Some rheumatic diseases, such as rheumatoid arthritis, occur when the immune system goes haywire and attacks the linings of joints, thereby causing inflammation, swelling and pain in the joints or muscles (Scott et al., 2010). A wide variety of rheumatic diseases lead to dysfunction of the liver (Rau et al., 1975; Webb et al., 1975; Mills et al., 1980; Abraham et al., 2004). Therefore, it is important for pharmacologists to understand liver dysfunction not only as a result of pharmacotherapy but also as a primary disorder associated with rheumatic diseases.

The existence of polymorphic cytochrome P450 (CYP) genes may contribute to variation in the metabolism of xenobiotics (Meyer and Zanger, 1997). However, other factors such as age, diet, hormonal status, disease and exposure to xenobiotics may also contribute to the wide variety of CYP1-4 family isoforms (Wilkinson, 1997; Lin and Lu, 2001). Certain pathological states, particularly those involving a host inflammatory response, have also been associated with lower drug metabolism in the body with decreased hepatic CYPs (Morgan, 1997, 2001), and may ultimately influence the therapeutic efficacy and toxicity of drugs. Furthermore, hepatocytes are influenced by inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1α, interleukin-1β and interleukin-6, which are produced in the liver. All of the present findings demonstrate that hepatic CYP3A and CYP2B subfamily enzymes are decreased during the development of MT adjuvant-induced arthritis and further suggest that the decreases are dependent on the production of inflammatory cytokines in the liver.

**Key words:** Adjuvant-induced arthritis, Cytochrome P450, Inflammatory cytokine, CYP3A, Rat liver
es in the expression of hepatic CYPs during the development of rheumatic diseases are currently available (Ling and Jamali, 2005, 2009).

Lewis rats are currently the most frequently used strain of rats for the induction of arthritis by subplantar injection of heat-killed Mycobacterium tuberculosis (MT) in mineral oil. After MT adjuvant injection, inbred Lewis rats develop much more severe and less variable adjuvant-induced arthritis, with an incidence of 92% for affliction with secondary polyarthritis, compared with outbred Sprague-Dawley rats (Rosenthal, 1970). However, the temporal changes in the expression of hepatic CYP enzymes during the development of the adjuvant-induced arthritis have not been clarified.

In the present study, we examined the changes in expression of CYP3A and CYP2B subfamily enzymes and in the production of inflammatory cytokines in the liver during the development of the MT adjuvant-induced arthritis in inbred male Lewis rats. We found that hepatic CYP3A and CYP2B subfamily enzymes were decreased at the mRNA, protein, and activity levels during the development of the arthritis and further found that the decreases were dependent on increases in inflammatory cytokines in the liver.

**MATERIALS AND METHODS**

**Animals and treatment**

Male 6-week-old Lewis/CrlCrj rats were obtained from Japan Charles River Co. Ltd. (Kanagawa, Japan), and housed at three or four rats per cage with free access to a commercial chow (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) and fresh water. The rats were maintained on a 12-hr/12-hr dark/light cycle (lights on, 07:00-19:00) in an air-controlled room (temperature, 23.0 ± 1°C; humidity, 55 ± 10%), and handled with care according to the animal care guidelines of Kaken Pharmaceutical Co. Ltd. (Shizuoka, Japan). Some of the rats were administered a single intradermal injection of 0.1 ml of a suspension of dead Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA) in liquid paraffin (MT adjuvant: concentration, 6 mg/ml) into the planar surface of the right hind-foot. Control animals were treated with 0.9% saline (0.1 ml).

**Paw volume measurement**

The swelling volumes of the hind-feet were measured with a micrometer (Plethysmometer; Ugo Basile, Varese, Italy), and the body weights were measured at the same time points.

**Gene expression levels of hepatic CYP subfamily enzymes and cytokines**

The liver was isolated from individual animals at 1, 12, and 25 days after the MT adjuvant injection and stored in RNA later® Solution (Applied Biosystems, Foster City, CA, USA). Total RNA was prepared from individual livers using an RNaseasy® Mini Kit (Qiagen, Valencia, CA, USA) and used to determine the mRNA levels of CYP3A and CYP2B subfamily enzymes. The amounts of these mRNAs were measured with a Nano Drop® system (Nanodrop Technologies Inc., Wilmington, DE, USA). Briefly, a portion (735 ng) of the total RNA was converted to cDNA in 20 μl of RT-reaction mixture using a PrimeScript™ RT Reagent Kit (TaKaRa Bio Inc., Tokyo, Japan) according to the manufacturer’s instructions. Real-time RT-PCR was performed with an Mini Opticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) in a 20-μl reaction mixture containing 10 μl of the Power SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of cDNA, and 100 nM of each primer set. The Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was amplified as an internal standard. The amplification protocols and primer sequences are shown in Table 1. The amount of each cDNA was assessed using a relative standard curve method, as described in Bio-Rad User Bulletin #2 (1997).

