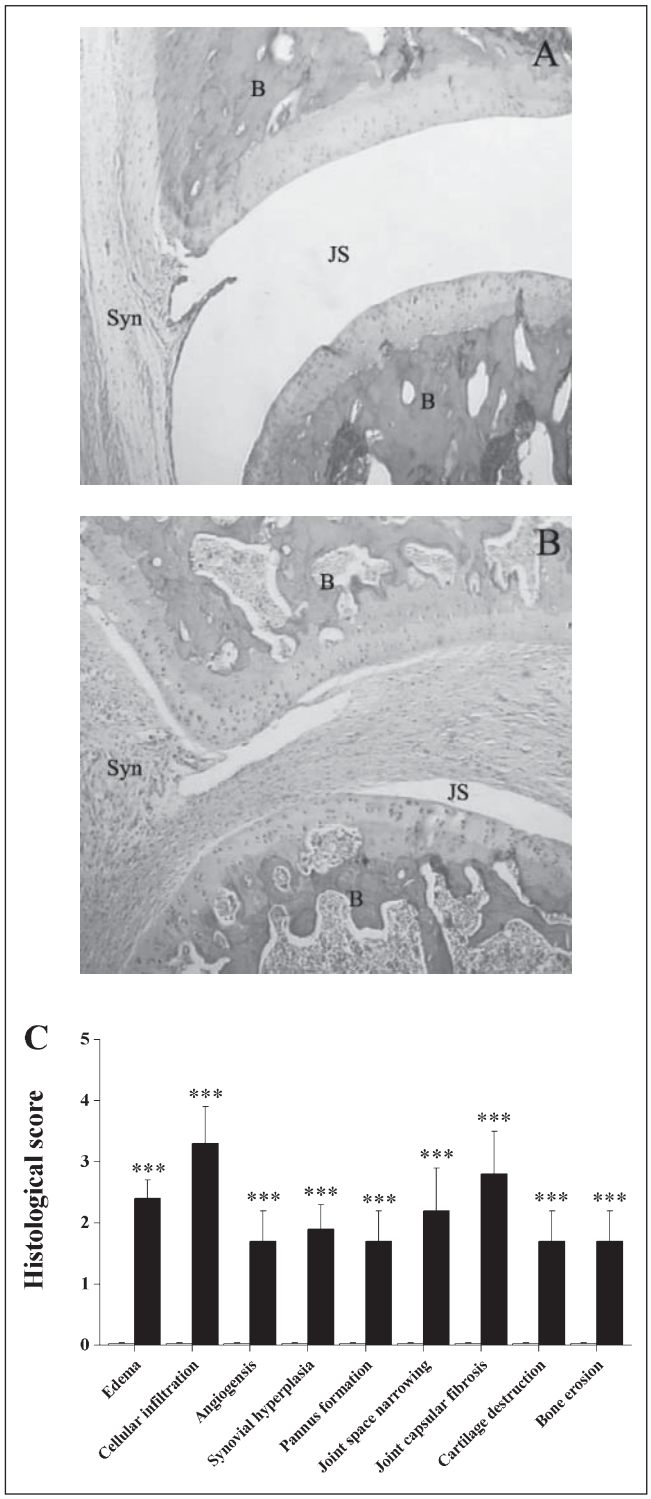

Fig. 7. Histopathological changes of the tibiotarsal joints of the SD control (A) versus AIA rats (B) inoculated with the ground MT suspension containing 62.5 µg MT. H&E stain (×40) of a representative arthritic joint manifests edema, cellular infiltration and angiogenesis. Expansion of the synovial lining (Syn) and pannus formation, narrowing of joint space (JS), and bone (B) destruction are evident in the arthritic joints. Significant differences in the histological score of edema, cellular infiltration, angiogenesis, synovial hyperplasia, pannus formation, joint capsular fibrosis and space narrowing, cartilage destruction and bone erosion (C) are observed between the AIA (■) and control rats (□). Data are expressed as mean ±SEM ($n = 10$). *** $p < 0.001$ versus control.

