Microarray Analysis of Lipopolysaccharide Potentiation of Trovafloxacin-Induced Liver Injury in Rats Suggests a Role for Proinflammatory Chemokines and Neutrophils

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ABSTRACT

Idiosyncratic drug toxicity refers to toxic reactions occurring in a small subset of patients and usually cannot be predicted during preclinical or early phases of clinical trials. One hypothesis for the pathogenesis of hepatic idiosyncratic drug reactions is that, in certain individuals, underlying inflammation results in sensitization of the liver, such that injury occurs from an agent that typically would not cause hepatotoxicity at a therapeutic dose. We explored this possibility by cotreating rats with nonhepatotoxic doses of bacterial lipopolysaccharide (LPS) and trovafloxacin (TVX), a drug that caused idiosyncratic hepatotoxicity in humans. The combination of LPS and TVX resulted in hepatotoxicity in rats, as determined by increases in serum alanine aminotransferase activity and hepatocellular necrosis, which were not observed with either agent alone. In contrast, treatment with LPS and levofloxacin, a fluoroquinolone without human idiosyncratic liability, did not result in these changes. Liver gene expression analysis identified unique changes induced by the combination of TVX and LPS, including enhanced expression of chemokines, suggestive of liver neutrophil (PMN) accumulation and activation. Consistent with a role for PMN in the hepatotoxicity induced by LPS/TVX, prior depletion of PMN attenuated the liver injury. The results suggest that gene expression profiles predictive of idiosyncratic liability can be generated in rats cotreated with LPS and drug. Furthermore, they identify gene expression changes that could be explored as biomarkers for idiosyncratic toxicity and lead to enhanced understanding of the mechanism(s) underlying hepatotoxicity induced by TVX.

Individual susceptibility plays an important role in determining whether or not a person develops an adverse drug reaction (ADR). Susceptibility to drug toxicity is determined by genetic predisposition as well as environmental factors. Among ADR, idiosyncratic drug reactions (IDR) are perhaps the most insidious. These reactions are unpredictable, bear no obvious relationship to the dose of drug, demonstrate inconsistent temporal patterns with regard to drug exposure, and are typically unrelated to the intended pharmacological effect (Park et al., 2000; Roth et al., 2003; Waring and Anderson, 2005). Furthermore, they occur in a small fraction (usually <5%) of people or animals, so that they are not predicted in preclinical studies in which limited numbers of animals are used, and they often are not identified in clinical trials. They typically become obvious only when drugs are used in a large human population (Knowles et al., 2000). IDR are often quite serious and sometimes fatal, and the liver has been a frequent target. Mechanisms underlying these reactions remain unknown, and models to predict hepatic IDR are lacking (Park et al., 2000; Shenton et al., 2004). Thus, the occurrence of IDR remains a perplexing human health problem and a significant economic problem for the pharmaceutical industry, because they often result in the removal of a drug.
from the market after significant resources have been spent on the discovery, development, and marketing of the compound.

Recent evidence from experimental models indicates that a episode of inflammation during drug treatment predisposes animals to tissue injury, suggesting that inflammation could be a factor for idiosyncratic drug toxicity in humans (Buchweitz et al., 2002; Luyendyk et al., 2003). A rat model has been developed that mimics human hepatic IDR by cotreating rats with a drug known to induce idiosyncratic liver injury in people and bacterial lipopolysaccharide (LPS) to induce modest inflammation. For example, the histamine-2 receptor antagonist ranitidine (RAN) has been associated with a low incidence of idiosyncratic liver toxicity in humans (Ramkrkhiani et al., 1998). Cotreatment of rats with nontoxic doses of LPS and RAN induced liver injury in a time- and dose-dependent fashion (Luyendyk et al., 2003). Interestingly, another histamine-2 receptor antagonist, famotidine, which has little or no association with idiosyncratic liver injury in humans, did not cause hepatotoxicity when coadministered with LPS (Luyendyk et al., 2003). Thus, this model of drug-inflammation interaction was able to distinguish a drug that causes idiosyncratic ADR from one in the same pharmacologic class that does not. Similar results have been observed with chlorpromazine, another drug for which idiosyncratic liver injury in humans is well documented (Buchweitz et al., 2002).

