The effects of chronic ethanol administration on the endocytosis of three representative cytokines were investigated in isolated rat hepatocytes. When hepatocytes were isolated from rats that were fed an ethanol liquid diet for 12 to 13 weeks, these cells exhibited a decreased ability to internalize and degrade transforming growth factor-α, tumor necrosis factor-α and interleukin-6, compared with hepatocytes from the pair-fed controls. This impaired endocytosis of all three cytokines was accompanied by significant decreases in the amount of hepatocyte surface-bound cytokine. Changes in cytokine binding to surface receptors and reduced rates of receptor-cytokine complex internalization into the cells seem to be major contributors to defective endocytosis in hepatocytes from the ethanol-fed rats. Impaired hepatocyte endocytosis could lead to altered steady-state levels of cytokines in the liver and modified physiological responses to cytokines. These changes could affect homeostasis among the various cell types in the liver and could contribute to liver dysfunction and injury.

Key Words: Ethanol, Hepatocytes, Cytokines, Endocytosis.

INTERCELLULAR COMMUNICATION among the various cell types of the liver plays an important role in regulating liver functions and maintaining physiological homeostasis. A complex network of mediator molecules, known as cytokines, seems to be an integral component of this communication system. Cytokines are soluble peptides that are produced and secreted by various cells within the liver and can act in a paracrine or autocrine fashion to generate a wide variety of biological responses by interacting with specific cell surface receptors. Alterations in cytokine levels and activity have been proposed to contribute to many of the injurious events associated with alcoholic liver injury, including inflammation, impaired regeneration, and fibrosis.

Regulation of cytokine levels and activity occurs at many levels in the liver. Expression of specific cytokine genes, conversion from inactive to active forms, presence of soluble inhibitors, and expression of specific surface receptors represent processes by which the biological action of cytokines can be controlled. In addition, the hepatocyte is a principal clearance site of cytokines, serving as a main scavenger for cytokines by degrading them via endocytosis. Thus, this endocytic step could serve as a major regulatory step in the action of various cytokines and could potentially be susceptible to ethanol-induced alteration. Because we have previously shown that chronic ethanol administration markedly impairs receptor-mediated endocytosis of asialoglycoproteins, epidermal growth factor, and insulin, the influence of ethanol intake on the endocytosis of cytokines by the hepatocyte could contribute to the modification of cytokine action during the development of alcoholic liver injury.

In this study, we have chosen to investigate the effects of chronic ethanol administration on three representative cytokines: tumor necrosis factor (TNF), interleukin-6 (IL-6), and transforming growth factor-α (TGF-α). Both TNF-α and IL-6 levels have been shown to be increased in patients with alcoholic hepatitis, and both have been suggested to be possible mediators of ethanol-induced liver damage. TGF-α binds to the same receptors as epidermal growth factor and acts in an autocrine manner as a potent stimulator of hepatocyte regeneration. Therefore, the purpose of this study was to investigate the effects of chronic ethanol treatment on the endocytosis of these three cytokines.

MATERIALS AND METHODS

Human recombinant TGF-α, human recombinant IL-6, human recombinant TNF-α, and bovine serum albumin (BSA) were purchased from Interogen Company (Purchase, NY). Collagenase (type IV), Percoll, HEPES, and phosphotungstic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant forms of 125I-TGF-α, 125I-IL-6, and 125I-TNF-α were purchased from Amersham Corporation (Arlington Heights, IL). Eagle's medium (Gibco Laboratories, Grand Island, NY; catalog no. 420-1400) was supplemented with 2.4 g/liter of HEPES (pH 7.4) and 0.22 g/liter of sodium bicarbonate. Eagle's BSA was Eagle's medium supplemented with 0.1% BSA. All other chemicals were reagent grade.

Male Wistar rats (140 to 160 g) were obtained from Charles River Laboratories (Raleigh, NC). Nutritionally adequate liquid diets were formulated according to the method of Leiber and DeCarli and purchased from Dyets, Inc. (Bethlehem, PA). The ethanol-containing diet consisted of 18% of total calories as protein, 35% as fat, 11% as carbohydrate, and 36% as ethanol. In the control diet, ethanol was replaced isoenergetically with carbohydrate.
Ethanol Treatment of Rats

Animals were initially maintained on a Purina rat chow diet until they reached body weights of 170 to 180 g and were then divided into two groups. The rats were housed in individual cages and acclimated to the Leiber-DeCarli control diet for 4 days. These rats were then weight-matched and paired so that one rat received the liquid diet containing ethanol as 36% of total calories, and the second animal was pair-fed the isocaloric control diet. The rats were pair-fed for 12 to 13 weeks. During the 24 hr before the isolation of hepatocytes, the liquid diets were given in three portions: one-fourth at 9 AM, one-half at 3 PM, and the final one-fourth at 7 AM. This meal-feeding regimen was used to minimize variations in feeding patterns between the ethanol-fed rats and their pair-fed controls before preparation of hepatocytes.

