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THE EFFECTS OF MURINE AIDS AND ETHANOL CONSUMPTION ON
THE SEVERITY OF MYOCARDIAL ISCHEMIC INJURY

By
Yinhong Chen

A Dissertation Submitted to the Faculty of the
GRADUATE INTERDISCIPLINARY PROGRAM
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For the Degree of
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In the Graduate College
THE UNIVERSITY OF ARIZONA

2001
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Yinhong Chen, entitled *The Effects of Murine AIDS and Ethanol Consumption on the Severity of Myocardial Ischemic Injury* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Ronald R Watson
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DEDICATION

This dissertation is dedicated to my husband and my daughter. Both have sacrificed through my graduate school. Their loves give me confidence to overcome all kinds of difficulty.

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ABSTRACT

Cardiovascular complications are prevalent in patients with AIDS. Cardiovascular complications, particularly ischemia-reperfusion injury, may be severe in AIDS patients. The pathology underlying cardiovascular complications in AIDS patients is unclear. Perhaps interplay of several pathologic factors amplifies the response to ischemia. Murine retrovirus (LP-BM5) induced murine AIDS is the best model of human AIDS research because LP-BM5 causes similar immune changes. Ethanol consumption has the advantage and disadvantage to health. The aim of this study was to determine if chronic ethanol consumption influences pathological changes caused by murine AIDS, specifically in cardiovascular complications, and if vitamin E supplementation could attenuate cardiovascular injury by murine AIDS. In our present study, we found that retrovirus infection enhanced neutrophil CD11b expression and ROS production, increased platelet CD62p and platelet microparticle formation, exaggerated coronary permeability to macromolecules and caused a severe myocardial ischemia-reperfusion injury. Chronic ethanol consumption down-regulated neutrophil CD11b expression, but neutrophil ROS production, platelet CD62p expression and platelet microparticle formation were enhanced. Chronic moderate ethanol consumption improved coronary microcirculation and attenuated ischemia-reperfusion injury. Our results indicate that neutrophil and platelet adhesion molecule expression increases in murine AIDS. Neutrophil and platelet-mediated severe ischemia-reperfusion injury may contribute to increased incidence of cardiomyopathy in AIDS. The cardiovascular protective effects of
moderate ethanol consumption may be related to modulation of neutrophil CD11b expression and improve coronary microcirculation. However, chronic ethanol consumption did not preserve myocardial damage by retrovirus infection. In this study, we also demonstrated that vitamin E attenuated AIDS-induced myocardial injury. Vitamin E may be a therapeutic adjuvant agent for preventing and treatment AIDS-induced cardiovascular diseases.
BACKGROUND AND RATIONALE

ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

1. Epidemiology

AIDS is a health crisis affecting more than 47 million people worldwide. 21.8 million people have died from AIDS since the epidemic began. During the year 2000, AIDS caused the deaths of an estimated 3 million people. HIV/AIDS has now become the fourth leading cause of mortality, and its impact is increasing [1]. According to the Joint United Nations Programs on HIV/AIDS, as of the end of 2000, 36.1 million people are estimated to be living with HIV/AIDS. The World Health Organization (WHO) estimated that as many as 15,000 people are newly infected HIV each day and that about 5.3 million more people worldwide were infected with HIV during 2000 [2]. In the U.S. alone, it is estimated that 40,000 new HIV infections occurred in the year 2000, plus more than 688,000 cases of AIDS have been reported since 1981. The prevalence of cardiac involvement in patients with HIV, both asymptomatic and symptomatic, ranges from 28% to 73% [3]. The cardiovascular mortality rate associated with HIV infection in the United States is estimated to be 1-6% [4]. After the introduction of highly active antiretroviral therapy (HAART), the survival rate of HIV infected patients was significantly enhanced. However, cardiovascular involvement in AIDS becomes more apparent. Indeed, HIV cardiomyopathy was reported to be the fourth leading cause of
dilated cardiomyopathy in the United States [5]. Congestive heart failure has become the leading cause of death in pediatric patients with AIDS, and half of them die within 6 to 12 months [6]. The incidence of myocardial infarction (MI) in HIV infected patients has increased from 0.86 in year 1983-86 to 3.41 in year 1995-98 respectively per 1000 patient [7,8]. The pathogenesis underlying cardiovascular manifestations in HIV infected patients is unclear, but it likely involves an interplay of several pathologic factors such as cytokine dysregulation, cardiovascular endothelial cell dysfunction, platelet activation associated with hypercoagulation and thrombosis formation, and excessive neutrophil activation amplifying the inflammatory response in the heart.


The murine model of LP-BM5-induced acquired immune deficiency exhibits many features of human AIDS [9]. Our laboratory [9-11] and others [12-15] have provided evidence that LP-BM5 murine retrovirus infection produces an immunodeficiency that resembles human HIV-1. Many investigators [9,14,15-19] have described the development and severity of cytokine dysregulation during murine retrovirus infection, much as occurs in human AIDS [20]. Murine retrovirus infection also enhances tumor growth [21,22] and induces lymphoma [13-15], the most common cancer in human AIDS. LP-BM5 retrovirus-infected mice demonstrate reduced resistance to opportunistic pathogens common to human AIDS patients: Candida albicans [23], Mycobacterium avium [24], Cytomegalovirus [25], Cryptosporidium parvum [26], and Giardia muris [27]. AZT, a highly active antiretroviral agent for human AIDS, also retarded
development of murine AIDS [28,29]. Thus, LP-BM5-induced murine AIDS is an excellent model for human AIDS research. It provides useful knowledge about mechanisms of pathogenesis that have relevance to human AIDS. The model is also useful in the development of therapy of HIV-associated cognitive impairment and adjunctive agents that can counteract the deleterious effects of HIV-induced immune activation.