previously observed SD rats only had an incidence of 60 % affliction with secondary polyarthritic symptoms after a subplantar injection of mycobacteria in mineral oil [8]. Banik's recent reexamination showed unsatisfactory incidence of AIA as low as 38 % in SD rats even when inoculated with a very high dose of *M. butyricum* (1,200 µg/rat) [9]. Our findings seem to contradict Rosenthale's and Banik's observations. However, in Rosenthale's study, the MT suspension with unknown particle number-size distribution was injected into the footpad of SD rats and then secondary polyarthritic symptoms in the non-injected paws were evaluated, but in our study, the MT suspension was injected into the base of the tail of SD rats, which is now the most frequently-used method to induce arthritis. Hence, differences between Rosenthale's study and our findings may largely arise from completely different inoculation pathways, different particle size and doses of MT in the CFA suspension. As for Banik's observations [9], although the inoculation route of AIA described is the same as used in our current study, the strain of mycobacteria (*M. tuberculosis* × *M. butyricum*), the preparative techniques for the CFA suspension and the sources of animals are quite different, which may have contributed to different susceptibility of SD rats to the induction of AIA. In our present studies, following intradermal injection of the ground MT suspension containing 500 µg MT, rats developed rather severe arthritis, while injection with the commercial CFA produced only very mild arthritis. These results indicate that although commercial CFA preparations are convenient to use, it is unsuitable for directly inducing AIA in SD rats. Using the classical method of grinding the bacteria and the optimal number-size distribution in MT suspension in study is a better method [4, 12].

In general, rats are inoculated at the base of the tail with CFA, most frequently through intradermally rather than subcutaneously. Alternatively, animals can be injected in the footpad as done in Rosenthale's report [8], but the injected paws may be easily infected and then develops serious ulceration, finally giving rise to the failure of the experiment. Comparing the two inoculation routes, i. e., intradermal and subcutaneous routes, in the induction of AIA, the results showed the same incidence and no significantly different severity of AIA. These observations imply that the inoculation routes may play a relatively minor role in the induction and severity of AIA. In this case, use of the subcutaneous route, which is far more convenient and consistently performed, is justified.

The epidemiology of human RA reveals that frequency is two or three times higher in women than in men [17]. In the present study, male SD rats were found to develop markedly more severe arthritis than female rats (Fig. 2). This result confirms some previous reports [5, 6], but contradicts Swingle et al. [7], who found no sex differences in SD, Holtzman, Charles River and Buffalo strains of rats. It is well-known that hormones, particularly those involved in the hypothalamic-pituitary-gonadal and -adrenal axes (HPG and HPA), play important roles in human RA and the related animal models such as AIA and collagen-induced arthritis (CIA). The HPA axis is intimately linked to the HPG axis and is sexually dimorphic. It was reported that estrogens stimulate higher corticosteroid responses in female rats [18]. Studies in high-responder Lewis and low-responder F344 rats to

streptococcal cell wall induced arthritis (SCWA) found that Lewis rats has markedly impaired plasma corticotropin and corticosterone responses to SCW [19]. Furthermore, Joe et al. reveal that the severity of AIA is regulated by both major histocompatibility complex (MHC) and non-MHC quantitative trait loci (QTLs) of which one QTL significantly down-regulates the disease severity of AIA in female rats, but not male rats [20]. Hence, the relative sex preferences in the present model may arise from the hormonal and genetic regulations.

In the present study, the dose of MT in the suspension was demonstrated as having a critical role in the induction of AIA, as it markedly affected the incidence and severity of AIA in a dose-dependent manner (Fig. 3). All animals inoculated with 500, 250 and 125 µg MT began to develop arthritis within 9 to 12 days after inoculation, while the arthritic signs appeared in all animals inoculated with 62.5 µg MT until days 9 to 15. When the dose of MT was reduced to 31.25 µg, it could not induce macroscopically visible arthritis.