Preparation of Hepatocytes

Hepatocytes were prepared by the collagenase perfusion method as described previously. Cells were further purified using continuous Percoll gradients. Viability was routinely >80% after centrifugation with Percoll. This treatment did not affect endocytosis of the cytokines as tested in this study. Purified hepatocytes were incubated in Eagle's medium, containing 0.1% BSA, at cell densities of 2.5 to 3.0 million cells/ml in a metabolic shaker at 37°C. For all the experiments, hepatocytes were first preincubated for 40 min at 37°C to increase and equilibrate the number of cell surface receptors before the endocytosis of each cytokine was studied.

Measurements of Surface Binding and Endocytosis

Determinations of surface binding and endocytosis of the cytokines were conducted after the basic procedures used previously in our laboratory to test the effects of ethanol feeding on the endocytosis of epidermal growth factor. These procedures were similar to those used by others to measure the endocytosis of TGF-α, IL-6, and TNF-α. Because endocytosis results in the specific cellular uptake and degradation of a given ligand, the amount of ligand endocytosed over a given time period can be represented by the sum of the ligand degraded and the amount of intact ligand present inside the cell. Therefore, measurements of cytokine degradation and amounts of intracellular intact cytokine were conducted, and the sum of these values represented the total amounts of cytokine endocytosed by hepatocytes.

Degradation of labeled TGF-α, IL-6, or TNF-α was followed by determining the amount of soluble radioactivity after precipitating protein in the cell suspensions with 1% (w/v) of phosphotungstic acid in 10% trichloroacetic acid. Intracellular, intact TGF-α, IL-6, or TNF-α was taken as the amount of radioactivity displaced by acid treatment (pH 2.5) of the presence of 125I-TGF-α, 125I-IL-6, or 125I-TNF-α at concentrations of 100 ng/ml. During incubation, the endocytosis of TGF-α was determined as described in “Materials and Methods.” Results are expressed as means ± SE for 7 to 8 experiments. Values significantly different from controls are indicated: *p < 0.05; **p < 0.01; ***p < 0.001.

Because some hepatocyte preparations may release cytokine-degrading activity into the medium and even though our system was developed to minimize this release, we tested the extent of extracellular degradation of cytokines in our hepatocyte preparations. Medium removed from cells incubated for 180 min, degraded <5% of added cytokine after an additional 180 min of incubation, and no significant differences were noted between hepatocyte preparations from ethanol-fed and control rats. Therefore, most of the degradation of cytokines in our cellular preparations occurred by intracellular processes.

General Procedures

Centrifugations of cell suspensions were performed at 4000 rpm for 30 sec. The 1% phosphotungstic acid and 10% trichloroacetic acid mixtures were chilled on ice for at least 15 min, then centrifuged for 7 min at 2000 rpm, and radioactivity in the supernatant and precipitate determined. Because some hepatocyte preparations may release cytokine-degrading activity into the medium, we tested the extent of extracellular degradation of cytokines in our hepatocyte preparations. Medium removed from cells incubated for 180 min, degraded <5% of added cytokine after an additional 180 min of incubation, and no significant differences were noted between hepatocyte preparations from ethanol-fed and control rats. Therefore, most of the degradation of cytokines in our cellular preparations occurred by intracellular processes.

RESULTS

TGF-α was very actively endocytosed by hepatocytes from the pair-fed controls, and most of the TGF-α that was added initially to the incubation media at both the low 5 ng/ml level and also at the high 100 ng/ml level was processed within 2 hr of incubation (Fig. 1). Hepatocytes from the ethanol-fed animals exhibited a decreased ability to endocytose TGF-α at both the low (Fig. 1A) and high (Fig. 1B) concentrations. Associated with this decrease in endocytosis, a decrease in hepatocyte surface binding of TGF-α was observed. For example, after 15
EFFECTS OF CHRONIC ETHANOL ADMINISTRATION ON ENDOCYTOSIS

Fig. 2. Effects of ethanol feeding on the endocytosis of TNF-α. Hepatocytes from pair-fed control (○) and ethanol-fed rats (●) were incubated at 37°C in the presence of 125I-TNF-α at concentrations of (A) 5 ng/ml and (B) 100 ng/ml. During incubation, the endocytosis of TNF-α was determined as described in "Materials and Methods." Results are expressed as means ± SE for 7 to 8 experiments. Values significantly different from controls are indicated: *p < 0.05; †p < 0.01; ‡p < 0.001.

Fig. 3. Effects of ethanol feeding on the binding of TNF-α to the surface membrane of isolated hepatocytes. 125I-TNF-α at concentrations of (A) 5 ng/ml and (B) 100 ng/ml were incubated at 37°C with hepatocytes isolated from pair-fed control (○) and ethanol-fed rats (●). Surface binding was determined at the indicated time periods as described in "Materials and Methods." Results are expressed as means ± SE for 7 to 8 experiments. Values significantly different from controls are indicated: *p < 0.05; †p < 0.01; ‡p < 0.001.