There is evidence that a variety of inflammatory mediators, including TNF-α, IL-1, IL-6, and platelet activating factor (PAF) increase in AIDS (9,14,15-19, 30-33). TNF-α, IL-1, and IL-6 are all potent neutrophil and endothelial cell mediators that initiate neutrophil and endothelial cell adhesion molecule expression. PAF is a versatile lipid inflammatory mediator that acts on G-protein coupled receptors in a variety of cells. PAF primes neutrophils, causing a dramatic increase in response to activating agents such as TNF-α, and IL-1 [34]. Priming causes an increase ROS generation and upregulation of CD11b/CD18 adhesion molecules. Upon subsequent stimulation, PAF not only mediates a number of inflammatory responses but also induces platelet activation by the PKC and arachidonic acid pathways. Recently, in-vitro data demonstrates that TNF-α- induced apoptosis can be prevented by coincubation with either a PAF antagonist or PAF acetylhydrolase, the catabolic enzyme for PAF [35]. This result suggests that TNF-α- programmed apoptosis may be enhanced by PAF receptor activation [32,35]. IL-6 is identified as a mediator of myocardial injury with reversible myocardial depressant
effects in vivo [36,37]. These effects appear to be regulated by nitric oxide (NO)[38-40]. Narcella et al [40] suggested that eNOS, nNOS, and inducible nitric oxide syntheses (iNOS) contribute 50%, 50% and 0% respectively to systemic NO production under baseline conditions. The increase in systemic NO production during stress is mediated by iNOS. Increased expression of iNOS was found in vitro in cardiac myocytes treated with TNFα, IL-1 and interferon (IFN-γ) [41]. Myocyte death in culture paralleled increased NO synthesis and reactive oxygen species (ROS). Long-term treatment of cardiomyocytes with IL-1 and TNFα reduced contractility and cyclic adenosine monophosphate (cAMP) accumulation by inhibition of adrenergic responsiveness. The myocardial depressant effects of TNFα infusion in dogs resulted in LV dysfunction [42]. Anti-TNFα antibodies reduced cardiac dysfunction during sepsis [43]. Barbaro et al [44] demonstrated greater intensity of TNFα and iNOS immunohistochemical stains of endomyocardial biopsies in cardiomyopathy patients with AIDS than patients without AIDS. Overall, proinflammatory mediators play a critical role on 1) neutrophil, endothelial and platelet activation; 2) ischemia-reperfusion injury; 3) myocyte apoptosis and necrosis; and 4) cardiovascular dysfunction.

4. Platelet and Neutrophil Activation in Murine AIDS.

Platelet adhesion molecule expression, followed by aggregation, constitutes the primary mediator of coronary microvascular thrombosis. Activated platelets attract leukocytes at the site of endothelial damage to promote inflammation and atherogenesis: Conversely, leukocyte adhesion to the dysfunctional endothelium can lead to platelet adhesion and
local thrombogenesis resulting in ischemic events [45,46]. Many studies found that there is a greater incidence of thrombosis among HIV-infected individuals [47,48]. These findings suggest that platelets circulate in an activated state in AIDS. In addition, if the PAF level is increased in AIDS, platelets will likely be activated. Upon platelet activation, CD62p (P-selectin) is translocated from α granule membranes to the surface of plasma membranes and mediates platelet adhesion to vascular endothelial cells and neutrophils. CD61 has fibrinogen binding sites and is consecutively expressed on platelets. Platelet activation may trigger platelet apoptosis and form platelet microparticles (PMP). CD61 expressed on PMP bind with neutrophils, vascular endothelial cells and platelets through a fibrinogen bridge. The PMPs-cell interaction may also induce thrombosis formation and a pathological inflammatory response. Neutrophil function studies were extensively investigated in AIDS. However, few investigators explore neutrophil adhesion molecule expression in AIDS, and much less is known about platelet, neutrophil activation and PMP formation mediated ischemia-reperfusion injury in murine AIDS hearts. CD11b, a neutrophil activating marker, is a key molecule involved in the adhesion of neutrophils to platelets and vascular endothelial cells. Increased ROS production in neutrophils confirms neutrophil activation and indirectly indicates the severity of the inflammatory response. A higher affinity CD11b expressed on activated neutrophils interacts with ICAM-1 on cardiovascular endothelial cells to facilitate neutrophil accumulation in the heart. Therefore, it is important to measure CD62P expression on platelets, PMP formation and CD11b expression and ROS production of neutrophils in murine LP-BM5-infected mice.
5. The Effect of Murine AIDS on Coronary Microvascular Permeability.

Cardiovascular involvement in AIDS may also contribute to coronary microvascular endothelial dysfunction. Coronary microvascular permeability change is an indicator of endothelial cell dysfunction. Indirectly, several studies report that the retrovirus impairs the blood-brain barrier [51-53]. The possible mechanisms include that 1) retroviruses directly attack endothelial cells; 2) elevated TNFα and PAF released by virus infected cells have cytotoxic to endothelial cells; and 3) overproduced NO by iNOS amplifies the endothelial cell dysfunction [54]. Indeed, Barbaro et al [44] demonstrated that TNFα and iNOS immunohistochemical stains of endomyocardial biopsies had greater intensity in cardiomyopathy patients with AIDS than in patients without AIDS. We suspect that LP-BM5 infected mice also have an increase in coronary microvascular permeability due to the same mechanisms impairing the blood-brain barrier. Permeability changes are a sensitive indicator of endothelial cell dysfunction. Therefore, it is possible that coronary microvascular permeability to macromolecules is increased in LP-BM5-infected mice.

6. Ischemia-Reperfusion Injury in LP-BM5 Infected Mouse Hearts.

Postischemic reperfusion may profoundly alter cardiac function. A principal mediator of this phenomenon is neutrophils. Neutrophils may plug coronary capillaries, mechanically blocking blood flow. Upon reflow, recruitment of neutrophils is accompanied by their adhesion molecule expression. A higher affinity β2-intergrin (CD11/CD18) expresses on activated neutrophils and ICAM-1 serves as a major endothelial ligand to CD11/CD18.
Therefore, CD11b/CD18 plays a key role in mediating firm adhesion of neutrophils to vascular endothelial cells prior to transmigration into ischemic heart tissues. Activated neutrophils release the proteolytic enzymes that directly induce heart tissue damage and proinflammatory mediators that amplify the local inflammatory reaction. The oxidative burst of neutrophil releases cytotoxic \( \text{H}_2\text{O}_2 \). Overall, neutrophil accumulation in heart tissue results in severe ischemia-reperfusion injury. Neutrophils may be activated in murine AIDS and become more reactive during an ischemic attack in murine AIDS. There are no direct reports of up-regulated ICAM-1 of endothelial cells in AIDS, but several investigators demonstrate an increased ICAM-1 expression on B cells in murine AIDS [57] and T cells in HIV-infected individuals [58]. These results imply that LP-BM5 infected mice may up-regulate ICAM-1 on endothelial cells as well. In support of this scenario, we will test our hypothesis that ischemia-reperfusion injury is worse in murine AIDS due to excessive inflammatory response.