Microarray analysis has been shown to be a useful tool in identifying molecular mechanisms underlying drug-induced toxicity (Buczynski et al., 2000; Waring and Halbert, 2002; Yang et al., 2004). We previously applied microarray analysis toward identifying gene expression changes induced by an antibacterial quinolone agent trovafloxacin (TVX) in isolated human hepatocytes. TVX is associated with human idiosyncratic toxicity, resulting in severe restrictions on its use (Ball et al., 1999; Bertino and Fish, 2000). In isolated human hepatocytes treated in vitro, TVX produced a unique gene expression profile compared with quinolones with less idiosyncratic potential. However, in vivo, TVX was not hepatotoxic to rats and hepatic gene expression analysis was not able to distinguish TVX from quinolone antibiotics devoid of idiosyncratic liability in people (Liguori et al., 2005).

We have expanded on these studies by applying gene expression analysis in the LPS-cotreated rat model to determine whether liver toxicity occurred and to understand the mechanism of idiosyncratic toxicity caused by TVX. Our results show that cotreatment of rats with LPS and TVX does result in hepatotoxicity. Furthermore, gene expression changes were identified that are associated with hepatotoxicity in LPS/TVX-treated rats and which therefore might be used to identify compounds with the potential to cause idiosyncratic hepatotoxicity.

**Materials and Methods**

**Study Design.** All studies in vivo were conducted at Michigan State University (MSU, East Lansing, MI). Rats received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (1996) prepared by the National Academy of Sciences, and procedures were approved by the MSU Committee on Animal Use and Care. Male Sprague-Dawley rats [Crl:CD (SD) IGS BR; Charles River, Portage, MI] weighing 250 to 350 g were used for these studies. Animals were fed standard chow (rodent chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. They were allowed to acclimate for 1 week in a 12-h light/dark cycle before use.

LPS derived from *Escherichia coli* serotype O55:B5 with an activity of $9.2 \times 10^6$ EU/mg was used (catalog number L-2880, Lot 024K4067; Sigma-Aldrich, Inc., St. Louis, MO). This activity was determined using a QCL Chromogenic LAL Endpoint Assay from Cambrex (East Rutherford, NJ). Doses of LPS and TVX were selected from preliminary range-finding studies. In brief, doses of LPS and TVX were identified that did not cause a significant increase in serum alanine aminotransferase activity which was determined spectrophotometrically at a dose calculated to result in a similar pharmacologic toxicity to the dose of TVX used (Lubasch et al., 2000). Rats fasted for 24 h were given 44.4 $\times 10^6$ EU/kg LPS or its saline vehicle (Veh) intravenously; food was then returned. Two hours later, 60 mg/kg TVX or 150 mg/kg LVX or vehicle (50/50 sterile saline/saline) was administered intravenously. TVX solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. To simplify treatment nomenclature, the following designations are used: Veh/Veh (LPS vehicle/quinoone vehicle), LPS/Veh (LPS/quinoone vehicle), Veh/TVX (LPS vehicle/TVX), Veh/LVX (LPS vehicle/LVX), LVX/LVX, and LVX/TVX. Six or 22 h later, rats were anesthetized with sodium pentobarbital (75 mg/kg i.p.). Plasma was collected by drawing blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%), and rats were then euthanized by exsanguination from the dorsal aorta. Blood collected from the aorta was allowed to clot at room temperature, and serum was collected and stored at $-20°C$ until use. Representative (3–4-mm) slices of the ventral portion of the left lateral liver lobe were collected and fixed in 10% neutral-buffered formalin. The right medial lobe of the liver was flash-frozen in liquid nitrogen for subsequent gene expression analysis. Rats were dosed with Veh/Veh ($n = 6$), LPS/Veh ($n = 10$), Veh/TVX ($n = 6$), Veh/LVX ($n = 10$), LVX/LVX ($n = 15$), or LVX/TVX ($n = 25$) in four separate studies. Each study comprised rats in each treatment group.

**ALT Activity and Histopathology Assessment.** Hepatic parenchymal cell injury was estimated by evaluating serum ALT activity, which was determined spectrophotometrically using Infinity™-ALT reagent from Thermo Electron Corp. (Louisville, CO). Formalin-fixed liver samples were routinely processed and embedded in paraffin. Paraffin sections (6 μm) were stained with hematoxylin and eosin. Resulting slides were coded before evaluation by a pathologist.

