Differential Effects of Ethanol on Permissive versus Nonpermissive Macrophages Infected with *Legionella pneumophila* (43606)

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Abstract. **The effect of ethanol treatment was studied in terms of effect on permissive versus nonpermissive macrophages for growth of *Legionella pneumophila*, which is an intracellular bacterium causing pneumonia in immunocompromised patients. It was found that ethanol treatment of permissive macrophages from *L. pneumophila*-susceptible A/J mice evinced a decrease in replication of the bacteria compared with nontreated infected macrophages. Whereas there was more than a 100-fold increase in Legionella growth over a 48-hr culture period in infected A/J mouse macrophages, treatment of the macrophages with 0.5% ethanol depressed the ability of the macrophages to be infected by Legionella approximately 45%. A lower concentration of ethanol had a lesser effect but still resulted in inhibition of the ability of the cells to replicate Legionella. In contrast to ethanol-induced inhibition of the A/J mouse macrophages to replicate Legionella, macrophages from Legionella-resistant BALB/c mice, which only minimally replicated Legionella (i.e., only a 2-fold increase or less over a 48-hr period), treatment with ethanol resulted in their greater replication of the Legionella. This effect was most marked with the 1.0% concentration of ethanol after 7 days of pretreatment, while the 0.5% and 0.1% concentrations of alcohol caused less enhancement of bacterial growth in the cells, but these concentrations still had a significant enhancement effect. Thus, ethanol had differing effects on growth of the opportunistic intracellular bacterium Legionella in macrophages from permissive versus nonpermissive mice. Studies on the mechanisms involved are in progress.** [P.S.E.B.M. 1993, Vol 203]

It is widely acknowledged that alcohol has marked effects not only on the central nervous system but also on the immune system (1, 2). Effects of alcohol on immunity are thought by some to be indirect, i.e., affecting the central nervous system to modulate neuropeptide production, which affects the immune system, or possibly by direct effects on cellular, humoral, or both forms of immunity associated with lymphocytes and soluble mediators. In this regard, it has been reported by various investigators there may be effects of alcohol on the immune response system both *in vivo* and *in vitro*. For example, impaired immunity in alcoholics has been reported in regard to both humoral immune mechanisms, including antibody production (3), and various aspects of cell-mediated immunity, such as delayed-type hypersensitivity reactions (4, 5) and lymphocyte proliferative responses to mitogens (6, 7). Furthermore, studies with experimental animals, especially rats and mice, have shown that administration of alcohol markedly suppresses many immune functions (8–12). Such experimental studies have suggested deleterious effects of alcohol on the immune system and supported clinical observations on immune disorders in alcoholics. It is also widely acknowledged there may be an increased incidence of infection in alcoholics, especially with bacterial pneumonia. In addition, recent reports have indicated a dysfunction in phagocytic cell activity, as well as lymphocyte function, induced by alcohol and possibly related to increased susceptibility to infection (13). However, there is only limited experimental evidence to support such a contention.

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Legionella pneumophila is a well-known intracellular bacterium that causes pneumonia in immunocompromised individuals (14). This organism replicates readily in monocytes from humans, who are considered only moderately susceptible to this organism, as well as macrophages from susceptible guinea pigs (15, 16). In recent studies, we reported that macrophages from genetically susceptible A/J mice are also highly susceptible to replication of *L. pneumophila*, although macrophages from other mouse strains, such as BALB/c, DBA/2, C3H/HeN, C57BL/6 and BDF1, are resistant to infection (17). Furthermore, it was shown that although macrophages are the preferential cell for growth of Legionella, lymphocytes are also important in the regulation of Legionella growth in macrophages (18). In the present study, the effects of ethanol (ETOH) treatment on the ability of macrophages to replicate *L. pneumophila* was studied in detail using macrophages from Legionella nonpermissive BALB/c mice as compared with macrophages from permissive A/J mice. ETOH treatment of the macrophages resulted in a decrease in *L. pneumophila* growth in the permissive macrophages from the A/J mice, but, in contrast, resulted in increased numbers of *L. pneumophila* in the cultures of the nonpermissive macrophages from the BALB/c mice.

