Impaired Bactericidal Activity and Host Resistance to *Listeria monocytogenes* and *Borrelia burgdorferi* in Rats Administered an Acute Oral Regimen of Ethanol

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A rat model was used to examine how ethanol ingestion may interfere with antimicrobial immunity both in vitro and in vivo. Nonimmune Long-Evans rats were given a short-course treatment orally with excessive amounts of ethanol. Their spleens were removed at the time of sacrifice, and separate spleen cell suspensions were prepared and tested in vitro for their ability to kill two bacterial pathogens, *Listeria monocytogenes* and *Borrelia burgdorferi*. After the bacteria were mixed separately with various concentrations of spleen cells, it was found that spleen cells from the ethanol-treated rats killed fewer bacteria than matching pair-fed controls, based on counts of the number of cultured CFU (for *Listeria*) or based on microscopic examination (for *Borrelia*). For the in vivo studies, ethanol-treated and control rats were infected intraperitoneally with *Listeria*, and then, 1 to 3 days later, they were assessed for systemic infection based on the numbers of organisms present in their livers and spleens. Numbers of bacterial CFU for both organs were significantly higher in the group fed ethanol for the first 2 days after listerial challenge. These results support the concept that acute exposure to high levels of ethanol can impair host defense mechanisms, especially those expressed at the cellular level, which could lead to increased susceptibility to certain types of infections.

Protection against most infectious agents has traditionally been attributed to the various arms of the host immune system, which includes antibody, complement, T lymphocytes, macrophages, and other phagocytic cells. Direct killing of microorganisms is due mainly to macrophages and neutrophils, often mediated or enhanced by T cells (9), which can also directly attack virally infected cells (30) and some extracellular bacteria (5, 14). In this context, little is known about alcohol's influence on these mechanisms of resistance to infectious agents, especially opportunistic pathogens. Nonetheless, there are several reports (1, 8, 27) linking excessive consumption of alcohol to increased susceptibility to infection, especially of the respiratory tract (3), which is often associated with a high rate of mortality. It should be noted, however, that within the alcoholic population, measuring alcohol's direct effects on the immune system with precision is difficult because of the frequently associated conditions of malnutrition, liver disease, and other lifestyle-related problems. Such coincidental disorders could also impact significantly on the proper functioning of the host immune system. It has been important, therefore, to develop suitable animal models in order to provide key insights into the extent to which the detrimental effects on resistance mechanisms are caused only by ethanol. For example, an alcohol-related decrease of protection has been reported to occur in response to viral infection (4) and to listerial infection in mice (24) and during salmonellosis in mice (25). Consistent with these findings are earlier reports of members of our group (21; C. M. Harris and C. S. Pavia, abstr. 670C from the Joint Scientific Meeting of the Research Society on Alcoholism and the International Society for Biomedical Research on Alcoholism 1996, Alcohol. Clin. Exp. Res. 20:115A, 1996) showing that after rats were fed intoxicating amounts of ethanol, they developed significant hematologic and immunologic changes, including decreased humoral and cellular immune responses to bacterial antigens.

To understand further the mechanisms by which ethanol limits cell-mediated immunity's participation in destroying microorganisms (primarily through spontaneous activity), we established an in vitro system in which spleen cells kill bacteria and combined this finding with a well-established animal model for measuring direct in vivo effects of ethanol intoxication on host resistance to bacterial infection. Accordingly, we used this combined in vitro-in vivo infectivity model to test the effect of ethanol on cellular immune responses against two genera of bacteria (*Listeria monocytogenes* and *Borrelia burgdorferi*).

MATERIALS AND METHODS

**Animals and ethanol treatment.** Long Evans rats, 6 to 12 months old, were obtained from Charles River Laboratories (Wilmington, Mass.). Prior to the beginning of the ethanol-treatment experiments, they were fed a standard diet of commercial laboratory chow and water ad libitum. After random assignment into separate experimental groups, the rats were pair fed, each day, with a fortified liquid diet containing either ethanol (8 to 10 g/kg of body weight) or on an isocaloric sucrose solution, following closely procedures already described by members of our group (21). The rats were fed intragastrically using a specially designed, tapered stainless steel or flexible, plastic feeding tube that had been attached to a 5-ml syringe. They received each diet in fractional doses (3.0 ml, four times daily, 4 h apart). The amount of ethanol given here made the rats moderately intoxicated, leading to limp axial musculature, a staggering gait, and elevated blood-alcohol levels, assessed as previously detailed (11, 21) using a commercially available diagnostic kit (#332-UV; Sigma, St. Louis, Mo.). For the in vitro experiments (described below), the rats were sacrificed on day 6, which was 1 day after receiving the last ethanol or control diet treatment. For the