**RNA Preparation.** Frozen liver samples (approximately 100 mg of tissue per sample) were immediately added to 2 ml of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and homogenized using a Polytron 300D homogenizer (Brinkman Instruments, Westbury, NY). One milliliter of the tissue homogenate was transferred to a microfuge tube, and total RNA was extracted via chloroform extraction followed by nucleic acid precipitation with isopropanol. The pellet was washed with 80% ethanol and resuspended in molecular biology grade water. Nucleic acid concentration was determined by O.D. 260 nm (Smart-Spec; Bio-Rad Laboratories, Hercules, CA), and RNA integrity was evaluated using a bioanalyzer (model 2100; Agilent Technologies, Foster City, CA).

**Gene Array Analysis.** Microarray analysis was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA). In brief, approximately 15 μg of total RNA was reverse-transcribed into cDNA using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen Life Technologies) according to the manufacturer’s instructions, with the exception that the primer used for the reverse transcription reaction was a modified T7 primer with 24 thymidines at the 5‘ end (Affymetrix). The sequence was 5‘-GGCGGATGAAATGTTAATAC-GACGCTCAGTATAGGGAGGCCG-dT24-3’. cDNA was purified via phenol/chlorform/isooamylalcohol (Invitrogen Life Technologies) extraction and ethanol precipitation. The purified cDNA was resuspended in molecular biology grade water. After this procedure, biotin labeled cRNA was synthesized from the cDNA according to the manufacturer’s instructions using the Enzo RNA Transcript Labeling Kit (ENZO Life Sciences, Farmingdale, NY). The labeled cRNA was then purified using RNeasy kits (QIAGEN, Valencia, CA). Subsequently, cRNA concentra-
tation and integrity were evaluated. Approximately 20 μg of cRNA was then fragmented in a solution of 40 mM Tris acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc at 94°C for 35 min. Fragmented, labeled cRNA was hybridized to an Affymetrix rat genome RAE230A array, which contains sequences corresponding to roughly 15,900 transcripts, at 45°C overnight using a Hybridization Oven 640 (Affymetrix). The array was subsequently washed and stained twice with streptavidin-phycocerythrin (Molecular Probes, Carlsbad, CA) using the Gene-Chip Fluidics Workstation 400 (Affymetrix). The array was then scanned using the GeneChip Scanner 3000 (Affymetrix). The microarray scanned image and intensity files (.CEL files) were imported in Rosetta Resolver gene expression analysis software, version 5.0 (Rosetta Inpharmatics, Seattle, WA).

**Evaluation of Hepatic Neutrophil Accumulation and Serum CINC-1 and MIP-2 Concentration.** Neutrophil (PMN) immunohistochemistry was performed on formalin-fixed, paraffin-embedded liver sections as described previously (Yee et al., 2003). Hepatic PMN accumulation was quantified by counting the average number of PMN in 20 randomly selected, high-power fields (400×). Serum CINC-1 and macrophage inflammatory protein-2 (MIP-2) concentrations were determined using commercially available ELISA kits (CINC-1; Assay Designs, Inc., Ann Arbor, MI; and MIP-2; Biosource International, Camarillo, CA).

**PMN Depletion.** PMN depletion was accomplished by administering PMN antisera before treatment with LPS/TVX. Rats were treated with vehicle (n = 8) or given 0.25 ml of either normal rabbit serum (control serum) (n = 11) or rabbit anti-rat PMN serum (anti-PMN serum; Intercell Technologies, Jupiter, FL) (n = 11) diluted 1:1 in sterile saline (total injection volume per rat, 0.5 ml i.v.) 18 h before administration of LPS. Previous studies in which anti-PMN serum was administered to rats demonstrated a selective and pronounced depletion of PMN from blood (Snipes et al., 1995; Luyendyk et al., 2005). Rats were killed 6 h after treatment with TVX, and citrated plasma was collected. Total blood leukocyte count was quantified using a Unopette white blood cell determination kit (Becton-Dickinson, Franklin Lakes, NJ) and a hemacytometer. Slides were prepared from whole blood and stained using the Hema 3 staining system (Fisher Scientific, Middleton, VA), and differential counting was performed.

