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# Regulation of Hepatobiliary Transport Activity and Noninvasive Identification of Cytokine-Dependent Liver Inflammation

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Many diseases are associated with cytokine release after inflammatory infiltration, which perturbs organ function. Radioligands capable of noninvasive tracking to assess the integrity of specific biochemical pathways offer potent ways to establish such perturbing mechanisms. **Methods:** To demonstrate regulation of hepatobiliary transport in disease, we used <sup>99m</sup>Tc-mebrofenin in a carbon tetrachloride-induced liver injury model in Fischer 344 rats. Healthy rats served as control animals. Image analysis was used to determine <sup>99m</sup>Tc-mebrofenin handling. Liver tests and histologic analysis were used for grading liver injury and hepatic fibrosis. To address the role of inflammatory cytokines, we used *in vitro* assays with <sup>99m</sup>Tc-mebrofenin-loaded primary rat hepatocytes. **Results:** In healthy rats, <sup>99m</sup>Tc-mebrofenin was promptly excreted, and after 1 h only 20% ± 5% (mean ± SD) of peak <sup>99m</sup>Tc-mebrofenin activity remained in the liver. In contrast, rats treated with carbon tetrachloride for 1 or 3 mo showed 84% ± 5% and 80% ± 7% (mean ± SD), respectively, of peak <sup>99m</sup>Tc-mebrofenin activity in the liver after 1 h (*P* < 0.001). Abnormal <sup>99m</sup>Tc-mebrofenin transport was associated with necroinflammatory activity and not hepatic fibrosis. This was examined directly in animals, where withdrawal of carbon tetrachloride for 2 wk after significant liver injury produced loss of inflammatory activity without affecting hepatic fibrosis. In this situation, <sup>99m</sup>Tc-mebrofenin transport returned to normal, indicating a central role of inflammatory activity in this process. *In vitro* assays showed impairment in <sup>99m</sup>Tc-mebrofenin excretion after incubation of cultured hepatocytes with interleukin-6 and further impairment with interleukin-6 plus tumor necrosis factor- $\alpha$ . **Conclusion:** The findings indicate that inflammatory cytokines regulate <sup>99m</sup>Tc-mebrofenin transport. This cytokine-mediated process establishes a paradigm for identifying and monitoring organ inflammation, including in viral or alcoholic hepatitis, fatty liver disease, allograft rejection, and responses to gene therapy vectors.

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**Key Words:** <sup>99m</sup>Tc-mebrofenin; liver; inflammation; cytokines

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**T**he integrity of discrete biochemical and metabolic pathways in cells is disturbed during disease. Although various pathophysiologic alterations are often helpful in establishing the presence or extent of organ damage, specific metabolic radioligands are of major interest because they permit noninvasive assessment of molecular pathways (1,2). For instance, <sup>99m</sup>Tc-mebrofenin (<sup>99m</sup>Tc-*N*-[3-bromo-2,4,6-trimethylacetanilide]iminodiacetic acid) and related compounds, widely used for assessing gallbladder and liver function, are incorporated exclusively in the liver followed by transport into bile (3–11). It is generally accepted that, because hepatic incorporation and biliary transport of <sup>99m</sup>Tc-mebrofenin depend on the prevailing mass of hepatocytes, <sup>99m</sup>Tc-mebrofenin handling is altered by the presence of liver disease. However, the mechanism of <sup>99m</sup>Tc-mebrofenin retention in chronic liver disease in the absence of gallbladder dysfunction or biliary obstruction is unclear. Because <sup>99m</sup>Tc-mebrofenin excretion in bile requires specific transporters (12), one possibility is that hepatic inflammation, cell damage, or fibrosis produces abnormal regulation of <sup>99m</sup>Tc-mebrofenin transporter activity. The roles of such mechanisms in the regulation of biliary transport activity offer potential for advancing insights into radiotracer biology.

We postulated that specific mechanisms could be established in animals, where progressive depletion of the parenchymal liver cell mass is associated with inflammatory and profibrogenic activity, as observed during repeated administration of carbon tetrachloride (CCl<sub>4</sub>) to rats (13). CCl<sub>4</sub> induces peroxidative liver injury after conversion to toxic intermediates by cytochrome P450 enzymes. Whereas discrete exposure to CCl<sub>4</sub> results in acute liver injury, continuous exposure to this toxin produces progressive liver injury

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and fibrosis, eventually causing cirrhosis, portal hypertension, and death (13,14). A noteworthy feature of CCl<sub>4</sub>-induced hepatotoxicity concerns reversal of liver disease after its withdrawal in rats. In that situation, necroinflammatory activity subsides rapidly, followed by regression of fibrosis, and liver histology gradually returns to normal (13). Here, we report studies in CCl<sub>4</sub>-treated rats to determine mechanisms regulating handling of <sup>99m</sup>Tc-mebrofenin transport in the liver. Our studies were aimed at establishing the role of hepatic necroinflammatory activity versus fibrosis and associated mechanisms in perturbing <sup>99m</sup>Tc-mebrofenin handling and analysis of disease-specific pathophysiological events—for example, the effect of specific inflammatory cytokines.

