Ethanol Feeding Inhibits Proinflammatory Cytokine Expression from Murine Alveolar Macrophages Ex Vivo

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The prolonged and excessive consumption of alcohol has been shown to predispose the host to a variety of infectious complications, which may be due, in part, to the inability to produce important activating and chemotactic cytokines. In this study, we assessed the effect of alcohol ingestion on the expression of tumor necrosis factor-α (TNF-α), and the chemokines macrophage inflammatory protein-2 (MIP-2) and macrophage inflammatory protein-1α (MIP-1α) from murine alveolar macrophages (AMs) cultured ex vivo. Two-week ethanol feeding resulted in substantial impairment in the lipopolysaccharide (LPS)-induced expression of TNF-α, MIP-2, and MIP-1α mRNA, and protein from LPS-stimulated AMs, compared with cytokine production from AMs obtained from CD-1 mice receiving an isocaloric control diet. These findings indicate that ethanol feeding results in diminished production of chemotactic and/or activating cytokines from AMs ex vivo that may contribute to the impairment in lung inflammatory responses and antimicrobial host defense that is observed in the setting of alcohol ingestion/intoxication clinically and experimentally.

Key Words: Tumor Necrosis Factor-α, Chemokines, Alcohol, Alveolar Macrophages, Cytokines.

HOST DEFENSE against lung pathogens requires the accumulation and activation of leukocytes, including neutrophils and mononuclear phagocytes, at the site of microbial invasion. Resident alveolar macrophages (AMs) are believed to play a critical role in the process of leukocyte recruitment/activation via the elaboration of important activating and chemotactic cytokines. Tumor necrosis factor-α (TNF-α) is one such cytokine produced by activated AMs that directly activates leukocyte microbicidal activity, and can mediate lung polymorphonuclear influx by upregulating leukocyte adhesion molecules and by serving as an affrent signal in the autocrine or paracrine induction of chemotactic molecules. AMs are also rich cellular sources of chemokines. The C-X-C chemokine family, which includes macrophage inflammatory protein-2 (MIP-2), exerts predominant neutrophil chemotactic and activating effects, whereas C-C chemokines—including macrophage inflammatory protein-1α (MIP-1α)—are chemotactic for mononuclear cells and eosinophils. TNF-α, as well as several members of the chemokine family, have been shown to be required for effective host defense against a variety of pulmonary pathogens. The induction of TNF-α, MIP-2, and MIP-1α gene expression in response to endotoxin requires the binding of a trans-NF-κB binding protein to the NF-κB DNA binding site, resulting in enhanced proinflammatory cytokine gene expression.

The prolonged and excessive consumption of alcohol predisposes the host to a variety of infectious complications, in particular bacterial infections of the lung. The alcohol-induced impairment in lung antimicrobial host defense is believed to be due, in part, to the inability to produce important activating and chemotactic cytokines. Specifically, the acute, but not chronic infusion of alcohol, has been shown to attenuate lipopolysaccharide (LPS)-induced increases in serum TNF-α. In addition, alcohol inhibits the induction of TNF-α detected in bronchoalveolar lavage (BAL) fluid after the intratracheal administration of LPS or live bacterial organisms. The incubation of AMs with alcohol in vitro suppresses the production of murine macrophage-derived interleukin (IL)-6 production, whereas prolonged alcohol feeding significantly inhibits the expression of interferon-γ and soluble IL-2 receptor from retroviral-infected murine splenocytes. These studies indicate that alcohol exposure globally suppresses the ability of immune cells to express leukocyte activating cytokines. Molecular mechanisms by which alcohol mediates this effect have not been characterized.

In this study, we assessed the effect of chronic alcohol consumption (2 weeks) on the expression of TNF-α, MIP-2, and MIP-1α from resting and LPS-stimulated AMs ex vivo. We have chosen to study these cells ex vivo, because these conditions allow for cells to be recovered and studied in pure populations, while closely reproducing the conditions of in vivo alcohol exposure.

MATERIALS AND METHODS

Reagents

Murine recombinant TNF-α, MIP-2, and MIP-1α were purchased from R&D Systems (Minneapolis, MN). Polyclonal antimurine TNF-α, MIP-2, and MIP-1α antiserum used in our ELISA were produced by immunization of rabbits with recombinant murine cytokines in multiple intradermal sites with complete Freund's adjuvant. Stock LPS (Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 200 μg/ml in sterile saline.
members of the murine chemokine family, including murine TNF-α, MIP-2, or MIP-1α from 1 pg/ml to 100 pg/ml. This ELISA terminated with 50 μl of 3 M H₂SO₄, and then exposed and intubated using a 1.7 mm o.d. polyethylene catheter.

