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Regulation of Hepatic Cytochrome P450 Expression in Mice with Intestinal or Systemic Infections of *Citrobacter rodentium*[S]

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ABSTRACT:

We reported previously that infection of C3H/HeOuJ (HeOu) mice with the murine intestinal pathogen *Citrobacter rodentium* caused a selective modulation of hepatic cytochrome P450 (P450) gene expression in the liver that was independent of the Toll-like receptor 4. However, HeOu mice are much more sensitive to the pathogenic effects of *C. rodentium* infection, and the P450 down-regulation was associated with significant morbidity in the animals. Here, we report that oral infection of C57BL/6 mice with *C. rodentium*, which produced only mild clinical signs and symptoms, produced very similar effects on hepatic P450 expression in this strain. As in HeOu mice, CYP4A mRNAs and proteins were among the most sensitive to down-regulation, whereas CYP4F18 was induced. CYP2D9 mRNA was also induced 8- to 9-fold in the C57BL/6 mice. The time course of P450 regulation followed that of colonic inflammation and bacterial colonization, peaking at 7 to 10 days after infection and returning to normal at 15 to 24 days as the infection resolved. These changes also correlated with the time course of significant elevations in the serum of the proinflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α, as well as of interferon-γ and IL-2, with serum levels of IL-6 being markedly higher than those of the other cytokines. Intraperitoneal administration of *C. rodentium* produced a rapid down-regulation of P450 enzymes that was quantitatively and qualitatively different from that of oral infection, although CYP2D9 was induced in both models, suggesting that the effects of oral infection on the liver are not due to bacterial translocation.

Bacterial infection and/or inflammation down-regulate both hepatic and extrahepatic cytochrome P450 (P450) expression and other proteins involved in drug metabolism and transport (for reviews, see Hoshino et al., 1999; Renton, 2004; Aitken et al., 2006; Petrovic et al., 2007; Morgan et al., 2008), leading to altered drug responses that may result in significant variations in clinical drug therapy. The bacterial endotoxin, lipopolysaccharide (LPS), has been used extensively as a sterile model of sepsis to study the down-regulation of P450s during inflammation or infection and has been an invaluable tool in understanding the importance and mechanisms of hepatic P450 regulation in inflammation. LPS is the major constituent of the outer membrane of Gram-negative bacteria that produces inflammation upon injection and elicits its actions through Toll-like receptor 4 (TLR4) (Poltorak et al., 1998) in association with several proteins, including LPS-binding protein, CD14, and MD-2 proteins (Beutler et al., 2001). However, it is hard to predict accurately from the LPS model how P450 enzymes will be regulated in other models of inflammation or in live bacterial infections. The fact that inflammatory cytokines differentially regulate P450 gene expression (Craig et al., 1990; Chen et al., 1995; Aitken and Morgan, 2007) led us to postulate that the LPS model and other chemical models of inflammation may not predict how P450 enzymes will be regulated in a live infection.

Citrobacter rodentium, a member of the family of enteropathogens, provides an excellent in vivo model to investigate pathogen-host interactions under physiological conditions with the ability to modify host/bacterial conditions. It is a natural murine pathogen that uses attaching and effacing lesions to colonize the host's gastrointestinal tract (Jurjus et al., 2004; Wales et al., 2005), and its infection in mice is equivalent to human enteropathogenic *Escherichia coli* (EPEC) infection (Schauer and Falkow, 1993) and colitis. EPEC is a specific serotype of *E. coli* and is the major cause of infantile diarrhea worldwide (Nataro and Kaper, 1998), which is associated with considerable mortality in developing countries. The colitis caused by *C. rodentium* infection is also characteristic of inflammatory bowel disease in mice and humans, and so *C. rodentium* may be a live model

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ABBREVIATIONS: P450, cytochrome P450; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; EPEC, enteropathogenic *Escherichia coli*; HeOu, C3H/HeOuJ; HeJ, C3H/HeJ; CFU, colony-forming unit; RT, reverse transcriptase; PCR, polymerase chain reaction; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MIP-1α, macrophage inflammatory protein 1α; MCP-1, monocyte chemotactic protein-1; KC, chemokine CXCL1; RANTES, chemokine CCL5; ANOVA, analysis of variance; p.i., postinfection.

for inflammatory bowel disease (Higgins et al., 1999; Caradonna et al., 2000; Goncalves et al., 2001; Gobert et al., 2004).

We previously investigated hepatic P450 expression during *C. rodentium* infection in wild-type C3H/HeOuJ (HeOu) mice and in C3H/HeJ (HeJ) mice deficient in functional TLR4, a critical component of LPS signaling. We found that *C. rodentium* infection produced effects on hepatic P450 expression that were more enzyme-selective than the effects produced by LPS injection and were largely independent of TLR4 activation (Richardson et al., 2006). HeOu mice are much more sensitive to the pathogenic effects of *C. rodentium* infection than are most other mouse strains, and the P450 down-regulation was associated with significant morbidity in the animals. Because the host and pathogen factors that differentially regulate P450 expression during the course of infection are not known, there could be strain differences in the P450 responses to infection. Therefore, the goals of this study were as follows: 1) to determine whether or not liver P450 expression is selectively modulated during *C. rodentium* infection in a mouse strain that exhibits minimal morbidity or mortality during infection; 2) to determine whether the effects of infection are reversible and to correlate the hepatic P450 regulation with serum cytokines and colonic inflammation by characterizing the time course of P450 regulation; and 3) to determine whether acute systemic infection with *C. rodentium*, resulting from i.p. injection, produced changes in the liver similar to those caused by oral infection.

For this study, we selected the C57BL/6 strain, because it has been well characterized as a host for *C. rodentium*, displaying relatively low mortality upon infection (Vallance et al., 2003), and because it serves as the genetic background for many targeted genetic mutants. We report that the pattern of hepatic P450 gene regulation in these mice that exhibited negligible clinical signs or symptoms was very similar to that observed previously in the highly susceptible HeOu strain. The regulation of P450 expression in the liver was reversible and followed the time course of colonic bacterial colonization and pathology. However, the pattern of acute P450 regulation produced by parenteral injection of *C. rodentium* was more characteristic of the less selective effects reported previously for LPS injection.

Materials and Methods

Bacteria. A wild-type strain of *C. rodentium* (no. 51116) was obtained from the American Type Culture Collection (Manassas, VA). Before infection, *C. rodentium* was grown in Luria broth without shaking overnight and harvested by centrifugation. Bacteria were resuspended in sterile phosphate-buffered saline, and the nominal concentration was calculated spectrophotometrically. Actual concentrations were determined by retrospective plating on MacConkey agar, on which *C. rodentium* forms small pink colonies with white rims.