## MATERIALS AND METHODS

### Animals

Thirty young adult Fischer 344 (F344) rats, 6–8 wk old and weighing 120–160 g, were used. Rats were divided into 5 groups of 6 animals each: 1 control group and 4 experimental groups. Rats in experimental groups were treated with CCl<sub>4</sub> under phenobarbitone priming, according to previously established protocols (13). Briefly, 0.5 mL/kg CCl<sub>4</sub> in mineral oil (1:1, v/v) was administered intramuscularly at 4- to 5-d intervals, along with 0.5 g phenobarbitone per liter of drinking water (Sigma Chemical Co.). Three groups of rats received CCl<sub>4</sub> and phenobarbitone for 1, 2, or 3 mo. One group of rats received CCl<sub>4</sub> and phenobarbitone for 3 mo followed by withdrawal of both for 2 wk. All animals were sacrificed after completing the studies.

### Surgical Procedures

Rats were anesthetized with ketamine and xylazine. The spleen was isolated via laparotomy, and a 23-gauge butterfly needle was inserted into the splenic pulp to inject 7.4 MBq <sup>99m</sup>Tc-mebrofenin in 1 mL. Hemostasis was secured with a ligature around the splenic pole (9). Liver samples were obtained for histology after completion of imaging.

### Nuclear Medicine Procedures

Mebrofenin was mixed with 185–222 MBq <sup>99m</sup>Tc-sodium perchlorate in 3 mL normal saline according to the manufacturer's instructions (Choletec; Bracco Diagnostics). A  $\gamma$ -camera (Argus; ADAC Laboratories), equipped with a low-energy, high-resolution, parallel-hole collimator and interfaced to a computer, was used for image acquisition. A 20% window centered on 140 keV was used for energy discrimination. Ten-second dorsal images were acquired for 60 min, using a 64 × 64 × 16 matrix at a zoom factor of 2. Data were analyzed using a conventional nuclear medicine workstation (Pegasys; ADAC Laboratories). Regions of interest were drawn over the liver, and hepatic time-activity curves were generated. The time at which maximal hepatic activity of <sup>99m</sup>Tc-mebrofenin occurred ( $T_{\text{peak}}$ ) as well as the time required for peak <sup>99m</sup>Tc-mebrofenin activity to decrease by 50% ( $T_{1/2 \text{ peak}}$ ) were determined. The percent hepatic retention of the peak activity at 10, 20, 30, and 60 min after <sup>99m</sup>Tc-mebrofenin administration was also determined. Residual <sup>99m</sup>Tc-mebrofenin activity in excised livers was measured with a  $\gamma$ -counter.

### Histologic Analysis

Liver samples were fixed in 10% buffered formalin. Paraffin-embedded sections were prepared and stained with hematoxylin-eosin or Gomori's 1-step trichrome staining using standard procedures. Rats with chronic liver disease show hepatocellular necrosis or apoptosis, steatosis, hydropic change, megalocytosis, mitosis, and fibrosis. These changes were graded according to the criteria of Batts and Ludwig (15). Hepatic disease activity was graded by analysis of portal or lobular inflammation and necrosis: grade 0, no disease activity; grade 1, minimal portal inflammation with minimal or patchy lobular inflammation and necrosis; grade 2, mild portal inflammation with mild lobular inflammation and little hepatocellular change; grade 3, moderate portal inflammation, moderate lobular inflammation and necrosis with noticeable hepatocellular damage; and grade 4, severe portal and lobular inflammation, necrosis with prominent diffuse hepatocellular damage. Hepatic fibrosis was graded as follows: grade 0, no fibrosis; grade 1, portal fibrosis; grade 2, periportal fibrosis; grade 3, septal fibrosis; and grade 4, cirrhosis. The cumulative histologic scores could have ranged from 0 (entirely normal) to 8 (severe disease with cirrhosis).

### Laboratory Tests

Sera stored at –20°C were analyzed for bilirubin, albumin, and alanine aminotransferase (ALT) with an automated microsystem (Bayer-Chem-1; Bayer Corp.).

