Alcoholic Liver Disease in Rats Fed Ethanol as Part of Oral or Intragastric Low-Carbohydrate Liquid Diets

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The intragastric administration of ethanol as part of a low-carbohydrate diet results in alcohol hepatotoxicity. We aimed to investigate whether comparable liver injury can be achieved by oral diet intake. Male Sprague-Dawley rats were fed ethanol as part of low-carbohydrate diets for 36–42 days either intragastrically or orally. Liver pathology, blood ethanol concentration, serum alanine amino transferase (ALT), endotoxin level, hepatic CYP2E1 induction, and cytokine profiles were assessed. Both oral and intragastric low-carbohydrate ethanol diets resulted in marked steatosis with additional inflammation and necrosis accompanied by significantly increased serum ALT, high levels of CYP2E1 expression, and production of auto-antibodies against malondialdehyde and hydroxyethyl free radical protein adducts. However, cytokine profiles differed substantially between the groups, with significantly lower mRNA expression of the anti-inflammatory cytokine interleukin 4 observed in rats fed low-carbohydrate diets orally. Inflammation and necrosis were significantly greater in rats receiving low-carbohydrate alcohol diets intragastrically than orally. This was associated with a significant increase in liver tumor necrosis factor α and interleukin 1β gene expression in the intragastric model. Thus, oral low-carbohydrate diets produce more ethanol-induced liver pathology than oral high-carbohydrate diets, but hepatotoxicity is more severe when a low-carbohydrate diet plus ethanol is infused intragastrically and is accompanied by significant increases in levels of proinflammatory cytokines. Exp Biol Med 229:351–360, 2004

Key words: ethanol; hepatoxicity; low carbohydrate; rats; oral diets; total enteral nutrition

A critical limitation to expanding our mechanistic knowledge of the pathogenesis of alcoholic liver disease is the difficulty of reproducing in experimental animals the hepatic injury caused in humans by alcohol abuse. Nutritional status and diet are important variables that influence cellular responses to toxins (1). This is particularly true in experimental models of ethanol-induced hepatotoxicity (2). When alcohol is mixed into traditional liquid diets, such as that originally formulated by Lieber and DeCarli (3, 4), laboratory rats will not typically consume sufficient diet to either maintain normal body weight gains or to achieve sufficient blood ethanol concentrations to produce pathology more severe than steatosis. To circumvent these problems, investigators have turned to intragastric infusion models in which diet and ethanol dosing can be completely controlled. Such systems have been demonstrated to produce steatosis accompanied by inflammatory infiltrates and focal necrosis (5–8) and to result in the development of fibrosis when extended over a period of months (9). Alcohol-induced liver damage (ALD) in some rodent intragastric infusion models has been reported to be accompanied by Kupffer cell stimulation as a result of enhanced plasma endotoxin levels (6, 7). In addition, the increased expression of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6; Ref. 9–12)

This work was supported in part by grants R21 AA12031 and R01 AA088645 (T.M.B.).

Presented in part at the Annual Meeting of the Society of Toxicology, San Francisco, CA, 2001.

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Received September 18, 2003.
Accepted December 16, 2003.

1535-3702/04/2294-0000$15.00

have been described. Such alterations in the cytokine profile have been suggested to be a key step in the pathogenesis of alcoholic liver disease, because they stimulate stellate cells to produce collagen.

However, even among different intragastric (IG) models of ALD, considerable differences in the time course and characteristics of liver pathology have been observed. In some rat IG models, the development of inflammation and necrosis is rapid and reversible by antibiotic treatment (6, 7), but, in others, the development of pathology additional to steatosis is much more gradual (9, 13). In some models, pathology is predominantly centrilobular, correlates with localization of CYP2E1 expression, and is reversed with CYP2E1 inhibitors (14), whereas, in others, both steatosis and inflammatory/necrotic foci are panlobular (2, 15).

Dietary composition is clearly also an important factor in the determination of pathological outcome in ALD. Saturated fat appears to protect against the development of pathology, whereas alcohol hepatotoxicity is significantly increased in diets that contain polyunsaturated fatty acids (16–18). Using a model in which ethanol is administered as part of an IG total enteral nutrition (TEN) system, we have recently demonstrated that low dietary carbohydrate (or a high unsaturated fat:carbohydrate ratio) is also a key component required for the production of ethanol-induced hepatic inflammation and necrosis (2).

Lindros and Jarvelainen (19) have published preliminary data on the development of ALD in the rat using a new low-carbohydrate oral diet. They reported that in this model, ethanol produced significantly greater liver pathology than traditional Liber-DeCarli oral diets, including inflammation, necrosis, and increased plasma alanine aminotrasferase (ALT) values. However, other reports in which low-carbohydrate oral diets have been used have reported steatosis but no inflammation or necrosis, despite increased ALT values (20). In the current study, we compared liver pathology produced by ethanol treatment as part of oral or IG low-carbohydrate diet models side by side. In addition, biochemical effects and changes in cytokine profile were compared in these models of ethanol exposure.