Chemicals, Animals, and Treatments. Unless otherwise specified, all of the reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were acclimatized to the animal facility for at least 1 week, and mice were 7 to 9 weeks of age at the time of infection. Mice were housed and sacrificed in a Biosafety Level-2 facility to prevent transmission of infection to other mouse colonies. Mice were housed in groups of six to a cage. Oral administration of *C. rodentium* was achieved by infection of the drinking water (with 20% sucrose) for 24 h at a nominal concentration calculated to result in a bacterial dose of 7×10^8 cells/mouse. The actual doses for each group were calculated from the volume consumed by each group of mice times the concentration of *C. rodentium* determined by retrospective plating. Actual doses for each group were 8, 7, 6, and 6×10^8 bacteria/mouse for the 7-, 10-, 15-, and 24-day groups, respectively. We included two control groups: normal control and pair-fed control. The amount of food consumed by each cage of infected mice was measured and the amount of food consumed per day per mouse was calculated. The same amount of food was offered to the pair-fed control group the next day. Both sets of control groups received 20% sucrose in their drinking water on the first day. Six mice from each group were sacrificed at 7, 10, 15, and 24 days after administration of sucrose or bacteria. Livers and a piece of distal colon were collected. Livers were rinsed in cold 1.15% potassium chloride and stored at −80°C for later RNA or microsome preparation. Colon samples were washed for fecal content in saline solution and fixed in 10% neutral buffered formalin solution until they were processed for histological analysis. They were stained with hematoxylin and eosin for histopathological evaluation. Blood was also collected from all of the animals and allowed to clot for 30 min. Serum was separated immediately by centrifugation and stored at −20°C until analyzed.

For the mice infected by i.p. administration of *C. rodentium*, groups of six ad libitum-fed, 10-week-old female C57BL/6 mice received injections of either saline (control group) or 10^4, 10^6, or 10^8 CFUs of *C. rodentium*, and they were killed 24 h later. In the time course experiment, mice received either saline or 10^8 CFUs and were killed at the indicated times. Livers, pieces of colon, and serum were collected and stored as stated above. All of the protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

Preparation of Total RNA. Total liver RNA was prepared using RNA-Bee isolation reagent according to the manufacturer's instructions (Tel-Test Inc., Friendswood, TX). Total RNA concentration was estimated spectrophotometrically at 260 nm, and RNA purity and integrity were confirmed by formaldehyde-agarose gel electrophoresis followed by visualization with ethidium bromide.

Preparation of Postmitochondrial and Microsomal Fractions. Postmitochondrial supernatants were prepared by centrifuging liver homogenates at 13,000*g* for 25 min. Liver microsomes were prepared from the supernatants by centrifugation at 250,000*g* for 45 min. The microsomal pellets were resuspended in 10 mM Tris acetate buffer (pH 7.4), containing 0.1 mM EDTA and 23% glycerol, and stored at −80°C. Protein concentrations of the fractions were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockport, IL) with bovine serum albumin as the protein standard.

cDNA Synthesis. Purified total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System for the reverse transcriptase (RT)-polymerase chain reaction (PCR) kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

Primer Sequences. Primers for CYP3A25 and CYP3A41 were from Martignoni et al. (2006), and for CYP2D9, CYP2D10, and CYP2D22 were from Blume et al. (2000). For mouse CYP4F mRNAs, specific sequences for PCR primers and dual-labeled fluorescent probes were designed at the region of the highest sequence specificity or intron/exon boundaries using Primer Express software (Applied Biosystems, Foster City, CA) and custom synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The isoform specificity of P450 primers and probes was confirmed through multiple alignments with the other members of the CYP4F subfamily as well as homology-searched to ensure that there was no cross-reactivity with other genes using a National Center for Biotechnological Information BLAST search. The primer and probe sequences were CYP4F18 forward 5′ AGAGCCTGGTGCGAACCTT 3′, reverse TGGAATATGCGGATGACTGG, and probe 56Fam-TGCATGCTGCT-GGTGGGTGG-Tamra; CYP4F15 forward CATGACATGGCTGGGTCCTA, reverse GAGGCATTGAGAACAGATCGA, and probe CCTATCATCAC-TCTGTGCCACCCTGACAT-3BHQ1; and cyclophilin forward CGATGAC-GAGCCCTTGG, reverse TCTGCTGTCTTTGGAACTTTGTC, and probe Fam-CGCGTCTCCTTCGAGCTGTTTGCA-Tamra.

All primers for other mouse P450s cytokines, acute-phase proteins, and glyceraldehyde phosphate dehydrogenase have been described and used by our laboratory previously (Richardson et al., 2006). All of the primers were custom-synthesized on a 50-nmol scale by MWG Biotech (High Point. NC) and obtained desalted and lyophilized. Primers were diluted to 100 μM in deionized water and stored at −80°C.

Quantitative Real-Time RT-PCR. With the exception of the CYP4Fs, real-time RT-PCR was performed using the ABI PRISM 7000 Sequence Detection System and SYBR Green to determine the expression of mRNAs of interest in mouse liver, as described previously (Richardson and Morgan, 2005). In brief, duplicate reactions were performed in a total volume of 25 μl using SYBR Green Master Mix reagent (Applied Biosystems, Bedford, MA); 2 μl of cDNA/sample was used as template for the reaction, with 10 μM forward and reverse primers. Thermal cycling conditions included 2 min at

50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 1 min at the appropriate annealing temperature. Results are expressed as relative levels of target mRNA, normalized to levels of the housekeeping gene glyceraldehyde phosphate dehydrogenase, as calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). The expression level in control samples was arbitrarily set at 1. All primer sets yielded a single PCR product of expected size as determined by agarose gel electrophoresis. Specificity was routinely monitored by checking product dissociation curves in each reaction well. Analysis of mouse P450 4Fs was achieved via TaqMan technology, as we described previously (Richardson et al., 2006). The relative amounts of each CYP4F mRNA in samples were determined by normalizing the values for copy number of the gene of interest to the copy number values of *m*-cyclophilin.

Western Immunoblotting. P450 protein levels in mouse hepatic microsomes or postmitochondrial supernatants were measured by SDS-polyacrylamide gel electrophoresis and Western blotting, as described previously by Richardson and Morgan (2005). Equal amounts of protein were loaded on the gels for each assay. Five to 10 μg of microsomes or 30 to 75 μg of postmitochondrial supernatants was used, depending on the sensitivities of each antibody. Antibodies to rat CYP3A, CYP4A, and CYP2E were generously provided by Dr. James Halpert (University of Texas Medical Branch, Galveston, TX), Dr. Gordon Gibson (University of Surrey, Guildford, UK), and Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden), respectively. Polyclonal antibodies to rat CYP3A, CYP4A, and CYP2E proteins were diluted 1:5000, whereas CYP2C antibody was diluted 1:20,000. Secondary antibodies were as follows: goat anti-rabbit, 3A, 2C, and 2E, and rabbit anti-sheep, 4A; dilution for each was 1:2500, with the exception of 2C, for which dilution was 1:10,000. All assays were performed within a linear range, and the intensity of stained bands was measured by laser densitometry.