Fig. 4. Effects of ethanol feeding on the endocytosis and surface binding of IL-6. Hepatocytes from pair-fed control (○) and ethanol-fed rats (●) were incubated at 37°C in the presence of 5 ng/ml of 125I-IL-6. (A) Endocytosis and (B) surface binding were determined at the indicated time periods as described in "Materials and Methods." Results are expressed as means ± SE for 7 to 8 experiments. Values significantly different from controls are indicated: *p < 0.05; †p < 0.01; ‡p < 0.001.

DISCUSSION

The results of this study demonstrate that chronic ethanol administration impairs the endocytosis of cytokines by the hepatocyte. Decreased endocytosis of TGF-α, TNF-α, and IL-6 by hepatocytes isolated from ethanol-fed rats was observed. Altered endocytosis of TGF-α is consistent with our previous findings showing an ethanol-induced impairment of endocytosis of epidermal growth factor,9 because TGF-α has a 30 to 40% homology with epidermal growth factor and binds to the same receptor.15 TGF-α, a potent stimulator of hepatocyte regeneration, was very efficiently taken up and degraded by the hepatocyte, but this process was significantly inhibited by ethanol treatment. Endocyto-
sis of TNF-α and IL-6 was much less efficient than TGF-α; however, ethanol feeding was still very effective in decreasing the endocytosis of these two cytokines.

Impaired endocytosis of all three cytokines was accompanied by significant decreases in the amount of hepatocyte surface-bound cytokine. Because the endocytosis of these ligands is likely receptor-mediated, altered binding to specific receptors may account in part to the observed reduction in processing of these ligands by hepatocytes isolated from the ethanol-fed rats. Deaciuc et al.22,23 have also reported changes in TNF-α and IL-6 binding properties to hepatocyte-surface receptors by chronic ethanol feeding, and our previous studies also indicated ethanol-induced changes in epidermal growth factor binding to cell-surface receptors.9 However, it should be pointed out that the net amount of surface-bound ligand observed during continuous endocytosis, as reported in this study, was not only caused by cytokine binding to receptor, but was also influenced by the rate of cytokine internalization into the cell. Impaired internalization of receptor-cytokine complexes into the hepatocyte could also be a major contributor to impaired endocytosis of cytokines by ethanol feeding. This is a likely possibility because our previous studies have shown that chronic ethanol administration selectively impairs the uptake of various ligands, including epidermal growth factor, that are internalized via the clathrin-coated pit pathway.24

The most apparent consequence of impaired hepatocyte endocytosis of cytokines would be a decrease in clearance (i.e., degradation) of cytokines, resulting in elevated cytokine levels in the liver. Increased levels of TNF-α and IL-6 have been reported in patients with alcoholic hepatitis.11-13 Increased production and secretion of cytokines by various cell types in the liver, especially Kupffer cells, induced by ethanol treatment have been shown to contribute to increased levels of TNF-α25-27 and other cytokines,28 and the subsequent biological effects of these elevated levels of cytokines have been implicated in the pathogenesis of alcoholic liver disease.4,29 Increased levels of TNF-α mRNA in ethanol-fed rats have been reported,30 suggesting an effect of ethanol on cytokine gene expression. However, in addition to cytokine synthesis and secretion, the degradation of cytokines also contributes to the steady-state levels of cytokines in the liver. Because the hepatocyte serves as a major clearance site for cytokines,2 the impaired endocytosis and subsequent decrease in degradation of cytokines, as reported in this study, could also be a significant factor in the ethanol-induced elevation of hepatic cytokine levels and their enhanced cytoxic activity.

In addition to simply decreasing cytokine clearance, impaired cytokine endocytosis could also alter the signal transduction process elicited by cytokines and potentially modify the biological response in hepatocytes of these various cytokines. In fact, previous studies by Akerman et al.31 have shown that chronic ethanol treatment alters the hepatic response to the regenerative effects of TNF-α. Future studies focused on the role of altered endocytosis, and cytokine signaling should yield important and relevant new information concerning the role of cytokines in the pathogenesis of alcoholic liver disease.

In summary, chronic ethanol administration impairs the hepatocyte endocytosis of three representative cytokines: TGF-α, TNF-α, and IL-6. Changes in hepatocyte-surface binding of cytokines and inhibited internalization of receptor-cytokine complexes into the cell seem to be the major steps of the endocytotic process affected by ethanol treatment. This impairment could lead to altered steady-state levels of cytokines in the liver and modified physiological response to cytokines. These ethanol-induced effects on the actions of cytokines could lead to a disruption of the intercellular communication system among the various cell types in the liver and could play an important role in the pathogenesis of alcoholic liver disease.

REFERENCES
15. Mead JF, Fausto N: Transforming growth factor α may be a physiological regulator of liver regeneration by means of an autocrine mechanism. Proc Natl Acad Sci USA 86:1558-1562, 1989