Materials and Methods

Animals. All animal studies described below were approved by Institutional Animal Care and Use (IACUC) committees at the University of Arkansas for Medical Sciences and at the National Public Health Institute, Finland. IG cannulae were surgically implanted into male Sprague-Dawley rats (weighing 300 g) at the Arkansas Children's Nutrition Center. After surgery, the rats were allowed to recover until their presurgical weight was attained. Groups of 8–10 rats were continually infused for 42 days with TEN (control) or TEN plus ethanol (EtOH) diets at an isocaloric intake of 187 kcal/kg$^{3/4}$/day; the National Research Council (NRC) recommended intake for optimal growth, as described elsewhere (2, 21). The only modification to previously published studies was that, instead of gradually replacing carbohydrate calories with ethanol as the amount of ethanol increased, rats were started at 10 g/kg/day of ethanol, and fat calories were reduced as the ethanol infusion increased in 0.5 g/kg/day steps to ~12 g/kg/day (33% of calories), to maintain carbohydrate levels in the ethanol-containing diets at 5% of total calories throughout the study. The final level of ethanol infusion was determined on an individual rat basis to remain below levels at which acute ethanol toxicity was evident. The average ethanol intake for the infused group over the entire study was 11.5 ± 0.7 g/kg/day. Oral ethanol diets were fed at the National Public Health Institute, Helsinki (Finland) using 8–10 male Sprague-Dawley rats (300 g). Rats were pair-fed for 42 days with oral low-carbohydrate liquid diets that contained 40% carbohydrate (control) or 5.5% carbohydrate plus 34.5% ethanol (EtOH), as described elsewhere (19). On the basis of the measurement of daily diet intake, these rats consumed an average of 171 kcal/kg$^{3/4}$/day and an average of 12.3 ± 0.5 g ethanol/kg/day. In addition, to compare CYP2E1 expression in the oral low-carbohydrate model with a traditional, higher carbohydrate, ethanol-containing liquid diet, 5 male Sprague-Dawley rats were fed traditional (11% carbohydrate) Lieber-DeCarli diets plus ethanol (4, 19).

Biochemical Analysis. Blood ethanol concentrations (BECs) were measured at sacrifice using an Analox Instruments GL5 analyzer or commercial kits from Sigma Chemical Co. (St. Louis, MO). Plasma ALT was measured at death using a commercial kit (Sigma Chemical Co.). For the analysis of plasma endotoxin levels, blood was collected by heart puncture into pyrogen-free tubes that contained heparin (Chromogenix, Mölndal, Sweden). Plasma was separated immediately in a refrigerated centrifuge at 800 *g* for 10 mins. Supernatant was stored at –80°C in pyrogen-free storage tubes (Biowhittaker QCL 1000, Walkersville, MD) until analysis. Endotoxin levels were assayed using the limulus amebocyte lysate (LAL) chromogenic assay (Biowhittaker), as described by Nosova *et al.* (22). Liver microsomes were prepared by differential ultracentrifugation (23). Microsomal carbon tetrachloride–dependent lipid peroxidation was assayed as a marker of CYP2E1-dependent monooxygenase activity according to the method of Johansson and Ingelman-Sundberg (24). CYP2E1 apoprotein expression was measured by Western immunoblot analysis, as described elsewhere (2) using a rabbit polyclonal antibody raised against purified rat CYP2E1. The absolute quantification of CYP2E1 was obtained by comparing the relative densitometric values from liver microsomes with those of known concentrations of purified CYP2E1 apoprotein in the same Western blot autoradiographs (25).

Pathological Evaluation. Liver pathology was assessed in hematoxylin-eosin–stained liver sections and scored using blinded samples by two independent board-certified pathologists (E.A. and S.K.), as described elsewhere (2). For statistical comparison, levels of steatosis (macro- and microvesicular), leucocyte infiltration, and necrosis were scored on a scale of 1–5, where 1 indicates

Table 1. Optimal PCR Conditions, Sequence of the Different Primers and Size of PCR Products Used for Cytokine mRNA Assays

Cytokine	Mimic (attmoles)	Annealing temp. (°C)	$MgCl_2$ (m*M*)	No. of PCR cycles	Sense, 5'-3'	Antisense, 5'-3'	Target, mimic (bp)
Cyclophilin	2.7×10^{-1}	53	1.7	36	TGAGCACTGGGGAGAAAG	AGGGGAATGAGGAAAATA	521, 210
TNF-α	3.0×10^{-4}	60	1.5	40	ACGCTCTTCTGTCTACTG	GGATGAACACGCCAGTCG	592, 256
IL-1β	3.0×10^{-2}	55	1.4	35	GACCTGTTCTTTGAGGCTGA	TTCATCTCGAAGCCTGCAGT	330, 613
IL-4	3.0×10^{-2}	67	0.9	40	AACACCACGGAGAACGAGCTCATC	AGTGAGTTCAGACCGCTGACACCT	152, 448
IL-10	1.2×10^{-4}	63	1.5	38	GAAGTGATGCCCCAGGCAGA	CACGTAGGCTTCTATGCAGT	240, 425

no pathology. The total pathology score was calculated as the sum of steatosis, inflammatory, and necrotic scores, with a baseline score of 3 representing no pathology.

