Ethanol Feeding Impairs Innate Immunity and Alters the Expression of Th1- and Th2-Phenotype Cytokines in Murine Klebsiella Pneumonia

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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 cytokines. In this study, we assessed the effect of chronic alcohol ingestion on bacterial clearance, survival, and cytokine mRNA and protein expression in mice with Klebsiella pneumonia. Two-week ethanol feeding resulted in substantial impairment in the clearance of K. pneumoniae and decreased survival, compared with CD-1 mice receiving an isocaloric diet without ethanol. No differences were noted between control and ethanol groups in the total numbers or percent of bronchoalveolar lavage fluid neutrophils or macrophages at 24 and 48 hr post-intratracheal K. pneumoniae. Importantly, the lungs of alcohol-fed mice with Klebsiella pneumonia displayed a decrease or delay in the expression of interleukin (IL)-12 p35 and p40 mRNA and interferon-γ mRNA, respectively, as well as reduced IL-12 and interferon-γ protein levels, compared with controls. Conversely, a time-dependent increase in lung IL-10 mRNA and protein was noted in ethanol-fed animals, compared with control animals challenged with K. pneumoniae. In summary, our studies indicate that ethanol ingestion results in a profound suppression of lung bacterial clearance and decreased survival in Klebsiella pneumonia, which occurs in association with a shift in the balance of lung cytokine mRNA and protein expression favoring Th2- rather than Th1-phenotype cytokines.

Key Words: Ethanol, Interleukin-12, Interleukin-10, Tumor necrosis factor, Interferon-γ

THE PROLONGED and excessive consumption of alcohol predisposes the host to a variety of infectious complications, particularly bacterial infection of the lung. The alcohol-induced impairment in lung antimicrobial host defense is due to several factors, including alteration in both innate and acquired immune responses. Specifically, acute ethanol intoxication has been shown to impair the mobilization, adherence, and metabolic function of neutrophils [polymorphonuclear leukocyte (PMN) cells] both in vitro and in vivo. In addition, ethanol exposure significantly attenuates the ability of alveolar macrophages to phagocytose and kill bacterial pathogens, in part, by reducing the generation of oxygen-derived free radicals. Finally, ethanol impairs cell-mediated immune responses by reducing the number and effector cell activities of various T-lymphocyte populations.

The specific cellular mechanism(s) by which ethanol exposure alters the recruitment and/or activation of leukocytes at the site of microbial invasion has not been clearly defined, but is believed to be due, in part, to the inability to produce important activating and chemotactic cytokines. Tumor necrosis factor is a cytokine that is required for effective lung innate and acquired immunity. The acute, but not chronic, infusion of alcohol has been shown to attenuate lipopolysaccharide (LPS)-induced increases in serum tumor necrosis factor (TNF) as well as the induction of TNF in bronchoalveolar lavage fluid (BALF) after the intratracheal administration of LPS or live bacterial organisms. We and others have recently shown that Th1-phenotype cytokines, including interferon-γ (IFN-γ) and interleukin (IL)-12, are required signals in host defense against bacterial, mycobacterial, fungal, and parasitic pathogens, whereas the expression of the Th2-phenotype cytokine IL-10 is detrimental to lung innate and cell-mediated immunity. Conflicting data exist regarding the effect of alcohol on the expression of Th1 cytokines. For example, Alak and associates found that prolonged alcohol feeding significantly inhibited the production of IFN-γ and soluble IL-2 receptor from retroviral-infected murine splenocytes. In contrast, acute ethanol treatment has recently been shown to augment the expression of IL-12 from activated human monocytes. The only study examining the effects of ethanol on Th2-phenotype cytokines indicates that the in vitro incubation of human blood monocytes can stimulate the constitutive and LPS-induced expression of IL-10 from human monocytes.

In this study, we assessed the effects of prolonged alcohol consumption (2 weeks) on the development of lung inflammation, bacterial clearance, and survival in mice inoculated with K. pneumoniae intratracheally (i.t.). In addition, we examined the effects of ethanol consumption on the time-
dependent expression of Th1 (IFN-γ and IL-12)- and Th2 (IL-10)-phenotype cytokine mRNA and protein in the lungs of animals with Klebsiella pneumonia.

MATERIALS AND METHODS

Animals
Specific pathogen-free CD-1 mice (6- to 12-week-old females, Charles River Breeding Labs.) were used in all experiments. All mice were housed in pathogen-free conditions within the animal care facility at the University of Michigan (ULAM) until the day of sacrifice.