As reported previously [12], the particle size of MT suspended in mineral oil had a significant influence on the severity of AIA in Wistar rats. In the present studies, three different preparative techniques, namely grinding, homogenizing and mixing, produced distinctly different particle size-number distribution of MT in the suspension, and these MT suspensions were then found to have markedly different arthritogenic abilities. The results showed that the ground, homogenized and mixed MT preparations contained more than 80, 70 and 60 % of particles that were less than 10 µm in diameter, and induced relatively severe, moderate and slight arthritis, respectively (Table 1 and Fig. 4). The clinical arthritic score and hind paw swelling were markedly different between these groups of animals (Fig. 4). The mixing technique was not an advisable alternative since the mixed suspension with 500 µg MT produced only slight arthritis at a low incidence of 50 % (Fig. 4). AIA has been generally believed to be the result of a delayed-type hypersensitivity (DTH) response to a disseminated antigen probably derived from the injected bacterial cell wall [21]. T cell specific for the mycobacterial 65 kDa heat-shock protein (hsp65) plays a pivotal role in the development of AIA. It is believed that AIA in rats is induced by a T cell clone specific for an epitope formed by amino acids 180–188 of the mycobacterial hsp65 [22, 23]. In addition, the smallest peptide subunit of mycobacterial peptidoglycan required for arthritogenicity of mycobacteria is molecularly defined as N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) [24]. Obviously, the fractions of mycobacteria responsible for the production of arthritis may be chemically arthritogenic in nature. Using dyed adjuvant, Newbould showed that the adjuvant must enter the lymphatic system to produce secondary inflammatory lesions [25]. Therefore, the smaller particle size of mycobacteria with the larger surface area makes the MT suspension more stable and possible for the systematic dissemination through lymphatics, preventing rapid structural destruction and assuring persistence of the antigen in lymph nodes.

Szekanecz et al. [26] investigated the correlation between clinical signs and the laboratory analysis along with time-dependent expression of pro-inflammatory cytokines in female Lewis rats, showing that the production of TNF-α, IL-1β and IL-6 was abundant during the course of AIA induced by subcutaneous injection of 1,500 µg *M. butyricum* suspended in mineral oil. However, Lussier et al. [27] showed that in Lewis rats, 500 µg of *M. butyricum* could effectively produce the maximal intensity of lesions. Those results indicate that the high doses of *M. butyricum* described may certainly induce a rather severe AIA that resembles the level of disease induced by 500 µg MT in our study. Thus, we decided to further characterize various features of the AIA model in SD rats using a relative low dose of MT rather than using a high dose, i. e., subcutaneous inoculation with 62.5 µg MT. In this case, 90 % incidence and moderate arthritic signs were successfully obtained (data available upon request). Even in this moderate AIA model, the ESR levels are in line with the changes of clinical arthritic signs. It peaked on day 18 and then fell sharply, indicating a relatively early but lasting stimulation of acute-phase protein production during disease progression (Fig. 5A). Furthermore, histopathological analysis of the synovial joints revealed evident edema with cellular infiltration, synovial hyperplasia, pannus formation, and joint space narrowing (Fig. 7). Also, both histological and radiological examinations demonstrated that AIA rats manifested presence of cartilage destruction and bone erosion – the ultimate hallmark of human RA (Figs. 6 and 7). These histopathological and radiological features observed in the current AIA model closely resemble the arthritic features of the AIA induced in Lewis rats [14, 28].

Recent studies have revealed the key roles of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, in the pathogenesis of RA [29]. IL-1β, IL-6 and TNF-α are elevated in synovial fluid of RA patients [30]. It has been suggested that those inflammatory cytokines are produced through continuous activation of T cells and interaction of the activated T cells and monocytes/macrophages in RA [31]. Moreover, anti-TNF, IL-1 and IL-6 therapies have been reported to be effective in the treatment of RA [32]. In this AIA model of SD rats, the levels of IL-1β, IL-6 and TNF-α are dramatically elevated in relation to the disease progression as compared to control rats (Fig. 5). Serum IL-1β levels in AIA rats remained significantly higher throughout the course of the disease, but the most abundant expression of IL-1β was detected only in the earlier phase (Fig. 5B). IL-6 levels in the serum markedly peaked on day 18 and thereafter showed a progressive decrease (Fig. 5C), while TNF-α levels were significantly elevated in the relatively later phase of AIA (Fig. 5D). In the study performed by Szekanecz et al., expression of IL-1β in the AIA Lewis rats was found to be similar to TNF-α, while the serum IL-6 levels were increased throughout the time course with abundant production occurring in the late phase of AIA [26]. Actually, these divergent cytokines profiles have been described previously as being due to different animal strains involved and different experimental protocols [33, 34].