Determination of Tissue Bacterial Loads. To measure the CFUs of *C. rodentium* in colon and liver, tissue samples weighing approximately 0.1 to 0.3 g were homogenized at low speed with a Tissuemizer (Thermo Fisher Scientific) in 1 ml of phosphate-buffered saline. The lysate was plated on MacConkey agar plates at various dilutions, and *C. rodentium* colonies were recognized as pink with a white rim as described previously (Vallance et al., 2003) and were counted after 20 h of incubation at 37°C to determine the CFUs per gram of tissue.

Colon and Liver Pathology. Paraffin-embedded colonic tissue sections stained with hematoxylin and eosin were examined by an observer blinded to the experimental condition. Tissues were scored (0, normal; 1, mild; 2, moderate; and 3, severe) for the following observations: edema, hyperplasia, ragged epithelium, neutrophil infiltration, goblet cell depletion, and obvious bleeding. For crypt height measurements, three measurements of well oriented crypts were taken in the distal colon for each mouse, using micrometry by a 200M microscope (Zeiss, Thornwood, NY) with a 20× NA1.4 lens and Slidebook (Intelligent Imaging Innovations, Denver, CO). The livers of the mice were removed and placed in 10% neutral buffered formalin for 2 days for fixation. The livers were sectioned by a microtome at 5 μm and stained with hematoxylin and eosin. Paraffin-embedded liver tissue sections stained with hematoxylin and eosin were evaluated by a veterinary pathologist blinded to the experimental condition for the presence of inflammation containing neutrophils and macrophages or degeneration and fibrosis and necrosis. The presence of liver changes was scored (0, normal; 1, minimal; 2, mild; 3, moderate; and 4, severe).

Cytokine Analysis. Serum samples were analyzed for cytokines using 25 μl of serum from each sample using xMAP technology (Luminex, Austin, TX) with a single anti-mouse LINCOplex kit (Linco Research, St. Charles, MO). The cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), granulocyte macrophage colony-stimulating factor, macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein-1 (MCP-1), chemokine CXCL1 (KC), chemokine CCL5 (RANTES), IL-9, and IL-13 were analyzed by following the protocol supplied with the kit. In brief, 96-well plate filter plates were washed with wash buffer, and samples (1:1 dilution, 50 μl final volume) were applied to each well. Antibody-coated beads were added to the wells, and the samples were incubated for 18 h at 4°C. After incubation, the plate was washed twice with buffer supplied with the kit. Biotinylated antibodies against the different cytokines and chemokines from the kit were added, and the mixture was incubated for 1 h at room temperature. Then the cytokine-antibody complexes were detected by addition of streptavidin coupled to phycoerythrin. For determination of the number of positive complexes, each sample was read in a Luminex XYP platform. Data were analyzed using MasterPlex 1.2 (Hitachi Software Engineering America, Ltd., MiraiBio Group, South San Francisco, CA), and the concentrations are expressed in picograms per milliliter.

Statistical Analysis. For data with continuous variables, differences between groups were determined by one-way ANOVA followed by Tukey's or Dunnett's test as appropriate. For discrete pathological data, a Wilcoxon Mann-Whitney rank-sum test or a Kruskal-Wallis test followed by a Wilcoxon Mann-Whitney rank-sum test with Bonferroni correction were used as dictated by the experimental design. The level of significance was set at $P < 0.05$.

Results

Studies on Oral Infection with *C. rodentium* in C57BL/6 Mice. C57BL/6 mice have been well characterized as a host for *C. rodentium.* Colonic colonization peaks at ~6 to 10 days in mice of this strain (Vallance et al., 2003). Inflammatory cell infiltration of the colon is maximal at days 10 to 18, and this corresponds with the observed colonic pathology (Deng et al., 2004). Therefore, we studied hepatic P450 expression at 7, 10, 15, and 24 days of infection to encompass the peak of colonization and colonic pathology and the resolution phase. To control for the possible effects of reduced food intake on P450 expression, we included both untreated control groups and pair-fed groups at each time point.

Pathology and bacterial loading of the liver and colon. Bacterial colonization of the colon was significant at 7 days postinfection (p.i.), reached its peak at 10 days (Supplemental Fig. 1A), and was negligible at days 15 and 24 p.i. Colon weights were not significantly increased until 10 days p.i., and the increase persisted at 15 days p.i. (Supplemental Table 1). Infection was associated with mucosal hyperplasia, ragged epithelia, edema, and increased crypt length on day 7 p.i. (Supplemental Fig. 2; Supplemental Table 2), each of which persisted through day 15 and normalized by day 24 p.i. (Supplemental Table 2). PMN infiltration was significant by day 10, and persisted through day 24 p.i. A significant increase in goblet cells was only observed on day 10 p.i. (Supplemental Table 2).

C. rodentium cells were detected in the liver after infection, and the time course paralleled that of colonic colonization (Supplemental Fig. 1B). However the bacterial load in the liver was 50,000 times lower than that in the colon. Infected mice had increases in liver weight of 37, 16, and 25% at 7, 10, and 15 days p.i., respectively (Supplemental Table 1). Livers of infected mice at 7 days p.i. had minimal to mild lymphocytic inflammation (Supplemental Table 3; Supplemental Fig. 3) characteristic of a bacterial infection. By day 10 p.i, inflammation was minimal (Supplemental Table 3).

Food intake and body weights. The food intakes of the infected mice were more variable from day to day than those of the control mice, but over the first 1 to 10 days p.i., they did not differ in a consistent manner from that of the untreated animals (Supplemental Fig. 4A). It is interesting to note that the infected mice tended to eat more than the untreated animals in the resolution phase of the infection. Consistent with previous observations (Vallance et al., 2002), the body weights of the infected mice dropped significantly at day 10 p.i. and recovered shortly thereafter (Supplemental Fig. 4B). No such effect was observed on the body weights of the pair-fed animals (Supplemental Fig. 4B).

Serum cytokines. Serum levels of IL-1β, IL-4, IL-5, IL-9, IL-10, IL-12, and granulocyte macrophage colony stimulating factor were not significantly different from those of pair-fed mice at any time point (data not shown). The time courses of serum cytokines that differed between infected and pair-fed mice were highly similar, peaking between 7 and 10 days and returning to baseline at 15 days p.i. (Fig. 1). IL-6 was the most abundant cytokine in the plasma of