**Isolation of Murine AMs**

CD-1 mice (Charles River Breeding Laboratories, Cambridge, MA) were maintained under specific pathogen-free conditions. The mice were then anesthetized and exsanguinated. BAL was performed to obtain AMs. Before BAL, the pulmonary vasculature was perfused with 1 ml of phosphate-buffered saline (PBS) via the right ventricle. The trachea was then exposed and intubated with a 1.7 mm o.d. polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1 ml aliquots. Approximately 10 ml of lavage fluid was retrieved per mouse, resulting in the isolation of 5 to 7 x 10⁵ AMs/mouse. The BAL fluid from mice was pooled. The AMs were washed using complete media, followed by cell counts and differential cell analysis. BAL cell differentials consisted of >97% AMs in both control and ethanol-fed animals. AM viability was >95% by trypan blue exclusion. 2 x 10⁵ AMs/200 μl were adherence-purified per well of a 96-well culture plate for protein analysis. For total RNA isolation, 1 x 10⁵ AMs/ml were adherence-purified for each 35 nm culture plate.

**Cytokine ELISAs**

Extracellular immunoreactive murine TNF-α, MIP-2, and MIP-1α was quantitated using a modification of a double ligand method as previously described. Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F, Denmark, Netherlands) were coated with 50 μl/well of rabbit antimurine TNF-α, MIP-2, or MIP-1α antibody (1 μg/ml in 0.6 M NaCl, 0.26 M H₃BO₃, and 0.08 N NaOH; pH 9.6) for 16 hr at 4°C and then washed with PBS (pH 7.5); 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin (BSA). Plates were washed four times, followed by incubation for 1 hr at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit anti-TNF-α, MIP-2, or MIP-1α antibody (3.5 μg/ml in PBS (pH 7.5); 0.05% Tween-20, and 2% fetal calf serum), and plates incubated for 30 min at 37°C. Plates were washed four times, streptavidin-horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA) added, and the plates incubated for 30 min at 37°C. Plates were again washed four times and chromogen substrate (Vector) added. The plates were then incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 M H₂SO₄ solution. Plates were read at 490 nm in an ELISA reader. Standards were ½ log dilutions of recombinant murine TNF-α, MIP-2, or MIP-1α from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine TNF-α, MIP-2, and MIP-1α concentrations above 25 pg/ml and did not cross-react with IL-1, IL-2, IL-4, IL-6, or interferon-γ. In addition, the ELISA did not cross-react with other members of the murine chemokine family, including murine KC, EDA-78, MCP-1, and murine MIP-1β.

**Protocol for Alcohol Feeding**

To assess the effects of alcohol on the expression and regulation of proinflammatory cytokines, two groups of 6- to 8-week-old female CD-1 mice received calorie-matched complete liquids diets (BioServ, Frenchtown, NJ), with the alcohol-fed animals receiving incremental increases in ethanol content in their diet as follows: ethanol 2.2% (v/v) x 4 days; then 4.4% x 4 days, then 6.6% x 6 days, for a total of 14 days of alcohol feeding. Serum ethanol levels were determined in the Clinical Toxicology Laboratory at the University of Michigan Hospital by gas chromatography. The ethanol level in alcohol-fed animals at the end of this period was 166.9 ± 19.4 mg/dl (range: 5.9 to 398.4 mg/dl). During the 2-week calorie-matched diet, the control mice gained 2.00 ± 0.18 g, whereas ethanol-fed animals lost 0.99 ± 0.15 g.

**Statistical Analysis**

Data were analyzed by a Macintosh II computer using Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as mean ± SEM and compared using a two-tailed Student’s t test. Data were considered statistically significant if p values were <0.05.

**RESULTS**

**Effect of Ethanol Feeding on TNF-α, MIP-2, and MIP-1α Expression from Murine AMs Ex Vivo**

Because AMs are the chief immune effector cells of the lung airspace, we assessed the effect of alcohol feeding on the expression of proinflammatory cytokines from murine AMs ex vivo. AMs were harvested from control and alcohol-fed mice, adherence-purified for 1 hr, challenged with various doses of LPS, and supernatants harvested after 24 hr in culture. This time point was chosen, as maximal accumulation of cytokines is observed at 24 hr post-LPS stimulation. As shown in Fig. 1, unstimulated AMs obtained from control mice produced low levels of TNF-α and MIP-1α, and moderate levels of MIP-2, whereas cells treated with graded doses of LPS from 100 ng to 10 μg/ml expressed substantial quantities of these cytokines in a dose-dependent fashion. Interestingly, AMs obtained from ethanol-fed mice expressed significantly less TNF when stimulated with maximal levels of LPS (10 μg/ml), whereas AMs obtained from ethanol-fed mice expressed significantly less MIP-2, and MIP-1α when stimulated with both 1 and 10 μg/ml LPS. Maximally, cells isolated from ethanol-fed mice challenged with 10 μg LPS producing 38.2%, 61.3%, and 43% less TNF-α, MIP-2, and MIP-1α, compared with similar numbers of AMs obtained from control animals. Interestingly, the effect of ethanol feeding was
reversible, as the expression of TNF-α and chemokines from LPS-treated AMs returned to control levels when alcohol feeding was discontinued for 7 days postethanol feeding (data not shown). These data indicate that ethanol feeding results in a marked reduction in the ability of AMs to express important activating and chemotactic cytokines in response to exogenous stimuli, and this inhibitory effect of alcohol is maintained even when these cells are removed from the environment of in vivo alcohol exposure.