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TABLE 1. Proliferative responses of fractionated spleen cells to T- and B-cell mitogens

| Treatment group | Cell group | cpm (10^3)* | No mitogen | PHA | LPS |
|-----------------|------------|-------------|------------|-----|-----|
| Control         | Unfractionated cells | 1.87 | 35.90 | 14.58 |
| Ethanol         | Unfractionated cells | 1.52 | 14.14 | 5.17 |
| Nonadherent (panned) | 1.34 | 55.74 | 2.33 |
| Nonadherent (panned) | 1.16 | 26.37 | 1.92 |

* Data are expressed as mean counts per minute (cpm) of a triplicate culture. Standard deviations for each test group were ±15% or less. Each culture contained 5 × 10^5 spleen cells in 0.2 ml of fortified RPMI 1640 culture medium with or without optimal mitogenic concentrations of PHA (3 μg/culture) or LPS (10 μg/culture). After 70 h in a humidified CO[sub]2[/sub] atmosphere for 4 to 6 h. The end of the incubation period, mixtures containing Listeria were remixed by gentle pipetting.

Infectivity experiments were described below, prior to being injected with bacteria, the rats were maintained on their respective ethanol or control diets for 4 to 6 days, followed by continuation with these feeding treatments for an additional one to 3 days postinfection. Uninfected and infected rats were kept in separate cages. All of the rats were housed in an air-filtered environment maintained at 20°C ± 2°C. These studies were approved by our institution's Animal Care and Use Committee and were carried out in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health (Bethesda, Md.).

**Bacteria.** Listeria monocytogenes ATCC strain 7644 was obtained from Chrisspe Technologies (Lake Charles, La.). Listeria bacteria were grown at 37°C in thiglycolate broth for 18 h to mid-log phase. Both cultures were diluted to the desired concentrations in sterile phosphate-buffered saline (PBS) (pH 7.4), after enumeration of CFU of the cultured bacteria on blood agar plates. B. burgdorferi (strains B31 and CA287) were maintained as broth cultures in antibiotics-free Barbour-Stoenner-Kelly (BSK) medium as previously described (23). After microscopic counts were done, the Borrelia bacteria were adjusted to the desired concentration using BSK medium. The diluted linterial and borrelial suspensions were then used either for measuring bacteriologic activity of the rat spleen cells or for the in vivo infectivity experiments (both described below).

**Bactericidal assays.** Bacterial killing by rat spleen cells was analyzed using a modified assay system originally designed to measure lymphocyte-mediated killing of Pseudomonas bacteria (14). Splen cells were removed aseptically from uninfected (noinmune) ethanol-treated (for 5 days) or control rats and suspended in 2.0 ml of BSK medium. The separate spleens were minced finely with sterile forceps and scissors, washed twice, and resuspended in 2.0 ml of BSK medium. Spleen cells were counted using a hemocytometer after being stained with crystal violet and adjusted to the desired concentration using BSK medium. The diluted listerial and borrelial suspensions were then used either for measuring bactericidal activity of the rat spleen cells or containing medium alone. The various cultures were incubated at 37°C in a humidified CO[sub]2[/sub] atmosphere for 4 to 6 h. At the end of the incubation period, mixtures containing Listeria were remixed by gentle pipetting. A 50-μl sample was removed from the cell mixtures, diluted serially, 10-fold up to 1/10,000 in PBS, and plated finally onto blood agar. After incubation at 37°C for 24 to 48 h, the number of CFU was determined and averaged for each dilution. Only plates having between 25 and 250 CFU were considered suitable for counting, and these were used for calculating the numbers of surviving organisms that remained in the original test mixtures. Data are reported as mean log_10 CFU values or as percent growth inhibition. For those mixtures containing Borrelia, after the 4- to 6-h incubation period, the test wells were examined for live, motile spirochetes based on microscopic (by dark-field or phase-contrast) examination as previously described (23). Data are reported as percent inhibition (or reduction) of bacterial growth calculated according to the following formula: [1 – (number of live bacteria in the presence of spleen cells/number of live bacteria present in medium alone)] × 100.

