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ORIGINAL ARTICLE

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The comparative study of Sprague–Dawley and Lewis rats in adjuvant-induced arthritis

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Abstract The outbred Sprague–Dawley (SD) rats, similar to the inbred Lewis (LEW) rats, have been recently demonstrated to be highly susceptible to adjuvant-induced arthritis (AIA). We herein compared AIA in SD and LEW rats in terms of clinical, histological, radiological, and immuno-inflammatory features. The results showed that, following inoculation with a ground *Mycobacterium tuberculosis* (MT) suspension, SD and LEW rats manifested closely similar disease progression, with 100% incidence and similar severity. The development of arthritis was accompanied by significantly higher erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels than in control rats. Radiographic examination of the hind paws showed that both SD and LEW AIA rats manifested conspicuous soft tissue swelling, bone matrix resorption, periosteal new bone formation and bone erosion, while histopathological analysis of the synovial joints revealed marked cellular infiltration, angiogenesis, synovial hyperplasia, pannus formation, narrowing of joint space, and cartilage and bone destruction. Moreover, in relation to disease progression, serum tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 levels were markedly overexpressed in both SD and LEW AIA versus control rats, and SD and LEW AIA rats exhibited divergent profiles for the expression of TNF-α and IL-1β. Taken together, these results demonstrated that the SD rat AIA model shares several arthritic features with the comparable model in LEW rats. Hence, given the more favorable characteristics of SD rats than LEW rats (i.e., lower cost, wider availability, and heterogenic background), this SD rat AIA model is more cost effective and advantageous for screening and testing novel anti-arthritic agents.

Keywords Adjuvant-induced arthritis · Sprague–Dawley rats · Lewis rats · *Mycobacterium tuberculosis* · Pro-inflammatory cytokines

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Introduction

Adjuvant-induced arthritis (AIA) was primarily established by Pearson (1956) when a Freund-type water-in-oil emulsion was used to inoculate different strains of rats. Since then, AIA has been used extensively as an experimental model for studying immuno-inflammatory processes of arthritic diseases in humans, in particular rheumatoid arthritis (RA), as well as for screening and testing novel anti-arthritic agents (Billingham 1983; Pearson 1963). However, AIA has 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 and convenient use.

The classical method of inducing AIA is to inject animals at the base of the tail with ground, heat-killed *Mycobacterium tuberculosis* (MT) H37Ra suspended in incomplete Freund's adjuvant (IFA), commonly known as complete Freund's adjuvant (CFA) (van Eden et al. 1994). Lewis (LEW) rats are currently the most frequently used strain of rats for induction of AIA. Rosenthale (1970) previously reported that, after a sub-plantar injection of mycobacteria in mineral oil, the inbred LEW rats developed much more severe and less variable AIA, at an incidence of 92% affliction with secondary polyarthritic signs, than the outbred Sprague–Dawley (SD) rats, which showed only a 60% incidence. The recent re-examination by Banik et al. (2002) showed unsatisfactory incidence of AIA as low as 38% in SD rats when inoculated with *M. butyricum* suspended in mineral oil, while the arthritic

signs appeared earlier, more severely and more consistently in all immunized LEW rats. In our recent studies, the roles of various variables in the induction of AIA in SD rats were investigated, and the results showed that the particle size and dose of MT in the CFA suspension played a dominant role in the induction and severity of AIA. A gender preference in rats for AIA was also revealed, with male SD rats developing markedly more severe arthritic signs than female rats. Following subcutaneous inoculation with the ground MT suspension (CFA), containing 500 μg MT, at the base of the tail, male SD rats developed severe and low variable arthritis at a 100% incidence. The results were readily reproducible. In parallel, when the same induction protocol was used, the same incidence and highly similar severity of AIA were observed in LEW rats. Further analysis suggests that differences between Rosenthale's and Banik's observations and our findings might largely arise from different experimental protocols for the induction of AIA (data were submitted for publication elsewhere).

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 than, in particular, the inbred LEW rats; in addition, SD rats are outbred with heterogenic background, i.e., genetically more like humans, such that the AIA model of SD rats possibly better mimics the genetic features of human RA than other experimental models of autoimmune arthritis. Hence, an SD rat AIA model would offer marked advantages for study. The current work, therefore, aimed to intensively and comprehensively analyze the features of the AIA model in the outbred SD and inbred LEW rats using examination of clinical, serological, radiological, and histological parameters, and thereby to provide scientific evidence as to whether this AIA model in SD rats can be reliably used for studies of the pathophysiology of arthritis in humans as well as of novel anti-arthritic drugs for humans.