Measurement of Antibody Titers Against Malondialdehyde and Hydroxyethyl Protein Adducts. Bovine serum albumin (BSA) adducts with malondialdehyde (MDA-BSA) and hydroxyethyl free radicals (HER-BSA) were prepared as described elsewhere (26). For antibody detection, polystyrene microwell plates (Immunolon IV; Nunc, Fisher Scientific, St. Louis, MO) were coated for 4 hrs at 37°C with 0.05 mg/mL of either modified BSA or native BSA solubilized in 0.1 *M* bicarbonate buffer (pH 9.6). To block nonspecific binding sites, 0.3 ml of coating buffer that contained BSA in phosphate-buffered saline (PBS; pH 7.4) was incubated for 1 hr at 37°C. The coated wells were washed three times with PBS that contained 0.25 Triton X-100. Rat serum (0.20 ml; dilution 1:50 in coating buffer) were added in duplicate to the appropriate wells and incubated for 1 hr at 37°C. After washing three times with PBS-0.25 Triton X-100, peroxidase-linked goat anti–rat IgG (dilution 1:6000) was added and incubated for 60 mins at 37°C. Antibody binding was measured at 490 nm, as reported elsewhere (26), and the results were expressed after subtraction of the background reactivity with unmodified BSA.

Cytokine Analysis. For the analysis of cytokine mRNA expression in liver samples, ~80 μg of total RNA was isolated from 30–40 mg of liver tissue by using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA). Total RNA was electrophoresed on 1.25% agarose gel that contained 20 m*M* guanidine thiocyanate (27). The quality of RNA was ascertained by the intactness of ribosomal RNA bands. Intact total RNA sample was reverse-transcribed to cDNA by using the Advantage RT-for-PCR kit (Clontech). Polymerase chain reaction (PCR) mimics were constructed according to the manufacturer's instructions, and the competitive reverse-transcription (RT) PCR was carried out as described elsewhere (12). Table 1 shows the sequence of PCR primers from rat cytokines and the sizes of the products (target and mimic). The optimal conditions for the quantitation of competitive RT-PCR under conditions of linearity are given in Table 1. PCR products run on agarose gels were scanned with the Fuji Las-1000 luminescent image analyzer (Fuji, Japan). The intensity data of the scanned bands were converted to ratios of target:mimic and then normalized by the ratio of cyclophilin, which served as a control for the quantity of mRNA and efficiency of RT.

Statistics. Data are expressed as mean ± standard error (SE). Groups were compared by one-way analysis of variance (ANOVA) followed by Student's Neuman-Keuls post hoc analysis, and significance was set at $P \leq 0.05$.

Results

Weight Gain. *Ad libitum* oral feeding of low-carbohydrate ethanol diets resulted in a very low growth

