ALCOHOL CONSUMPTION DURING MURINE ACQUIRED IMMUNODEFICIENCY SYNDROME ACCENTUATES HEART PATHOLOGY DUE TO COXSACKIEVIRUS

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Abstract — Alcohol, especially after prolonged and excessive consumption, results in marked alteration of host immunity and increased susceptibility to infection. To determine whether ethanol consumption exacerbates coxsackievirus B3 cardiomyopathy during murine acquired immunodeficiency syndrome (AIDS), female C57BL/6 mice were infected with LP-BM5 retrovirus and administered 40% ethanol both in water and in solid agar-based form. Cardiac histopathology was semi-quantitatively assessed for lesion severity and induced production of splenocyte: interleukin (IL)-2, IL-4, IL-6, tumour necrosis factor-α and interferon-γ were determined. Ethanol consumption during murine retrovirus infection increased coxsackievirus-induced myocarditis in 85% of the animals and also exacerbated the lesion severity. Mice infected with retrovirus and co-infected with coxsackievirus showed significant heart lesions. Retrovirus infection suppressed Th1 responses, causing cytokine dysregulation and immunosuppression, which facilitated coxsackievirus-induced myocarditis. Our data suggest that ethanol consumption heightens the cytokine imbalance to favour a Th2 response by enhancing Th2 and/or by suppressing Th1 function. In conclusion, murine AIDS facilitated severe cardiotoxicity during coxsackievirus infection, while non-retrovirus-infected mice were resistant. These effects were accentuated by ethanol consumption.

INTRODUCTION

The prolonged or excessive consumption of alcohol results in a marked alteration in host immunity and an increased susceptibility to infection. Specifically, alcohol consumption in animals modifies the immune system and enhances susceptibility to viral, intracellular bacterial, and fungal infections. Alcohol misuse in humans significantly compromises host defences resulting in increased incidences of infection, morbidity and mortality. Of those who misuse alcohol, populations with human immunodeficiency virus (HIV) infections are of growing concern. In the USA, HIV-seropositive individuals are more likely to consume alcohol (82%) and be categorized as alcohol misusers (41% with a MAST score >5) than the general population (Lefevre et al., 1995). Furthermore, ethanol (EtOH) consumption among HIV-infected individuals accentuates HIV because EtOH-induced liver disease increases the expression of pro-inflammatory cytokines, implicated as promoters of HIV replication. Fong et al. (1994) published a case report in which a heavy alcohol misuser rapidly progressed to AIDS shortly after seroconversion. Thus, EtOH consumption in large amounts has a profound effect on adaptive immune responses to bacteria and viruses that require T-cell-dependent immune responses for resistance to primary and secondary infections.

Alcohol also acts on the cardiovascular system to promote hypertension, cerebrovascular disease, and stroke. This may occur in part via the promotion of immunosuppression, which facilitates infection and increased pathogenesis. Chronic EtOH consumption in mice causes harmful effects on the host immune response, oxidative balance stress, and nutritional status of the animals (Lee et al., 1999). EtOH reduces the T-cell response to mitogens, suppresses natural killer cell function, reduces granulocyte migration, and lowers macrophage phagocytic activity (Wang and Watson, 1995a,b,c; Wang et al., 1997). Thus, EtOH can act to enhance the host’s susceptibility to retroviral infection and to accelerate the onset of murine aquired immunodeficiency syndrome (MAIDS). These factors make it paramount to understand the impact of alcohol consumption on the progression of HIV disease.

There is a high incidence of cardiopathy in AIDS which is not related to HIV, but may be related to other pathogens such as coxsackievirus. Coxsackievirus B3 (CVB3), an enterovirus in the Picornaviridae family, is an aetiological agent of virus-induced myocarditis (Woodruff, 1980; Tracy et al., 1991). Patients with myocarditis have elevated antibody levels against enterovirus proteins and enterovirus nucleic acids (Bowles et al., 1986; Easton and Eglin, 1988; Tracy et al., 1991). CVB3-induced myocarditis is an immunopathological disease in which the host’s immune system, once triggered by the virus, is the major contributor to heart pathology (Woodruff, 1980). Suggested mechanisms include the development of heart neoantigen (Paque et al., 1979), the cross-reactivity between virus and heart myosin (Gauntt et al., 1991, 1993a,b), and the development of heart-reactive cytolytic T-cells (Friedman et al., 1998; Seko et al., 1998). Clearly, the immune system is required for virus clearance, as severe combined immunodeficient mice develop cardiac necrosis with a high rate of mortality during CVB3 infection (Chow, 1993).