Materials and methods

Preparation of mycobacteria suspension A ground MT suspension (CFA, 5 mg/ml) was freshly prepared according to the method described by van Eden et al. (1994). Briefly, the heat-killed MT H37Ra (Difco, Detroit, Mich., USA) was put into a roughened mortar and ground intensively until its color changed from gray to white, then mineral oil (Sigma, St. Louis, Mo., USA) was added gradually and grinding was continued until a paste was formed. The grinding time was normally not less than 5 min.

Induction of AIA Specific pathogen-free outbred male SD and inbred male LEW rats (Laboratory Animal Unit, the University of Hong Kong, Hong Kong, China), aged 6–7 weeks, were used. Rats were housed four per cage in rooms maintained at 20±0.5°C with alternating 12-h light–dark cycles. Food and water were provided ad libitum throughout the experiments. Rats were acclimated to their surroundings over 1 week to eliminate the effect of stress prior to initiation of the experiments.

The AIA model was induced on day 0 by a single subcutaneous injection of 0.1 ml of the ground MT suspension (CFA), containing 500 μg MT, at the base of the tail of the rats. Control rats were injected with saline solution. All 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 the USA and the Department of Health of the Hong Kong Special Administrative Region.

Clinical evaluation of the development of AIA Rats were inspected daily for the onset of arthritis characterized by edema and erythema in the paws. Disease progression and severity were evaluated by arthritic scoring and measurements of bi-hind paw volumes and body weight on days 0, 9, 12, 15, 18, 21, 24, 27, and 30 after CFA injection. Lesions on all 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 edema and erythema of the periarticular tissues; 16 was the potential maximum of the combined arthritic scores per animal (van Eden et al. 1994). The hind paw volumes were measured with a plethysmometer chamber (7140 UGO. Basile, Comerio, Italy) and expressed as the mean volume of both hind paws of the rats. The body weight of the rats was monitored with a 0.1 g precision balance (Sartorius AG, Goettingen, Germany).

Determination of erythrocyte sedimentation rate, C-reactive protein and pro-inflammatory cytokine levels Blood samples were collected from the arteries of the tails for laboratory tests on days 0, 6, 12, 18, 24, and 30 after CFA injection. Erythrocyte sedimentation rate (ESR) was determined by a modified method based on International Council for Standardization in Haematology (ICSH) selected methods (Bull et al. 1993). 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 C-reactive protein (CRP) and the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 in blood serum were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits for CRP (Helica Biosystems, Fullerton, Calif., USA), TNF-α and IL-6 (BD Biosciences, San Diego, Calif., USA), and IL-1β (Pierce Biotechnology, Rockford, Ill., USA), according to the manufacturers' recommendations.

Radiological and histopathological studies At the end of the experiments the rats were killed 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 were evaluated for soft tissue swelling, bone matrix resorption, periosteal new bone formation and bone erosion, and were scored in a blind fashion by two independent observers on a scale of 0 (normal), 1 (mild changes), 2 (moderate changes), and 3 (severe changes) (Esser et al. 1995). Total radiological scores were calculated from the sum of both hind paws, with a maximum possible score of 6 for each radiological parameter per rat.

After the X-ray check, the hind paws were fixed in 10% phosphate-buffered saline (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), angiogenesis, synovial hyperplasia, pannus formation, narrowing of joint space, cartilage destruction, and bone erosion of the ankle joints were examined in a blind fashion by two independent observers using a semiquantitative scale from 0 (normal), 1 (mild changes), 2 (moderate changes), and 3 (severe changes) (Cai et al. 2005; McCartney-Francis et al. 2003). Histological scores were combined and expressed as the sum of both tibiotarsal joints, with 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 differences between groups. Statistical significance was accepted for $P<0.05$ (two-tailed).

Results

Clinical progression of AIA in SD and LEW rats

Following inoculation with the ground MT suspension (CFA) containing 500 μg MT at the base of the tail, the inflammatory and arthritic lesions characterized by edema and erythema in the paws appeared in all LEW rats around days 7 to 9 and in all SD rats within 9–12 days. From day 9 onwards both strains of rats experienced closely similar disease progression of arthritis, showing very significant increase in arthritic score (Fig. 1a) and hind paw volume (Fig. 1b) as well as marked loss of body weight (Fig. 1c) as compared to control rats. At the end of the experiment, both SD (Fig. 2c) and LEW (Fig. 2d) AIA rats displayed severe soft tissue swelling and ankylosis in the paws in comparison with SD (Fig. 2a) and LEW (Fig. 2b) control rats.