### Cell Culture Assays

Primary hepatocytes were isolated from F344 rats by 2-step collagenase perfusion as described previously (13). Cell viability was estimated by trypan blue dye exclusion. Cell culture dishes were coated with type-1 collagen isolated from the tail of F344 rats, as described previously (16). Cells were cultured in dishes with 5–6 × 10<sup>4</sup> cells per 24-well dish (Nalge Nunc International) in RPMI 1640 medium (Life Technologies) containing 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% fetal bovine serum (Atlanta Biologicals). To analyze <sup>99m</sup>Tc-mebrofenin incorporation and release, 370 kBq <sup>99m</sup>Tc-mebrofenin were added to each well containing cells after culture for >40 h. Cells were incubated with <sup>99m</sup>Tc-mebrofenin for 30 min at 37°C and washed 3 times with cold phosphate-buffered saline, pH 7.4. Medium was collected from 6 replicate wells, each after 30, 60, 90, and 120 min, and cells were lysed in 10 mmol/L ethylenediaminetetraacetic acid and 0.5% sodium dodecyl sulfate for measuring <sup>99m</sup>Tc-mebrofenin activity. To determine nonspecific <sup>99m</sup>Tc-mebrofenin binding, cells were pretreated with 5 mmol/L of the nonhydroxamate iron chelator diethylenetriaminepentaacetic acid (DTPA) or potassium cyanide. The cellular protein content was measured by the Bradford assay (Bio-Rad).

Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) (Intergen Co.) were added to cells after 20–24 h of culture to perturb <sup>99m</sup>Tc-mebrofenin handling. In initial studies, cells were primed with 100 or 200 ng/mL actinomycin D (Sigma) for 30 min before adding 10–20 ng/mL cytokines, according to Jones et al. (16). To demonstrate perturbations in cell viability, utilization of the thiazolyl blue dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide [MTT]; Sigma) was analyzed, as described previously (16). In aliquots, total cellular protein was measured by the Bradford assay. Final cell culture studies used 10 ng/mL TNF- $\alpha$  and IL-6. To demonstrate that cytokines were bioactive, an initial experiment was performed, in which hepato-

**TABLE 1**  
Liver Test Abnormalities in Rats Studied

Parameter	Untreated	CCl <sub>4</sub> for 1 mo	CCl <sub>4</sub> for 2 mo	CCl <sub>4</sub> for 3 mo
Total serum bilirubin (mg/dL)	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Serum albumin (g/dL)	3.7 ± 0.4	2.9 ± 0.2	2.3 ± 0.3*	2.0 ± 0.1*
Serum ALT (IU/L)	70 ± 17	1,861 ± 780*	4,803 ± 1,206*	6,699 ± 594*

\**P* < 0.05 vs. normal controls.

cytes were incubated with TNF- $\alpha$  with or without actinomycin D priming. All studies used 6 replicate conditions and were performed with cells from separate normal F344 rats on 3 occasions.

### Statistical Analysis

Data are presented as mean  $\pm$  SD and were analyzed with statistical software (SigmaStat 2.0; Jandel Scientific). To analyze the significance of differences, the Student *t* test was used for intergroup comparisons and ANOVA was used for multigroup comparisons. Multiple linear regression was used to establish dependencies among discrete variables. *P* < 0.05 was considered significant.

## RESULTS

### Laboratory Tests

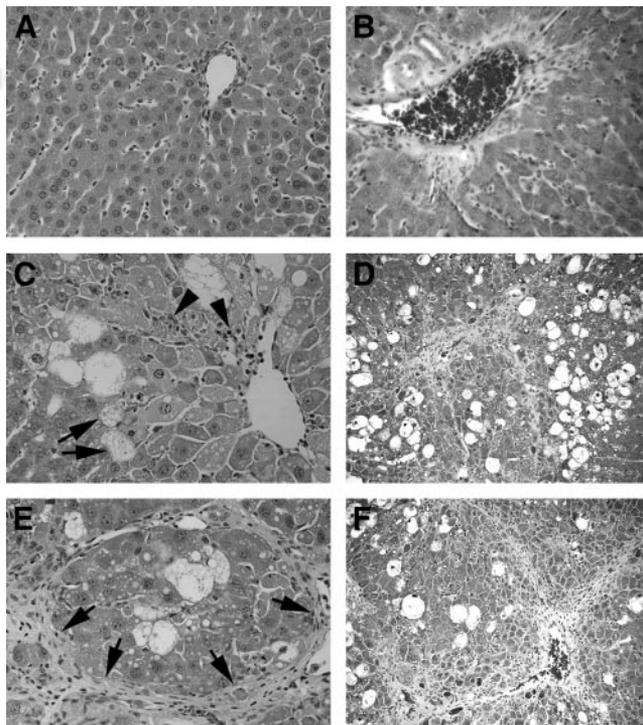
Serum bilirubin levels were normal in all animals studied. Serum albumin levels decreased with increasing duration of CCl<sub>4</sub> treatment, such that albumin levels were significantly lower in CCl<sub>4</sub> recipients treated for 2 and 3 mo than in normal untreated rats. Serum ALT levels were significantly elevated in CCl<sub>4</sub>-treated rats (Table 1).