The present study was designed to determine whether retrovirus-induced murine AIDS, alone or with EtOH consumption, would accentuate the pathological outcome of CVB3 infection, using a coxsackievirus-resistant strain of mouse.

MATERIALS AND METHODS

Virus proliferation

The cardiovirulent coxsackievirus B3 (CVB3/59) was a generous gift from Dr Melinda Beck at the University of North Carolina at Chapel Hill, NC, USA. Coxsackievirus stocks were propagated in HeLa cell monolayers in minimum essential medium supplemented with 10% fetal bovine serum.
Infection and treatment of mice

Four-week-old female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, DE, USA). They were housed in polycarbonate cages with stainless-steel wire lids (four mice per cage) in the Central Animal Facility of the University of Arizona. Animals were cared for as required by the University of Arizona Animal Care and Use Committee. The housing facility was maintained at 20–22°C and 30–70% relative humidity, with a 12-h light/12-h dark cycle. Mice were maintained for 4 months in the facility on an NIH-31-modified mouse sterilizable diet (mouse diet #7001; Teklad, Madison, WI, USA) and water ad libitum. Mice were then randomly assigned to one of the following groups: uninfected control mice; uninfected mice given EtOH (v/v) in drinking water; mice infected with retrovirus; mice infected with retrovirus given EtOH; mice infected with retrovirus and coxsackievirus; mice infected with retrovirus and coxsackievirus given EtOH; mice infected with coxsackievirus; and mice infected with coxsackievirus given EtOH. The EtOH concentration was increased in increments of 10%, at 1-week intervals, from 10% to a final concentration of 40% (v/v) in drinking water. The mice drinking EtOH were also given 25 g agar gel containing EtOH (40% v/v plus 2% peanut butter) for 16 weeks, five times a week. This dietary approach to EtOH supplementation in mice was based on its development by Bautista in rats (Batey and Patterson, 1991; Bautista, 1997). Pair-fed control mice received isocaloric water and agar gels in which EtOH was substituted with sucrose. Administration of EtOH and/or water supplemented with sucrose, as well as agar, began 2 weeks after LP-BM5 infection. Fresh drinking water and/or EtOH were provided twice weekly and an agar block every day.

LP-BM5 retrovirus was administered intraperitoneally to mice in 0.1 ml of minimum essential medium with an esotropic titre of 4.5 log<sub>10</sub>, plaque forming units × 10<sup>6</sup>/l, which induces disease with a time course comparable with that previously published (Wang and Watson, 1994a,b). Infection of female C57BL/6 mice with LP-BM5 murine leukaemia virus leads to the rapid induction of clinical symptoms with virtually no latent phase (Liang et al., 1996). The mice were infected 2 weeks before initiation of treatment as done previously in numerous studies (Wang and Watson, 1994a,b, 1995a,b,c,d; Wang and Liang, 1997). The infection period was 20 weeks, while the treatment went on for 18 weeks for all groups. After 16 weeks of EtOH treatment, the appropriate groups of mice were inoculated intraperitoneally with 3 × 10<sup>4</sup> TCID<sub>50</sub> of CVB3/59 in 0.1 ml of Roswell Park Memorial Institute 1640 medium. Mice were killed 12 days after inoculation.

When murine AIDS had developed at 16 weeks after retrovirus infection, all mice in all groups were killed the same week while under ether anaesthesia. Spleens were then removed, and kept at 4°C.