Moreover, the development of arthritis was accompanied by significantly high elevation of ESR and CRP levels in both SD and LEW AIA versus control rats. As shown in Fig. 3a and b, the levels of ESR and CRP peaked on day 12 and, thereafter, showed progressive decrease.

Radiological analysis of AIA in SD and LEW rats

As seen in the representative radiographs taken on day 30 of the disease, when the inflammatory process characterized by edema and erythema were almost inactive, both SD (Fig. 4c) and LEW (Fig. 4d) AIA rats manifested severe soft tissue swelling, pronounced decrease in bone density, marked destruction of bones, and abnormal ossifications in the tarsal, metatarsal, and interphalangeal regions as compared to SD (Fig. 4a) and LEW (Fig. 4b) control rats. Statistically significant differences in the radiological scores, assessing soft tissue swelling, bone matrix resorption, periosteal new bone formation, and bone erosion between the AIA and control rats, are depicted in Fig. 4e.

Histopathological analysis of AIA in SD and LEW rats

Representative H&E-stained sections of the ankle joints from SD and LEW control and AIA rats are shown in Fig. 5. Histological examination of the ankle joints of control rats revealed a clear space between the bones and thin synovial membrane (Fig. 5a and b). In contrast, both SD and LEW AIA rats manifested the presence of predominant cellular infiltration, angiogenesis, intense synovial proliferation, and massive pannus formation. Dramatic loss of joint space and severe cartilage and

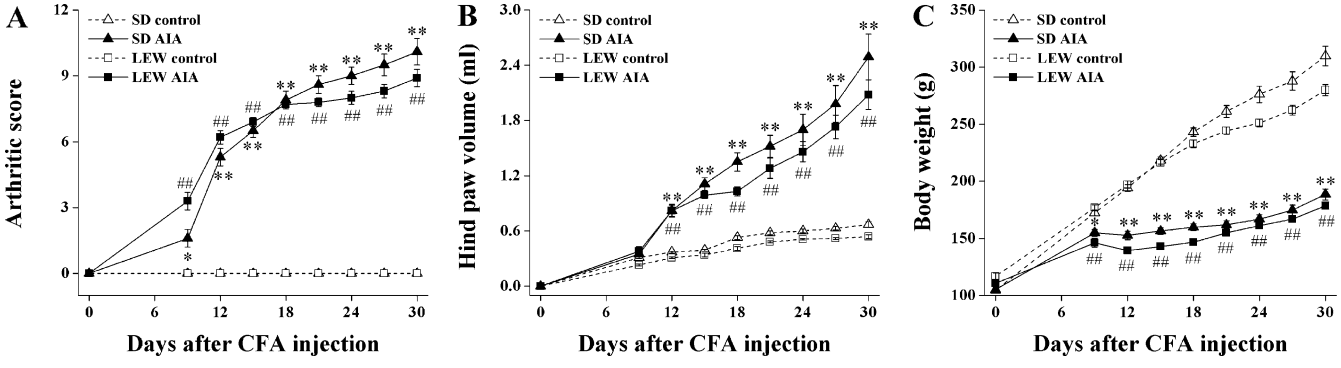

Fig. 1 Clinical progression of AIA in SD and LEW rats during a 30-day course. Data are expressed as mean ± SEM (n=7–8). **$P<0.01$ versus SD control rats; ##$P<0.01$ versus LEW control rats