### Histopathologic Changes

Animals treated with CCl<sub>4</sub> for 1 mo showed macro- and microvesicular hepatic steatosis, hepatocellular necrosis, nuclear abnormalities, inflammatory cell infiltrates, and portal fibrosis (Fig. 1). Disease activity was most pronounced in perivenous areas of the liver lobule, as expected, since CCl<sub>4</sub> is preferentially metabolized in perivenous hepatocytes with greater cytochrome P450 enzyme activity. The magnitude of liver injury increased after additional CCl<sub>4</sub>. Hepatic fibrosis was most pronounced in animals treated for 3 mo, with fibrotic septae extending from portal-to-portal or portal-to-perivenous areas (bridging fibrosis), along with fibrotic nodules. Extensive hepatic reorganization found in established cirrhosis and ascites or extensive nodules were not observed. Liver abnormalities were absent in normal untreated control rats. Histologic grading indicated significant disease activity in animals treated with CCl<sub>4</sub> (Table 2). Similarly, the grade of fibrosis advanced with greater cumulative doses of CCl<sub>4</sub>. Analysis of histologic changes demonstrated that disease activity and fibrosis increased in animals between 1 and 3 mo after CCl<sub>4</sub> (*P* = 0.005, ANOVA; Dunnett pairwise comparisons 1, 2, and 3 mo after CCl<sub>4</sub> vs. controls, *P* < 0.05) (Table 2).

### <sup>99m</sup>Tc-Mebrofenin Handling

In normal control rats, <sup>99m</sup>Tc-mebrofenin appeared promptly in the liver and bile, with rapid clearance from the liver (Fig. 2). Hepatic <sup>99m</sup>Tc-mebrofenin uptake was not affected by CCl<sub>4</sub> treatment, regardless of the treatment duration (*T*<sub>peak</sub>: 140  $\pm$  7 s, normal controls vs. 165  $\pm$  70 s, CCl<sub>4</sub> for 3 mo, *P* = not significant [NS]). <sup>99m</sup>Tc-Mebrofenin clearance, in contrast, was markedly delayed in CCl<sub>4</sub>-treated rats, although the overall pattern of <sup>99m</sup>Tc-mebrofenin retention in the liver was not different in animals treated with CCl<sub>4</sub> for various times. In normal untreated control rats, the *T*<sub>1/2</sub> of <sup>99m</sup>Tc-mebrofenin excretion was 356  $\pm$  86 s (Fig. 3).



**FIGURE 1.** Liver injury in animals treated with CCl<sub>4</sub>. (A and B) Untreated control rats with normal liver histology. (C and D) Rat treated for 1 mo with CCl<sub>4</sub>. Note extensive fatty change (C, arrows), cell necrosis, and inflammatory activity (C, arrowheads), along with fibrosis. (E and F) Rat treated for 3 mo with CCl<sub>4</sub> shows inflammation, cell necrosis, and extensive fibrosis (E, arrows). (A, C, and E: hematoxylin–eosin stain,  $\times$ 200; B, D, and F: trichrome stain for fibrosis,  $\times$ 100)

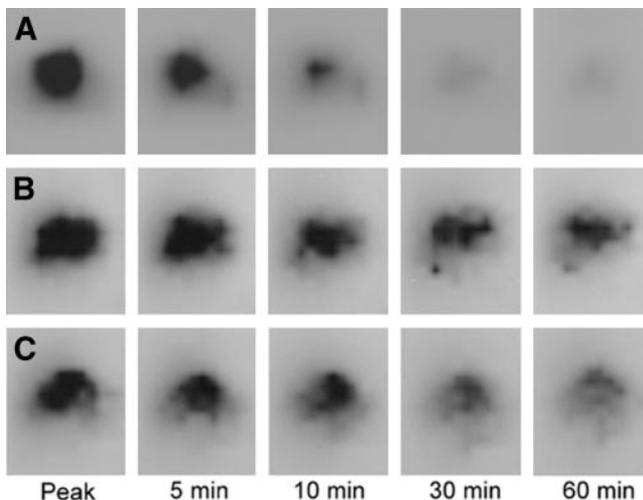
**TABLE 2**  
Histologic Grading of Liver Lesions