**Preparation of hepatic microsomes**

Microsomal fractions were prepared from liver homogenates by differential centrifugations as described previously (Degawa, et al., 1989). Briefly, livers from individual rats were homogenized with a Teflon homogenizer (Iuchi, Osaka, Japan) in three volumes (v/w) of 1.15% KCl, and each liver homogenate was centrifuged at 9,000 × g for 20 min at 4°C. Each supernatant was further centrifuged at 105,000 × g for 1 hr at 4°C, and the resultant pellet was homogenized with a stock solution (1.15% KCl containing 20% glycerol) and used as a microsomal fractions. The amount of microsomal protein was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

**Western blot analysis of CYP proteins**

Each hepatic microsomal preparation (10 μg protein/ lane) was separated by 10% SDS-PAGE. The separated proteins were transferred from the gel to a PVDF membrane (Bio-Rad Laboratories), and immunostained a primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibody. Positive signals were detected with an Immu-Star™ HRP Chemiluminescent Kit (Bio-Rad Laboratories) according to the manufactur-
Table 1. The primer sets used in this study

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sets</th>
<th>Reaction condition</th>
<th>GenBank accession number</th>
<th>Reference or supplier</th>
</tr>
</thead>
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<tr>
<td>CYP2B1</td>
<td>5'- ATCGACACTACCTTCTGCG -3' (Forward)</td>
<td>Denaturation 95°C</td>
<td>50°C</td>
<td>72°C</td>
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<tr>
<td></td>
<td>5'- ATCAGTGTAGGCGATTTACTGCGG -3' (Reverse)</td>
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<tr>
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<td>1 min</td>
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<tr>
<td>CYP3A1</td>
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<td>Denaturation 95°C</td>
<td>60°C</td>
<td>15 sec</td>
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<tr>
<td>CYP3A2</td>
<td>Sequence data not shown</td>
<td>Annealing 1 min</td>
<td>1 min</td>
<td>1 min</td>
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<tr>
<td>TNF-α</td>
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</tr>
<tr>
<td></td>
<td>5'- GTACCACCAGTTGGTTCTTTGA -3' (Reverse)</td>
<td>Annealing 15 sec</td>
<td>1 min</td>
<td>1 min</td>
</tr>
<tr>
<td>IL-6</td>
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<td>15 sec</td>
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<tr>
<td></td>
<td>5'- GTGCACTCAT CGCTGTACTCAACTCA -3' (Reverse)</td>
<td>Annealing 15 sec</td>
<td>1 min</td>
<td>1 min</td>
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<tr>
<td>IL-1α</td>
<td>5'- GAAAGCAAGCTGCTGGTTCTGAA -3' (Forward)</td>
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<tr>
<td></td>
<td>5'- AGGGTCGTCCTCTAACCTGCTGATG -3' (Reverse)</td>
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<tr>
<td>IL-1β</td>
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<td>Denaturation 95°C</td>
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<td>15 sec</td>
</tr>
<tr>
<td></td>
<td>5'- AGGTCGTCATCATCCACGGAG -3' (Reverse)</td>
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<td>1 min</td>
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<tr>
<td>GAPDH</td>
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<td>Denaturation 95°C</td>
<td>60°C</td>
<td>15 sec</td>
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<tr>
<td></td>
<td>5'- ATGGTGGTGGAAGACGCCAGTA -3' (Reverse)</td>
<td>Annealing 15 sec</td>
<td>1 min</td>
<td>1 min</td>
</tr>
</tbody>
</table>

* QuantiTect Primer Assay (QiaGen). ** Perfect real time support system (TaKaRa Bio Inc.).
er’s instructions. Rabbit anti-rat CYP3A and goat anti-rat CYP2B antibodies (Nosan Corporation, Kanagawa, Japan) were used as the primary antibodies for the CYP3A and CYP2B subfamily enzymes, respectively. HRP-conjugated goat anti-rabbit (Bio-Rad Laboratories) and HRP-conjugated rabbit anti-goat IgG-+l (Bethyl Laboratories Inc., Montgomery, TX, USA) antibodies were used as the secondary antibodies for the CYP3A and CYP2B subfamily enzymes, respectively.