Materials and Methods

Animals. Female A/J and BALB/c mice were purchased from Jackson Laboratories, Bar Harbor, ME, and were used at 6–10 weeks of age in this study.

Bacteria. *L. pneumophila*, Serogroup 1, was originally obtained from a case of legionellosis at Tampa General Hospital, Tampa, FL, and cultured on buffered charcoal yeast extract agar (Gibco Laboratories, Madison, WI). After 48 hr of culture on buffered charcoal yeast extract agar at 37°C, the bacteria were suspended in pyrogen-free saline and the concentration was determined by spectrophotometry, as described previously (19).

Macrophages. Cultures of peritoneal macrophages from the peritoneum of the mice were prepared as described previously (19). In brief, mice were injected intraperitoneally with 3 ml of thioglycolate broth (Difco Laboratories, Detroit, MI). Four days later, peritoneal exudate cells were collected and suspended in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) containing 10% heat-inactivated fetal calf serum (Hyclone Laboratory, Logan, UT). The macrophage suspensions were adhered to 24-well tissue culture plates (Costar Co., Cambridge, MA) for 2 hr at 37°C in 5% CO_2 and then nonadherent cells were removed by washing. The macrophage monolayers were further incubated with or without various concentrations of ETOH in 10% fetal calf serum-RPMI 1640 medium for 3 hr to 7 days. The concentrations of ETOH used (diluted in medium) are given as v/v percentages. Since ETOH is volatile, every 24 hr the macrophage culture medium containing ETOH was changed with freshly prepared medium. After treatment with ETOH, the macrophage monolayers were infected with *L. pneumophila* (infectivity ratio of 10 bacteria/macrophage), incubated for 30 min at 37°C, washed to remove nonphagocytized bacteria, and then further incubated for up to 48 hr in the presence of ETOH. The number of viable bacteria per culture was assessed by lysing the macrophages with sterilized water and assaying for colony-forming units by standard plate counts on buffered charcoal yeast extract agar, as described previously (19).

Statistics. All studies were performed at least three times. Representative data from these experiments were presented as mean ± SD. Significance was determined by Student's *t* test.

Results

As is apparent in Table I, *L. pneumophila* grew vigorously in macrophages from permissive A/J mice, but did not grow well in macrophages from BALB/c mice. There was more than a 100-fold increase in the number of *L. pneumophila* in permissive A/J mouse macrophage cultures over a 48-hr period. Nonpermissive macrophages from the BALB/c mice cultured in the same manner did not support significant growth of these bacteria. As shown in Table II, macrophages from the A/J mice treated with doses of ETOH ranging from 0.1% to 2.0% for 24 hr and then infected with *L. pneumophila* showed differing numbers of bacteria 24 hr after infection. Whereas the control cultures without ETOH showed a relatively large number of bacteria recovered in macrophage lysates, those treated with 0.5% ETOH had approximately 45% fewer bacteria. This was evident when the number of Legionella in the cultures was assessed at 24 hr (Table II) or even when the cultures were assayed at 48 hr (data not shown).

There was no significant difference between ETOH-treated macrophages versus control cultures in terms of viability as determined by the trypan blue stain

Table I. Fate of *L. pneumophila* in Permissive A/J versus Nonpermissive BALB/c Mouse Macrophages[a]

Macrophage source	Time after infection[b]		
	0 hr	24 hr	48 hr
A/J	38 ± 3	780 ± 120	6900 ± 819
BALB/c	39 ± 1	60 ± 1	32 ± 9

[a] Macrophage monolayers (1×10^6 cells/well) were infected with 1×10^7 bacteria for 30 min at 37°C, washed to remove nonphagocytized bacteria, and then incubated further for 24 hr to 48 hr in 10% fetal calf serum-RPMI 1640 medium.