7. Vitamin E, a Potential Therapeutic Agent, Attenuates Ischemia-Reperfusion Injury in Murine AIDS Due to Modified Platelet, Neutrophil and Vascular Endothelial Cell Function and Its Antioxidant Activity.

Vitamin E is best known for its antioxidant activity. Vitamin E prevents coronary heart disease [59,60] based on the assumption that atherosclerosis progression is determined by oxidative modification of LDL, which is then taken up by macrophages of atherosclerotic plaque. Platelet adhesion, the principal event of platelet activation, provides a crucial step in the development of cardiovascular events such as myocardial infarction and ischemic...
stroke. Vitamin E is capable of inhibiting platelet aggregation by a variety of agonists [61,63]. Some evidence [61-65] suggests that vitamin E interferes with platelet activation via influencing: 1) arachidonic acid metabolism; 2) PLC pathway; and 3) antioxidant activity. Protein kinase C plays key roles in the early phase of platelet activation and in the secretary phase of platelet aggregation. It has been shown that vitamin E can inhibit protein kinase C activity, IP₃ formation, Ca²⁺ mobilization, and thromboxane A2 formation by platelets [63]. Therefore, we expect that vitamin E could significantly reduce the incidence of strokes and recurrent transient ischemic attacks. Recently, vitamin E has been identified as an adhesion molecule modifier. Vitamin E inhibits adhesion molecule expression of leukocytes and vascular endothelial cells stimulated by a number of mediators such as oxidized LDL, interleukin-1, N-formyl-methionyl-leucyl-phenylalanine (fMLP), and LPS [66-70]. Vitamin E may exert its benefits on leukocyte accumulation through several different ways: (1) an increased resistance of LDL to oxidation; (2) a decreased production of several chemokines IL-6, 8, monocyte chemoattractant protein-1 (MCP-1) that are involved in the recruitment of immune cells to the site of stimulation [66,71]; and (3) inhibition of CD11b/CD18 adhesion molecule expression on leukocytes, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin adhesion molecules on endothelial cells [68-70]. Thus, vitamin E can decrease in the interaction of leukocytes and endothelial cells, blockage of leukocyte migration into extravascular spaces. If LP-BM5 infected mice increase proinflammatory cytokine secretion, platelet and neutrophil activation and cardiac oxidation, then vitamin E will be an excellent candidate for attenuating ischemia-reperfusion injury due to its multi-
beneficial effects on cardiovascular events in murine AIDS. In addition, HIV infected people were found conclusively to lose vitamin E as the progression of AIDS occurred, which also happens in murine AIDS [18]. Supplementation with vitamin E slowed this process [72]. Vitamin E supplements at extremely high levels (15-450 times the normal intake) significantly normalized cytokines produced by splenocytes without toxicity [16,71]. Therefore, the high degree of safety and tolerability of vitamin E warrant larger and longer efficacy trials.

CHRONIC ETHANOL CONSUMPTION.

1. Epidemiology
Ethanol consumption is the third leading cause of preventable death. It is estimated that 5.9% of the U.S. population are heavy drinkers and ethanol contributes to approximately 19,515 deaths annually [73]. Ethanol is the major cause of non-ischaemic cardiomyopathy in Western society [74]. Prolonged ethanol abuse can also result in alcoholic heart muscle disease [74]. However, regular moderate drinkers tend to have an advantage to health. The relation of ethanol intake with mortality seems to resemble a J-shape curve. The higher the intake, the higher the mortality, excepting abstainers have an increased mortality compared to moderate drinkers [75]. Epidemiological studies [75-80] also demonstrated that moderate consumption of ethanol reduces the risk of coronary heart disease, sudden cardiac death, and ischemic stroke.
2. Ethanol and Proinflammatory Cytokines.

There is evidence that ethanol may interfere with the level of cytokines has been recognized. However, the results are controversial [81]. This controversy may be due to different research designs. Most studies [81,82,83,84] measured the level of TNF-α, IL-1, and IL-6. Recently, Arbabi et al [84] found that ethanol inhibited IL-8 secretion. All of them are proinflammatory cytokines that are involved to neutrophil activation. Ethanol also suppresses cytokine-induced iNOS expression [85], which may involve a mechanism of cardiovascular cell apoptosis. Overall, chronic ethanol consumption may modulate functional neutrophils and exhibit certain effects on ischemia-reperfusion injury.

3. Ethanol Modulates Neutrophil and Platelet function.

MacGregor’s studies [86,87] indicated that ethanol intoxication has profound effects on neutrophil kinetics. Most significant is the inhibition of neutrophil mobilization to sites of inflammation. Although bactericidal activity was normal in ethanol intoxication, mean chemotaxis was significantly below normal and phagocytic activity was less than half that of control subjects. Most of these effects were likely through the mechanism of inhibited adherence. Neutrophil CD11b/CD18 is a key adhesion molecule. Expression CD11b/CD18 is necessary for neutrophil migrating to site of infection. Chronic ethanol consumption may inhibit neutrophil CD11b expression. If neutrophil accumulation in the hearts for response to ischemia-reperfusion injury is impeded by ethanol consumption, myocardium damage due to ischemia-reperfusion will be decreased. Therefore, regular
ethanol consumption may exhibit the advantage and disadvantage effects in the different situation.

An effect of ethanol on platelet function has been observed. Acute ethanol intoxication decreased platelet survival time and impaired platelet production in the bone marrow [88]. Ethanol decreases platelet aggregation in response to diverse agonists such as thrombin, ADP, epinephrine, and collagen [89]. Nguyen [90] found that ethanol inhibits collagen-induced platelet aggregation, secretion, arachidonate mobilization, and thromboxane A2 formation but does not inhibit platelet adhesion. Phospholipase A2 may be a major site for ethanol-induced platelet inhibition. Additional signal transduction pathways are likely targets for ethanol including phosphoinositide-specific phospholipase C and cyclic AMP [91]. Epidemiological studies have linked an inhibition of platelet aggregation to the cardioprotective effects of moderate ethanol consumption, but few investigators noticed the effect on platelet adhesion molecule, CD62p, by chronic ethanol consumption.