In conclusion, An AIA model in SD male rats has been successfully developed with optimal grinding manipulation techniques and 500 µg of MT in the CFA suspension, showing 100 % incidence, low variability in clinical signs, and high similarity to the arthritic features of the Lewis rat AIA model as well as human RA. Results indicate that the induction of AIA in SD rats depends not only on the genetic susceptibility of animals but also on the manipulation techniques of investigators. Moreover, we recently used this well-developed SD

rat AIA model to test a novel anti-arthritic botanical drug in which indomethacin served as positive control. The results showed that it was susceptible to such therapeutic intervention at the clinical, serological, histological, and radiological levels [15]. Hence, this AIA model in SD rats is an efficient and cost-effective arthritis model available for studying the pathophysiology of arthritis in humans as well as for screening and testing novel anti-arthritic agents.

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References

[1] Pearson CM. Development of arthritis, periarthritis and periostitis in rats given adjuvant. Proc Soc Exp Biol Med 1956; 91: 95–101.
[2] Pearson CM. Experimental joint disease observations on adjuvant-induced arthritis. J Chronic Dis 1963; 16: 863–74.
[3] Billingham ME. Models of arthritis and the search for anti-arthritic drugs. Pharmacol Ther 1983; 21: 389–428.
[4] van Eden W, Wagenaar-Hilbers JPA, Wauben MHM. Animal models for autoimmune and inflammatory disease: adjuvant arthritis in the rat. In: Colagin JE, editor. Current Protocols in Immunology. New York: John Wiley, 1994: Unit 15.4: 1–11.
[5] Glenn EM, Gray J. Adjuvant-induced polyarthritis in rats: biologic and histologic background. Am J Vet Res 1965; 26: 1180–94.
[6] Griffiths MM. Immunogenetics of collagen-induced arthritis in rats. Int Rev Immunol 1988; 4: 1–15.
[7] Swingle KF, Jaques LW, Kvam DC. Differences in the severity of adjuvant arthritis in four strains of rats. Proc Soc Exp Biol Med 1969; 132: 608–12.
[8] Rosenthale ME. A comparative study of the Lewis and Sprague Dawley rat in adjuvant arthritis. Arch Int Pharmacodyn Ther 1970; 188: 14–22.
[9] Banik RK, Kasai M, Mizumura K. Reexamination of the difference in susceptibility to adjuvant-induced arthritis among LEW/Crj, Slc/Wistar/ST and Slc/SD rats. Exp Anim 2002; 51: 197–201.
[10] Battisto JR, Smith RN, Beckman K, Sternlicht M, Welles WL. Susceptibility to adjuvant arthritis in DA and F344 rats: a dominant trait controlled by an autosomal gene locus linked to the major histocompatibility complex. Arthritis Rheum 1982; 25: 1194–200.
[11] Kohashi O, Kuwata J, Umehara K, Uemura F, Takahasi T, Ozawa A. Susceptibility to adjuvant-induced arthritis among germ-free, specific pathogen-free, and conventional rats. Infect Immun 1979; 26: 791–4.
[12] Best R, Christian R, Lewis DA. Effect of particle size of dried mycobacteria on adjuvant induced arthritis in the rat. Agents Actions 1984; 14: 265–8.
[13] International Council for Standardization in Haematology (Expert Panel on Blood Rheology). ICSH recommendations for measurement of erythrocyte sedimentation rate. J Clin Pathol 1993; 46: 198–203.
[14] Esser RE, Hildebrand AR, Angelo RA, Watts LM, Murphey MD, Baugh LE. Measurement of radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. Arthritis Rheum 1995; 38: 129–38.
[15] Cai X, Zhou H, Wong YF, Xie Y, Liu ZQ, Jiang ZH et al. Suppressive effects of QFGJS, a preparation from an anti-arthritic herbal formula, on rat experimental adjuvant-induced arthritis. Biochem Biophys Res Commun 2005; 337: 586–94.