[b] Number of viable bacteria (colony-forming units $\times 10^3$) in macrophage lysates was measured at indicated time after infection by plate count method; data represent the mean ± SD in triplicate macrophage cultures.

Table II. Effect of ETOH Treatment on the Growth of *L. pneumophila* in Permissive A/J Mouse Macrophages

Macrophage treatment[a]	Time after infection[b]	
	0 hr	24 hr
None	55 ± 7	1290 ± 180
ETOH		
2.0%	44 ± 4	630 ± 150[c]
1.0%	79 ± 13	990 ± 60
0.5%	70 ± 11	720 ± 10[c]
0.1%	77 ± 19	1110 ± 60

[a] Macrophage monolayers were treated with indicated concentrations of ETOH for 24 hr and then infected with *L. pneumophila*.
[b] Number of viable bacteria (colony-forming units × 10^3) in macrophage lysates was measured at indicated time after infection by plate count method; infectivity ratio was 10 bacteria per macrophage and data show mean ± SD in triplicate cultures.
[c] $P < 0.05$.

Table III. Effect of Preincubation Periods with ETOH on the *L. pneumophila* Growth in BALB/c Mouse Macrophages

Macrophage treatment[a]	Incubation with ETOH[b]		
	3 hr	2 day	6 day
None	44 ± 9	141 ± 15	109 ± 0.7
ETOH			
1.0%	36 ± 1	136 ± 21	164 ± 16[c]
0.5%	44 ± 6	153 ± 16	178 ± 12[c]
0.1%	41 ± 4	166 ± 19	136 ± 2

[a] Macrophage monolayers were treated with indicated concentrations of ETOH for 3 hr to 6 days at 37°C in 5% CO_2 and then infected with *L. pneumophila*; infectivity ratio was 10 bacteria per macrophage and culture medium was changed every 24 hr with fresh ETOH solution.
[b] Macrophage lysates, at 24 hr after infection, were prepared and measured; the number of viable bacteria (colony-forming units × 10^3) was determined by plate count method and data show the mean ± SD in triplicate macrophage cultures.
[c] $P < 0.05$.

assay. The least number of Legionella was evident when the cultures were treated with 2.0% ETOH, but this concentration of alcohol caused no significant loss in viability of the macrophages. The 1.0% concentration of ETOH caused somewhat less inhibition of growth of Legionella in the macrophages than the 0.5% concentration. There was no difference in the number of bacteria taken up by the macrophages at zero time in cultures treated with the various amounts of ETOH as compared with the nontreated control cultures.

In additional experiments, it was found that A/J mouse macrophages pretreated with ETOH for 3 hr showed essentially similar results as cells pretreated for 24 hr. Relatively longer preincubation periods with ETOH, such as 3 or 6 days, did not result in greater differences in *L. pneumophila* growth in macrophages after ETOH treatment (data not shown).

In contrast to the suppression of Legionella growth in the permissive A/J mouse macrophages treated with ETOH, there was an enhancement of growth in nonpermissive macrophages from BALB/c mice. There was much less growth of the bacteria in BALB/c macrophages infected with Legionella, i.e., approximately 3 × 10^4 bacteria/culture as compared with 6.9 × 10^6 bacteria at 48 hr in cultures of the same number of macrophages from the A/J mice. However, when the macrophages from the BALB/c mice were treated with ETOH for 6 days, but not 3 hr or 2 days, there was approximately a 65% increase in bacterial growth at 24 hr with the 0.5% concentration of ETOH (Table III). This increase was similarly evident when the cultures were incubated for 48 hr (data not shown). The 0.1% dose of ETOH resulted in a consistent increase in bacterial growth, but this was less than the increase resulting from the 0.5% ETOH concentration.