Animal group	Disease activity (grade 0–4)	Liver fibrosis (grade 0–4)
Normal controls (n = 6)	0 ± 0	0 ± 0
CCl <sub>4</sub> for 1 mo (n = 6)	2.8 ± 1.7*	1.5 ± 0.8*
CCl <sub>4</sub> for 2 mo (n = 6)	3.7 ± 0.8*	2.3 ± 0.8*
CCl <sub>4</sub> for 3 mo (n = 6)	3.8 ± 0.4*	2.8 ± 1.0*

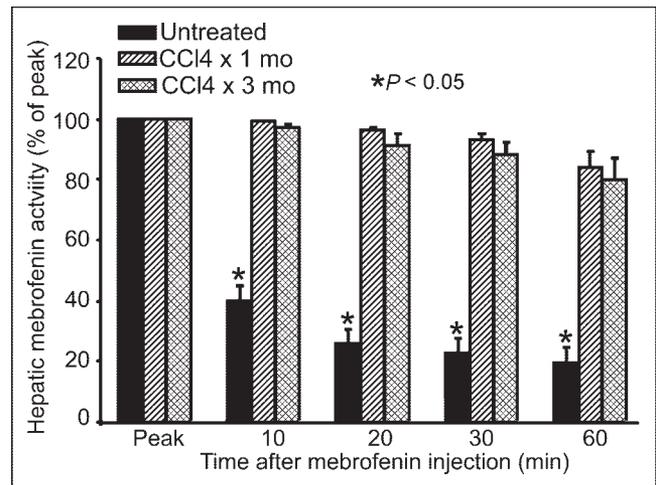
\*P < 0.05 vs. normal controls.

In contrast, <sup>99m</sup>Tc-mebrofenin excretion was severely impaired in animals treated with CCl<sub>4</sub>. Because of extremely slow <sup>99m</sup>Tc-mebrofenin excretion in these animals, as indicated in Figure 3, the slopes were not amenable for estimating T<sub>1/2</sub> values of <sup>99m</sup>Tc-mebrofenin excretion.

In normal control rats, at 20 and 60 min after intrasplenic injection, only 26% ± 3% and 20% ± 5%, respectively, of the peak <sup>99m</sup>Tc-mebrofenin activity remained in the liver. In contrast, 96% ± 1% and 91% ± 4% of the <sup>99m</sup>Tc-mebrofenin activity was still present in the liver after 20 min in animals treated with CCl<sub>4</sub> for 1 and 3 mo, respectively (P < 0.001 vs. controls, *t* tests). At 60 min, animals treated with CCl<sub>4</sub> for 1 and 3 mo still retained much of the <sup>99m</sup>Tc-mebrofenin in the liver, 84% ± 5% and 80% ± 7%, respectively (P < 0.001, *t* tests). Similarly, measurement of the retained <sup>99m</sup>Tc-mebrofenin activity in excised livers obtained after imaging revealed that rats treated with CCl<sub>4</sub> for 1 or 3 mo contained on average 7.3-fold and 5.2-fold greater activity than that of untreated normal rats (P = 0.002, Mann–Whitney rank sum tests) (1.29 ± 1.09 × 10<sup>5</sup> counts



**FIGURE 2.** Scintigraphy shows hepatic <sup>99m</sup>Tc-mebrofenin uptake and excretion. Sequential images are shown from same animals. (A) Normal rat shows rapid <sup>99m</sup>Tc-mebrofenin clearance. In contrast, rats treated with CCl<sub>4</sub> for 1 mo (B) or 3 mo (C) show markedly delayed <sup>99m</sup>Tc-mebrofenin clearance.



**FIGURE 3.** Analysis of <sup>99m</sup>Tc-mebrofenin retention in liver of normal untreated rats and rats treated for 1 or 3 mo with CCl<sub>4</sub>. Note that largest fraction of <sup>99m</sup>Tc-mebrofenin was cleared from liver of normal rats within 10 min, whereas <sup>99m</sup>Tc-mebrofenin was retained in animals after liver injury.

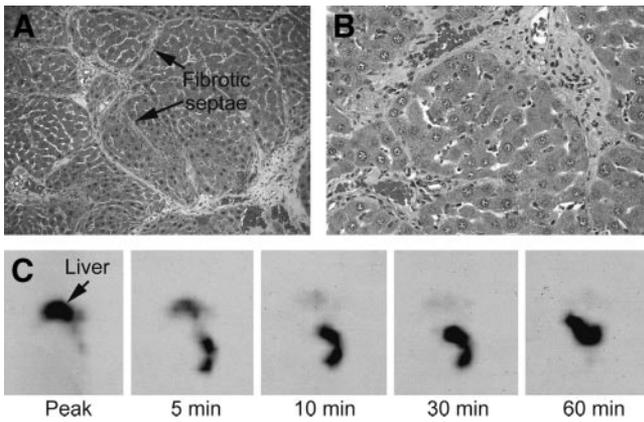
per minute [cpm]/g liver, controls, vs. 9.43 ± 1.42 × 10<sup>5</sup> cpm/g liver and 6.71 ± 2.19 × 10<sup>5</sup> cpm/g liver, 1 and 3 mo after CCl<sub>4</sub>, respectively).