**Infectivity experiments.** Rats were inoculated intraperitoneally with 5 × 10^7 CFU of mid-log-phase L. monocytogenes suspended in 0.3 ml of PBS. These injections were given 24 to 72 h prior to sacrifice of the ethanol-treated (described above) and control rats. At the time of sacrifice, the rats were lightly anesthetized with ketamine hydrochloride (50 mg/kg), followed by exsanguination via cardiac puncture. Bacterial burden in the liver and spleen was determined based on viable counts of cultured extracts and used as a measure of the extent of systemic infection. After the rats were bled out, their livers and spleens were removed and weighed. Separate extracts, on the portions of each tissue that were used, were prepared by mincing them with scissors and forceps in PBS, followed by partial homogenization done by repeated insertion and expulsion of semiquick tissue material through a 3- or 5-ml syringe. The spleen was resuspended finally in 2.0 ml of PBS, and the liver was resuspended in 10.0 ml of PBS. Both suspensions were kept in screw-cap tubes. The partially homogenized tissue suspensions were mixed gently for 50 to 60 min, before 50-μl aliquots of samples of undiluted and diluted (10-fold, in PBS) tissue suspension was removed and spread out onto blood agar plates. The plates were incubated at 37°C, and bacterial colonies were counted and data reported as described above.

**Statistical analysis.** The data between the various untreated and ethanol-treated test groups were analyzed for statistical significance based on Student's t test. P values of <0.05 were considered significant.

**RESULTS**

**In vitro killing of Listeria and Borrelia.** We first investigated the cell combinations and conditions that might lead to spleen-cell-mediated killing of two different genera of bacteria. Spleen cells were chosen because they represent a unique mixture of T and B lymphocytes and macrophages, all of which play crucial roles in basic immune responses and in host defense against infection. In preliminary experiments (Harris and Pa-via, Alcohol. Clin. Exp. Res. 20:1115A, 1996), it was found that spleen cells taken from normal, untreated, nonimmune rats exhibited significant spontaneous bactericidal activity. This result led to testing of whether in vivo treatment with ethanol altered such naturally occurring inhibitory effects. As is shown in Fig. 1 and 2, ethanol pretreatment significantly reduced the spontaneous listericidal activity (P < 0.025) and the borrelial-acid killing capabilities (P < 0.005) of rat spleen cells, relative to untreated controls. Inhibition of growth occurred at two different effector/target cell ratios (10:1 and 100:1) that had been established in the test wells of the microtiter plates. These results were based on analysis of supernatants of well-mixed short-term cultures. Subsequent reduced growth, after culturing onto blood agar or in BSK medium, would be reflective of true bactericidal activity, irrespective of whether or not some of the bacteria had adhered to, or were ingested by, some of the effector spleen cells. Adhering or ingested bacteria, but not killed bacteria, would be expected to grow out and yield detectable CFU.
We next compared bacterial killing by spleen cells enriched for T cells and a few residual macrophages with whole spleen cell populations. B cells were effectively removed (Table 1), using the modified panning technique, after their adherence to antibody-coated plates (22, 29). The data (Table 2) indicate that compared with medium controls, 62% of the *Listeria* and 23% of the *Borrelia* bacteria were killed after being mixed with the B-cell-depleted preparations derived from untreated rats. In contrast, only 36% of the *Listeria* and 10% of the *Borrelia* were killed in wells containing B-cell-depleted populations derived from the ethanol-treated rats, even though proportionally the same number of treated cells was used for each of the cell mixtures.

**In vivo killing of *Listeria***. With the preceding data showing significant ethanol-related impairment of bactericidal activity, the last set of experiments was designed for the purpose of determining whether ethanol exposure would lead to an overwhelming infection in *Listeria*-challenged rats. Blood ethanol levels in the ethanol-treated rats were measured 1 h after the last treatment and were found to be highly elevated (Table 3). In these rats, *Listeria* grew at a much higher rate or remained viable longer relative to the number of organisms present in controls (Table 3). Over a 24- to 72-h period, this growth phase was marked by significantly higher numbers of *Listeria* bacteria being found in the livers and spleens of the ethanol group. For both groups of rats, *Listeria* had been cleared from both organ sites by the fourth or fifth day of challenge (data not shown), based on little or no growth of cultured extracts on blood agar. Although some of the rats from both groups showed signs of illness (such as slight fever, lethargy, slight weight loss), these were considered mild since there were no spontaneous lethal infections that could be attributed to any severe adverse complications resulting from uncontrollable listerial growth.