The increase in the growth of *L. pneumophila* was most evident in cultures pretreated with the graded amounts of ETOH for 7 days and then infected with the bacteria (Table IV). All ETOH concentrations resulted in a marked increase in Legionella growth at both 24 and 48 hr after infection. There was a greater than 4-fold increase in the number of viable bacteria in these macrophages after treatment of the cultures with 1.0% ETOH, but even the 0.1% and 0.5% concentrations caused an increase. The concentration of ETOH used did not affect the uptake of the bacteria at zero time. Furthermore, there was no detectable effect on the viability of the macrophages treated with the ETOH for 7 days, as determined by the trypan blue dye exclusion assay and microscopic examination for detachment of cells from tissue culture plates (data not shown).

Table IV. Effect of ETOH Treatment for 7 Days on the Growth of *L. pneumophila* in BALB/c Mouse Macrophages

Treatment[a]	Time after infection		
	0 hr	24 hr	48 hr
None	28 ± 6	65 ± 4	71 ± 3
ETOH			
1.0%	30 ± 5	105 ± 12[b]	320 ± 122[b]
0.5%		119 ± 19[b]	144 ± 25[b]
0.1%		125 ± 19[b]	126 ± 15[b]

[a] Macrophage monolayers were treated with indicated concentrations of ETOH for 7 days at 37°C in 5% CO_2 and then infected with *L. pneumophila*; culture medium was changed every 24 hr with fresh ETOH solution and number of viable bacteria (colony-forming units × 10^3) in macrophage lysates was measured at indicated time after infection by plate count method. Infectivity ratio was 10 bacteria per macrophage and data show the mean ± SD in triplicate cultures.
[b] $P < 0.05$.

Discussion

It is widely acknowledged that increased incidence of infection in alcoholics, especially bacterial pneu-

monia, may be evident (13). Such increased infections are believed to be linked to a possible immunodeficiency induced by alcohol (1). A recent study by Bermudez and Young (20) showed a possible direct effect of alcohol on infection. They reported that cultured human monocyte-derived macrophages or murine Kupffer cells exposed to ETOH *in vitro* showed significantly greater intracellular *Mycobacterium avium* growth as compared with control cultures. Such results suggest that ETOH may directly affect the anti-Mycobacterium activity of macrophages. Several other studies concerning the effect of alcohol on phagocytic cells have also shown deleterious effects *in vitro* and *in vivo*. For example, ETOH treatment of mice induced an impaired response of phagocytic cells and caused a decreased ability to clear bacteria from the lung (21). *In vitro* treatment of macrophages with alcohol affects the ability of the cells to evince adherence and phagocytosis (22). Furthermore, Fc receptor binding of macrophages was also found affected by treatment with alcohol (23, 24). These reports suggest that certain concentrations of alcohol may affect the function of macrophages involving anti-Mycobacterium activity. However, it is still unclear whether other antimicrobial activities of macrophages are affected by alcohol. Other possible mechanism of alcohol effect on bacterial growth in macrophages is due to affection of alcohol metabolites on the replication of the intracellular bacteria, since alcohol is metabolized inside macrophages.