**Statistical Methods.** ALT activity, CINC-1, and MIP-2 ELISA data were analyzed by two-way ANOVA with Tukey’s post hoc test. For Fig. 6A, changes in cytokine/chemokine mRNAs were compared among treatment groups after angular transformation using one-way ANOVA followed by Holm-Sidak post hoc test or ANOVA on Rank’s test followed by Dunn’s test for data that were not normally distributed. For PMN depletion studies, ALT activity, blood cell counts, and PMN accumulation were analyzed by one-way ANOVA with Tukey’s post hoc test. The criterion for significance was a p value less than or equal to 0.05 for all studies.

For microarray analysis, error models were applied and ratios were built for each treatment array versus its averaged respective vehicle control using the Rosetta Resolver system. A p value was calculated for every analysis change using the Rosetta Resolver error model (Rajagopalan, 2003). Gene expression was considered significantly changed if the p value was less than or equal to 0.05. Agglomerative cluster analysis was performed using the average link heuristic criteria and the Euclidean distance metric for similarity measure. Principal component analysis (PCA) reduces a large set of genes into several components, for which each component is a weighted linear combination of all genes. By selecting the first n components as predictors, one is able to vastly reduce the dimension of gene expression data and, at the same time, retain the major information (Yang et al., 2004). Both agglomerative clustering and principal component analysis of microarray data were performed using Rosetta Resolver software.

**Results**

**Serum ALT.** Treatment of rats with TVX or LVX alone did not cause an increase in serum ALT activity at 6 h (Fig. 1).

Likewise, no increase in ALT activity was evident with the dose of LPS used in these studies. This dose of LPS has been shown to cause some hepatic PMN accumulation and increased tumor necrosis factor α (TNF-α) in plasma but little to no hepatocellular injury (Luyendyk et al., 2003). ALT activity was increased approximately 3-fold in rats cotreated with LPS and TVX, indicating hepatocellular injury (Fig. 1). In contrast, LVX coadministered with LPS did not result in an increase in serum ALT (Fig. 1). Serum ALT activity among LPS/TVX-treated animals was somewhat variable, such that some animals displayed high ALT activity, whereas others showed almost no change compared with controls. Liver injury was also evaluated 22 h after administration of TVX, and ALT activity was still increased at this time in rats cotreated with LPS and TVX, although the magnitude of the response was less than that seen at 6 h after drug treatment (Table 1).

**Histopathology.** From the results of combined studies presented in Fig. 1, one study was selected for histopathology and microarray evaluation. No significant lesions were observed in the Veh/Veh, Veh/TVX, and Veh/LVX treatment groups (Fig. 2). All LPS-treated groups showed evidence of mild leukocyte infiltration, occasional apoptotic hepatocytes, and minimal kupffer cell hyperplasia and hypertrophy, indicative of activation. However, in the LPS/TVX-treated rats, the severity of these changes was more pronounced and the quantity of apoptotic hepatocytes was slightly increased (Fig. 2). In addition, in the majority of the LPS/TVX-treated rats evaluated for histopathology (six of nine), there was evidence of multifocal, randomly distributed small foci of coagulative necrosis. This histologic finding was not observed in any of the livers from rats treated with LPS alone, and only one minute focal area of necrosis was observed in one of six LPS/LVX-treated rats.

**Gene Microarray Results.** Liver samples were analyzed for global gene expression changes on Affymetrix RAE230A rat genomic arrays. The complete list of gene expression changes is included in supplemental Table 1. There were

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of Animals</th>
<th>ALT Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh/Veh</td>
<td>7</td>
<td>39.3 ± 14.8</td>
</tr>
<tr>
<td>LPS/Veh</td>
<td>10</td>
<td>31.7 ± 10.0</td>
</tr>
<tr>
<td>Veh/TVX</td>
<td>7</td>
<td>37.7 ± 14.3</td>
</tr>
<tr>
<td>LPS/TVX</td>
<td>11</td>
<td>77.1 ± 23.2*</td>
</tr>
</tbody>
</table>

* Significantly different from the LPS/Veh and the Veh/TVX groups.
relatively few gene expression changes when either TVX or LVX was given alone compared with Veh/Veh-treated rats (Fig. 3). As can be seen from the dendrogram at the left of Fig. 3, treatment with LPS alone or in combination with LVX resulted in similar gene expression patterns. By contrast, a distinct pattern was observed in LPS/TVX-treated rats, which clustered separately from the other treatment groups.