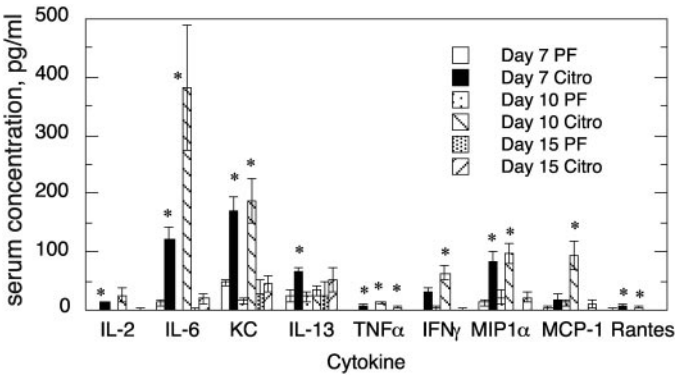

FIG. 1. Serum cytokine profile during *C. rodentium* (Citro) infection in mice. Mice were infected with *C. rodentium* p.o., and blood was collected at sacrifice for measurement of serum cytokines as described under *Materials and Methods*. Values represent means ± S.E.M. of six mice per group, with the exception of the 15-day pair-fed group (five mice). *, $P < 0.05$ compared with pair-fed (PF) control. Differences between groups were determined by Student's *t* test.

infected mice, peaking at 380 pg/ml (Fig. 1). Levels of the chemokine KC (mouse IL-8 analog) were approximately 200 pg/ml, whereas levels of MCP-1, MIP-1α, IL-13, and IFN-γ peaked between 50 and 100 pg/ml. Peak levels of IL-2, RANTES, and the proinflammatory cytokine TNF-α were all <25 pg/ml.

Hepatic CYP4A expression. Our previous work showed that enzymes of the CYP4A subfamily exhibited the most profound changes in response to *C. rodentium* infection of C3H/HeOuJ or HeJ mice. As shown in Fig. 2A, the mRNA for CYP4F18 was induced 8-fold in the infected mice but not in the pair-fed C57BL/6 mice at 7 and 10 days p.i. Its expression was still significantly induced by 2.75-fold at 15 days p.i., but had returned to only 1.4-fold of control by day 24. CYP4A12 mRNA was unaffected (Fig. 2A), whereas CYP4A10, 4A14, and 4F15 mRNAs were down-regulated at 7 and 10 days p.i., with each mRNA returning to control (or slightly above) within 15 days p.i (Fig. 2B). The down-regulation of hepatic microsomal CYP4A proteins followed a time course similar to that of the CYP4A mRNAs, except that the down-regulation of the proteins persisted at 15 days p.i. (Fig. 2, C and D). Unlike the CYP4A mRNAs, which were slightly induced at 7 days in the pair-fed mice, CYP4A proteins were unaffected at 7 days of pair-feeding and slightly suppressed at 15 days of infection (Fig. 2, B–D). On the basis of observations that most of the parameters measured had returned to baseline or near baseline by day 15, we limited further measurements to the 7, 10, and 15 day p.i. samples only.

Expression of hepatic drug-metabolizing P450 subfamilies 1, 2, and 3. Of the 11 members of the drug-metabolizing P450 families whose mRNAs we measured, four exhibited significant differences between the untreated and pair-fed control groups at 7 and 10 days p.i.: 2A5 and 2E1 (Fig. 3A) and 3A25 and 3A41 (Fig. 3B). Pair-feeding reduced CYP3A41 mRNA expression but induced the 2A5, 2E1, and 3A25 mRNAs. All of the family 1, 2, and 3 P450 mRNAs examined, with the exception of CYP3A13 (Fig. 3B) and 2D9 (Fig. 3C), were down-regulated in *C. rodentium*-infected mouse livers (Fig. 3). CYP1A2, 2B9, 2C29, 2D22, 3A11, 3A25, and 3A41 were suppressed whether compared with pair-fed or untreated controls, whereas CYP2A5 and CYP2E1 were only suppressed when compared with pair-fed mice. Most of the down-regulated P450 mRNAs were suppressed to ~50% of untreated levels, except for CYP2B9, which was suppressed to 25% of the level in untreated mice (Fig. 3). In each case, the degree of suppression was approximately equivalent in the 7- and 10-day p.i. groups and had returned to control levels by 15 days p.i. The exception was CYP1A2, which was not affected until 10 days p.i. and remained below control levels at 15 days. In contrast, CYP2D9 mRNA was induced 9.5- and 4.8-fold in the livers of infected mice at 7 and 10 days p.i., respectively (Fig. 3C). CYP3A13 mRNA was unchanged in the day 7 and day 15 p.i. mice, although it was significantly induced 1.4-fold at 10 days p.i. (Fig. 3B).

C. rodentium infection caused a down-regulation of CYP2B, 2C, 2E, and 3A proteins relative to both untreated and pair-fed controls at 7 days p.i (Fig. 4). Whereas CYP2B, 3A, and 2E proteins were still down-regulated at 10 days p.i. and returned to control levels at 15 days p.i., CYP2C proteins were not different from controls at 10 days p.i. (Fig. 4). CYP2B proteins were significantly elevated after 7 days of pair-feeding compared with untreated mice. The CYP2D9 antibody recognized two proteins in mouse liver microsomes, and it appeared that both bands were affected similarly by infection (Fig. 4A). At 7 days p.i., CYP2D proteins were down-regulated in both pair-fed and infected mice, whereas CYP2D protein expression increased progressively at later time points to become significantly induced at 15 days p.i. (Fig. 4).

Hepatic cytokine and acute-phase mRNAs. Hepatic expression of the acute-phase protein α_1-acid glycoprotein was induced by 6- and 8-fold, respectively at 7 and 10 days p.i. but returned to near baseline by 15 days (Fig. 3C). Hepatic mRNAs for IL-1, IL-6, and TNFα were each induced 3-fold, but only at day 10 p.i. (Fig. 3C).

Studies on Parenteral Infection with *C. rodentium*. Because we detected low, but significant, levels of *C. rodentium* cells in the livers of infected mice, we next studied the effect of systemic infection with *C. rodentium* on hepatic cytochrome P450 expression. In the first experiment, mice were treated i.p. with various doses of *C. rodentium* cells and killed 24 h later for analysis.

Mice that received injections of the lowest dose of *C. rodentium* cells (10^4/mouse) did not show any behavioral differences compared with the control group, but at the highest dose of bacteria (10^8/mouse) the mice became very weak and sluggish. Only the highest dose of bacteria (10^8/mouse) caused a significant decrease in body weight of 15% (Supplemental Fig. 5A). The total bacterial contents of the livers of the infected animals were significantly elevated in the two highest dose groups (Supplemental Fig. 5B).

The bacterial dose dependencies of the effects of i.p. *C. rodentium* injection on hepatic P450 mRNA expression are shown in Fig. 5. The mRNAs of CYP1A2, 2A5, 2B9, 2C29, 3A11, 3A25, and 3A41 showed similar patterns of down-regulation, each reaching a nadir of 10 to 20% of control levels at the highest bacterial dose (Fig. 5A). CYP1A2 and CYP3A25 mRNAs were significantly down-regulated at the lower doses as well (Fig. 5A). CYP4A14 and CYP2E1 mRNAs were unaffected at any bacterial dose (Fig. 5B), whereas CYP4A10 and CYP2D22 were down-regulated to 34 and 39% of control, respectively, at the highest dose. CYP 3A13 mRNA was significantly induced 1.5-fold at the 10^6 cell dose (Fig. 5B), whereas CYP2D9 was induced 2.5- and 4-fold by the 10^6 and 10^8 cell doses, respectively (Fig. 5C). The mRNAs of the acute-phase proteins α_1-acid glycoprotein and fibrinogen-α were induced with dose dependencies similar to that of CYP2D9, whereas angiotensinogen was unaffected (Fig. 5C). Of the hepatic cytokine mRNAs, that of TNF-α was induced to a much greater extent (20-fold) than those of IL-1 and IL-6 (~3-fold) (Fig. 5D). TNF-α was also significantly induced (2.8-fold) at the 10^6 cell dose.