**Enzyme activities of hepatic CYP enzymes**

Hepatic microsomal activity for pentoxyresorufin O-dealkylation, which is mainly mediated by CYP2B enzymes (Lubet *et al.*, 1985; Burke *et al.*, 1994), was measured by the microassay method of Lagueux *et al.* (1997). Briefly, a 160-μl reaction mixture of 0.1 M sodium phosphate buffer at (pH 7.4) containing hepatic microsomes (80 μg) and an NADPH-generating system was preincubated at 37°C for 10 min in a 96-well deep well plate (BM Equipment Co. Ltd., Tokyo, Japan). The reaction was started by the addition of 40 μl of pentoxyresorufin (25 μM) containing 5% dimethylsulfoxide. After incubation at 37°C for 30 min with orbital shaking, 200 μl of cold ethanol was added to stop the reaction, and 100 μl of each reaction mixture was transferred to a 96-well half-area black plate (Corning Inc., Corning, NY, USA). The amount of resorufin formed in the reaction mixture was measured with a Wallac 1420 ARVOsx Multilabel Counter (Perkin Elmer, Waltham, MA, USA) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Hepatic CYP3A activity was determined using a P450-GloTM CYP3A4 Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. In this system, luciferin-6’-pentafluorobenzyl ether (Luciferyl-PFBE) was used as a substrate for CYP3A subfamily enzymes (Cali *et al.*, 2006). Briefly, a 25-μl reaction mixture of 0.1 M sodium phosphate buffer (pH 7.4) containing hepatic microsomes (20 μg) and Luciferin-PFBE (100 μM) was placed in each well of a 96-well plate (Corning Inc.) and preincubated at room temperature for 10 min under light shielding. The reaction was started by the addition of 25 μl of an NADPH-generating system solution to each well of the plate. After incubation at room temperature for 30 min under light shielding, 50 μl of Luciferin Detection Reagent was added to each well of the 96-well plate, and the plate was further incubated at room temperature for 20 min under light shielding. After the incubation, 90 μl of each reaction mixture was transferred to a 96-well half-area white plate (Corning Inc.), and the amount of luciferin formed was measured with the Wallac 1420 ARVOsx Multilabel Counter in the luminescence detection mode.

**Expression levels of hepatic inflammatory cytokines**

The amounts of hepatic TNF-α, IL-6, IL-1α and IL-1β proteins were determined by the method as described previously (Kojima *et al.*, 2005, 2009). Briefly, the livers from individual rats were homogenized with three volumes (v/w) of 1.15% KCl. Each liver homogenate was centrifuged at 9,000 × g for 20 min at 4°C, and the supernatant was further centrifuged at 105,000 × g for 1 hr at 4°C. The resultant supernatant (S-105, 10-50 μl/well) was used to determine the amounts of cytokines using Rat Cytokine ELISA Kits for TNF-α, IL-1β, and IL-6 (Thermo Fisher Scientific Inc.) and a Rat IL-1α Platinum ELISA Kit (eBioscience, San Diego, CA, USA). The amount of protein in each S-105 fraction was determined with the BCA Protein Assay Kit. The amounts of cytokines were represented as pg per mg S-105 protein.

**Statistical Analysis**

The significance of differences between values was evaluated by Student’s *t*-test using Microsoft Excel program. Values of *P* < 0.05 were considered to indicate statistical significance.

**RESULTS**

**Paw volume**

The pattern of events during a typical 25-day MT adjuvant-induced arthritis experiment is shown in Fig. 1A. Significant swelling of the MT adjuvant-injected right hind-foot was observed at day 1. The swelling of the foot remained virtually unchanged from days 1-8. However, beginning on day 9, a steady increase in the paw volume was observed up to day 13, and thereafter, the volume was maintained up to day 25. Furthermore, involvement of the uninjected left hind-foot became apparent by day 12. The increases in the left hind-foot paw volume occurred in a time-dependent manner up to day 15, and the increased levels were subsequently maintained up to day 25.