Regular moderate drink tends to have cardioprotective effects. The underlying mechanisms are not well established. It is possible that the cardioprotective effects of ethanol are through vascular relaxation, HDL elevation, lowering fibrinogen level, adjusting cytokine level, modulating neutrophil, platelet function, or diminishing thrombus formation on damaged arterial walls [92,93]. Moderate levels of ethanol also
induce expression of vascular endothelial growth factor and stimulate angiogenesis [94].

Overall, moderate ethanol consumption may improve cardiovascular microcirculation and reduce myocardial ischemia-reperfusion injury.

HIV infected individuals experience significant behavior change. Chronic ethanol consumption in AIDS patients is common. Indeed, 14% of HIV infected patients abuse alcohol [95]. HIV and ethanol may interact in a complex manner on the circulatory system. To clarify how the combination factors affect the cardiovascular system may assist physicians to design clinical trails.
SPECIFIC AIMS

Cardiovascular involvement is prevalent in patients with AIDS. Cardiovascular manifestation, particularly ischemic heart disease, may be severe in AIDS patients. The pathology underlying ischemic heart disease in AIDS patients is unclear. Perhaps interplay of pathologic factors amplifies the response to ischemia. Murine retrovirus (LP-BM5) induced murine AIDS is an excellent model of human AIDS research because LP-BM5 causes similar immune changes. The hypotheses of this project are that retrovirus infection impairs the integrity of coronary endothelial cells, and activates neutrophils platelets. Neutrophil activation amplifies the inflammatory response, enhancing ischemia-reperfusion injury. Since AIDS patient have been found to greatly influence behavior change and many HIV infected patients have ethanol abuse, we will test the hypothesis that ethanol consumption enhances these pathological changes in murine AIDS. To test these hypotheses, we will assess neutrophil CD11b expression and ROS production, platelet CD62p expression, coronary microvascular permeability, and the severity of myocardial ischemia-reperfusion injury in murine AIDS in the presence or absence of chronic ethanol consumption. We will also test the potentially therapeutic vitamin E on ischemia-reperfusion injury in murine AIDS by using an ischemia-reperfusion heart model in mice.
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CHAPTER 1. MURINE RETROVIRAL INFECTION MODULATES NEUTROPHIL ACTIVATION DURING CHRONIC ETHANOL CONSUMPTION

1.1. ABSTRACT

The major role of neutrophils (PMNs) is to protect the host against infectious agents. Functional activity of PMNs, such as chemotaxis, phagocytosis, and microbicidal function, have been profoundly researched in AIDS. However, few investigators utilized PMN adhesion molecule CD11b, reactive oxygen species (ROS), and myeloperoxidase (MPO) to evaluate the functional activity of PMNs in murine AIDS, to monitor the progressive retrovirus infection. The aim of this study was to determine if PMNs are activated in murine AIDS in the presence or absence of chronic ethanol consumption. Four groups were studied: control, murine AIDS, ethanol, and ethanol plus murine AIDS. We induced murine AIDS by infection with retrovirus complex LP-BM5. Ethanol (final concentration 30%) was added to the drinking water for the ethanol consumption group. PMN activation was assessed by the expression of CD11b and ROS production using flow cytometry. Blood samples were added f-Met-Leu-Phe (fMLP) in whole blood ex vivo to determine the capability of PMN response to bacterial infection. We found that PMNs lost their responsiveness to fMLP due to ethanol or retrovirus exposure compared to control. One month after retrovirus infection, PMN CD11b expression was up-regulated (p<0.05) along with a significant increase in ROS (p<0.001). After two months PMN CD11b and ROS decreased. However, after three months, the infected mice demonstrated
a general malaise. PMN CD11b expression (p<0.01) increased to a new level along with increased ROS, perhaps due to a secondary infection. These findings suggest that the retrovirus initially causes PMN activation but subsequently impairs their function. In the ethanol consumption group, PMN CD11b expression was down-regulated after two months (p<0.05), but ROS production increased in the first and third months (p<0.05). In the murine AIDS plus ethanol group, there were significant increases in both ROS (p<0.001) and CD11b expression (p<0.01) during the three-month observation period. Hyperactivated PMNs accumulated in hearts after a three-month observation period. The pattern of PMN CD11b expression and ROS production might help to predict the stage of murine AIDS. It may become promising prognostic markers. PMN infiltration in hearts may contribute to high incident cardiomyopathy in AIDS.

1.2. INTRODUCTION

PMNs are the first line of defense against invading foreign microorganism. Impairment of PMN functional activity [1-4] may contribute to the onset of certain life-threatening bacterial and fungal infections in AIDS. However, PMNs are also the principal mediators contributing to ischemia-reperfusion injury. Activated PMNs expressed a higher affinity of β2-integrin (CD11/CD18) to mediate firm adhesion of PMNs to vascular endothelial cells prior to transmigration into extra-vascular sites. PMN CD11b expression and reactive oxygen species (ROS) production are inflammatory responses to mechanical, bacterial and viral injury. Activated PMNs release proteolytic enzymes and cytotoxic


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\text{H}_2\text{O}_2 \text{ that are critical for microbial killing. However, in certain circumstances, for instance, PMN accumulation in heart tissue results in severe ischemia-reperfusion injury. We therefore tested PMN CD11b expression and ROS production.}
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In physiologic conditions, circulating PMNs are in the resting state. CD11b, a component in the CD11b/CD18 adhesion protein complex, is a sign of PMN activation by upregulation of its expression. The presence of this adhesion complex allows PMNs to migrate from the blood circulation to tissue [5]. MPO is an enzyme that is specific to PMNs. MPO activity in tissues has been identified as a marker of PMN infiltration and sequestration in that tissue [6,27]. Accumulated PMNs in the heart result in degranulation and release cytotoxic contents that damage heart tissue and impede cardiovascular functions.