The regulation of hepatic P450 protein levels in the parenterally infected mice is shown in Fig. 6. CYP2C and CYP3A proteins were down-regulated to 59 and 31% of control at the highest bacterial dose, correlating with the down-regulation of CYP2C29 and most of the CYP3A mRNAs studied. However, CYP2B, 2D, and 4A proteins levels were unaffected. CYP2E1 protein was induced >2-fold at the

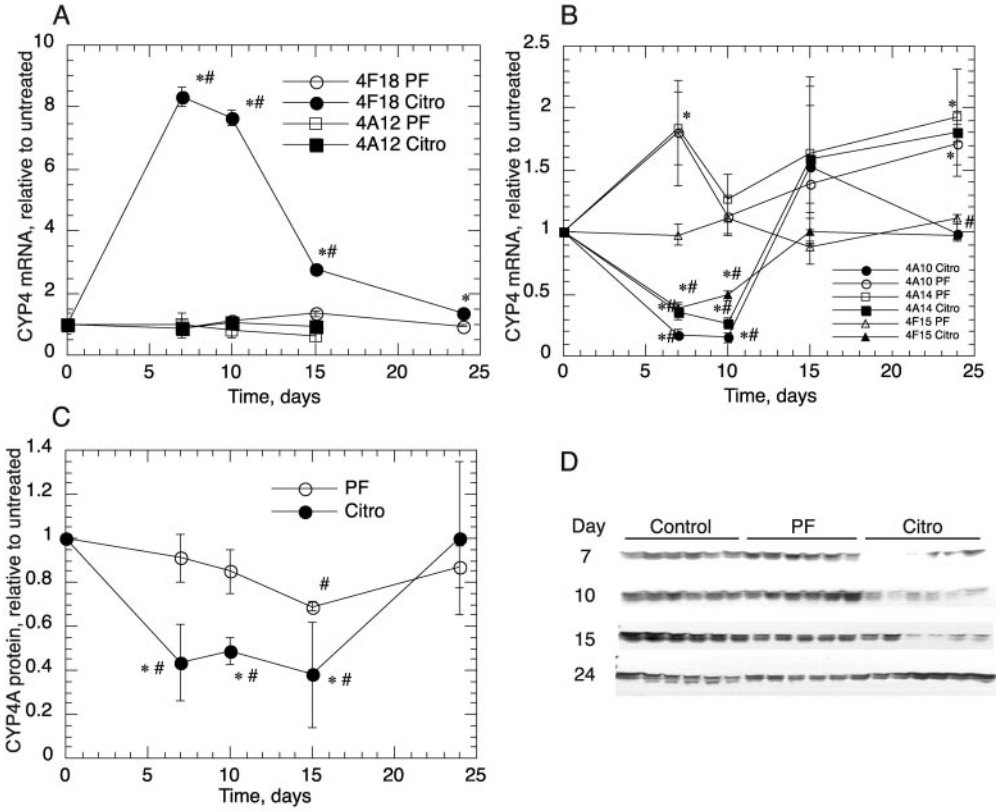

FIG. 2. Effect of oral *C. rodentium* (Citro) infection on CYP4A family mRNAs and proteins in mouse liver. Mice were infected with *C. rodentium* p.o., and livers were harvested at the indicated times for measurement of CYP4 mRNA and protein levels as described under *Materials and Methods*. A, CYP4F18 mRNA; B, CYP4A10, 4A14, and 4F15 mRNAs; C, CYP4 family proteins; D, Western blots from which the data in C were obtained. Values represent means ± S.E.M. of six mice per group, with the exception of the 15-day pair-fed (PF) group (five mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1. *, $P < 0.05$ compared with untreated; #, $P < 0.05$ compared with pair-fed. Differences among groups were determined by one-way ANOVA followed by Tukey's test.

two highest bacterial doses. Of cytokines measured in the serum 24 h after parental infection, TNF-α was moderately elevated at the highest bacterial dose, IL-1β was unaffected, and IL-13 was reduced (Supplemental Fig. 6). IL-6 was not measured in these samples.

We next studied the time course of P450 regulation in mice that received injections of the maximally effective dose of *C. rodentium*. CYP2A5 and CYP3A11 mRNAs were significantly down-regulated as early as 6 h after injection, whereas CYP2B9 was not significantly affected until 24 h after injection (Fig. 7A). CYP4A14 was not significantly affected at any time point, although there was a trend toward a decrease. The induction of CYP2D9 mRNA was first detected at 12 h after injection, and CYP3A13 was also significantly induced at that time point. TNF-α was the predominant proinflammatory cytokine induced in the liver after parenteral injection, peaking at 6 h (Fig. 7B). In contrast to CYP2B9 mRNA, CYP2B proteins were significantly down-regulated to 50% of control levels within 12 h of injection (Supplemental Fig. 7). However, CYP2D9, 3A, and 4A proteins were not affected at this time point (Supplemental Fig. 7).

Discussion

These studies demonstrate that subclinical oral infection of mice with the enteropathogenic bacterium *C. rodentium* causes selective and in some cases profound down-regulation of hepatic cytochrome P450 mRNA and protein levels. Moreover, these effects are reversed after the organism has been cleared from the gastrointestinal tract. In contrast, acute systemic infection with the same organism causes rapid and less selective down-regulation of most P450s studied.

C. rodentium infection of C57BL/6 mice (this study) and of HeOu mice (Richardson et al., 2006) shows a very similar profile of P450 regulation (Fig. 8), with the exception of CYP2A5. This is remarkable because HeOu mice are highly sensitive to the infection and showed substantial morbidity, whereas C57BL/6 mice exhibited no behavioral changes, and the only clinical signs were loose stool and perianal fecal staining. Reproduction in C57BL/6 mice of the findings from the sensitive HeOu strain will be crucial to future studies to elucidate the mechanisms of the observed down-regulation using genetically modified strains.

The small and variable effects of pair-feeding were more likely to be due to stress caused by competition for a limited food supply than to reduced caloric intake, because the food intake of mice was minimally affected by infection (Supplemental Fig. 1). Because any changes in feeding behavior are part of the physiological response of the animal to infection, we conclude that untreated controls are more appropriate for these studies.