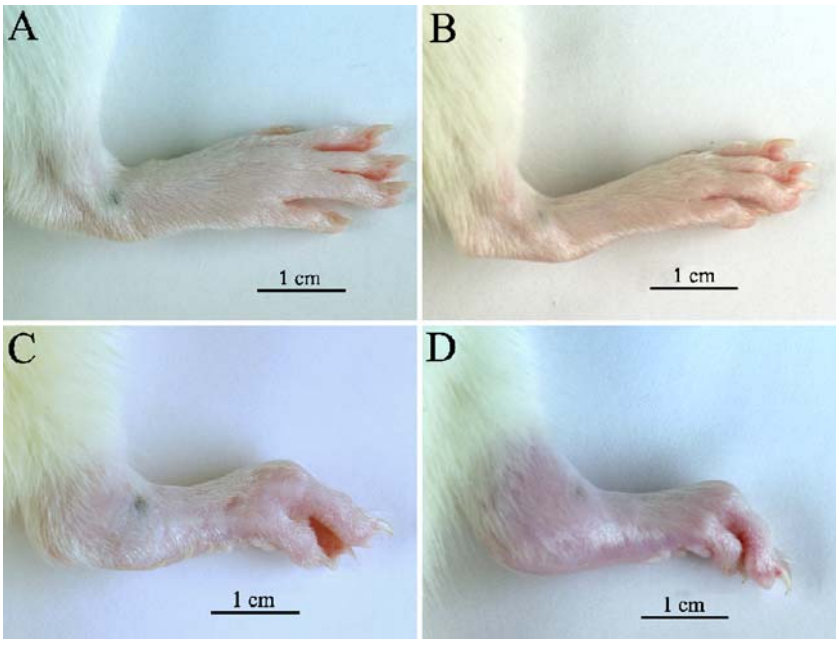

Fig. 2 Clinical appearance of the hind paws of SD and LEW AIA rats. Shown are representative photographs of the hind paws from SD (**a**) and LEW (**b**) control rats, and SD (**c**) and LEW (**d**) AIA rats, taken on day 30 after the immunization with CFA. Note diffuse soft tissue swelling and complete ankylosis in the hind paws of both SD and LEW AIA rats compared to control rats

bone erosion were also noted in the synovial joints of the arthritic rats (Fig. 5c and d). Further histopathological scoring shown in Fig. 5e demonstrates that both SD and LEW AIA rats showed very significant cellular infiltration, angiogenesis, synovial hyperplasia, pannus formation, narrowing of joint space, and cartilage destruction and bone erosion as compared to SD and LEW control rats.

Expression profiles of serum cytokines levels of AIA in SD and LEW rats

A time–course expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in blood serum of SD and LEW AIA versus control rats was investigated. The results showed that, in relation to disease progression, TNF-α, IL-1β, and IL-6 in the serum of both SD and LEW AIA rats were markedly overproduced as compared to control rats from days 12 to 30 after the inoculation of MT, and similar kinetics of serum TNF-α, IL-1β, and IL-6 levels were observed in both strains of rats throughout the time course of the experiment (Fig. 6).

In detail, the abundant production of TNF-α in the serum of SD AIA rats appeared more evident than in that of LEW AIA rats. Statistical difference was attained only for serum TNF-α levels of SD AIA versus control rats from days 12 to 30, with a peak on day 18 after CFA injection (Fig. 6a). In contrast, serum IL-1β levels were more prominently detected in LEW AIA rats than in SD AIA rats. Compared with control rats, LEW AIA rats revealed significant elevation of IL-1β levels in the serum on days 12, 18 and 30, as did SD AIA rats on days 12, 18, 24 and 30 after CFA injection (Fig. 6b). Moreover, highly increased serum IL-6 levels in SD and LEW AIA rats were observed during disease progression of AIA, especially in the late phase of the disease. Serum IL-6 levels remained markedly higher in arthritic versus control rats from days 12 to 30 after CFA injection (Fig. 6c).

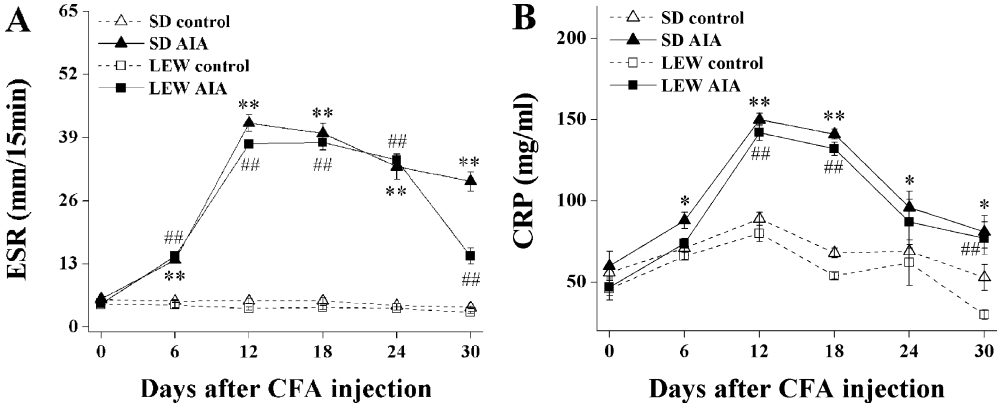

Fig. 3 Kinetics of ESR (**a**) and serum CRP levels (**b**) of SD and LEW AIA versus control rats during a 30-day course. Data are expressed as mean ± SEM (n=7–8). *P<0.05, **P<0.01 versus SD control rats; ##P< 0.01 versus LEW control rats