Enzyme-linked immunosorbent assays (ELISA) for cytokines

The production of interleukin (IL)-2, IL-4, IL-6, interferon (IFN)-γ, and tumour necrosis factor (TNF)-α from mitogen-stimulated splenocytes was determined as described previously (Chouaib et al., 1985). Briefly, spleens were gently teased with forceps in culture medium (CM; RPMI 1640 containing 10% fetal bovine serum, 2 mmol/l glutamine, 1 × 10<sup>6</sup> units/l of penicillin and streptomycin) producing a suspension of spleen cells. Red blood cells were lysed by incubation with a lysis buffer (0.16 mol/l ammonium chloride Tris buffer, pH 7.2) at 37°C for 3 min. The cells were then washed twice with culture media (CM). Cell concentrations were determined and adjusted to 1 × 10<sup>6</sup> cells/ml. Splenocytes [0.1 ml/well (1 × 10<sup>5</sup> cells/ml)] were cultured in triplicate on 96-well flat-bottomed culture plates (Falcon 3072, Lincoln Park, NJ, USA) with CM. Splenocytes were then stimulated with concanavalin A (Con A, 10 mg/l, 0.1 ml/well; Sigma Chemical Co., St Louis, MO, USA) to determine the production of IL-2 and IL-4 after 24 h and of IFN-α after 72 h in a 37°C, 5% CO<sub>2</sub> incubator. Splenocytes were also incubated for 24 h after the addition of lipopolysaccharide (LPS; 10 mg/l; Gibco) to induce IL-6 and TNF-α production. After incubation, supernatants were collected and stored at −70°C until analysis. Cytokines were determined by sandwich ELISA as described previously (Lee et al., 1999). Rat anti-murine IL-2, IL-4, IL-6 and TNF-α purified antibodies; rat anti-murine IL-2, IL-4, IL-6, IFN-γ and TNF-α biotinylated antibodies; and recombinant murine IL-2, IL-4, IL-6, IFN-γ and TNF-α were obtained from Endogen (Cambridge, MA, USA).

Histopathology

Upon the death of mice, hearts were removed, rinsed in saline and transversely cut in halves. One half of each heart was immediately placed in Histochoice tissue fixative (Amresco, Solon, OH, USA) and stored at 0°C. Fixed heart tissues were sectioned (6 μm) on a Zeiss HM 505 N cryostat (Carl Zeiss, Inc., Thornwood, NY, USA) and stained with haematoxylin and eosin. The extent of inflammatory lesions within the myocardium was graded by a pathologist without knowledge of the other experimental variables. The grading was performed in a semi-quantitative manner according to the relative degree (from heart to heart) of mononuclear cell infiltration and the extent of necrosis (Fig. 1). Mild damage is considered as <10% of heart tissue affected, moderate = 10–25% and severe as >25% of heart tissue affected.

Statistics

All variables were compared using a one-way analysis of variance (ANOVA), followed by a two-tailed Student’s t-test.
for comparison between any two groups. Differences between two groups were considered significant at \( P < 0.05 \).

RESULTS

**Body weight and EtOH intake**

There were no significant changes in EtOH, water or agar consumption due to retrovirus or coxsackievirus infection (data not shown). Mice consuming EtOH in water had a mean ± SD intake of 3.6 ± 0.41 ml/mouse/day over the period during which they received 40% EtOH, whereas controls consumed 3.7 ± 0.31 ml/mouse/day of sucrose in water. We applied the agar-block feeding procedure to provide more frequent consumption of EtOH. Mice consumed 18.5 ± 3.7 g/four mice/day of agar gel containing EtOH, which was not significantly different from controls (22.5 ± 2.8 g/four mice/day). Spleen and lymph node weights (190 days post-infection) were significantly \( P < 0.05 \) elevated in infected mice (data not shown). This correlated with severe immunodeficiency and aberrant cell accumulation or proliferation, as reported previously (Chouaib et al., 1985; Wang and Watson, 1995a,b,c,d; Wang et al., 1997). Body weight was not significantly changed (data not shown).