**Body weight**

The body weights of control rats gradually increased in a time-dependent manner throughout the experimental periods. On the other hand, the body weights in the MT adjuvant-injected rats decreased markedly, compared with the corresponding control rats, along with the progression of arthritis and significant increases in the hind-paw volumes (Fig. 1B).
Decreases in hepatic CYP enzymes in the rats with arthritis

Fig. 1. MT adjuvant-induced arthritis in rats. Rats were injected with either saline or MT adjuvant, and their hind paw volumes and body weights were measured at the indicated time points (n = 5 in each group). (A) Changes in the hind paw volume in the MT adjuvant- and saline-treated rats. ○, Saline treatment (injected foot): ●, MT adjuvant treatment (injected foot): △, Saline treatment (uninjected foot): ▲, MT adjuvant treatment (uninjected foot). (B) Changes in the body weights of MT adjuvant- and saline-treated rats. ○, Saline treatment: ●, MT adjuvant treatment.

Fig. 2. Changes in the gene expression levels of hepatic CYP2B1, CYP2B2, CYP3A1 and CYP3A2 after the MT adjuvant-injection. Rats were injected with either saline or MT adjuvant and killed after 1, 12, and 25 days. Total hepatic RNA was extracted from each animal, and the expression levels of the CYP mRNAs were measured by real-time RT-PCR. Open columns, saline-treated rats: closed columns, MT adjuvant-treated rats. The data represent the ratios relative to the corresponding time-matched controls (saline-injected group), and are shown as mean ± S.D. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from the corresponding controls.
Expression of hepatic CYP3A and CYP2B subfamily enzymes

We examined the alterations in the expression levels and activities of hepatic CYP3A and CYP2B subfamily enzymes after the MT adjuvant injection. The changes in the mRNA levels of hepatic CYP3A (CYP3A1/3A2) and CYP2B (CYP2B1/2B2) subfamily enzymes are shown in Fig. 2. The expression levels of CYP3A1 and CYP3A2 decreased to 30% and 65% at day 1, 47% and 34% at day 12, and 17% and 57% at day 25, respectively, relative to the corresponding time-matched controls. Likewise, the expression levels of CYP2B1 and 2B2 decreased to about 43% and 19% at day 1, 21% and 6% at day 12, and 51% and 37% at day 25, respectively. The changes in the protein levels of the CYP3A and CYP2B subfamily enzymes are shown in Fig. 3A. After MT adjuvant injection, the protein levels of CYP3A1 and CYP3A2 were not clearly changed at day 1, but were significantly decreased at days 12 and 25. On the other hand, the protein levels of the CYP2B subfamily enzymes, especially CYP2B2, were clearly decreased at day 1 and markedly decreased at days 12 and 25. The activities (Luciferin-PFBE activation) of hepatic CYP3A enzymes at 1, 12, and 25 days after MT adjuvant injection were decreased to 41%, 20%, and 37%, respectively, relative to the corresponding time-matched controls. Likewise, the activities (pentoxyresorufin O-dealkylation) of the CYP2B enzymes at 1, 12, and 25 days after MT adjuvant injection were decreased to 76%, 38%, and 54%, respectively, relative to the corresponding time-matched controls.

Amounts of hepatic inflammatory cytokines

The changes in the expression levels of hepatic inflammatory cytokines after MT adjuvant injection were examined at the mRNA and protein levels. Significant increases in the expression levels of hepatic TNF-α mRNA in MT adjuvant-injected rats were observed at all the time points examined (Fig. 4), while a significant increase in its protein level was only observed at 1 day (Fig. 5). At all time points after MT adjuvant injection, significant increases in the expression levels of hepatic IL-1α and IL-1β were observed at both the mRNA and protein levels. At 12 and 25 days, but not 1 day, after the MT adjuvant injection, the expression mRNA and protein expres-

![](image_url)
sion levels of hepatic IL-6 were significantly increased, compared with the corresponding time-matched controls (Figs. 4 and 5).

**DISCUSSION**

A wide variety of rheumatic diseases lead to a hepatitis-like pathogenesis and dysfunction of the liver (Rau, 1975; Webb et al., 1975; Mills et al., 1980; Abraham et al., 2004). In this study, we examined the changes in the expression levels of hepatic CYPs, especially CYP3A subfamily enzymes, which play a major role in the metabolism of many drugs (Guengerich, 1999), during adjuvant-induced arthritis in male Lewis rats.

First, we confirmed the development of arthritis following an intradermal injection of an MT suspension in liquid paraffin into the right hind-foot of rats (Newbould, 1963). The edema volume of the injected paw, as the primary lesion, developed within 1 day after the injection and persisted at this level up to day 8. Beginning on day 9, the volume further increased up to day 13 and persisted at the level up to day 25. Furthermore, beginning on day 12, swelling of the uninjected left hind-foot, as a secondary lesion, indicating systemic inflammation occurred and reached a maximum at day 15 that persisted up to day 25.