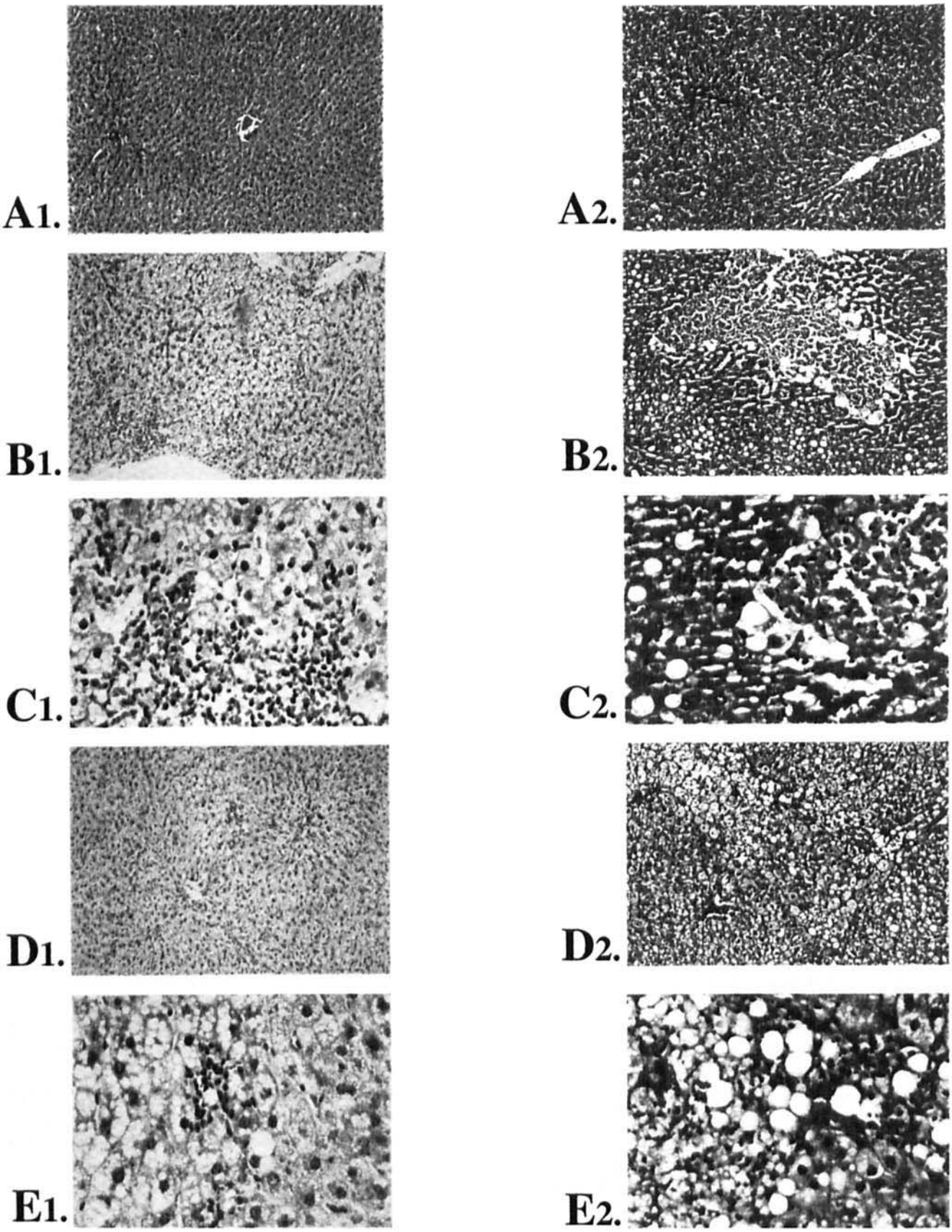

Figure 1. Liver pathology associated with ethanol exposure in the low-carbohydrate intragastric model (series A1–E1) and low carbohydrate oral model (series A2–E2). Representative liver sections were stained with hematoxylin-eosin. (A) Control liver, magnification ×10. (B and D) Liver sections from rats fed ethanol, magnification ×10. (C and E) Liver sections from rats fed ethanol, magnification ×40.

rate of ~0.6–0.9 g/day. Pair-fed controls grew at a rate of 1.2 g/day, a level that is much below that seen in rats fed control IG liquid diets or chow *ad libitum* (~3 g/day). This is the result of the well-known aversion of rats to alcohol-containing liquid diets and, consequently, a lower than *ad libitum* caloric intake (171 vs. 187 kcal/kg$^{3/4}$/day). Conversely, rats infused with an IG low-carbohydrate diet that contained ethanol at the optimal 187 kcal/kg$^{3/4}$/day level grew at 2.9 g/day, which is only slightly lower than their relative controls and comparable with rats fed chow *ad libitum*.

Liver Injury and Pathology. Rats fed low-carbohydrate diets plus ethanol orally developed pan-lobular micro- and macrovesicular steatosis, inflammatory infiltrates of monocytes, polymorphonucleated granulocytes, and lymphocytes and occasional foci of necrosis (Fig. 1B2–E2). These pathological alterations were qualitatively similar to those observed in the livers of rats that received a low-carbohydrate diet plus ethanol via intragastric infusion (Fig. 1B1–E1). However, although steatosis scores (macro- and microvesicular) were comparable, greater levels of inflammation and necrosis were observed in the livers of rats fed IG ethanol ($P \leq 0.05$; Fig. 2). Baseline serum ALT values were higher in control rats infused intragastrically than those fed orally. No pathology was observed in control rats of either group (Fig. 1, A1 and A2). In both low-carbohydrate models, ethanol caused a threefold increase in serum ALT concentrations over values in control animals ($P \leq 0.05$; Table 2).

Blood Ethanol. Even though the mean BECs attained were comparable between the oral feeding and the IG models (Table 2), in the latter, ethanol concentrations demonstrated a characteristic pulsatile pattern of high peaks around 500–600 mg/dl and the nadirs of almost zero previously observed by ourselves and other investigators (28–30).

Autoimmunity. Analysis of the immune response induced by HER or lipid peroxidation adducts with hepatic proteins demonstrated a significant ($P \leq 0.05$) elevation in the titers of antibodies recognizing MDA and HER antigens after ethanol exposure in both oral and IG low-carbohydrate models (Table 3). However, greater antibody titers toward MDA adducts were observed in the rats that received IG ethanol (Table 3).

CYP2E1. The induction of CYP2E1 apoprotein expression by ethanol relative to expression in control microsomes was significantly greater in the IG than in the oral low-carbohydrate model (Table 2). However, when absolute expression of CYP2E1 apoprotein was assessed after ethanol treatment, using purified CYP2E1 as an internal standard, CYP2E1 expression in the two models was the same (0.53 ± 0.03 vs. 0.53 ± 0.08 nmol/mg microsomal protein in the intragastric vs. oral model), and comparable levels of carbon tetrachloride-dependent lipid peroxidation were also observed (777 ± 155 vs. 735 ± 125 pmol thiobarbituric acid reactive products formed/mg/min).