Effect of Ethanol Feeding on TNF-α, MIP-2, and MIP-1α mRNA Expression from Murine AMs Ex Vivo.

To examine the molecular level at which alcohol suppressed proinflammatory cytokine expression from endotoxin-challenged AMs, we assessed TNF-α, MIP-2, and MIP-1α mRNA levels from AMs obtained from control and ethanol-fed animals after 4 hr in culture. This time point was chosen because maximal accumulation of chemokine mRNA is observed at that time point in vitro, whereas TNF-α mRNA continues to be expressed in high levels at 4 hr post-LPS stimulation. Both the constitutive and LPS-induced expression of TNF-α (Fig. 2) and MIP-1α (Fig. 3) mRNA were markedly reduced, compared with cytokine mRNA expression from control AMs. Whereas the constitutive expression of MIP-2 mRNA was not altered by ethanol feeding, the LPS-induced expression of MIP-2 levels was diminished, compared with that observed in control AMs (Fig. 4). Maximally, steady-state levels of TNF-α, MIP-1α, and MIP-2 mRNA from ethanol-exposed AM were 47%, 51%, and 48% less than steady-state mRNA levels in control AM, respectively.

DISCUSSION

Alcohol exposure has previously been shown to result in significant impairment in inflammatory cytokine expression. The incubation of murine peritoneal macrophages in 1% v/v ethanol in vitro inhibited the LPS-induced production of IL-6.20 In vivo acute alcohol intoxication resulted in significant attenuation of TNF-α release in response to either endotoxin or intrapulmonary challenge with Klebsiella pneumoniae.17,18 In contrast, the chronic administration of ethanol (6 weeks) had either no effect or actually enhanced the peak expression of serum TNF-α postendotoxin administration.16,19 Our study is the first to examine the effect of in vivo alcohol feeding on the ability of pure populations of AMs to express important proinflammatory
cytokines ex vivo. Two-week alcohol feeding resulted in significant inhibition of AM TNF-α mRNA and protein expression in response to LPS, with cells demonstrating a relative insensitivity to LPS, compared with control AMs. In addition, we observed a marked inhibition of C-X-C and C-C chemokine expression in AMs from alcohol-fed mice in a fashion similar to that observed with TNF-α. It is of interest that the suppressive effects observed after 2-week exposure to alcohol was similar to that observed in acute ethanol intoxication,17,18 rather than the enhancing effects observed after chronic alcohol ingestion.20 Furthermore, our data suggest that AMs are susceptible to reversible alcohol suppressive effects, and the observed impairment in TNF production in the airspace of acutely ethanol-intoxicated animals challenged with *K. pneumoniae*19 is likely due to impairment in TNF production from AMs.

The molecular mechanism(s) by which alcohol suppresses macrophage-derived cytokine production had not previously been defined. Our studies indicate that alcohol feeding suppresses cytokine mRNA levels from endotoxin-stimulated AMs, indicating that the effects of ethanol occur as a result of decreased mRNA synthesis and/or accelerated mRNA degradation. Preliminary studies in our laboratory using gel shift analysis suggest that nuclear NF-κB activity is attenuated in LPS-treated AMs from alcohol-fed mice, compared with control AMs. Indeed, impaired NF-κB activity would account for diminished LPS-induced synthesis of TNF-α, MIP-2, and MIP-1α mRNA—all of which require for NF-κB activation.24,25 Our preliminary findings are consistent with the findings of Fox and colleagues,26 who noted decreased NF-κB activity in Kupffer cells exposed to 100 mM ethanol in culture. In vivo, however, acute ethanol administration or chronic ethanol ingestion followed by acute rechallenge has been associated with enhanced NF-κB activity in rat brain,27 indicating that disparity exists between those observations made in vitro to those made in the in vivo settings. Although these studies suggest ethanol suppresses cytokine mRNA transcription, we cannot exclude a component of ethanol-induced acceleration of cytokine mRNA degradation.

Despite strict calorie-matching of complete liquid diets, alcohol-fed mice experienced modest weight loss during alcohol feeding, whereas control animals gained weight during the experimental period. Disparity in weight gain while on isocaloric diets has been noted previously.18 Therefore, it is possible that nutritional factors could con-
Additional studies indicate that alcohol treatment inhibits the ability of neutrophils and/or macrophages to kill bacterial organisms such as \textit{S. aureus}, \textit{P. mirabilis}, and \textit{Legionella pneumophila}. Collectively, these observations suggest that defects in leukocyte recruitment and antimicrobial activity may be due to insufficient expression of required chemotactic and activating cytokines. The observation of alcohol-induced changes in TNF-\(\alpha\) and chemokine release by activated AMs would readily explain observations made previously. Further studies are required to determine the duration of alcohol effects on ex vivo cytokine expression, as well as effects of alcohol on chemotactic and activating cytokine expression during the evolution of innate and acquired immune responses in vivo.

**REFERENCES**