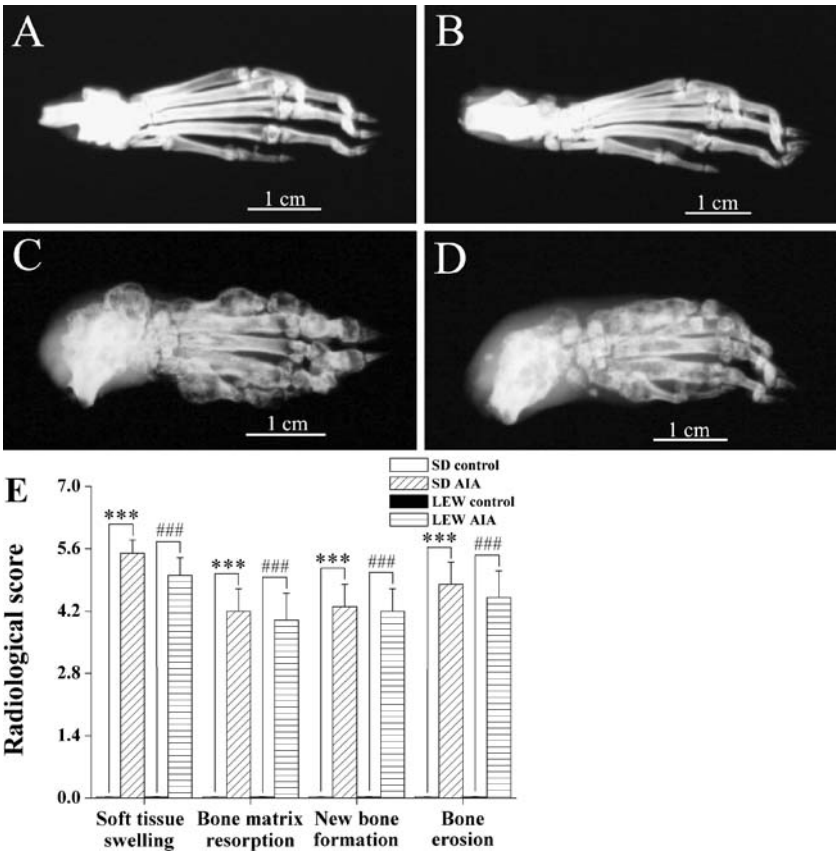

Fig. 4 Radiographic evidence of the hind paws of SD and LEW AIA rats. Shown are representative radiographs of the hind paws from SD (**a**) and LEW (**b**) control rats, and SD (**c**) and LEW (**d**) AIA rats, taken on day 30 after the immunization with CFA. Note severe soft tissue swelling, bone matrix resorption, periosteal new bone formation, and bone erosion in the tarsal, metatarsal, and interphalangeal regions of both SD and LEW AIA rats compared to control rats. Radiological analysis demonstrates very significant differences in these radiological parameters between the AIA and control rats (**e**). Data are expressed as mean ± SEM (n=7–8). ***P<0.001 versus SD control rats; ###P< 0.001 versus LEW control rats

Discussion

AIA is a rather aggressive and monophasic form of arthritis; usually, the disease is quite severe and finally leads to complete ankylosis and permanent joint malformations. Therefore, AIA is most frequently used as a model for screening and testing anti-arthritic agents, especially non-steroidal anti-inflammatory drugs (NSAIDs), as the inflammation associated with AIA is very dependent on prostaglandin E_2 (PGE_2) generated by cyclooxygenases (COXs) (Anderson et al. 1996; Billingham 1983). Compared with other experimental models of autoimmune arthritis, AIA is unique in that no autoantigens are particularly well-defined, although proteoglycans are likely candidates (van Eden et al. 1985; van Vollenhoven et al. 1988). In this respect, AIA can be used for studying basic mechanisms of how external triggering may lead to undesired self-recognition.