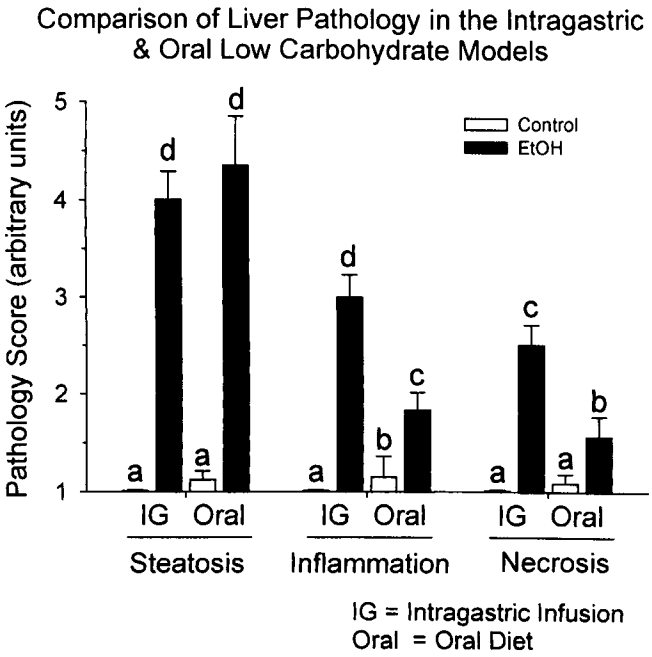

Figure 2. Liver pathology scores for steatosis, inflammation, and necrosis in male rats exposed to ethanol using the low-carbohydrate intragastric TEN model or the low-carbohydrate oral liquid diet model. Data are presented as mean ± SE for 8–10 rats/group. Pathology scores were determined as described in "Materials and Methods" on blinded samples by two independent pathologists. Means with differing superscripts are significantly different ($P \leq 0.05$) using one-way ANOVA followed by Student's Neuman-Keuls post hoc analysis.

In contrast, liver microsomes from rats fed ethanol orally as part of a high-carbohydrate Lieber DeCarli diets had significantly lower CYP2E1 expression (0.32 ± 0.06 nmol/mg) and CYP2E1-dependent carbon tetrachloride metabolism (358 ± 17 pmol/mg/min; Table 4).

Endotoxin. A small increase in plasma endotoxin over controls was observed in rats fed the low-carbohydrate ethanol diet orally ($P \leq 0.05$). However, the endotoxin level in these rats was still very low (9 pg/ml) and was actually lower than that in control rats fed IG low-carbohydrate diets. No significant increases in plasma endotoxin were observed with ethanol in the IG model (Table 2).

Cytokine Analysis. The results of quantitative RT-PCR analysis of cytokine mRNA expression in the livers of rats that received ethanol in low-carbohydrate diets are shown in Figure 3. In the IG model, ethanol increased the expression of proinflammatory cytokines IL-1β and TNF-α and the expression of IL-10 mRNA ($P \leq 0.05$). This was accompanied by a significant fall in anti-inflammatory IL-4 mRNA expression. In contrast, in the oral-feeding model, ethanol had no effects on IL-4 expression and caused significant reductions ($P < 0.05$) in IL-1β, TNF-α, and IL-10 mRNA. Of interest, the basal expression of IL-4 in control rats that received the oral low-carbohydrate diet was drastically lower than that observed in the controls of the IG model.

Table 2. Effects of Ethanol Feeding in Rats in Combination with Low-Carbohydrate Diets Delivered Intragastrically or Orally[a]

Treatment	Body wt. (g at death)	Liver wt. (g at death)	% of liver as body wt. at death	Ethanol dose (g/kg/day)	Mean BEC (mg/dl)	ALT (SFU/ml)	Total pathology score	CYP2E1 (% of control)	Endotoxin (pg/ml)
IG model									
Control	452 ± 22***	12.6 ± 1.0**	2.8 ± 0.1*	0	0	112 ± 10***	3.0 ± 0.3*	100 ± 36*	13 ± 4[b]
EtOH	430 ± 11***	21.2 ± 0.9***	5.0 ± 0.2***	11.5 ± 0.7	182 ± 4	292 ± 43****	10.4 ± 0.4***	1,400 ± 193***	4 ± 7[a,b]
Oral model									
Control	351 ± 5**	9.0 ± 0.5*	2.6 ± 0.1*	0	0	14 ± 1*	3.0 ± 0.3*	100 ± 25*	3 ± 1[a]
EtOH	327 ± 13*	11.0 ± 0.7**	3.3 ± 0.2**	12.3 ± 0.5	172 ± 61	60 ± 10**	8.0 ± 0.5**	840 ± 60**	9 ± 2[b]

[a] Data are presented as mean ± SE for 8–10 animals/group. Means with asterisks as superscripts differ by $P \leq 0.05$ using one-way ANOVA followed by Student's Neuman-Keuals post hoc analysis, * < ** < *** < ****.