**Mitogenesis of splenocytes**

The proliferation of splenocytes stimulated by LPS and Con A was significantly \( P < 0.05 \) decreased by LP-BM5 retrovirus (Fig. 2A and B). EtOH supplementation for 49 days increased T- and B-cell mitogenesis in uninfected mice, but decreased T-cell and increased B-cell mitogenesis in coxsackievirus infected mice \( P < 0.05 \). EtOH-treated retrovirus-infected mice showed a significant decrease in both T- and B-cell mitogenesis, when compared to EtOH-treated uninfected mice \( P < 0.05 \). Infection with coxsackievirus increased the proliferation of T-cells in the non-retrovirus-infected group; on the other hand, B-cells mitogenesis in the same group was decreased, when compared to the ethanol-treated CVB infected group. Both non-treated and EtOH-treated retrovirus-infected and coxsackievirus-superinfected groups showed a significant decrease in their T- and B-cell mitogenesis, when compared to uninfected and/or CVB infected groups treated or not treated with EtOH \( P < 0.05 \).

**Cytokine production by splenocytes**

Production of IFN-\( \gamma \) by Con A-stimulated splenocytes was significantly \( P < 0.05 \) inhibited by retrovirus infection and CVB superinfection (Table 1). IFN-\( \gamma \) produced by Con A-stimulated splenocytes was not affected by EtOH consumption.
Splenocytes from retrovirally infected mice supplemented with EtOH produced significantly less IFN-γ than those from uninfected controls ($P < 0.05$). Spleen cells from mice infected with coxsackievirus produced significantly less IFN-γ compared to cells from uninfected controls ($P < 0.05$). Splenocytes from mice infected with both retrovirus and coxsackievirus showed a significant decrease in IFN-γ production when compared to all other groups ($P < 0.05$ at least). EtOH consumption in general down-regulated production of IFN-γ.

IL-2 production showed no significant differences between groups (Table 1).

Production of Th2 cytokines by LPS-stimulated splenocytes from retrovirally infected mice was significant; IL-4 showed no significant difference, whereas IL-6 showed a marked increase, when compared to untreated uninfected mice (Table 2).

Coxsackievirus infection considerably decreased the secretion of IL-6, but not IL-4, in non-retrovirus-infected mice when compared to non-coxsackievirus non-retrovirally infected mice ($P < 0.05$). Splenocytes from mice infected with both coxsackievirus and retrovirus and supplemented with EtOH produced significantly more IL-6 than cells from any of the following groups ($P < 0.05$): uninfected controls; uninfected controls + EtOH; coxsackievirus-infected; and coxsackievirus-infected + EtOH. Conversely, the cells from mice infected with both coxsackievirus and retrovirus and supplemented with EtOH produced significantly less TNF-α than did cells from uninfected mice or those mice infected with retrovirus or coxsackievirus. TNF-α secretion was also decreased in mice infected with both retrovirus and coxsackievirus when compared to uninfected controls ($P < 0.05$).

**DISCUSSION**

Infection of mice with the murine leukaemia retrovirus mimics HIV infection in humans (Liang et al., 1996). A shift favouring Th2 cells has been observed both in human as well as in murine AIDS (Shearer and Clerici, 1993; Clerici et al., 1994). This experimental model is used to understand the effects of retroviral infection on the immune system.
Table 2. Effect of coxsackievirus B3 infection and ethanol (EtOH) intake on Th2 cytokine and tumour necrosis factor (TNF-α) production by concanavalin A (Con A)- or lipopolysaccharide (LPS)-stimulated splenocytes in murine acquired immunodeficiency syndrome

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*Splenocytes (1 × 10⁷ cell/ml Roswell Park Memorial Institute medium) were incubated with Con A [interleukin (IL)-4] and LPS (IL-6 and TNF-α) for 24 h at 37°C. After collecting supernatants, rat anti-murine IL-4, IL-6, and TNF-α antibodies were used to detect murine IL-4, IL-6, and TNF-α. The concentration of cytokines was measured by enzyme-linked immunosorbent assay at 450 nm. Every sample was determined in triplicate. Values are mean ± SD for eight mice per group. *P < 0.05 compared with untreated uninfected mice; b*P < 0.05 compared with untreated infected mice; c*P < 0.05 compared with CVB3 infected mice.