Multiple linear regression established a significant relationship between hepatic disease activity and <sup>99m</sup>Tc-mebrofenin retention (Table 3) when serum ALT, disease activity, and liver fibrosis were assigned as independent variables and <sup>99m</sup>Tc-mebrofenin retention was assigned as a dependent variable. These findings indicated that hepatic disease activity played a significant role in <sup>99m</sup>Tc-mebrofenin clearance. Study of rats administered CCl<sub>4</sub> for 3 mo, followed by CCl<sub>4</sub> withdrawal for 2 wk, showed extensive fibrosis but absence of liver inflammation (Fig. 4). In agreement with these findings, liver tests showed significant improvement, with nearly normal serum ALT (122 ± 116 IU/L), improved serum albumin (2.8 ± 0.1 g/dL), and normal serum bilirubin (0.3 ± 0.15 mg/dL) levels. The disease activity grade decreased to 1 ± 0, although the fibrosis grade remained elevated at 2.4 ± 0.5, which was virtually similar to that after 3 mo of CCl<sub>4</sub>. Hepatic <sup>99m</sup>Tc-mebrofenin clearance in these animals was normal (Fig. 4). The T<sub>1/2</sub> of <sup>99m</sup>Tc-

**TABLE 3**  
Associations Between Clinical Parameters and <sup>99m</sup>Tc-Mebrofenin Excretion

Independent variable	Hepatic <sup>99m</sup> Tc-mebrofenin retention after 60 min (P)
Serum ALT	0.28
Hepatic fibrosis, grade	0.495
Hepatic disease activity, grade	<0.001

Multiple linear regression was used.



**FIGURE 4.** Normalization of  $^{99m}\text{Tc}$ -mebrofenin excretion in animals after  $\text{CCl}_4$  withdrawal. (A and B) Rats show persistent fibrosis (A, arrows; trichrome stain,  $\times 40$ ) but resolution of inflammatory activity (B, hematoxylin-eosin stain,  $\times 100$ ). (C) Scintigraphy shows rapid  $^{99m}\text{Tc}$ -mebrofenin clearance in a rat (compare with Fig. 2A).

mebrofenin excretion was  $322 \pm 33$  s, similar to that of normal untreated control rats ( $P = \text{NS}$ ), and residual  $^{99m}\text{Tc}$ -mebrofenin activity 20 and 60 min after peak  $^{99m}\text{Tc}$ -mebrofenin accumulation was  $25\% \pm 5\%$  and  $20\% \pm 6\%$ , respectively, which was similar to that of normal untreated control rats ( $P = \text{NS}$ ).

#### Role of Cytokines in Hepatic $^{99m}\text{Tc}$ -Mebrofenin Excretion in Primary Rat Hepatocytes

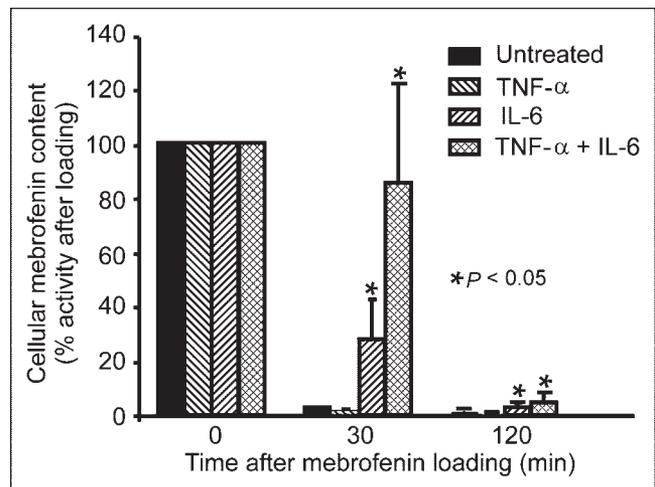
Incubation of cells with  $\text{TNF-}\alpha$  alone had no effect on MTT dye utilization. However, after culture with  $\text{TNF-}\alpha$  plus actinomycin D, cell viability declined to  $45\% \pm 4\%$  of that of untreated controls or controls treated with  $\text{TNF-}\alpha$  alone ( $P < 0.001$ ,  $t$  test), which was an appropriate and expected response (17). In further studies, loading of cells with  $^{99m}\text{Tc}$ -mebrofenin was successful and provided a suitable assay to analyze the kinetics of  $^{99m}\text{Tc}$ -mebrofenin excretion in vitro.