Table 3. Antibody Formation Against Malondialdehyde and Hydroxyethyl Free Radical Protein Adducts After Ethanol Treatment in Rats in Combination with Low-Carbohydrate Diets Delivered Intragastrically or Orally

Treatment	Anti–MDA-BSA IgG[a]	Anti–HER-BSA IgG[b]
IG model		
Control	0.36 ± 0.1*	0.11 ± 0.03*
EtOH	0.57 ± 0.1***	0.17 ± 0.02**
Oral model		
Control	0.22 ± 0.02*	0.05 ± 0.01*
EtOH	0.40 ± 0.02**	0.15 ± 0.02**

[a] Optical density (OD) at 490 nm in an enzyme-linked immunosorbent assay (ELISA) using BSA modified by reaction with 50 m*M* malondialdehyde (MDA).
[b] OD at 490 nm in an ELISA using BSA reacted with hydroxyethyl radicals generated from the decomposition of 1,1′-dihydroxyazoethane (see "Materials and Methods"). Data are presented as mean ± SE for 8–10 animals/group. Means with asterisks as superscripts differ by $P \leq 0.05$ using one-way ANOVA followed by Student's Neuman-Keuals post hoc analysis, * < ** < ***.

Discussion

Ethanol consumed *ad libitum* as part of oral high-carbohydrate (Lieber DeCarli) diets results in steatosis but no further pathology (3, 4, 19). Recently, a number of laboratories have reported that diets low in carbohydrate (or with high fat:carbohydrate ratios) exacerbate the liver pathology produced by ethanol exposure (2, 19, 20). However, the additional pathology described was highly variable. One of these studies (2) used an IG infusion model, whereas, in the latter two (19, 20), oral liquid diets were fed. In addition, although all of the diets used had low carbohydrates, they differed substantially in composition. For example, the low-carbohydrate ethanol IG diet of Korourian *et al.* (2) contained 36% ethanol, hydrolyzed whey protein at 16% of total calories, and corn oil at 42% calories. The oral low-carbohydrate diet of Li *et al.* (20) contained 40% ethanol but only 29% corn oil and was based on lactalbumin as a protein source at 25% of total calories. In contrast, the oral low-carbohydrate diet of Lindros and Javelainen (19) was a modified Lieber DeCarli diet with 35% ethanol, 44% fat (corn oil, safflower oil, and olive oil), and 16% protein, with 0.4% carboxymethylcellulose added as a stabilizer. The liver pathology was also scored by different pathologists. All of these variables may have contributed to the wide range of reported alcohol pathology. To gain a better insight into the differences in liver pathology produced by ethanol in the various low-carbohydrate rodent models and the molecular mechanisms underlying ethanol effects, we conducted side-by-side studies using a low-carbohydrate oral ethanol diet and the IG low-carbohydrate ethanol diet. In addition to pathology, for the first time, ethanol-induced biochemical and cytokine profiles have also been compared in these different models.