ROS is another PMN activation marker. PMN-derived ROS may cause an intracellular oxidant stress by retroviral infection. ROS production by PMNs is also critical for microbial killing. When PMNs are stimulated, the membrane-bound NADPH oxidase catalyzes the production of superoxide anions and hydrogen peroxide. However, PMN activation is tightly regulated by many factors. The underlying mechanisms remain limited. It may involve cytokine dysregulation, viral products, and secondary infection in AIDS. Indeed, many investigators [7-13] have described the development and severity of cytokine dysregulation in murine AIDS, which has been considered an excellent model for human AIDS research. Increased cytokine production by Th2 cells and infected
macrophages is a hallmark of murine AIDS. Production of IL-4, IL-6, IL-10 and TNF-alpha was dramatically elevated [7-13]. Retrovirus-infected macrophages can further release abnormally large amounts of IL-1, TNF-alpha, and platelet activating factor (PAF) [14-16]. IL-1β, TNF-α and PAF are potent PMN activating factors. We therefore hypothesize that the basal level of PMN CD11b adhesion molecule and the production of ROS are elevated in murine AIDS.

Since several cytokines involved in PMN activation are secreted mainly by immune cells, such as T, B cells, and macrophages, the levels of cell destruction in the different stages of murine AIDS may influence cytokine levels. Eventually, it will reflect on PMN activities, such as PMN CD11b adhesion molecule expression and ROS production. Levels of PMN CD11b expression and ROS production may become useful prognostic markers to monitor progressive stages of AIDS.

The lifestyle of AIDS patients may modulate the retrovirus actions. MacGregor’s studies [17-19] indicated that ethanol intoxication has profound effects on PMN kinetics. Most significant is the inhibition of PMN mobilization to sites of inflammation. Most of these effects were likely through the mechanism of inhibited PMN adhesion molecular expression through cytokine dysregulation. Indeed, Arbabi [20] and Elbim [21] noted that acute ethanol intoxication inhibits the production of IL-8 and TNF-α. Ethanol consumption could directly increase production of ROS in PMNs by ethanol dehydrogenase, microsomal oxidation systems and catalase. However, few investigators
used both PMN CD11b expression and ROS production for PMN kinetic and/or functional studies in chronic ethanol consumption. Since HIV infection has been found to greatly influence individuals’ behavior changes, and 14% of HIV infected patients abuse ethanol [22], we also investigated how both factors influence PMN CD11b expression and ROS production.

1.2. MATERIALS AND METHODS

**Reagents.** The reagents and sources were as follows: LDS-751 and 2’7’-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR); mouse fluorescein isothiocyanate (FITC)-conjugated anti-CD11b mAb was purchased from Pharmingen (San Diego, CA); f-Met-Leu-Phe (fMLP), hexa-1,6-bis-decyltrime-thylammonium bromide (HTAB), 3,3’-5,5’-tetramethylbenzidine (TMB) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS) was filtered using a 0.45 um pore filter prior to all experiments.

**Animals.** Female C57BL/6N mice (National Cancer Institute) at 8-12 weeks of age and weighting about 20-22.5 g were randomly assigned to 4 different groups: control, murine AIDS, ethanol, and murine AIDS plus ethanol. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20° to 22° C with constant humidity and a 12:12 hour light dark cycle. Murine AIDS was induced by LP-BM5 murine leukemia retrovirus infection. The LP-BM5 viruses were administered intraperitoneally with 0.1 ml
of an LP-BM5 innoculum with titer of 4.5 log 10 plaque forming units/ml, as done previously by our lab [10,12,23,24]. Infection leads to the rapid induction of clinical symptomatology that was similar to human AIDS. In the first week, 10%(v/v) of ethanol in autoclaved tap water was made available to the chronic ethanol group mice in a 300 ml plastic bottle with a stopper. The ethanol concentration was increased in increments of 10% at one week intervals, from an initial 10% to a final concentration of 30% (v/v) and kept at 30% (v/v) for the rest of the treatment periods. The average of ethanol intake per mouse was 2.8ml/day. Mice are active at night; therefore, we took blood samples in night (after lights turned off three and half hours) to estimate blood ethanol concentration (Sigma Diagnostics Alcohol Kit). The average of blood ethanol concentration was 62.6mg/dl (0.0626%).

**Hematology Parameter.** Blood cell concentration was determined by an automated cell counter (Serono Diagnostics, Allentown, PA, Model 9018 CP). Leukocyte differential counts were verified manually on Wright stained blood smears.

**PMN CD11b Expression and ROS Production.** Flow cytometry was used to identify PMNs and their activity in whole blood. Freshly drawn, citrated whole blood was mixed with the vital nucleic acid stain, LDS-751 (1 ug/ml). Labeling leukocytes with LDS-751 allowed for the investigation of leukocyte characteristics in whole blood and thus avoided leukocyte isolation procedures, which are known to cause artificial leukocyte activation [25]. This mixture of whole blood and LDS-751 was used for all subsequent leukocyte
experiments. PMN CD11b and ROS measurements were performed by incubating saturating concentrations of FITC-labeled mouse CD11b antibody, and 80 mM DCFH-DA with the whole blood/LDS-751 mixture. Samples were protected from light by using foil wrapped round bottom polypropylene tubes (Becton Dickinson) and incubated in a 37°C water bath for 15 min, then diluting with 0.5 ml of cold PBS, and placing it on ice until data acquisition in flow cytometer. During FACScanning (Becton Dickinson, FACScan Clinical Flow Cytometry), a 488nm argon laser light was used for excitation, and fluorescence emission was detected as forward scatter (FSC), which was a measure of cell size, and side scatter (SSC), which was a measure of cell granularity. In addition, a threshold fluorescence was set on the LDS-751 signal that allowed list-mode data collection on leukocytes in whole blood without interference from erythrocytes. Thus, PMN subpopulations could be separated on the basis of their dot plots pattern on FSC, SSC, and LDS-751 in the FL3 channel (figure 1.1). The fluorescence intensity due to bound FITC-labeled CD11b antibody was monitored in FL1 channel. For measurement of ROS, this method [26] used the properties of DCFH-DA, which rapidly diffused across the cell membrane and was then trapped within the cell by a deacetylation reaction. In the presence of hydrogen peroxide, this compound was oxidized to DCF, which was highly fluorescent in the FL1 channel. To determine the capacity of PMNs to upregulate the CD11b adhesion molecule surface expression or ROS generation, inflammatory mediators (fMLP, 10^-6M of final concentration) were added to the samples for an additional 10-minute incubation. After 25 minutes total incubation, samples were diluted with ice-cold PBS, and kept on ice until analysis by flow cytometry. The data
from the FACS processing was further analyzed using WinMDI 2.8. Data were expressed as total fluorescence intensity (TFI=mean channel of fluorescence × % of positive events).