In untreated control hepatocytes,  $96.8\% \pm 1.0\%$  of the accumulated  $^{99m}\text{Tc}$ -mebrofenin activity was cleared after 30 min and only  $1.2\% \pm 0.5\%$  of  $^{99m}\text{Tc}$ -mebrofenin activity was left in cells after 3 h (Fig. 5). Similarly, cells treated with  $\text{TNF-}\alpha$  alone promptly excreted  $^{99m}\text{Tc}$ -mebrofenin. However, treatment of cells with IL-6, and especially with the combination of IL-6 and  $\text{TNF-}\alpha$ , induced significant  $^{99m}\text{Tc}$ -mebrofenin retention ( $P < 0.001$ ,  $t$  tests). In this situation, 30 min after completion of cell loading, IL-6 was associated with retention of  $28\% \pm 15\%$  of  $^{99m}\text{Tc}$ -mebrofenin activity, whereas IL-6 plus  $\text{TNF-}\alpha$  resulted in retention of  $85\% \pm 36\%$  of  $^{99m}\text{Tc}$ -mebrofenin activity ( $P < 0.001$ ,  $t$  test). Essentially no  $^{99m}\text{Tc}$ -mebrofenin was incorporated in cells incubated with either DTPA or potassium cyanide, indicating the absence of nonspecific  $^{99m}\text{Tc}$ -mebrofenin adherence to cells under the assay conditions.

## DISCUSSION

Our findings demonstrate that abnormal retention of  $^{99m}\text{Tc}$ -mebrofenin in the liver requires the presence of inflammation and is independent of chronic hepatocellular damage and liver fibrosis. The data further establish that the process affecting  $^{99m}\text{Tc}$ -mebrofenin transport in inflammation is mediated by cytokines. This mechanism in the regulation of  $^{99m}\text{Tc}$ -mebrofenin handling will enable further rational applications of  $^{99m}\text{Tc}$ -mebrofenin imaging in health and disease.

We found that  $^{99m}\text{Tc}$ -mebrofenin was rapidly extracted by the liver in both normal and diseased animals, as shown by the  $T_{\text{peak}}$  values, which was similar to that of LEC rats with significant copper toxicosis-related chronic liver disease (9). In agreement with the 2-compartment modeling, taking into account fluxes of  $^{99m}\text{Tc}$ -mebrofenin between systemic and hepatic blood pools and hepatocyte uptake (3,4), we previously showed that the hepatic extraction efficiency for  $^{99m}\text{Tc}$ -mebrofenin follows first-order kinetics and is extraordinarily high. Also, our previous findings established that depletion of the reference systemic (cardiac) blood pool of  $^{99m}\text{Tc}$ -mebrofenin at early times, before the commencement of biliary excretion in normal rats and LEC rats with liver disease, was similar, indicating that hepatic  $^{99m}\text{Tc}$ -mebrofenin extraction was equally efficient in the presence of chronic liver disease (9). Our present findings provide further support that hepatic extraction of  $^{99m}\text{Tc}$ -mebrofenin is unperturbed in chronic liver disease. The fractional excretion of  $^{99m}\text{Tc}$ -mebrofenin in normal control rats, as judged by the  $T_{1/2}$  measurement of  $^{99m}\text{Tc}$ -mebrofenin excretion, as well as  $^{99m}\text{Tc}$ -mebrofenin retention at various times for up



**FIGURE 5.** Cytokine-induced perturbation of  $^{99m}\text{Tc}$ -mebrofenin handling in cultured hepatocytes. Data are from a typical experiment with prompt clearance of  $^{99m}\text{Tc}$ -mebrofenin in cultured hepatocytes after  $^{99m}\text{Tc}$ -mebrofenin loading for 30 min. Exposure of cells to  $\text{TNF-}\alpha$  did not affect  $^{99m}\text{Tc}$ -mebrofenin clearance, whereas exposure to either IL-6 alone or IL-6 plus  $\text{TNF-}\alpha$  resulted in significant  $^{99m}\text{Tc}$ -mebrofenin retention in cells.

to 60 min, was also similar to that of previous studies (9) and was in general agreement with observations in healthy people (18). However,  $^{99m}\text{Tc}$ -mebrofenin excretion was impaired markedly in animals with  $\text{CCl}_4$ -induced liver disease. Of note was the extensive perturbation of  $^{99m}\text{Tc}$ -mebrofenin excretion in animals at early stages of liver inflammation.  $^{99m}\text{Tc}$ -Mebrofenin retention was obvious within 20 min and remained markedly elevated after 60 min, indicating profound perturbations in the regulation of  $^{99m}\text{Tc}$ -mebrofenin transport in bile.