In the present study, following a single injection of the ground MT suspension (CFA) containing 500 μg MT at the base of the tail, both SD and LEW rats developed pronounced arthritis in the paws, showing 100% incidence and low variability in clinical signs (Fig. 1). At the end of the experiment, both SD and LEW AIA rats were characterized by diffuse soft tissue swelling with complete ankylosis in the paws (Fig. 2). Although the inflammatory and arthritic lesions occurred earlier in LEW rats (days 7–9) than in SD rats (days 9–12), the severity of disease in SD rats appeared to increase more sharply than in LEW rats from day 15 onwards (Fig. 1). 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 (Waksman et al. 1960). Using dyed adjuvant, Newbould (1964) showed that the adjuvant must enter the lymphatic system to produce secondary inflammatory lesions. Therefore, the current observation would imply that the systematic dissemination of the injected mycobacteria through lymphatics in LEW rats might occur more quickly than in SD rats, but the ability of both strains of rats to respond immunologically to the disseminated antigen could be similar.

ESR, the rate at which erythrocytes settle out of unclotted blood in a certain time period, is influenced by an increase in the plasma concentration of acute-phase reactant proteins (e.g., fibrinogen, α- and β-globulin), and, therefore, provides a measure of the acute phase response to inflammatory diseases (Bull et al. 1993; Wolfe and Michaud 1994). CRP is an acute-phase protein that has no direct effect on the ESR in physiological concentrations and has been identified as an important biomarker for various inflammatory, degenerative, and neoplastic diseases (Pepys and Hirschfield 2003; van Leeuwen and van Rijswijk 1994). Elevated levels of ESR and CRP have been found in the blood during virtually all diseases associated with active inflammation or tissue destruction, particularly in patients with RA (Kushner 1991; van Leeuwen and van Rijswijk 1994; Wolfe and Michaud 1994). Therefore, ESR