The time course of P450 regulation in the liver after oral infection with *C. rodentium* was correlated with the time course of bacterial colonization of the colon. Thus, expression of most P450s had returned to normal or had begun to recover by day 15 when the majority of the infection had been cleared from the colon. Residual down-regulation of some P450s at 15 days may be due to the residual inflammation seen in the colons (Supplemental Tables 1 and 2).

C. rodentium attach to cells of epithelial nature and modulate intracellular signaling processes (Vallance et al., 2003). Although *C. rodentium* cells were detected in the livers of our mice after oral infection, it is unlikely that the bacteria are attaching to hepatocytes to affect hepatocyte P450 expression. The peak bacterial content of the livers was 25,000 cells/g of liver at 10 days of infection. With a conservative estimate of 3×10^7 hepatocytes/g of liver (Kim et al., 1997), this corresponds to approximately 1 bacterial cell/1000 hepatocytes. Because we observed mild to moderate inflammation in the liver at 7 days p.i., it is possible that bacterial activation of Kupffer cells caused the P450 down-regulation. Arguing against this possibility is the fact that we have already demonstrated that the P450 down-regulation is independent of bacterial LPS signaling via TLR4

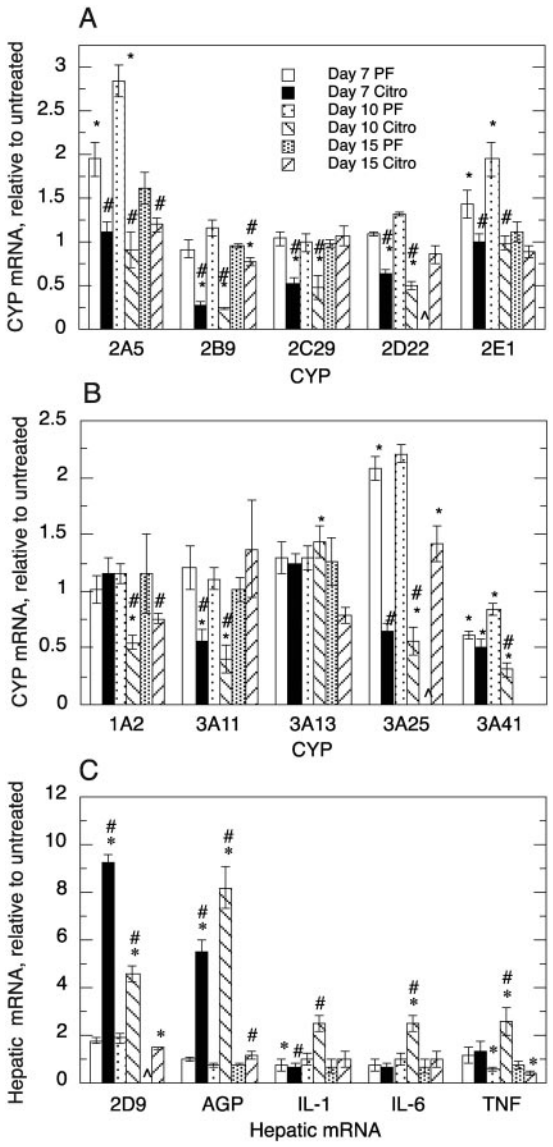

FIG. 3. Effect of oral *C. rodentium* (Citro) infection on P450, cytokine, and acute-phase protein mRNAs in mouse liver. Mice were infected with *C. rodentium* p.o., and livers were harvested at the indicated times for measurement of P450 mRNA levels as described under *Materials and Methods*. A, CYP2 family mRNAs; B, CYP1 and CYP3 family mRNAs; C, CYP2D9, cytokine and acute-phase mRNAs. Values represent means ± S.E.M. of six mice per group, with the exception of the 15-day pair-fed (PF) group (five mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1. *, $P < 0.05$ compared with untreated; #, $P < 0.05$ compared with pair-fed. ∧, not determined. Differences among groups were determined by one-way ANOVA followed by Tukey's test. AGP, α_1-acid glycoprotein.

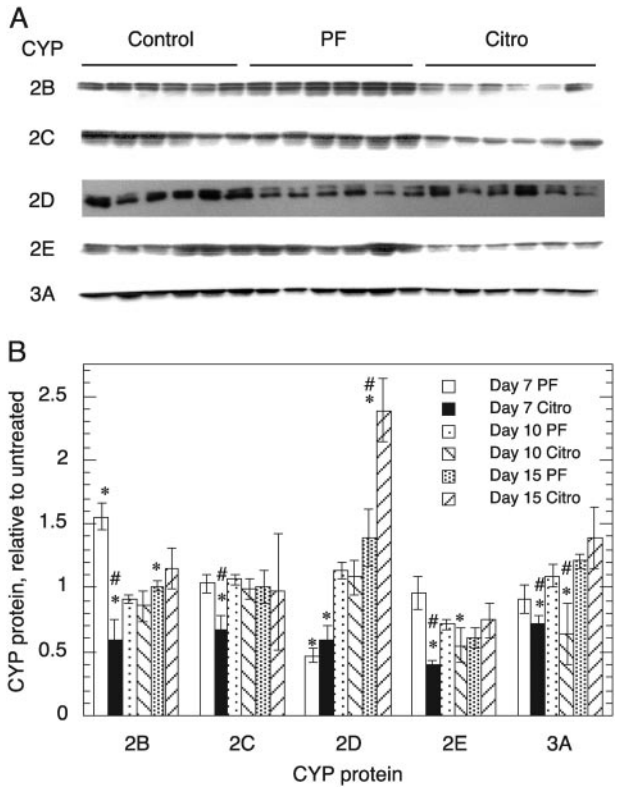

FIG. 4. Effect of oral *C. rodentium* (Citro) infection on P450 family 2 and 3 proteins in mouse liver. Mice were infected with *C. rodentium* p.o., and livers were harvested at the indicated times for measurement of P450 protein levels by Western blotting as described under *Materials and Methods*. A, Western blots of samples from day 7 infected and control liver microsomes; B, quantitative analysis of the data in A. Values represent means ± S.E.M. of six mice per group, with the exception of the 15-day pair-fed (PF) group (five mice), and are calculated relative to the mean of the saline-injected control group, which was set at 1. *, $P < 0.05$ compared with untreated; #, $P < 0.05$ compared with pair-fed. Differences among groups were determined by one-way ANOVA followed by Tukey's test. AGT, angiotensinogen; FGA, fibrinogen α polypeptide; AGP, α_1-acid glycoprotein.

(Richardson et al., 2006). In addition, the down-regulation at 7 days occurred in the absence of increased expression of hepatic proinflammatory cytokine mRNAs, when the mild lymphocytic inflammation was at its peak, and at 10 days these cytokine mRNAs were minimally elevated.