**MPO Measurement for Cardiac Tissue.** MPO activity was assessed for the quantification of PMN accumulation in tissues. We modified MPO assays from Kuebler et al [27]. Each heart was homogenized (Polytroa Type PT 10/35 Kinematica GmbH PHC) in an ice bath in 1 ml of cooled 0.02 M potassium phosphate buffer (pH 7.4). Positive control was performed by using control heart adding 250 ul of mouse blood. After addition of another 1 ml of cooled 0.02 M potassium phosphate buffer, the homogenate was centrifuged at 4 °C for 15 minutes at 20,000×g (RC-5 supperspeed Refrigerated Centrifuge, DU PONT Instruments SORVALL) in order to pellet the cellular components. The pellets were incubated for 2 hour at 60°C. Following incubation, pellets were resuspended in 1 ml of cooled 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% HTAB. The suspension was sonicated (ACE-Δ506-10) for 10 seconds, freeze-thawed 3 times and again sonicated for 10 seconds. The samples were centrifuged at 4 °C for 15 minutes at 20,000 g again and supernatants were saved at -70 °C for MPO assay.

In vitro MPO assay utilized the H₂O₂-dependent oxidation of TMB, thus converting the originally colorless TMB into its blue-green oxidized state. The assay employed 150 ul TMB, dissolved in dimethy sulfoxide (DMSO) in a final concentration of 1.6 mM, 150 ul
H₂O₂, dissolved in a cooled 0.08 M sodium phosphate buffer (pH 5.4) to a final concentration of 0.6 mM and 300 μl of supernatant from tissue sample processing. The reaction was started at 25 °C in 96-well flat-bottom plates (Falcon 3072, Lincoln Park, NJ) by adding the supernatant. MPO activity was expressed as initial velocity of absorbance increase at 620 nm and registered continuously by a Vmax Kinetic Microplate Reader. One unit of MPO activity was defined as the amount of enzyme reducing 1 μmol peroxide/min at 25°C. Data were expressed as mU/g of heart.

Statistical Analysis. Data were collected and tabulated on spreadsheets (Microsoft Excel 7.0). All values were expressed as mean ± standard error. All statistics were calculated using Prism Statistical software (version 3.0). Comparisons between groups was made using ANOVA with Newman-Keuls post-hoc testing when significant difference was observed. A probability of less than 0.05 was considered statistically significant.

1.4. RESULTS

Total White Blood Cell (WBC) and PMN Counts. The results of WBC, PMN (%), and PMNs are in the table 1.1. Although total WBC counts were not significantly different among the groups, the percent of PMN and absolute PMNs counts were significantly increased in three-months of murine AIDS and murine AIDS plus ethanol consumption compared to the control group (p<0.05). Moreover, there was a line trend increase of
PMN (%) and PMNs from one to three months of murine AIDS mice. In the group of murine AIDS plus ethanol consumption, there was significant decrease of PMN (%) and PMNs in two-month compared to in the control group (P<0.05). However, after three months PMN (%) and PMNs were dramatically increased.

**PMN CD11b Expression and ROS Production.** In LP-BM5-induced murine AIDS, circulating PMN CD11b (figure 1.2) was increased, even in the first month of infection (P<0.05). In the following month of murine AIDS, PMN CD11b expression went back to the control level. Three months post retrovirus infection, CD11b expression reached a new high point (p<0.001). PMN-derived ROS, another marker for PMN activation and killing activity, increased after one-month LP-BM5 of infection (p<0.001) (figure 1.3). Thereafter, ROS production decreased. Increased PMN ROS production was coupled with CD11b upregulation in one-month murine AIDS mice. After two months of LP-BM5 infection, PMN CD11b expression and ROS production both fell back to unstimulated control levels. After three months of infection, murine AIDS mice exhibited general malaise. At this time, PMN CD11b expression dramatically increased, but ROS production remained consistent.

In the chronic ethanol consumption group, PMN CD11b expression was down-regulated, especially after two months of ethanol consumption (P<0.05) (figure 1.2). ROS production significantly increased after one month (P<0.05), returned to the control level
after two months, and then increased again after three months of ethanol consumption (p<0.05) (figure 1.3). After one month of ethanol consumption, an increase PMN ROS production was not accompanied by CD11b upregulation. After two months, PMN CD11b was down-regulated, but PMN ROS production did not change. After three months, PMN ROS production was highly induced by chronic ethanol consumption, but PMN CD11b expression was not affected.

In the murine AIDS with chronic ethanol consumption group, no significant difference in PMN CD11b expression (figure 1.2) was observed in the first or second month. However, a significant increase in PMN CD11b expression occurred (p<0.01) during the three-month observation periods. The PMN ROS production (figure 1.3) had a complex pattern. It increased in the first month (P<0.001), then tended to decrease in the second month and then increased (P<0.001) again after three months. In the first month, PMNs induced a higher level of ROS without CD11b up-regulation. No significant difference of PMN CD11b and ROS was observed in the two-month group. An increased PMN CD11b expression was parallel to ROS production in the three-month murine AIDS plus ethanol consumption group.

**FMLP Stimulated PMN CD11b Expression and ROS Production.** To determine the capability of the PMN response to bacterial infection, we used fMLP, a bacterial peptide, to stimulate PMNs for 15 minutes ex vivo. For PMN CD11b expression (figure 1.4:A-C), stimulation caused a significant increase (P<0.05) exhibited in the control group only.
No significant differences were found in all other groups in the presence of fMLP. For PMN ROS production (figure 1.5, A-C), similar results were found. There was a significant increase of PMN ROS production ($P<0.05$) after being stimulated by fMLP in the control group and the early first month of ethanol consumption. However, no significant difference of PMN ROS production was found after stimulation by fMLP in the different stages of murine AIDS, murine AIDS in the presence and absence of ethanol consumption, and chronic moderate ethanol consumption alone.