[16] McCartney-Francis NL, Chan J, Wahl SM. Inflammatory joint disease: clinical, histological, and molecular parameters of acute and chronic inflammation and tissue destruction. In: Winyard PG, Willoughby DA, editors. Inflammation Protocols. New Jersey: Humana Press, 2003: 147–59.
[17] Gabriel SE. The epidemiology of rheumatoid arthritis. Rheum Dis Clin North Am 2001; 27: 269–81.
[18] Wilder RL. Hormones and autoimmunity: animal models of arthritis. Baillieres Clin Rheumatol 1996; 10: 259–71.
[19] Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW et al. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. Proc Natl Acad Sci USA 1989; 86: 2374–8.
[20] Joe B, Cannon GW, Griffiths MM, Dobbins DE, Gulko PS, Wilder RL et al. Evaluation of quantitative trait loci regulating severity of mycobacterial adjuvant-induced arthritis in monocogenic and polycongenic rats: identification of a new regulatory locus on rat chromosome 10 and evidence of overlap with rheumatoid arthritis susceptibility loci. Arthritis Rheum 2002; 46: 1075–85.
[21] Waksman BH, Pearson CM, Sharp JT. Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. J Immunol 1960; 85: 403–17.
[22] van Noort JM, Anderton SM, Wagenaar JP, Wauben MH, van Holten C, Boog CJ. Differential rat T cell recognition of cathepsin D-released fragments of mycobacterial 65 kDa heat-shock protein after immunization with either the recombinant protein or whole mycobacteria. Int Immunol 1994; 6: 603–9.
[23] Gaston JS, Life PF, Jenner PJ, Colston MJ, Bacon PA. Recognition of a mycobacteria-specific epitope in the 65-kD heat-shock protein by synovial fluid-derived T cell clones. J Exp Med 1990; 171: 831–41.
[24] Chang YH, Pearson CM, Chedid L. Adjuvant polyarthritis V: Induction by N-acetylmuramyl-L-alanyl-D-isoglutamine, the smallest peptide subunit of bacterial peptidoglycan. J Exp Med 1981; 153: 1021–6.
[25] Newbould BB. Role of lymph nodes in adjuvant-induced arthritis in rats. Ann Rheum Dis 1964; 23: 392–6.
[26] Szekanecz Z, Halloran MM, Volin MV, Woods JM, Strieter RM, Haines GK et al. Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. Arthritis Rheum 2000; 43: 1266–77.
[27] Lussier A, De Médicis R, Tétreault L. Adjuvant arthritis: influence of the adjuvant volume and composition on the established arthritis. Int J Tissue React 1984; 6: 105–10.
[28] Stanescu R, Lider O, van Eden W, Holoshitz J, Cohen IR. Histopathology of arthritis induced in rats by active immunization or by systemic transfer of T lymphocyte lines: a light and electron microscopic study of the articular surface using cationized ferritin. Arthritis Rheum 1987; 30: 779–92.
[29] Feldmann M, Brennan FM, Maini RN. Role of cykokines in rheumatoid arthritis. Annu Rev Immunol 1996; 14: 397–440.
[30] Szekanecz Z, Koch AE, Kunkel SL, Strieter RM. Cytokines in rheumatoid arthritis: potential targets for pharmacological intervention. Drugs Aging 1998; 12: 377–90.
[31] Panayi GS, Lanchbury JS, Kingsley GH. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. Arthritis Rheum 1992; 35: 729–35.
[32] Maini RN, Taylor PC. Anti-cytokine therapy for rheumatoid arthritis. Annu Rev Med 2000; 51: 207–29.
[33] Philippe L, Gegout-Pottie P, Guingamp C, Bordji K, Terlain B, Netter P et al. Relations between functional, inflammatory, and degenerative parameters during adjuvant arthritis in rats. Am J Physiol 1997; 273: R1550–6.
[34] Silva JC, Rocha MF, Lima AA, Brito GA, de Menezes DB, Rao VS. Effect of pentoxifylline and nabumetone on the serum levels of IL-1β and TNF in rats with adjuvant arthritis. Inflamm Res 2000; 49: 14–9.