A pathogenesis involving host inflammatory responses (e.g., bacterial and viral infections) has been associated with lower drug metabolism in the body together with decreased hepatic CYPs (Morgan, 1997, 2001). Furthermore, inflammatory cytokines, such as TNF-α, IL-1, and IL-6, are well known to downregulate the gene expression of a wide variety of CYP 1-4 family enzymes (Morgan, 2001; Nyagode et al., 2010).

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**Fig. 4.** Changes in the gene expression levels of hepatic TNFα, IL-6, IL-1α and IL-1β after the MT adjuvant injection. Rats were injected with saline or MT adjuvant and killed after 1, 12, and 25 days. Total hepatic RNA was extracted from each rat, and the expression levels of the cytokine mRNAs were measured by real-time RT-PCR. The expression level of each cytokine mRNA was normalized by the expression level of the GAPDH mRNA. Open columns, saline-treated rats: closed columns, MT adjuvant-treated rats. The data represent ratios relative to the corresponding time-matched controls (saline-injected group), and are shown as mean ± S.D. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from the time-matched controls.
Therefore, we examined the changes in the activities and expression levels of hepatic CYP3A and CYP2B subfamily enzymes and in the amounts of hepatic inflammatory cytokines, namely TNF-α, IL-1α, IL-1β and IL-6, at 1, 12, and 25 days after MT adjuvant injection. The hepatic activities of CYP3A and CYP2B subfamily enzymes were assessed using the substrates, Luciferin-PFBE for CYP3A activities (Cali et al., 2006) and pentoxyresorufin for CYP2B activities (Lubet et al., 1985; Burke et al., 1994). All of the enzyme activities examined were drastically decreased, even at 1 day after MT adjuvant injection, and the decreases persisted up to day 25. Likewise, the expression levels of hepatic CYP3A and CYP2B subfamily enzymes, especially CYP3A1 and CYP2B2, at both the mRNA and protein levels were drastically decreased at 1-25 days after MT adjuvant injection. On the other hand, the inflammatory cytokines, TNF-α, IL-1α, IL-1β and IL-6, which down-regulate the gene expression levels of hepatic CYP isoforms including CYP2B1 and CYP3A1/3A2 (Clark et al., 1995; Fukuda et al., 1992; Abdel-Razzak et al., 1995), were significantly induced throughout the 25-day experimental period after the MT adjuvant-injection.

Several reports have suggested that the pharmacokinetics of drugs such as atorvastatin (Kruger et al., 2009), erythromycin (Rivory et al., 2002), verapamil (Mayo et al., 2000) and propranolol (Ling and Jamali, 2005), which are metabolized by CYP3A subfamily enzymes, are altered in the patients with inflammatory diseases. For example, the serum concentrations of antihypertensive drugs, such as verapamil and propranolol, are higher in patients with rheumatoid arthritis than in healthy subjects or with mild disease (Mayo et al., 2000; Schneider et al., 1981). Likewise, decreases in the clearance of these antihypertensive drugs have been reported in rats with adjuvant-induced arthritis (Guirguis and Jamali, 2003; Ling and Jamali, 2005). These previous findings and our present data suggest that rheumatoid-like inflammatory diseases cause decreases in the clearance of CYP3A substrate drugs and certain side effects via downregulation of hepatic CYP3A enzyme expression.

In conclusion, we have demonstrated that decreases

Fig. 5. Changes in the levels of hepatic TNFα, IL-6, IL-1α, and IL-1β proteins after the MT adjuvant injection. Rats were injected with saline or MT adjuvant and killed after 1, 12, and 25 days. Hepatic S-105 fractions were prepared from individual rats and subjected to ELISA, measurements of cytokines. Open columns, saline-treated rats; closed columns, MT adjuvant-treated rats. The data represent means ± S.D. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, significant differences from the corresponding controls (saline-injected group).
in the expression levels of hepatic CYP3A and CYP2B subfamily enzymes occurred according to increases in the hepatic inflammatory cytokines, TNF-α, IL-1α, IL-1β and IL-6 during the development of MT adjuvant-induced arthritis in rats. The MT adjuvant-induced arthritis rat model is useful for not only the development of antirheumatoid drugs but also the prediction of inflammation-mediated expression of the toxicities of xenobiotics including drugs.

REFERENCES