An examination of the molecular regulation of hepatic bile acid and organic anion transporters by cytokines should be appropriate for explaining the relationship between inflammatory activity and  $^{99m}\text{Tc}$ -mebrofenin excretion. Although  $^{99m}\text{Tc}$ -mebrofenin is a bifunctional chelating agent, with one end conjugated to  $^{99m}\text{Tc}$  and the other to the lidocaine analog acetanilide, the identity of the specific transporter(s) involved in the hepatic excretion of  $^{99m}\text{Tc}$ -mebrofenin is unknown. On the other hand, the list of bile salt and organic anion transporters has grown extensively and significant complexities have emerged (12). Among the hepatic uptake mechanisms, though the  $\text{Na}^+$ /taurocholate cotransporting polypeptide (ntcp) is a major player in bile salt incorporation, a variety of sodium-independent transporters have also been found, including for bilirubin, organic anions, and multiple drugs—for example, organic anion transporters OAT-2, OATP-A, OATP-B, OATP-C, and OATP8. Because  $^{99m}\text{Tc}$ -mebrofenin incorporation was unchanged in our rats with liver injury, these transporters are not likely incriminated, and adenosine triphosphate-dependent proteins involved in bile canalicular transport and basolateral export of organic anions may be more relevant. The bile canalicular transporters include the bile salt excretory pump (BSEP), which probably plays small role in organic anion transport, and the multidrug-resistance-related protein MRP2, as well as basolateral exporters, including MRP1 and MRP3, which may play more significant roles in organic anion transport.

Recent studies in rats treated with  $\text{CCl}_4$  showed that  $\text{TNF-}\alpha$  downregulated expression of ntcp, OATP1, and OATP2 at the messenger RNA (mRNA) level (19). In mice,  $\text{TNF-}\alpha$  affected mRNA levels of MRP2, MRP3, and OATP2 but not OATP1 or BSEP, whereas IL-6 decreased MRP2, OATP1, OATP2, and BSEP mRNA expression (20). Our results, which showed that  $\text{TNF-}\alpha$  alone did not impair  $^{99m}\text{Tc}$ -mebrofenin excretion, whereas IL-6 inhibited  $^{99m}\text{Tc}$ -mebrofenin excretion, both directly and synergistically with  $\text{TNF-}\alpha$ , suggest that the list of cytokine-regulated transporters responsible for these perturbations could be pared down. Thus, further analysis will be appropriate to determine what types of inflammatory activity will perturb hepatic  $^{99m}\text{Tc}$ -mebrofenin handling in vivo. Similarly, manipulation of liver gene regulation by cytokines offers potent ways to identify the  $^{99m}\text{Tc}$ -mebrofenin transporters. Although cultured hepatocytes have previously been

shown to incorporate  $^{99m}\text{Tc}$ -mebrofenin and related compounds (21), our in vitro assays should help demonstrate further mechanisms regulating  $^{99m}\text{Tc}$ -mebrofenin excretion. For instance, nuclear hormone receptors regulate transcription of bile salt and organic anion transporters, including the farnesoid X receptor, which regulates BSEP transcription, and the hepatocyte nuclear factor (HNF)-1, HNF-4, retinoid X receptor/retinoic acid receptor, and other transcription factors, which are downregulated during cytokine-mediated acute liver inflammation, regulate expression of organic anion transporters, including MRP-2 (19).

Our findings indicate that fibrosis alone does not alter hepatic  $^{99m}\text{Tc}$ -mebrofenin handling. Similarly, hepatic necrosis alone was unlikely to be responsible for altering  $^{99m}\text{Tc}$ -mebrofenin handling, as suggested by the lack of an association between serum ALT levels and  $^{99m}\text{Tc}$ -mebrofenin retention. On the other hand, numerous conditions are associated with cytokine release, ranging from inflammatory states—for example, acute cholecystitis, viral or alcoholic hepatitis, fatty liver disease, and so forth. It should be appropriate to undertake studies of  $^{99m}\text{Tc}$ -mebrofenin handling in clinical situations, where hepatic inflammation is driven by specific populations of inflammatory cells—for example, neutrophilic infiltrates in alcoholic hepatitis, lymphocytic infiltrates in viral hepatitis, and plasma cell accumulation in autoimmune hepatitis.

## CONCLUSION

Use of  $^{99m}\text{Tc}$ -mebrofenin imaging in the context of cytokine activity will be effective for qualitative and quantitative analysis of liver inflammation. This will be potentially valuable for noninvasively assessing therapeutic responses in inflammatory liver diseases, which often necessitates repeated liver biopsies.

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