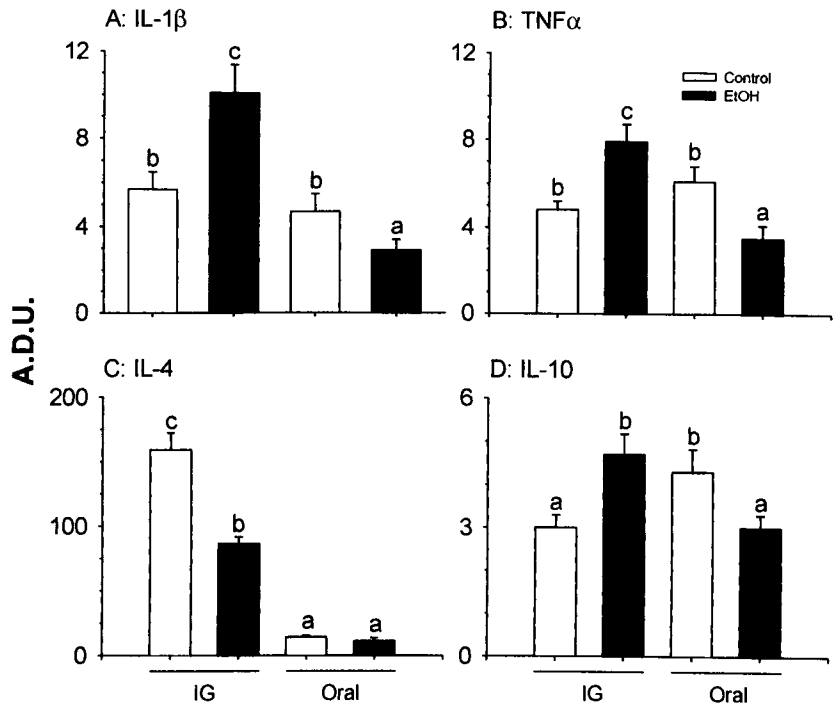

Figure 3. Relative expression of hepatic cytokine mRNAs in rats fed control or ethanol diets using the intragastric low-carbohydrate model or oral low-carbohydrate model. IG, intragastric model; Oral, oral model. Values are presented as ADU on the basis of densitometric analysis of RT-PCR products generated as described in "Materials and Methods." Data are mean ± SE for 8–10 rats/group. Means with different letters are significantly different ($a < b < c$), $P \leq 0.05$ using one-way ANOVA followed by Student's Neuman-Keuls post hoc analysis.

Results obtained using the low-carbohydrate oral feeding model developed by Lindros and Javelainen confirmed a greater pathology score than observed when ethanol is fed as part of traditional Lieber-DeCarli diets (3, 4, 19), accompanied by variable additional inflammation and necrosis and significantly increased plasma ALT values. In addition, significantly greater CYP2E1 expression was observed in liver microsomes from rats fed a low-carbohydrate ethanol diet orally compared with those fed Lieber DeCarli diets. We have demonstrated that carbon tetrachloride is a more specific substrate for CYP2E1 than either *p*-nitrophenol or chlorzoxazone (31). Carbon tetrachloride–dependent lipid peroxidation values correlated well with CYP2E1 expression in our results. Although controversial, it has been suggested that the oxidative metabolism of ethanol via CYP2E1 contributes significantly to the development of ALD through the generation of reactive oxygen species (32, 33). The high levels of CYP2E1 expression that we observed are likely related to the carbohydrate intake, because IG ethanol feeding in combination with a high-carbohydrate (21% of total calories) diet has been reported to limit CYP2E1 induction to three- to ninefold over the control levels, compared with the 20–30-fold induction observed in rats receiving a similar amount of ethanol plus a low-carbohydrate (3% of total calories) diet (2). It appears that, even during oral feeding, low carbohydrate intake enhances the ethanol induction of CYP2E1 to levels sufficient to promote hepatic necrosis and inflammation. The molecular mechanisms whereby low dietary carbohydrate increases CYP2E1 expression synergistically with ethanol are not understood.

Additional liver damage may occur as the result of increased oxidative stress. Indeed, protein adducts derived from HER and the lipid peroxidation product MDA have been observed in livers from rats fed ethanol in IG low-carbohydrate diets (15), and auto-antibodies against these protein adducts, a reliable marker for the development of free radical damage during ethanol intoxication (34), were significantly increased in ethanol-treated rats that received either oral or IG low-carbohydrate diets in the current study.

It is noteworthy that, in rats that received ethanol and low-carbohydrate diets by either oral or IG feeding developed hepatic necro-inflammatory lesions in the absence of a high concentrations of plasma endotoxin. The levels reported in the current study (3–14 pg/ml) are comparable to the 8–10 pg/ml endotoxin observed in human alcoholics (35, 36) and are considerably lower than the 40–100 pg/ml observed in some previous reports with IG ethanol feeding models or when ethanol was administered as a daily 5 g/kg bolus by gavage (37). Endotoxin concentrations were not significantly increased by ethanol in the IG low-carbohydrate model that had the greatest pathology (6, 7). The current data suggest that, although elevated endotoxin may exacerbate ALD, ethanol itself appears to be the major cause of liver injury in these low-carbohydrate rodent models (38, 39).

Although more labor intensive and expensive to maintain, the low-carbohydrate TEN IG model has the

Table 4. Effects of Ethanol in Rats Fed Low-Carbohydrate or Traditional Lieber DeCarli Diets Orally[a]

Treatment EtOH	Body wt (g at death)	Liver wt (g at death)	% of liver as body wt (at death)	Ethanol dose (g/kg/day)	Mean BEC (mg/dl)	ALT (SFU/ml)	Total pathology score	CCl_4 (pmol/mg/min)	CYP2E1 (nmol/mg microsomal protein)
Low carbohydrate	237 ± 34*	10.0 ± 2.1	4.2 ± 0.4**	11.7 ± 0.3	292 ± 41**	129 ± 68**	7.0 ± 0.5**	735 ± 125**	0.53 ± 0.08**
Lieber DeCarli	296 ± 15**	10.3 ± 0.7	3.5 ± 0.2*	12.4 ± 0.3	219 ± 29	55 ± 14*	5.1 ± 0.3*	358 ± 17*	0.32 ± 0.06*

[a] Data are presented as mean ± SE for 8–10 animals/group. Means with asterisks as superscripts differ by $P < 0.05$ using one-way ANOVA followed by Student's Neuman-Keuls post hoc analysis, * < **.

advantage of producing significantly greater liver pathology, especially inflammation and necrotic lesions, over the same period of time than the oral feeding of low-carbohydrate ethanol diets, while maintaining normal growth rates. Although control animals fed low-carbohydrate diets intragastrically had elevated endotoxin and ALT values and levels of HER-and MDA-adduct auto-antibodies compared with orally fed controls, which was indicative of some oxidative stress in the absence of ethanol, this was not associated with any liver pathology. We are unsure why control ALT values were high in the IG controls in the current study. Controls in this model generally exhibit ALT values of 40–60 SFU/ml (2). In humans, plasma ALT is generally poorly correlated with ethanol-induced liver pathology (40). ALT values are only increased an average of two- to eightfold in human ALD, similar to the ethanol-induced increases observed in the current study (40). This is in contrast to toxic or ischemic liver injury, where values increase >40-fold.