Protocol for Alcohol Feeding
To assess the effects of alcohol on lung innate immunity, two groups of 6- to 8-week-old female CD-1 received calorie-matched complete liquid diets (Bioserve, Frenchtown, NJ), with the alcohol-fed animals receiving incremental increases in ethanol content in their diet as follows: ethanol 2.2% (v/v) × 4 days, then 4.4% × 4 days, and then 6.6% × 6 days. The mice were challenged with K. pneumoniae on day 14 of ethanol feeding, and were continued at 6.6% ethanol until the time of organ harvest or death. The blood ethanol level in alcohol-fed animals at the time of K. pneumoniae administration (2 weeks of ethanol feeding) was 124.7 ± 29.4 mg/dL. During the 2-week calorie-matched diet, the control mice gained 2.10 ± 0.11 g, whereas ethanol-fed animals lost 1.07 ± 0.19 g.

Klebsiella pneumoniae Inoculation
We chose to use K. pneumoniae strain 43816, serotype 2 (ATTC, Rockville, MD) in our studies because K. pneumoniae is a common cause of pneumonia in chronic alcohol abusers, and this particular strain has been shown to induce an impressive inflammatory response in mice.1,26-28 K. pneumoniae was grown in tryptic soy broth (Difco, Detroit, MI) × 18 hr at 37°C. The concentration of bacteria in broth was determined by measuring the amount of absorbance at 600 nm. A standard of absorbencies based on known colony-forming units (CFU) was used to calculate inoculum concentration. Bacteria were pelleted by centrifugation at 3200 × g × 30 min, washed × 2 in saline, and resuspended at the desired concentration. Animals were anesthetized with 1.8 to 2 mg pentobarbital per animal intraperitoneally. The trachea was exposed, and 30 µl of inoculum or saline were administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Determination of Lung K. pneumoniae CFU
At the time of sacrifice, plasma was collected, the right ventricle perfused with 1 ml of phosphate-buffered saline, then lungs removed aseptically, and placed in 3 ml of sterile saline. The tissues were then homogenized with a tissue homogenizer under a vented hood. Plasma and lung homogenates were placed on ice, and serial 1:10 dilutions were made. The lung homogenates were placed on ice, and serial 1:10 dilutions were made. The trachea was exposed, and 30 µl of inoculum or saline were administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Isolation and Amplification of Cytokine mRNAs
Whole lungs were harvested and immediately “snap-frozen” in liquid nitrogen and stored at −70°C. Total cellular RNA from the frozen lungs was isolated by homogenizing the organs with a tissue homogenizer in a solution containing 25 mM Tris (pH 8.0), 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M of 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris (pH 8.0), 10 mM EDTA, and 1.0% sodium dodecyl sulfate. The mixture was then extracted two times each with phenol-chloroform and chloroform-isooamyl alcohol. The RNA was alcohol-precipitated and the pellet dissolved in diethylpyrocarbonate water. Total RNA was determined by spectrophotometric analysis at 260 nm wavelength. One microgram of total RNA was reverse-transcribed into cDNA utilizing a reverse transcription kit (BRK) and oligo (dT) 12-1 primers. The cDNA was then amplified using specific primers for murine TNF, IFN-γ, IL-10, IL-12 p35, and IL-12 p40, with β-actin primers serving as the control housekeeping gene. The sense and antisense primers used had the sequence 5'-CCT-GTA-GCC-CAC-GTC-GTA-GC-3' and 5'-TTG-ACC-TCA-GGC-CTG-AGT-TG-3' for TNF; 5'-CAG-CGA-CTC-CTT-TTC-CGC-TT-3' and 5'- CCT-CAG-CTT-TGA-AGT-CT-3' for IFN-γ; 5'-CTA-TGC-TGC-CTG-CT-CTC-TTA-3' and 5'-ATG-GCC-TTG-TAG-ACA-CCT-3' for IL-10, 5'-ACC-TGC-TGA-AGA-CCA-CAG-AT-3' and 5'-GAT-TCT-GAA-GTG-CTG-CTG-TG-3' for IL-12 p35, 5'-ATG-TTG-TAG-TGG-TGG-ACT-3' and 5'-GGA-CTG-CTA-CTG-CTG-TTG-AT-3' for IL-12 p40, and 5'-ATG-GAT-GAC-GAT-ACCT-GCT-G-3' and 5'-GAT-CTA-ATC-GGG-GGG-3' for IL-10 and β-actin, respectively. After amplification, the samples were separated on a 2% agarose gel containing 0.3 mg/ml (0.003%) of ethidium bromide and bands visualized and photographed using UV transillumination.