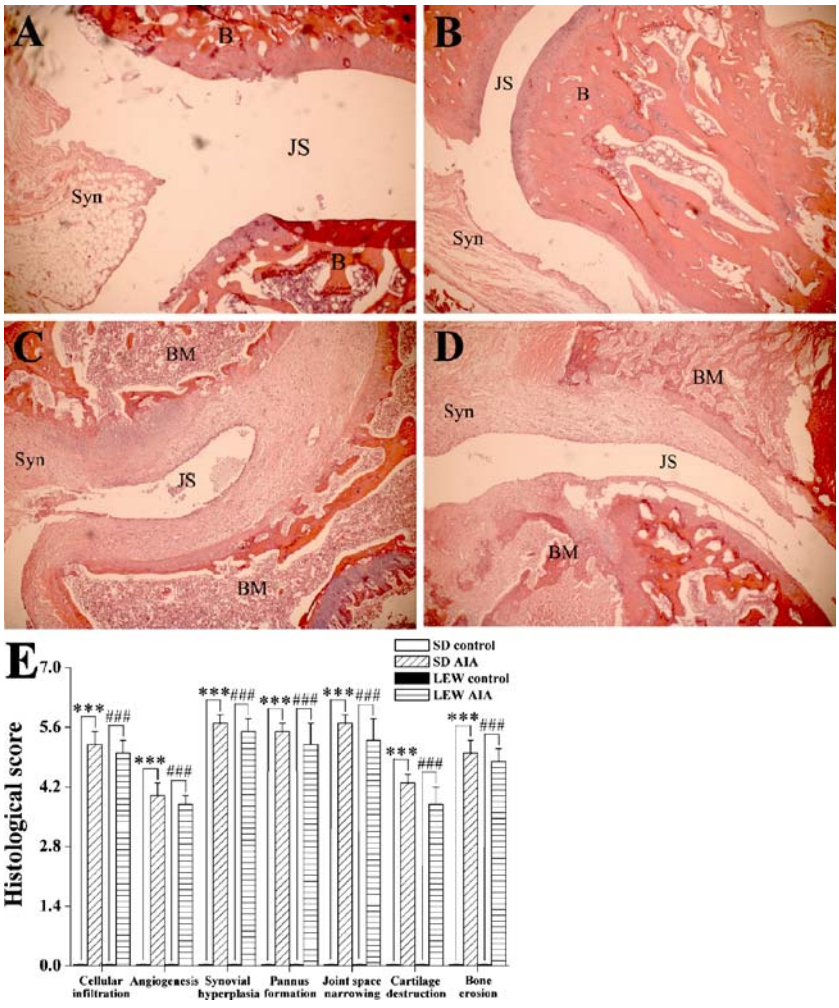

Fig. 5 Histopathological changes in the tibiotarsal joints of SD and LEW AIA rats. Shown are representative H&E stained sections (×40) of the tibiotarsal joints from SD (**a**) and LEW (**b**) control rats, and SD (**c**) and LEW (**d**) AIA rats. Note marked cellular infiltration, angiogenesis, synovial hyperplasia (*Syn*), pannus formation, narrowing of joint space (*JS*), and cartilage destruction and bone (*B*) erosion in the synovial joints of SD and LEW AIA rats compared to control rats. Histopathological analysis demonstrates very significant differences in these pathological events between the AIA and control rats (**e**). Data are expressed as mean ± SEM (n=7–8). ***P<0.001 versus SD control rats; ###P< 0.001 versus LEW control rats

or CRP or both are measured clinically to assist in establishing the presence of RA and other inflammatory disorders and in assessing the activity of disease and its response to treatment. In this AIA model of both SD and LEW rats, both ESR and CRP were found to be markedly associated with the development of the disease, and significantly elevated ESR and CRP levels were noted throughout the course of the experiment as compared to control rats (Fig. 3). In contrast to the change in clinical arthritic signs (i.e., joint swelling continuously increased from day 9 after CFA injection to the end of the experiment, as shown in Fig. 1), the ESR and CRP levels peaked on day 12 and then fell gradually, indicating a relatively early but lasting production and stimulation of acute-phase proteins during disease progression (Fig. 3).

RA is characterized by the presence of an immune-mediated inflammatory synovitis that is conveyed by a transendothelial influx and/or local activation of a variety of mononuclear cells as well as by new vessel formation. Destructive inflammation of the synovial membrane progressively invades and destroys the extracellular matrices of cartilage and bone of joints over the course of the disease, as predominantly manifested by the presence of focal erosions of subchondral bone and at joint margins in areas of direct pannus invasion in RA patients (McGonagle et al. 1999). The inflammatory process may also lead to systemic effects on bone remodeling that are associated with generalized osteopenia and osteoporosis of the axial and appendicular skeleton (Deodhar and Woolf 1996; Goldring 1996). In this AIA model of both SD and LEW rats, histological sections of the ankle joints showed extensive cellular infiltration, intense connective tissue proliferation with subsynovial angiogenesis and erosive pannus formation, dramatic loss of joint space, and marked destruction of cartilage and bone (Fig. 5). Radiological analysis revealed diffuse soft tissue swelling, pronounced bone loss with periosteal new bone formation, and severe bone erosion in the tarsal, metatarsal, and interphalangeal