It is possible that the additional hepatic inflammation and necrosis occurring in the TEN intragastric system, at the same level of ethanol intake and a comparable average blood ethanol concentration to that observed in the oral feeding model, might be the result of higher peaks of blood ethanol (up to 500 mg/dl) attained during the characteristic cycling of BECs in the TEN and other IG models (2, 28–30). In the oral feeding model, ethanol concentrations do not cycle in any regular fashion (20, 41). Alternatively, the additional pathology may be related to hypoxia/reoxygenation damage associated with cycling BECs (20, 30, 31, 41, 42). There is some evidence for the occurrence of cyclic drinking patterns in animal models of voluntary consumption, such as monkeys or pigs (43, 44), and it is possible that data from IG infusion models may have relevance to risks of alcoholic injury in binge drinkers. In addition, the high caloric intake in the infused animals, which, as in *ad libitum* fed rats, result in enhanced triglyceride synthesis and obesity, may also contribute to the additional ethanol-induced liver pathology observed in these animals.

Changes in pro- and anti-inflammatory cytokine profiles are a feature of human alcoholic liver disease (45) and have been suggested to play a key role in the development of ALD and cirrhosis (6, 8–12). Hepatic pathology in the IG low-carbohydrate model was accompanied by significant increase in proinflammatory TNF-α and IL-1β mRNAs. This is consistent with previous observations in the TEN IG model that demonstrated that ethanol-induced increases in proinflammatory cytokine expression were dependent on CYP2E1 activity (12). The up-regulation of proinflammatory cytokines induced by alcohol in the IG model was also accompanied by a decreased concentration of the cytokine IL-4. IL-4 is a pleiotropic cytokine produced by activated T lymphocytes, basophils, and mast cells that has important immunoregulatory functions on B and T cells (46). In particular, IL-4 acts as a growth factor for preactivated B and T lymphocytes, enhances the expression of class II MHC on the B cell surface,

and stimulates antibody-dependent Th2 immune responses, which are protective (46). A decrease in IL-4 production by peripheral blood T lymphocytes associated with an enhanced IL-2 and interferon γ (Th1) proinflammatory response has been recently observed in patients with advanced ALD (47). This suggests the possibility that the development of alcoholic liver injury might involve an imbalance in cytokine signaling toward the inhibition of a T cell–mediated Th2 immune response and induction of a Th1 response similar to that observed in other inflammatory diseases such as rheumatoid arthritis (48). The drastic loss of IL-4 in the oral low-carbohydrate model, even in the absence of ethanol, may be associated with greatly reduced caloric intake or with particular dietary components such as the presence of carboxy-methylcellulose. Because the loss of IL-4 will inhibit the Th2 immune response, it is possible that animals fed this diet are sensitized to ethanol-induced injury and develop mild inflammation and necrosis even in the absence of increased levels of proinflammatory cytokines. The significant increase in the expression of IL-10 in the IG model may be interpreted as an attempt to counteract the chronic proinflammatory stimulation induced by alcohol. The same effect has previously been reported in human alcoholics (49). Consistently, in a recent paper, Hill *et al.* (50) have reported that IL-10 knockout mice are more susceptible to proinflammatory stimuli and to liver damage induced by the concomitant administration of ethanol and lipopolysaccharide. The decrease in IL-10 in the oral feeding model may again be related to an interaction between ethanol and particular dietary components or to reduced nutrition and is consistent with the absence of increases in proinflammatory cytokines.

In conclusion, we show that the oral administration of ethanol in a low-carbohydrate diet according to the method of Lindros and Jarvelainen (19) replicates some of the liver pathology observed using low-carbohydrate IG feeding even in the absence of the increased synthesis of pro-inflammatory cytokines. However, by causing significantly greater liver pathology accompanied by proinflammatory changes in the cytokine pattern in the context of normal growth rates, the IG low-carbohydrate TEN model remains a more valuable experimental system for investigating the pathogenic mechanisms of ALD.

We thank the following for excellent technical assistance during this project: Terry Fletcher, Matt Ferguson, Shanda Ferguson, Cindy Mercado, Jared Eros, Misty Reeves, Elisa Mottaran, Maria Grazia Moretti, Gunilla Ronnholm, and Anna Uro.

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