Statistical Analysis
Data were analyzed by a Macintosh II computer using Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA). Survival data were compared using the x² analysis. All other 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 Consumption on Survival in K. pneumoniae
To assess the effect of ethanol feeding on outcome in Gram-negative bacterial pneumonia, CD-1 mice were fed either a complete liquid diet containing graded doses of ethanol or an isocaloric control diet and then administered 10² CFU K. pneumoniae i.t. As shown in Fig. 1, no lethality was observed in control animals until 48 hr post-K. pneumoniae administration, with a gradual decline in survival thereafter. No lethality was observed after 10 days, with nearly 50% of control animals surviving long-term. In contrast, a marked decrease in both early and long-term survival was noted in animals receiving the ethanol diet, with only 5% of animals surviving past 7 days.

Effect of Ethanol Consumption on Bacterial Clearance in K. pneumoniae
To determine if the increased lethality observed in ethanol-fed mice with Klebsiella pneumonia was attributable to impaired lung bacterial clearance, K. pneumoniae CFU were determined in plasma and lung homogenates obtained from control and ethanol-fed mice 48 hr after the intratracheal challenge with 10² CFU K. pneumoniae. Com-
Effect of Ethanol Consumption on the Generation of Pulmonary Inflammation 24 and 48 Hr after K. pneumoniae Administration

Having determined that ethanol ingestion markedly impaired bacterial clearance and survival in experimental Klebsiella pneumonia, we next performed studies to assess the effect of alcohol on the influx of inflammatory cells into the airspace post-K. pneumoniae administration. As shown in Table 1, the administration of K. pneumoniae 10^2 CFU to control mice resulted in an increase in BALF PMN, which was maximal 48 hr post-K. pneumoniae administration. Interestingly, we observed no significant differences in either the percent PMN or the total number of BALF PMN at 24 and 48 hr in ethanol-fed animals, compared with their nonethanol-fed counterparts. In addition, ethanol- and nonethanol-fed mice had similar increases in total lung myeloperoxidase activity (as a measure of total PMN content) at 24 and 48 hr post-K. pneumoniae challenge (data not shown). Finally, no differences in the percentage or total numbers of BAL macrophages was noted between the two groups at either time point. These studies indicate that alcohol-induced decreases in bacterial clearance and survival was not attributable to diminished influx of leukocytes early in the course of Klebsiella pneumonia.

Effect of Ethanol Consumption on the Time-Dependent Expression of Th1- and Th2-Phenotype Cytokine mRNAs and Protein Levels during the Evolution of K. pneumoniae

Experiments were performed to correlate alcohol-induced alterations in bacterial clearance with the time-dependent expression of important regulatory cytokines in Klebsiella pneumonia. Inoculation of control diet-fed CD-1 mice with K. pneumoniae resulted in the expression of TNF mRNA in lung homogenates by 1 day postadministration, with maximal expression occurring at 6 days post-Klebsiella administration (Fig. 3). Interestingly, the expression of TNF mRNA in the lungs of alcohol-fed mice was greater at all time points, with maximal TNF mRNA levels ~3.4-fold greater than that observed in the lungs of control animals. In contrast, there was a decrease in the expression of both IL-12 p35 and p40 mRNA in alcohol-fed mice at 1, 3, and 6 days postinoculation (maximal 66% and 42% decrease, respectively), and a substantial delay in the maximal expression of IFN-γ mRNA in the lungs of alcohol-fed mice, compared with controls. Furthermore, a notable induction of IL-10 mRNA was noted at 3 and 6 days post-K. pneumoniae.
administration in ethanol-fed mice, whereas no appreciable induction of IL-10 mRNA was noted in control animals challenged with *K. pneumoniae*. To correlate alcohol-induced alterations in lung cytokine mRNA expression with cytokine production, we determined cytokine protein levels in the lungs of alcohol and control-fed animals with *Klebsiella* pneumonia at 48 and 72 hr post-*Klebsiella* administration. As shown in Table 2, there was a significant decrease in the levels of lung IL-12 in the alcohol-fed group, compared with controls at 48 hr postbacterial challenge, and a trend toward decreased IL-12 expression at 72 hr (*p* = 0.07). Similarly, IFN-γ levels were reduced by ~33% at 72 hr in alcohol-fed animals, compared with animals receiving control diet (*p* < 0.05). Conversely, a 1.7- and 1.5-fold increase, respectively, in lung IL-10 levels, were noted in alcohol-fed mice, compared with controls at 48 and 72 hr post-*K. pneumoniae* inoculation (*p* < 0.05).