L. pneumophila is considered an opportunistic bacterium that infects preferentially macrophages from susceptible individuals, including humans, guinea pigs, and the A/J strain of mice (15–17). These bacteria grow rapidly in infected macrophages, reaching peak numbers at 24–48 hr depending on the infectivity ratio. Studies in this and other laboratories have shown that macrophages from A/J mice are highly permissive for growth of *L. pneumophila*, but macrophages from other mouse strains, including BALB/c mice, are relatively nonpermissive (17, 25). In the present study, the effect of ETOH on Legionella growth in permissive versus nonpermissive macrophages was examined. Various concentrations of alcohol, ranging from 0.1% to 2.0%, markedly affected the ability of the macrophages to replicate Legionella without affecting the viability of the cells during the 48-hr culture period. Such effects of ETOH on Legionella growth in macrophages, however, were different between permissive versus nonpermissive macrophages. Nonpermissive macrophages from BALB/c mice treated with 1.0% ETOH and infected with Legionella showed approximately a 4-fold increase of bacteria growth within 48 hr. Even treatment with relatively low doses of ETOH, such as 0.1%, still resulted in a significant increase of Legionella growth in the macrophages. Such a concentration of ETOH, i.e., 0.1% ETOH, corresponds to 790 μg/ml or 17 m*M*, similar to those in previous reports that showed impairment of phagocytic cell function. For instance, incubation of macrophages with ETOH *in vitro* reduces Fc receptor binding at 55 m*M* (0.32%) to 220 m*M* (1.29%) (23). It should be noted the effect of ETOH on Legionella growth required a relatively long incubation period with ETOH, such as 6 or 7 days, in nonpermissive BALB/c mouse macrophages, but not for permissive A/J mouse macrophages. For example, treatment of macrophages from these mice with ETOH for as short a time as 3 hr or 2 days resulted in no enhancing effect on BALB/c mouse macrophages, but resulted in a suppressive effect on A/J mouse macrophages. This difference was noted when Legionella growth was assessed in the cultures at either 24 or 48 hr. The different incubation periods required for modulation of Legionella growth in macrophages from the two strains of mice by ETOH might depend on the nature of the permissiveness of the macrophages. It is plausible that there are independent mechanisms for supporting or restricting Legionella growth in macrophages.

It is noteworthy that ETOH, at the concentrations used, did not affect the overt viability of the macrophages as determined by trypan blue staining and microscopic examination at the termination of culture, i.e., 48 hr. Also, the concentrations of ETOH used had no significant effect on the viability or growth of *L. pneumophila* in medium (unpublished data). Thus it appears that ETOH has a direct effect on the ability of macrophages to replicate this intracellular pathogen, but the effects were different between permissive versus nonpermissive macrophages, i.e., enhancement of Legionella growth occurred in ETOH-treated nonpermissive BALB/c mouse macrophages versus suppression of growth in permissive A/J mouse cells.

These differences may reflect differences in the nature of the macrophages from the permissive versus nonpermissive mice in terms of replication of Legionella. Macrophages from BALB/c mice, as well as from other nonpermissive mice, do not permit significant replication of the bacteria. This might be due to an innate inability of the macrophages from these strains of mice to provide the appropriate environment for the growth of the bacteria because of necessary nutritional mechanisms. Moreover, the macrophages from these mouse strains evince a genetic predisposition to mount an inhibitory effect against Legionella growth (26–28). Although there did not appear to be killing of these bacteria by the nonpermissive macrophages during 24–48 hr of cultures, the bacteria did not evince a vigorous or prolonged growth. In contrast, macrophages from permissive A/J mice permitted the Legionella to replicate vigorously. Macrophages from A/J mice, similar to macrophages from permissive guinea pigs or blood monocytes from humans, do not inhibit growth of Legionella unless first stimulated with activating

factors such as γ-interferon or bacterial lipopolysaccharide (29, 30).

It is also possible that metabolic pathways in the macrophages directly related to antimicrobicidal or microbistatic activities could be affected by ETOH treatment and this results in inhibition of macrophage activity permitting greater Legionella replication. Studies in progress in this laboratory are designed to determine which, if any, pathway ETOH may affect in macrophages from these two different mouse strains. Studies are also in progress to extend these *in vitro* experiments by feeding mice of these strains with ETOH and then determining *in vivo* their susceptibility to Legionella. Human monocytes/macrophages are also required to be used in similar experiments, especially from chronic alcohol users versus nonusers, since the murine system is different in humans. Nevertheless, the availability of genetically susceptible versus resistant mouse strains to an intracellular bacterial pathogen such as *L. pneumophila*, which replicates in macrophages, permits a model system to dissect in detail effects of ETOH on antimicrobial resistance. Such studies should provide useful information as to how alcohol influences an important immune mechanism involved in resistance to an opportunistic microbe similar to those important as infectious agents in alcoholic individuals.

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