Reducing the genomic data to three variables using PCA yielded additional evidence for a unique pattern of hepatic gene expression in LPS/TVX-treated rats (Fig. 4). In this analysis, the gene expression pattern from LPS/TVX-treated rats segregated from that of rats treated with LPS/LVX, either drug alone or with LPS alone, again suggesting that a unique pattern of gene expression exists for the LPS/TVX population.

Statistical comparison of gene expression in livers from LPS/TVX-treated rats to all other treatment groups identified approximately 220 gene expression changes that were regulated to a greater extent after LPS/TVX treatment (Fig. 5A). A number of these genes are involved in functions that have been proposed as a cause of TVX-induced idiosyncratic toxicity. For instance, genes involved in RNA transcription from polymerase II are differentially regulated in the LPS/TVX-treated rats (Fig. 5B), as well as genes involved in oxidative stress response, such as p53, c-fos, and glutathione reductase (data not shown). Genes coding for chemokines, such as CINC-1 and MIP-2, and cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6), were regulated to a greater extent in the LPS/TVX-treated rats than in any of the other treatment groups (Fig. 6A). Increases in serum concentrations of CINC-1 and MIP-2 were confirmed by ELISA (Fig. 6B). The results show significantly increased blood levels of these chemokines in the LPS/TVX-treated group compared with treatment with LPS alone or with LPS/LVX.

**PMN Depletion Studies.** The differential regulation of chemokines suggested a role for PMN in the hepatotoxicity in LPS/TVX-treated rats. However, neutrophil involvement could be a cause or effect of the liver injury. To determine whether PMN are involved causally in LPS/TVX hepatotoxicity, rats were pretreated with either control serum or anti-PMN serum before treatment with Veh/Veh or LPS/TVX. Cotreatment with LPS and TVX led to increased numbers of circulating PMN band cells (immature PMN) but no change in mature PMN. Both mature PMN and PMN band cells were reduced in circulating blood from rats treated with PMN-depleting antiserum (Table 2). Cotreatment with LPS and TVX caused accumulation of PMN in the liver, and this was reduced approximately 75% by pretreatment with anti-PMN serum (Fig. 7A). Liver injury was assessed by measuring serum ALT activity. PMN depletion significantly attenuated the liver injury induced by LPS/TVX treatment (Fig. 7B).
addition, histopathologic examination of the livers revealed a decrease in both the incidence and severity of the coagulative necrosis in LPS/TVX-treated rats that were PMN-depleted compared with those that were not (data not shown).

Discussion

We have applied a rat model of drug-inflammation interaction toward understanding hepatotoxicity induced by the quinolone antibiotic TVX. The predictive potential of the LPS-pre-treatment model has previously been suggested using RAN, famotidine, and chlorpromazine (Buchweitz et al., 2002; Luyendyk et al., 2003). Potentially, this model could be used to evaluate new drug entities to identify or predict liver toxicity liabilities in humans. Our data indicate that cotreatment of rats with normally noninjurious doses of TVX and LPS results in liver injury. This hepatotoxic interaction with LPS does not occur with LVX cotreatment, a drug that is in the same pharmacologic class but is devoid of idiosyncratic toxicity liabilities. Both hierarchical clustering and PCA analysis of gene expression from livers from LPS/TVX-treated rats revealed a unique expression profile compared with all other treatment groups.

It is presently not clear whether TVX enhances LPS hepatotoxicity or vice versa. However, the hepatic injury observed in rats cotreated with LPS and TVX was different at the histologic level from that occurring in rats treated with high doses of LPS alone. In our experience (Brown et al., 1996), acute histologic changes occurring after intravenous administration of LPS at toxic doses consist of panlobular leukocyte (mostly PMN) infiltration and widespread midzonal hepatic necrosis. In the current model, the histologic changes noted after the administration of LPS and TVX consisted of randomly distributed (i.e., without a particular zonal distribution) focal areas of coagulative necrosis with frequent subcapsular localization. This difference in pattern of necrosis suggests that the LPS/TVX co-treatment might have resulted in a unique form of liver injury.

The variability that was observed in serum ALT activity in LPS/TVX-treated rats was not reflected in the gene array data; i.e., all of the LPS/TVX animals clustered together irrespective of the serum ALT activity and were clearly separated from the other groups. In the LPS/TVX-treated group, ALT activity ranged from 48 (normal) to over 200 U/l. Despite this, the hepatic gene expression patterns from these rats were quite similar. Thus, at least for this model, gene expression analysis seems to be a less variable indicator of liver toxicity than serum ALT.