**DISCUSSION**

Alcohol exposure has been previously shown to predispose the host to a variety of infectious complications, particularly bacterial infection of the lung.1-9 In this study, we observed that experimental alcohol ingestion had a profound effect on survival in mice inoculated with *K. pneumoniae* i.t. The increased mortality observed in the ethanol-fed group was attributable to impaired bacterial clearance, as we observed a substantial increase in numbers of *K. pneumoniae* CFU in the lungs of alcohol-fed mice, compared with those receiving control diet. Alcohol-induced decreases in bacterial clearance and survival were not attributable to a diminished influx of leukocytes to the site of bacterial invasion during the course of *Klebsiella* pneumonia, because ethanol- and nonethanol-fed mice had similar increases in total lung myeloperoxidase activity, and no differences in the percentage or total numbers of BALF PMN and macrophages were noted between the two groups. Our findings are consistent with chronic, but not acute, effects of alcohol as reported by Nelson and colleagues. Specifically, these investigators observed reductions in the numbers of BALF PMN in acute ethanol intoxication 3 hr after the intratracheal administration of LPS or *K. pneumoniae*, but no change in BALF PMN in chronic alcoholic rats challenged with LPS i.t.6,7 Similarly, Lister and colleagues29 observed that treatment of rats with alcohol resulted in a significant impairment in the clearance of *Streptococcus pneumoniae* from the lungs of rats, even though lung PMN influx was unaltered. Our observations indicate that chronic alcohol consumption diminishes bacterial clearance in the lung by a mechanism other than altered leukocyte recruitment to the site of bacterial infection, and suggest that chronic alcohol use impairs the ability of both resident alveolar macrophages and/or recruited neutrophils to phagocytose and kill bacterial pathogens within the lung, resulting in early dissemination of infection to the bloodstream.30 This inability of the host to effectively eliminate invading microbes may be due to alcohol-induced alterations in the expression of important activating cytokines required for effective host defense.

TNF is an essential cytokine required for effective lung innate and acquired immunity against a variety of microbes, including *K. pneumoniae*.31-33 The effect of alcohol consumption on TNF expression has been previously investigated. In vivo, acute alcohol intoxication has resulted in significant attenuation of TNF release in response to either endotoxin or intrapulmonary challenge with *K. pneumoniae*.34-36 In contrast, the chronic administration of ethanol (6 weeks) had either no effect or actually enhanced the

<table>
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<tr>
<th>Cytokine</th>
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<th>48 Hr</th>
<th>72 Hr</th>
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<tr>
<td>TNF</td>
<td>Saline</td>
<td>0.12 ± 0.02</td>
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<td>ND</td>
<td>1.62 ± 0.12</td>
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Control, animals receiving control diet; ETOH, animals receiving ethanol diet; Kleb, animals challenged with *K. pneumoniae* 10⁷ CFU; ND, not done. Experimental, n = 3/saline group, 8-16 per *Klebsiella*-challenged mice.

*p* < 0.05, compared with control-fed animals.
peak expression of serum TNF postendotoxin administration. In agreement with the later study, we determined that the expression of TNF mRNA and protein in the lungs of alcohol-fed mice was greater or tended to be greater than that observed in the lungs of control animals at all time points post-*Klebsiella* administration, suggesting an alternative mechanism(s) by which chronic ethanol feeding impairs host defense against bacterial infection of the lung. However, because ethanol has been shown to inhibit the binding of TNF to PMN and other effector cells, we cannot exclude a component of impaired TNF bioactivity despite enhanced TNF expression.