**MPO Activity in the Heart.** PMNs consist of approximately 6.8% MPO stored in their azurophilic granula, which is on the order of 3 magnitudes higher than mononuclear blood cells. As a result, MPO activity is mainly associated with the number of PMN filtration in hearts. MPO activity in control hearts ($n=6$) was considered as a baseline level. There was a significant linear trend ($P<0.05$) among murine AIDS in the absence and presence of chronic consumption, and ethanol consumption alone during three-month observation periods (figure 1.6).

1.5. DISCUSSION

The major role of PMNs is to protect the host against infectious microorganisms. To accomplish this task, the PMN must first sense infection, migrate to the site of the infecting organisms, and then destroy them. PMN CD11b expression plays a key role in mediating firm adhesion of PMNs to vascular endothelial cells prior to transmigration.
into their target sites. Thereafter, activated PMNs induce oxidative bursts, releasing ROS that is toxic to microorganisms. To determine the ability of the PMN response to invading organisms, whole-blood was incubated ex vivo with fMLP (10^{-6}M), a structural analog of bacterial metabolic production. PMNs from control mice demonstrated a normal response to fMLP stimulation, which significantly increased CD11b expression and ROS production. Although PMN numbers increased during the different stages of LP-BM5 infection, PMNs lost the ability to respond to acute fMLP stimulation. This phenomenon was also observed in human AIDS. Elbim et al [28] reported fMLP, TNF, or IL-8 alone barely increased ROS production by PMNs from HIV-infected patients. This result indicates that PMN functional activity may have been disrupted by LP-BM5 infection. PMN dysfunction might contribute to the inability to fight threatening bacterial and fungal infections in AIDS individuals.

Many investigators [17-19, 29-31] reported that chronic ethanol intoxication impairs PMN functions. We found that PMN CD11b expression was impaired, but ROS production was normal in response to fMLP stimulation during the first month of moderate ethanol consumption. This result suggested that short-term of moderate ethanol drink affected PMN mobilization without impairment of PMN bacterial killing activity. At this point, PMNs may have the ability against bacterial and fungal infection, but PMNs likely can not migrate to the site of infection to perform their function. After prolonged exposure to moderate ethanol, the mobilization and killing activity of PMNs were both impaired even though PMN numbers did not significantly change. Diminished
PMN function was also exhibited in murine AIDS in the presence of chronic ethanol consumption. The PMN functional abnormalities in this study may be due to several factors, such as (1) viral particles from LB-BM5 infection, (2) ethanol or its metabolites, (3) cytokine dysregulation, or (4) PMN receptor desensitization. In conclusion, the capability of the PMN response to foreign invasion is aborted in the different stages of murine AIDS, chronic moderate ethanol consumption, and murine AIDS with ethanol consumption.

Excessive or inappropriate stimulation of PMNs can cause tissue injury. Recently, PMNs have been found to be a major mediator of cardiovascular ischemia-reperfusion injury [32]. PMNs sequester in ischemic tissue [5,32]. Highly cytotoxic ROS and proteolytic enzymes released by PMNs induce myocardial damage. The recruitment of PMNs into the heart may be associated with the high incidence of cardiomyopathy in AIDS. Our results indicate that circulating PMNs are highly activated, especially after three months of LP-BM5 infection in the presence and absence of ethanol consumption. Hyperactivated PMNs may accumulate in hearts in greater numbers, increasing heart damage. To access the number of PMNs in hearts, we measured MPO activity to estimate the degree of PMN filtration. A linear trend was observed in the murine AIDS group in the absence and presence of chronic ethanol consumption, and in the chronic ethanol consumption groups. This data indicates that more PMNs accumulate in hearts in the later stages of LP-BM5 infection and chronic ethanol consumption. PMN accumulation in heart tissue may affect heart performance.
During the progression of LP-BM5 infection, certain viral products and cytokines may directly or indirectly exert effects on the modulation of PMN function. Cytokines released from infected cells are a hallmark during LP-BM5 infection. Proinflammatory cytokines such as TNF-α, IL-1, IL-6, and platelet activating factor (PAF) increase in AIDS [7-16]. They are all potent PMN stimulators. In our study, we found that increased PMN ROS production was accompanied by PMN CD11b upregulation in the first month of LP-BM5 infection, even though the PMN response to fMLP was diminished. At this time, upregulation of PMN CD11b and ROS may be due to cytokine stimulation. After two of months LP-BM5 infection, both PMN CD11b expression and ROS production returned to the baseline level. This decrease may be due to (1) a decrease in cytokine production and (2) PMN receptor desensitization. A decreased cytokine level may be related to a massive destruction of cytokine producing cells due to rapid viral replication. PMN desensitization could be also related to cytokine dysregulation [33]. Thus, normal PMN CD11b expression and ROS production do not suggest the improvement of disease in that stage; in contrast, it may indicate that the disease gets worse. In the later stages of LP-BM5 infection, PMN CD11b and ROS were further upregulated. This increase may be caused by secondary infection. The extremely high ROS in circulating PMNs may induce apoptosis before PMNs reach the target sites. In conclusion, the pattern of PMN CD11b expression and ROS production could predict the different stages of murine AIDS. PMN CD11b and ROS may become promising prognostic markers.
Ethanol intoxication has been observed to decrease the adherence of PMNs to endothelial cells [17,19]. PMN mobilization to sites of inflammation is diminished [18]. Our results further support this observation because PMN CD11b adhesion molecules decrease after moderate ethanol consumption. Arbabi et al [20] found that ethanol intoxication inhibited inflammatory cytokines including TNF-α and IL-8 production. The impairment of PMN CD11b expression may be related to decreased proinflammatory cytokine production. This may explain why ethanol induces defects of PMN delivery into tissue sites of infection. It is well known that ethanol induces oxidative stress in many cell types [34,35]. PMN ROS may be induced directly by the microsomal ethanol oxidizing systems or indirectly by inflammatory cytokines. In our study PMN ROS do not remain increased. A "V" pattern of PMN ROS production was observed during the different stages of ethanol consumption. An initial peak of PMN ROS production may be dominated by ethanol oxidizing systems. After two months of ethanol consumption, a reduced level of PMN ROS with down-regulated PMN CD11b suggests that decreased proinflammatory cytokines override the effect of ethanol oxidizing systems. As mice continually drink, an increase of PMN ROS production with normal PMN CD11b expression suggests that PMNs are tolerant to response to proinflammatory cytokines; however, ethanol oxidizing systems in PMNs may be not affected. ROS is a necessary agent for bacterial killing. However, at this point, ROS in PMNs may exhibit its adverse effects. ROS may trigger PMN apoptosis before PMNs reach the inflammatory site. This phenomenon suggests that chronic ethanol consumption increases susceptibility to infection.
Ethanol has been proposed to be a cofactor that may further compromise PMN function in AIDS [30]. We do find that circulating PMNs are highly activated, especially PMN ROS production which is extremely elevated during three-month observation periods. These results suggest that PMNs are more susceptible to apoptosis in murine AIDS mice chronic exposure to ethanol. Apoptosis may contribute to exacerbating the progression of murine AIDS.