TNF-β and granulocyte macrophage colony stimulating factor (GM-CSF) promote an increase in HIV-1 replication, whereas IFN-α and IFN-β decrease replication (Matsuyama et al., 1991). This supports the hypothesis that a strong cell-mediated response (Th1) may be beneficial in resistance to HIV, whereas a shift towards Th2 predominance results in progression to AIDS.

Alcohol (or any variable that could result in a shift in Th1/Th2 profiles) could serve as a co-factor in the onset and progression of AIDS (Shearer and Clerici, 1993). EtOH can alter immune function either directly through its action on immunocompetent cells (Jerrels and Sibley, 1995; Lopez et al., 1997; Chen et al., 1998; Ahluwalia et al., 2000; Hosseini et al., 2000) or indirectly through modulation of different neuroendocrine hormones (Doll, 1998) and neurotransmitters (De Witte, 1996) that regulate the immune system.

Coxsackieviruses cause a wide spectrum of clinical diseases, including aseptic meningitis, colds, myalgia, myocarditis, pharyngitis, conjunctivitis, hand-foot-and-mouth disease, and possibly some cases of juvenile diabetes mellitus. Ten million Americans become infected by group B coxsackieviruses every year. At least 5% of these people experience heart infection, with an unknown proportion developing myocarditis. About 10–65% of biopsies are coxsackievirus-positive for patients with clinical diagnoses of myocarditis or unexplained heart failure (Gauntt and Pallansch, 1996). These percentages might be much higher, as screening for coxsackievirus infections is not usually carried out.

In our study, the mice co-infected with retrovirus and CVB3 and treated with EtOH exhibited more severe cardiopathology, when compared to the same group without EtOH treatment (Fig. 2). This correlates with an accentuation of suppression of Th1-cells and cytokine production due to EtOH treatment in retrovirus-immunosuppressed mice. LP-BM5 infection in mice, as well as HIV infection in humans, suppresses the Th1-cell response (Liang et al., 1996), which should increase coxsackievirus-induced myocarditis. We observed that the decreased production of IL-2 by splenocytes from mice with murine AIDS was further reduced by EtOH consumption, as with coxsackievirus infection. INF-γ production was also significantly inhibited in the group of mice with AIDS treated with EtOH when compared to the healthy animals. INF-γ production was considerably decreased in the murine AIDS group superinfected with coxsackievirus. This pronounced decrease correlates with the degree of cardiopathology observed in this group. In turn, Th2 cytokine secretion, specifically of IL-4 and IL-6, was increased in retrovirus-infected mice treated with EtOH, as well as in controls fed sucrose water.

Our data suggest that EtOH shifts the cytokine balance in favour of a Th2 response by enhancing Th2 function and/or by suppressing Th1 function (Tables 1 and 2). EtOH may thereby promote the conditions that favour the opportunistic viral infections that cause cardiac pathology.

In mice infected with the murine retrovirus, there was an increase in the secretion of Th2 cytokines facilitating suppression of the cellular immune response (Wang et al., 1997). This type of response promotes an antibody-mediated immunity. The cardiac pathology observed may have occurred via several mechanisms: (1) due to infection with CVB3 which induces synthesis of antibodies to the pathogen, some of the antibodies may cross-react with cardiac myosin, inducing myocarditis (Fujinami et al., 1983; Gaunt et al., 1991, 1993a, b; Barnet and Fujinami, 1992; Cunningham, 1993; Gaunt and Pallansch, 1996); (2) myocyte damage by the coxsackievirus with the concomitant release of myosin by the myocyte cells.

![Fig. 3. Histopathology scores of CVB3/59-inoculated C57BL6 mice treated with ethanol and/or infected with LP-BM5 retrovirus.](image)


Wang, Y. and Watson, R. R. (1994) Chronic ethanol consumption before retrovirus infection is a cofactor in the development of immune dysfunction during murine AIDS. *Alcoholism: Clinical and Experimental Research* 18, 976–981.