**DISCUSSION**

Past studies from our laboratory (21, 23) and those from others (6, 13, 15, 20) have shown the importance of both humoral and cellular immunity in the host defense against the Lyme disease spirochete, *B. burgdorferi*, and against the causative agent of listeriosis. In other related studies (21), members of our group have shown that ethanol interferes with the ability of rats to mount optimal humoral and cellular immune responses against borrelial antigens. Because ethanol-associated immunosuppression closely resembles short-term immune system impairment (induced by certain types of drugs), it was therefore of interest to explore further the impact that ethanol might have on two diverse bacterial pathogens. Indeed, it is well documented (12) that one of them (*L. monocytogenes*) can cause a serious, life-threatening illness, primarily in immunocompromised patients and in the developing fetus during pregnancy. Our findings of increased bacterial burden in *Listeria*-
challenged rats fed intoxicating amounts of ethanol are in close agreement with the reported results of others (24) showing that ethanol ingestion lowers resistance in *Listeria*-infected mice. Similar data, showing increased bacterial colonization of the liver, have been found (16) with rats consuming ethanol and infected with a related intracellular organism, *Mycobacterium bovis*.

Limited clinical studies (1, 27) exist on the direct effects that alcohol has in interfering with protection against infections without having to consider the possible role of other confounding contributing factors, such as malnutrition, heavy smoking, and liver cirrhosis, that often occur in the alcoholic patient. Accordingly, because of such coexisting conditions, it becomes difficult to fully analyze whether sufficient alcohol has been consumed in order to hinder host defenses to certain infectious agents, thus making the use of appropriate animal models essential for determining more precisely alcohol’s overall contribution.

In this report we also describe the in vitro conditions under which previously unstimulated spleen cells can kill *Listeria* and *Borrelia*. Not surprisingly, this spontaneous bactericidal activity appeared to be mediated both by T cells and possibly by some of the few remaining macrophages that were not entirely removed by the panning technique. The important protective role for activated T cells and macrophages against listeriosis is well established (20), whereas protection from Lyme disease has been attributed mostly to antibody and complement (6, 23, 26), although macrophages are capable of ingesting and adequately destroying *B. burgdorferi* (17, 18). With this in vitro bactericidal assay, it was significant to find that both unFractionated, naive spleen cells and those depleted of B cells, from the ethanol-fed rats, were less effective in killing our two test pathogens than cells from control rats. The spontaneous inhibitory activity associated with T cells here was most likely attributable to the secretion of an antimicrobial peptide, such as granzulysin (10), which is currently the only defined pathway by which previously unstimulated T cells can directly kill a variety of microorganisms. This finding also correlated well with the higher bacterial burden occurring in the infected rats exposed to ethanol, although they were generally able to fully clear their listerial infection as readily as the control rats. It should be noted, however, that in this context, our rats received only a short-term treatment regimen with ethanol combined with a sublethal dose of *Listeria*, which could account for the induction of only subtle ethanol-induced changes in host immunity that were insufficient to have life-threatening consequences. Another possible consideration that could be operative here is that the acute ethanol dosing regimen has significantly shifted the ratios of some of the cell populations in the lymphocyte and macrophage subsets (8) and thus could account for some reduction in bactericidal activity in the ethanol rat spleens. Further studies are planned in which a more chronic course of ethanol treatment will be given (for about 2 to 3 weeks) followed by a larger bacterial challenge.

Although our results closely resemble those previously reported for ethanol-treated mice challenged with *Listeria* (13), a few important distinctions should be pointed out. Listeriosis is not as severe a disease in rats as it is in mice. The 50% lethal dose (LD50) in mice (13, 24) is about a thousandfold less than the amount of *Listeria* (5 × 10^7 CFU) that was used here to infect our rats, yet all of our test rats survived, including a few that were observed for an additional 2 to 3 weeks after challenge. This would seem to suggest that we might be using an avirulent strain of *Listeria*. Contrary to this possibility, however, we have found (C. S. Pavia and C. M. Harris, unpublished observations) that normal, untreated adult mice or young (<4-week-old) rats die within 2 to 3 days after challenge with a low inoculum, having from 10^5 to 10^6 *Listeria* CFU and belonging to the same strain that was used here. Thus, such variables as age and species of animal need to be considered when analyzing and comparing ethanol's effects in vivo on immune function.