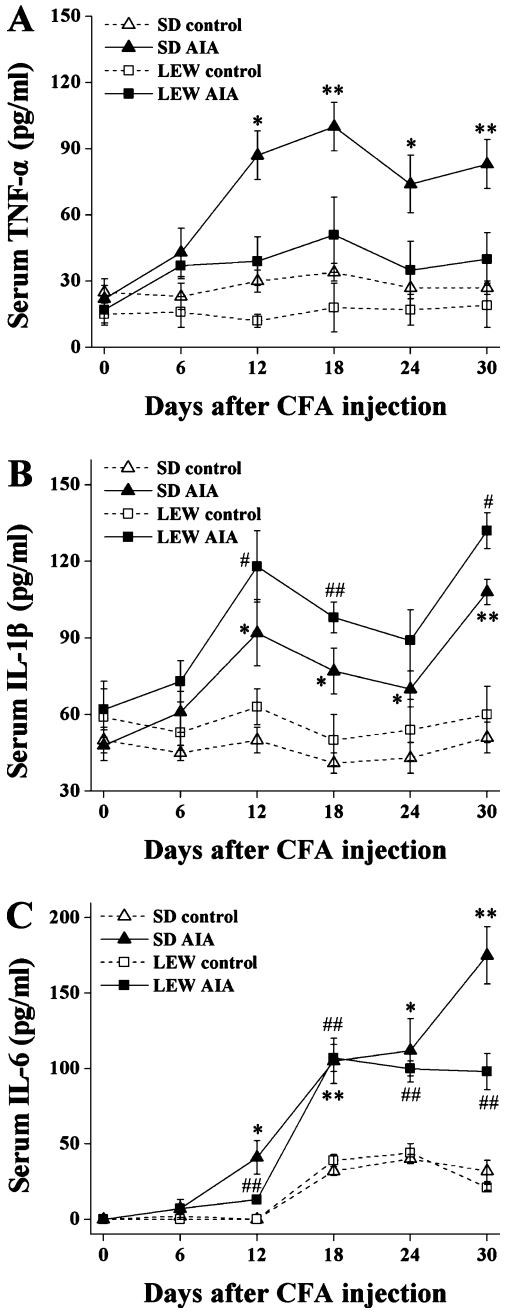

Fig. 6 Expression profiles of the pro-inflammatory cytokines TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) in the blood serum of SD and LEW AIA versus control rats during a 30-day course. Data are expressed as mean ± SEM (n=7–8). *P<0.05, **P<0.01 versus SD control rats; #P<0.05, ##P< 0.01 versus LEW control rats

regions (Fig. 4). The presence of marked bone matrix resorption in conjunction with heterotopic bone formation strongly suggests the presence of a disordered pattern of bone remodeling in the SD and LEW arthritic rats. Thus, our data obtained in the AIA model of both SD and LEW rats are in line with the observations on disease characteristics in human RA.

Recent studies have revealed the key roles of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in the pathogenesis and disease progression of RA (Feldmann et al. 1996; Gravallese and Goldring 2000). TNF-α, IL-1β, and IL-6 have been shown to be abundant in synovial fluid and joint tissues of RA patients (Feldmann et al. 1996), while anti-TNF, anti-IL-1 or anti-IL-6 therapies have been reported to be effective in the treatment of RA (Maini and Taylor 2000; Szekanecz et al. 1998). It has been suggested that these pro-inflammatory cytokines are produced by monocytes/macrophages and synovial fibroblasts that are activated by T cells through cell–cell contact and activation by different cytokines (Panayi et al. 1992). These soluble molecules, when engaged with their receptors, help to propagate a local or systemic inflammatory process and to induce biosynthesis and secretion of matrix metalloproteinases (MMPs) and osteoclasts that play a critical role, respectively, in the degradation of extracellular matrix and the destruction of bone (Burger et al. 1998; Gravallese and Goldring 2000; Smolen and Steiner 2003). In the current study, the serum levels of TNF-α, IL-1β, and IL-6 in both SD and LEW AIA rats were dramatically up-regulated in relation to disease progression as compared to control rats (Fig. 6). However, the production of TNF-α in the serum of SD AIA rats was much more marked than in that of LEW AIA rats. Indeed, in comparison with control rats, serum TNF-α levels were significantly higher in SD AIA rats, but not LEW AIA rats, throughout the course of the disease (Fig. 6a). In contrast to TNF-α, serum IL-1β levels were more evident in LEW AIA rats than in SD AIA rats (Fig. 6b). Moreover, the expression profiles of serum IL-6 in SD and LEW AIA rats were similar, showing marked elevation throughout the course of the disease compared to control rats (Fig. 6c). Our results indicate that SD and LEW rat AIA models have divergent profiles for the expression of TNF-α and IL-1. Several lines of evidence indicate that TNF-α might reside at the apex of the particular pro-inflammatory cytokines cascade in RA, and TNF antagonists have proven to be the most efficient therapy for RA so far (Feldmann and Maini 2001). However, Pascual et al. (2005) recently reported that IL-1 was a major mediator of the inflammatory cascade that underlies systemic onset juvenile idiopathic arthritis (SoJIA), and those SoJIA patients that were identified to be unresponsive to anti-TNF therapy responded favorably to recombinant IL-1 receptor antagonists. Therefore, based on our observations and the findings of Pascual et al., we suspect that AIA in SD rats is TNF-driven, whereas, in LEW rats, it is IL-1-driven; this hypothesis needs to be tested in the near future.

In conclusion, the SD rat AIA model has been demonstrated to share several clinical, hematological, radiological, histological, and immuno-inflammatory features with the comparable model in LEW rats, as well as, in a pattern, to closely resemble the human counterpart, RA. Hence, given the more favorable characteristics of SD rats as compared with LEW rats, i.e., lower cost, wider availability, and heterogenic background, this SD rat AIA model is much more cost effective and advantageous for studying the pathophysiology of arthritis in humans as well as for screening novel anti-arthritic agents.

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