Proinflammatory cytokines, as well as interferons, can selectively modulate P450 expression in cultured hepatocytes (Craig et al., 1990; Thal et al., 1994; Chen et al., 1995; Tinel et al., 1995; Donato et al., 1997; Aitken and Morgan, 2007). In the present study, the time courses of serum elevations in IL-6, TNF-α, IFN-γ, and IL-2 paralleled those of the down- or up-regulation of P450s in the liver. That IL-6 concentrations were the highest of the 16 cytokines measured is of particular interest, because this cytokine can down-regulate multiple P450s in cultured hepatocytes (Aitken and Morgan, 2007), and IL-6-null mice have impaired down-regulation of some hepatic P450s (Siewert et al., 2000). However, the importance of other cytokines or indeed of other blood-borne signals cannot be discounted. IL-13, MIP-1α, MCP-1, RANTES, and KC are chemokines involved in chemotaxis of immune cells, and their effects on P450 expression in hepatocytes are unknown.

In this study, we observed an even greater induction of CYP4F18 (9-fold) in infected mice than we saw previously in the HeOu strain. Because CYP4Fs are the most efficient catalysts of leukotriene B_4 inactivation (Kalsotra and Strobel, 2006), it is tempting to speculate that induction of CYP4F18 contributes to the resolution of inflammation in the liver (and perhaps systemically) by removing this inflammatory eicosanoid.

The marked induction of CYP2D9 mRNA in mice infected with *C. rodentium* is a novel observation. Human CYP2D6 is an important enzyme for the clearance of a large number of therapeutic agents, but it is one of the few drug-metabolizing hepatic P450s that appear not to be significantly induced by drugs or chemicals. Therefore, it will be important to determine whether CYP2D9 and/or CYP2D6 is induced in other inflammatory models and to elucidate the mechanism of this induction. Expression of CYP2D9 is sexually differentiated in mouse liver (Harada and Negishi, 1985) in response to the sex-specific growth hormone secretory pattern (Jarukamjorn et al., 2006; Waxman and O'Connor, 2006). LPS causes down-regulation of the hepatic

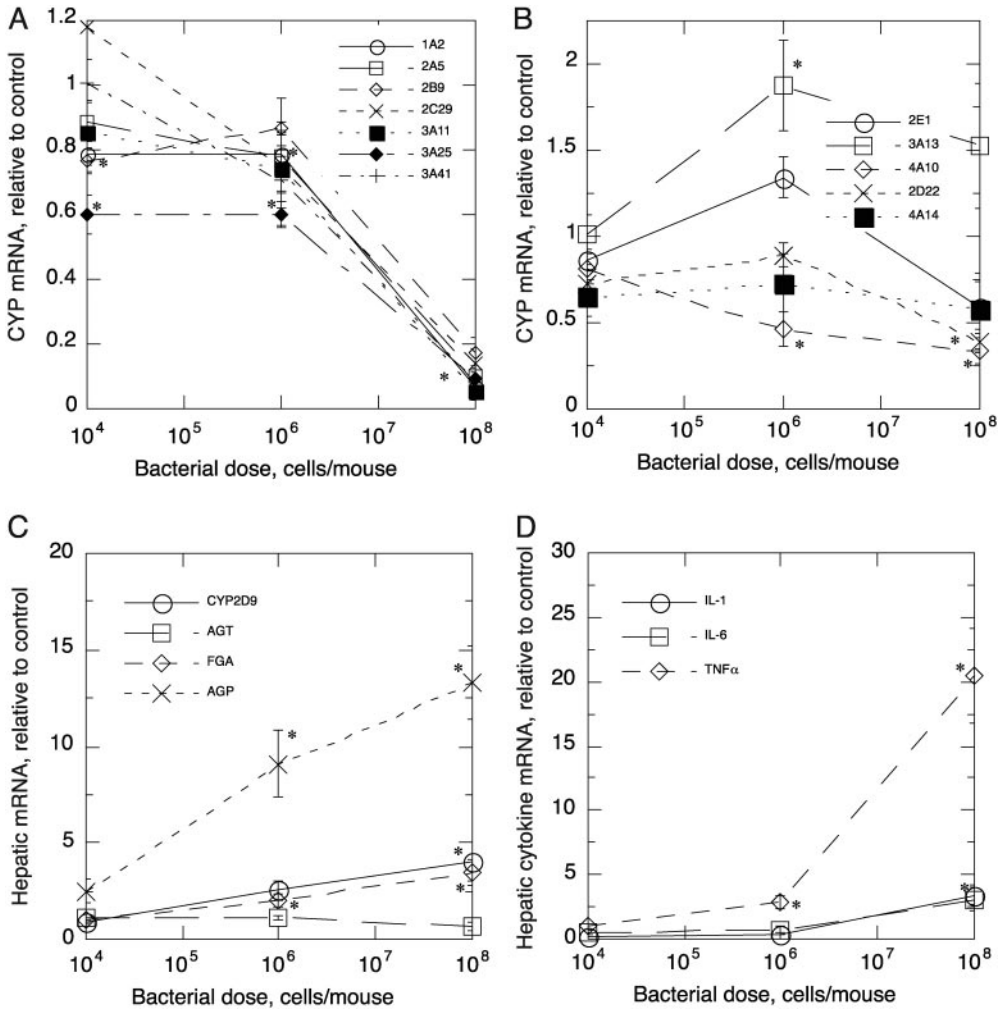

FIG. 5. Dependence on bacterial dose of parenteral infection of mice with *C. rodentium* on hepatic P450, cytokine, and acute-phase mRNAs. Mice received i.p. injections with the indicated number of *C. rodentium* cells and were killed 24 h later. Livers were harvested for measurement of liver mRNA levels by real-time RT-PCR. A, P450s 1A2, 2A5, 2B9, 2C29, 3A11, 3A25, and 3A41; B, P450s 2E1, 3A13, 4A10, 2D22, and 4A14; C, hepatic acute-phase protein mRNAs and CYP2D9; D, cytokine mRNAs. Values represent means ± S.E.M. of six mice per group and are calculated relative to the mean of the saline-injected control group, which was set at 1 for each mRNA. *, $P < 0.05$ compared with control. Differences between treated groups and control were determined by one-way ANOVA followed by Dunnett's test.