IL-10 and IL-12 are cytokines that were initially identified as being instrumental to the generation of Th2- and Th1-phenotype immune responses, respectively. More recently, these two proteins have been shown to play an important role in controlling the magnitude of host inflammatory responses. IL-10 exerts potent anti-inflammatory effects both in vivo and in vitro, in part by directly deactivating PMN and macrophages, and by downregulating the expression of several proinflammatory cytokines, including TNF, IFN-γ, IL-12, and several members of the chemokine family. Recent studies in our laboratory indicate that passive immunization with anti-IL-10 antibodies in mice with *Klebsiella* pneumonia resulted in improved survival and bacterial clearance, compared with control animals. Conversely, the administration of IL-10 to animals with pneumococcal pneumonia results in significant attenuation of bacterial clearance and decreased survival. In contrast to IL-10, IL-12 promotes Th1 immune responses and represents an important component of acquired immunity against intracellular pathogens. In addition, we have shown that IL-12 is required in the innate immune response against Gram-negative bacterial pathogens, because inhibition of IL-12 decreases bacterial clearance and survival, whereas transient lung IL-12 overexpression utilizing intratracheal adenoviral gene therapy enhances survival in murine *Klebsiella* pneumonia. Conflicting data exists regarding the effect of alcohol on the expression of Th1-type cytokines. Specifically, it has been demonstrated that ethanol treatment inhibits IFN-γ production in vitro, and prolonged alcohol feeding significantly inhibits the production of IFN-γ from retiroviral-infected murine splenocytes. In contrast, acute ethanol treatment in vitro has recently been shown to augment the expression of IL-12 from activated human monocytes. In this study, we observed a decrease in the expression of IL-12 p35 and p40 mRNA, and reduced protein levels in the lungs of alcohol-fed mice. In addition, we detected a considerable delay in the maximal expression of IFN-γ message and a significant reduction in IFN-γ protein levels in the lungs of alcohol-fed mice, compared with controls. Furthermore, a substantial induction of IL-10 mRNA and protein expression was noted after *K. pneumoniae* administration in ethanol-fed mice, whereas no apparent induction of this cytokine was noted in control animals. Collectively, these findings indicate that chronic alcohol feeding appears to shift the balance in favor of an anti-inflammatory Th2-type immune response by inhibiting the expression of IL-12 and IFN-γ, while at the same time enhancing the expression of IL-10. Because IL-10 directly inhibits the production of IL-12 and IFN-γ, we are unable to determine if ethanol mediates direct suppressive effects on Th1-phenotype cytokine expression, or rather indirectly inhibits the expression of these cytokines by directly inducing the in vivo production of IL-10. Furthermore, because IL-12 is a potent inducer of IFN-γ, it is likely that ethanol-induced reduction in IL-12 is directly linked to the attenuation of IFN-γ expression in vivo.

Our data suggests that ethanol-fed animals die due to impaired clearance of bacterial organisms, which we feel is attributable, in part, to enhanced Th2- and diminished Th1-phenotype cytokine responses. However, we cannot exclude the possibility that ethanol-fed mice may be more sensitive to the lethal effects of *K. pneumoniae*-derived LPS, compared with animals receiving a control diet. Enhanced IL-10 expression/administration, or inhibition of Th1-phenotype cytokines, has been shown to be protective in animals challenged with endotoxin. Therefore, these observations would suggest that alcohol-induced changes in cytokine profiles would diminish, rather than augment endotoxin effects.

The mechanism(s) whereby chronic alcohol feeding promotes Th2-type immune responses is not entirely clear. Hormonal changes associated with chronic alcohol consumption may shift the balance in favor of a Th2-phenotype response analogous to that which has been observed in pregnancy. Alternatively, alcohol-induced alterations on the production and release of arachidonic acid metabolites and, in particular, prostaglandin E2 (PGE2), may be responsible for the shift in the cytokine response. Conflicting data exists regarding the effect of alcohol on the expression of PGE2 and other eicosanoids. Ethanol has been shown to increase the release of PGE2 and other prostaglandins from stimulated human blood monocytes and cultured rat Kupffer cells. In contrast, ethanol had an inhibitory effect on the zymosan-stimulated production of PGE2 and other eicosanoids by cultured murine peritoneal macrophages, whereas chronic alcohol ingestion resulted in no differences in the synthesis of PGE2, leukotriene B4 or 5-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid by stimulated rat peritoneal macrophages and lung tissue homogenates. There is growing evidence that prostaglandins of the E series, especially PGE2, alter the expression of important pro- and anti-inflammatory cytokines. Endogenous or exogenous PGE2 inhibits the release of TNF, IFN-γ, and IL-12 from various leukocyte populations. Conversely, PGE2 has recently been shown to induce the expression of IL-10 from LPS-stimulated peritoneal macrophages. Hence, ethanol-induced changes in prostaglandin profiles favoring increases in PGE2 production/bioactivity could account for most, but not all, of the findings observed. A
notable exception is the apparent induction of TNF in ethanol-fed mice, which may occur as result of desensitization to PGE2 inhibitory effects, or as a result of regulatory influences unrelated to prostaglandins. Preliminary studies in our laboratory indicate that alveolar macrophages from ethanol-fed mice cultured ex vivo produce substantially greater quantities of PGE2 constitutively and when challenged with LPS, compared with similarly treated control alveolar macrophages. Similarly, whole lung PGE2 levels are greater in alcohol-fed animals than that detected in animals receiving an isocaloric control diet (T. Standiford, unpublished observations).

In summary, our studies indicate that ethanol ingestion results in a profound suppression of lung innate immunity in association with a shift in the balance of lung cytokine mRNA and protein expression favoring Th2-, rather than Th1-phenotype cytokines. Further studies are needed to establish causal relationships and to determine if targeted immunotherapy to correct this imbalance will aid in the outcome of ethanol-fed mice with Gram-negative pneumonia.

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