Similar to the gene expression results obtained in TVX-treated human hepatocytes (Liguori et al., 2005), we observed that numerous genes involved in RNA processing and regulation of transcription were uniquely regulated in the LPS/TVX-treated rats relative to other treatments (Fig. 5B). Some quinolone agents have been shown to interact with eukaryotic type II topoisomerase (Anderson and Osheroff, 2001; Sissi and Palumbo, 2003). Ciprofloxacin, for example, is a modest enhancer of DNA cleavage mediated by eukaryotic topoisomerase II (Anderson and Osheroff, 2001). It is possible that TVX has a higher affinity for eukaryotic polymerase II system than the other quinolone agents tested. This effect coupled with other factors, such as an inflammatory response, might result in a hepatotoxic reaction.

Previously, we have reported that, unlike responses in human hepatocytes, gene expression profiles from rats treated with TVX or LVX alone did not show differential regulation of genes involved in RNA processing and regulation of transcription (Liguori et al., 2005). Thus, LPS cotreatment in rats seemed to provoke gene expression changes similar to what was observed in isolated human hepatocytes. One possibility for this similarity could be that the isolated hepatocytes might have come from human donors that had experienced an inflammatory reaction in vivo prior to the hepatocyte isolation. If so, then the hepatocytes would have been exposed to inflammatory mediators before isolation, thus explaining why their response to the drugs looks more like that which occurs in a modestly inflamed rat liver. Another possibility is that the in vitro conditions, such as stress, decreased cell-to-cell contact, etc., might
Fig. 5. Selective expression of genes in rats treated with LPS/TVX. Heatmap (top) showing the expression of approximately 220 genes that were differentially regulated in LPS/TVX-treated rat livers versus other treatment groups. The genes were identified by performing a t test/ANOVA, comparing the LPS/TVX treatment group versus all other of the treatment groups and selecting genes with a p value of 0.000001 or less. Bottom, heatmap showing regulation of genes involved in RNA transcription.
mimic the LPS-treatment in vivo. Further studies are needed to address this possibility.

Several genes were regulated in the LPS/TVX-treated rats that raise interest as potential biomarkers to identify compounds that have IDR liability or that could provide clues to mechanisms of the pathogenesis. One gene product that was differentially regulated by LPS/TVX treatment is IL-6. IL-6 was shown to be a reliable biomarker for severe sepsis in humans (Damas et al., 1992), and mice given lethal or non-lethal doses of LPS could be distinguished based on the levels of circulating IL-6 (Kelly and Cross, 1992). In our study, the expression of IL-6 was greater in all LPS/TVX-treated rats compared with other treatment groups. Interestingly, one rat treated with LPS/LVX showed increased expression of IL-6, and this rat was the only one in that treatment group that had an area of coagulative necrosis in the liver.

Another gene of interest was GM-CSF, which was up-regulated relative to Veh/Veh control in all rats in the LPS/TVX treatment group and was not regulated by any of the other treatments (Fig. 6A). GM-CSF could be a key modulator in the hepatotoxicity seen in LPS/TVX-treated rats. Pretreatment of mice with GM-CSF markedly potentiated systemic hepatotoxicity induced by a subtoxic dose of LPS (Tiegs et al., 1994). It might be that certain drugs activate GM-CSF under some situations, which would make an individual more susceptible to endotoxin-mediated hepatotoxicity. Exactly how GM-CSF increases sensitivity to endotoxin is not certain. One possibility is that it modulates TNF-α formation or release (Bundschuh et al., 1997). Although differential expression of the gene encoding TNF was not observed in LPS/TVX-treated rats, the gene encoding TNF-induced protein was up-regulated uniquely in the LPS/TVX-treated group, suggesting that TNF-α activity may have been increased. Other genes that are regulated by GM-CSF were also differentially regulated by LPS/TVX treatment, including IL-6 (Lemaire et al., 1996), matrix metalloproteinase-9 (Takafuji et al., 2003), and mitogen-activated protein kinase kinase-1 (Suzuki et al., 1999).