*L. monocytogenes* and *B. burgdorferi* are significant human and animal pathogens in which an intact immune system is crucial in affording protection and resistance against the diseases that they cause, along with providing key diagnostic indicators when the infecting agent is not readily culturable. Certain animal infection models of listeriosis (20) and Lyme disease (2), including those that use the rat (7, 19), have provided key insights into the pathogenesis and mechanisms of

### TABLE 2. Comparison of bacterial killing by effector spleen cells depleted of B cells

| Group | Effector cell treatment | No. of bacterial target cells surviving<sup>a</sup> | Listeria | *Borrelia*<sup>b</sup> |
|-------|-------------------------|-----------------------------------------------|----------|-----------------------|
| 1     | None (media control)    | 5.17 ± 3.94                                   | 5.01 ± 3.75 |
| 2     | Whole spleen cells (from control rats) | 4.34 ± 3.64 (85) | 4.87 ± 3.62 (28) |
| 3     | Anti-IgG-treated control spleen cells | 4.75 ± 3.58 (62) | 4.90 ± 3.63 (23) |
| 4     | Whole spleen cells (from ethanol rats) | 4.84 ± 3.71 (53) | 4.95 ± 3.68 (12) |
| 5     | Anti-IgG treated ethanol spleen cells | 4.97 ± 3.83 (36) | 4.99 ± 3.71 (10) |

<sup>a</sup> Effector/target cell ratios were 10:1, established by mixing 10<sup>6</sup> spleen cells with 10<sup>5</sup> bacteria in wells of microtiter plates. Spleen cells were derived from six rats from each group.

<sup>b</sup> Strain B31 of *B. burgdorferi*.

<sup>c</sup> Mean log<sub>10</sub> ± standard error of the mean of surviving bacteria in three observations that normal, untreated adult mice or young (<4-week-old) rats die within 2 to 3 days after challenge with a low inoculum, having from 10<sup>5</sup> to 10<sup>6</sup> *Listeria* CFU and belonging to the same strain that was used here. Thus, such variables as age and species of animal need to be considered when analyzing and comparing ethanol's effects in vivo on immune function.

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### TABLE 3. The effect of ethanol ingestion on the course of *Listeria* infection in rats

| Treatment group | Time after infection (h) | Bacterial count<sup>a</sup> in: |  |  |  |  |  |  |
|-----------------|--------------------------|-------------------------------|---|---|---|---|---|---|
|                 |                          | Liver | Spleen | P value<sup>c</sup> for: | Liver | Spleen |
| Control diet    | 24                       | 5.40 ± 4.26 | 4.83 ± 4.11 | <0.025 | <0.05 |
|                 | 48                       | 5.37 ± 4.16 | 4.93 ± 4.23 | <0.025 | <0.05 |
|                 | 72                       | 3.18 ± 2.35 | 3.14 ± 2.51 | >0.2   | >0.2  |
| Ethanol diet    | 24 (0.31)<sup>d</sup>    | 5.78 ± 4.28 | 4.97 ± 4.25 |         |       |
|                 | 48 (0.27)                | 5.60 ± 4.14 | 5.23 ± 4.17 |         |       |
|                 | 72 (0.33)                | 3.21 ± 2.57 | 3.19 ± 2.60 |         |       |

<sup>a</sup> Rats (six per group) were fed ethanol or a control diet for 3 or 4 days and were then infected intraperitoneally with 5 × 10<sup>7</sup> CFU of *L. monocytogenes*.

<sup>b</sup> Mean log<sub>10</sub> ± standard error of the mean of *L. monocytogenes* bacteria cultured per gram of tissue.

<sup>c</sup> This value equals the significance of the difference between the number of culturable *Listeria* bacteria in the tissues of the control rats versus the ethanol-treated rats, for each of the matching time points.

<sup>d</sup> Values in parentheses indicate the mean percent blood ethanol levels found in the treated rats, measured 1 h after ingestion of the final dose of ethanol.
immunity to both diseases. With the clear-cut demonstration here of decreased antimicrobial activity following ingestion of intoxicating levels of ethanol, it is also possible that under similar conditions, vaccination against certain infectious diseases could be hampered, diagnostic and normally measurable antibodies could go undetected, or that treatment with certain types of antibiotics may be less effective.

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