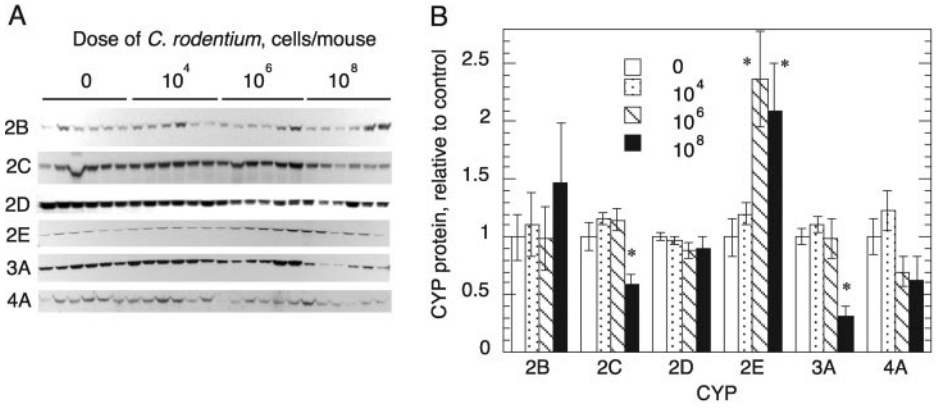

FIG. 6. Effect of parenteral infection with *C. rodentium* on mouse hepatic P450 protein levels. Mice received i.p. injections of the indicated doses of *C. rodentium* and were killed 24 h later. Livers were harvested, and postmitochondrial supernatants were prepared for measurement of P450 protein levels by Western blot analyses. A, images of the Western blots. B, quantitative analysis of the Western blot data. Values represent means ± S.E.M. of six mice per group and are calculated relative to the mean of the saline-injected control group. *, $P < 0.05$ compared with control. Differences between treated groups and control were determined by one-way ANOVA followed by Dunnett's test.

growth hormone receptor via proteolysis (Wang et al., 2008). Whether the up-regulation of CYP2D9 in the infected mice is due to changes in growth hormone secretion or growth hormone receptor expression remains to be determined. Down-regulation of rat hepatic P450 mRNAs by LPS was not due to changes in growth hormone secretion, because hypophysectomized rats infused with growth hormone responded in the same manner as intact animals (Morgan, 1993).

Although mice represent an attractive model to study the mechanisms of P450 regulation, few tools are available to study the regulation of specific P450 proteins or their attendant activities. Thus, we were able to confirm that P450 proteins reacting with antibodies to rat liver CYP2B, 2C, 2E1, 3A, and 4A are indeed down-regulated in the livers of infected mice, but our inability to identify specific immunoreactive proteins precludes making close associations between mRNA and protein regulation except for CYP2E1. CYP2E1 protein was down-regulated to a greater extent than its mRNA, relative to results in untreated controls at both 7 and 10 days p.i., suggesting that CYP2E1 protein degradation may be accelerated or its translation inhibited under these conditions.

The effects of i.p. injection of *C. rodentium* were rapid and pro-

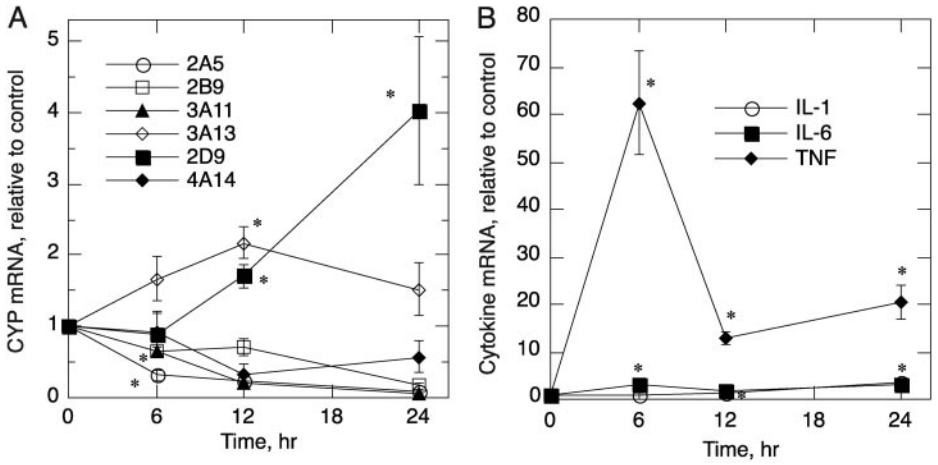

FIG. 7. Time courses of hepatic P450 and cytokine mRNA regulation after parenteral infection of mice with *C. rodentium*. Mice received i.p. injections of 10^8 cells of *C. rodentium* or with the same volume of sterile saline and were killed 6, 12, or 24 h later. A separate group of control animals was used for each time point. Livers were harvested for measurement of hepatic mRNA levels by real-time RT-PCR. A, P450 mRNAs; B, cytokine mRNAs. Values represent means ± S.E.M. of six mice per group and are calculated relative to the mean of the respective saline-injected control groups, which was set at 1 for each mRNA. *, $P < 0.05$ compared with control. Differences between treated groups and control were determined by Student's *t* test. Twenty-four-hour data are from the study in Fig. 5.

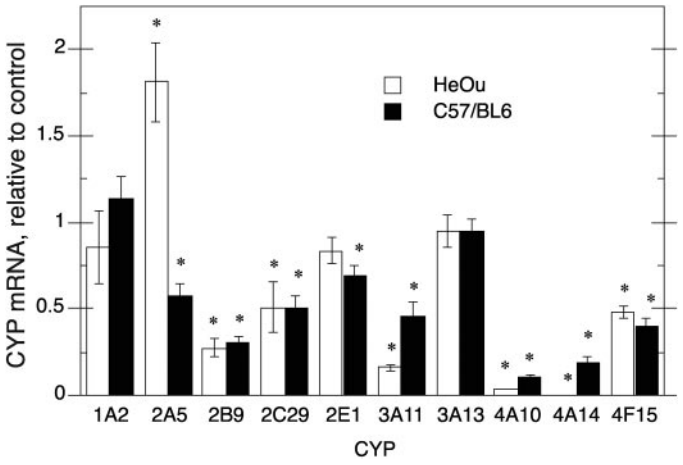

FIG. 8. Comparison of hepatic P450 mRNA regulation during *C. rodentium* infection in HeOu and C57BL/6 mice. Data for C57BL/6 mice are from this study (Figs. 4 and 5), and data for HeOu mice are from Richardson et al. (2006). The levels of each P450 mRNA relative to pair-fed controls are shown. *, $P < 0.05$ compared with pair-fed.

duced a more profound down-regulation of many of the P450s that are moderately down-regulated by oral infection. Strikingly, the CYP4As that are the most sensitive to down-regulation by oral infection were less sensitive to down-regulation by i.p. injection. These results suggest that the mechanisms of down-regulation are different in the two models and, therefore, that bacterial translocation to the blood and liver is not responsible for the majority of the effects caused by oral infection. However, i.p. injection of *C. rodentium* caused a rapid increase in CYP2D9 expression, similar in magnitude to that elicited by oral infection. It is tempting to speculate that CYP2D9 induction may be caused by bacterial LPS acting on the liver.

In conclusion, a gastrointestinal infection causing colonic inflammation that produces minimal clinical signs or symptoms exerts selective effects on hepatic P450 expression that resolve as the infection is cleared and the inflammation subsides. This selectivity is markedly different from that of systemic sepsis caused by the same bacterium, which has important implications for drug therapy in disease states and perhaps in different stages of the same infection. On the other hand, expression of CYP2D9 is markedly induced after either oral or systemic infection. A better understanding of the mechanisms involved will facilitate studies to determine whether the observed regulation of hepatic CYP2D9 has importance for the metabolism of the many drugs that are substrates of CYP2D6 in humans.

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