Two additional genes that might serve as biomarkers are CINC-1 and MIP-2. LPS/TVX treatment up-regulated the expression of these genes in the liver, and both were elevated selectively at the protein level in serum from LPS/TVX-treated rats but not from LPS/LVX-treated animals. Thus, if this model was to be used to screen new chemical entities, ELISA for CINC-1 and MIP-2 might be performed on serum from treated rats to identify whether liver injury, similar to that seen with LPS/TVX, might occur.

The differential regulation of chemokine members, such as CINC-1 and MIP-2, and the up-regulation of GM-CSF suggest a role for PMN in the injury induced by LPS/TVX treatment. The C-X-C chemokine family comprises chemottractants that contribute to hepatic PMN accumulation during

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leukocytes (cells/μL)</th>
<th>Mature PMNs (cells/μL)</th>
<th>Monocytes (cells/μL)</th>
<th>Band Cells (cells/μL)</th>
<th>Lymphocytes (cells/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control serum/Veh/Veh</td>
<td>754 ± 430</td>
<td>1238 ± 183</td>
<td>249 ± 28</td>
<td>51 ± 32</td>
<td>5932 ± 367</td>
</tr>
<tr>
<td>Control serum/LPS/TVX</td>
<td>4773 ± 423*</td>
<td>1166 ± 182</td>
<td>159 ± 26</td>
<td>666 ± 85*</td>
<td>2770 ± 283*</td>
</tr>
<tr>
<td>Anti-PMN serum/LPS/TVX</td>
<td>2316 ± 258***</td>
<td>107 ± 47*</td>
<td>71 ± 8***</td>
<td>157 ± 30*</td>
<td>1959 ± 232*</td>
</tr>
</tbody>
</table>

* Significantly different from Control serum/Veh/Veh-treated group; † significantly different from Control serum/LPS/TVX-treated group (p < 0.05).
endotoxemia (Calkins et al., 2002). Treatment with GM-CSF causes PMN accumulation (Shinohara et al., 2000), and PMN have been implicated in several liver injury processes, including endotoxemia, hepatic ischemia-reperfusion, and sepsis (Jaeschke et al., 1990, 1991; Hewett et al., 1993; Molnar et al., 1997). During endotoxemic reactions, PMN can cause hepatotoxicity in part through generation of reactive oxygen species (Gujral et al., 2004). Consistent with this, genes involved in oxidative stress were up-regulated in LPS/TVX-treated rats relative to Veh/Veh controls. Collectively, these observations were consistent with a role for PMN in injury from cotreatment with LPS/TVX.

PMN depletion prevented hepatocellular injury in LPS/TVX-treated rats. A similar result was seen with rats cotreated with LPS and RAN (Luyendyk et al., 2005). The PMN antiserum caused approximately 90% depletion of blood PMN and markedly reduced liver PMN accumulation, strongly suggesting that its hepatoprotective effect was due to depletion of these cells. Although selective, the anti-PMN serum also caused a more modest but statistically significant decrease in blood monocytes, thus a role for these cells in the liver injury cannot be ruled out. Nonetheless, the results with TVX and RAN do suggest a role for PMN in these drug-inflammation interaction models. Potentially, a combination of an inflammatory stimulus concurrent with exposure to certain drugs could lead to accumulation and activation of PMN, which could cause hepatocyte death. Further studies are needed to identify the potential for drugs that cause human IDR to promote PMN accumulation and/or activation during a concurrent inflammatory response.

In summary, TVX, a drug that causes idiosyncratic hepatotoxicity in humans, was found to cause liver injury in rats cotreated with a nontoxic dose of LPS. By contrast, LVX, which is devoid of idiosyncratic liver liability, did not interact with LPS to produce liver injury. We identified several differentially regulated genes associated with the toxicity induced by LPS/TVX cotreatment. The selective up-regulation of genes encoding for chemokines identified in the gene array study led to the finding that PMN are critical for liver injury. Ultimately, similar drug-inflammation models might be used to predict liver toxicity liabilities of new chemical entities in humans. Further steps in the characterization of such models include the evaluation of additional IDR-associated drugs and their pharmacologically comparable negative comparators. Using this approach, it may be possible to narrow in on genes more specifically correlated with idiosyncratic toxicity. Ultimately, these investigations may be extended to the clinic to confirm and validate the hypotheses generated from these studies.

References