In summary, our study indicates that the PMN response to bacterial infection is decreased in the different stages of AIDS with or without chronic ethanol consumption. The PMN response to bacterial infection is also decreased with chronic ethanol alone. Moderate ethanol consumption does not have synergistic effects on PMN CD11b expression in murine AIDS. PMN CD11b expression and ROS production may become promising prognostic markers to predict progressive murine AIDS. ROS production from circulating PMNs alone can not be used to examine functional PMNs in ethanol consumption. Hyperactivated PMNs accumulating in hearts may contribute to high incidence of cardiomyopathy in AIDS.
1.5. LIST OF FIGURES AND TABLES

Table 1.1 Effect of Murine AIDS and Ethanol on Hematology Parameters

<table>
<thead>
<tr>
<th>Groups</th>
<th>Month</th>
<th>Total WBC (K/ul)</th>
<th>PMNs (%)</th>
<th>PMNs(K/ul)</th>
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<td>0.44 ± 0.07*</td>
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<td>3</td>
<td>5.06 ± 1.18</td>
<td>26.83 ±4.97*</td>
<td>1.36 ± 0.54*</td>
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</table>

Control group as a baseline of level.

* P<0.05, control verse three-months of murine AIDS, one-month of murine AIDS verse three-month of murine AIDS, two-months of murine AIDS verse three-months of murine AIDS.

** P<0.001, control verse two (decrease) or three-months (increase) of murine AIDS plus ethanol consumption, three-months of murine AIDS plus ethanol consumption verse one or two-month of murine AIDS plus ethanol consumption.
Figure 1.1. Representative FACScans for PMN subpopulation.

Dotplots (A) gives representative side scatter (SSC) vs. forward scatter (FSC) from unlysed blood samples. Dotplots (B) gives representative SSC and fluorescence intensity characteristics of the nucleated cells with positive LDS-751 stain. A sortrect region R1 is made compared to SSC vs. FSC dotplots. It is easily identified lymphocyte population from SSC vs. FSC (ellipse) and SSC vs. LDS dotplots because more than 70% of leukocytes are lymphocytes. PMNs contain more granules than monocytes and lymphocytes do, the region R1 is PMN subpopulation. Designated R2 in SSC vs. LDS dotplots is lymphocyte and monocyte subpopulations.
Figure 1.2. Effect of murine AIDS and ethanol on PMN CD11b expression.

PMN CD11b expression significantly increased after one month of LP-BM5 infection (P<0.05). There was a decrease in PMN CD11b expression (P<0.05) after two months ethanol consumption. After three months, CD11b expression reached to a much significant high level (P<0.001) in murine AIDS, and murine AIDS plus ethanol groups (P<0.01).
Figure 1.3. Effect of murine AIDS and ethanol on PMN ROS production.

PMN ROS production significantly increased (P<0.001, P<0.001, <0.05), respectively, one month after LP-BM5 infection in either the presence or absence of ethanol consumption, or ethanol consumption alone. After two months, ROS production in all three different groups had no significant change compared to control. However, there were significant increases of PMN ROS in the murine AIDS plus ethanol (P<0.001), and ethanol (P<0.05) groups after three months.
Figure 1.4. CD11b expression from unstimulated and stimulated PMNs in all groups during three month of periods.

A. CD11b expression from unstimulated and stimulated PMNs during the different stages of murine AIDS.

B. CD11b expression from unstimulated and stimulated PMNs during the different stages of murine AIDS in the presence of chronic ethanol consumption.

C. CD11b expression from unstimulated and stimulated PMNs during the different stages of ethanol consumption.

Blood incubated with FITC-CD11b antibody only represented as unstimulated groups.

Blood incubated with FITC-CD11b antibody and fMLP represented as stimulated groups.

* P<0.05, significant difference exhibited only in the control group compared unstimulated to stimulated blood.
Figure 1.5. ROS expression from unstimulated and stimulated PMNs in all groups during three month of periods.

(A). ROS expression from unstimulated and stimulated PMNs during the different stages of murine AIDS. (B). ROS expression from unstimulated and stimulated PMNs during the different stages of murine AIDS in the presence of chronic ethanol consumption. (C). ROS expression from unstimulated and stimulated PMNs during the different stages of ethanol consumption.

Blood incubated with DCFH-DA only represented as unstimulated groups. Blood incubated with DCFH-DA and fMLP represented as stimulated groups. * P<0.05, significant difference was observed in the control and the first month of ethanol consumption groups compared unstimulated to stimulated blood.
Figure 1.6. MPO activity of hearts after three months of retrovirus and ethanol exposure. N=6 hearts for each group. Linear trend significant difference (P<0.05) among four different groups was found.

ACKNOWLEDGEMENT

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CHAPTER 2. PLATELET CD62p EXPRESSION AND MICROPARTICLE FORMATION IN MURINE AIDS AND CHRONIC ETHANOL CONSUMPTION

2.1. ABSTRACT

Platelets are involved in many pathological and physiological events. Abnormal platelet counts have been noticed in AIDS patients. However, the actual state of platelets in AIDS is unclear. We hypothesize that platelets are activated and platelet-derived microparticles increase in murine AIDS. To elucidate the ethanol effects on platelet in murine AIDS, we studied four groups: control, murine AIDS, ethanol, and ethanol plus murine AIDS. We used platelet CD62p as a platelet activation marker and CD61 positive microparticles as platelet microparticles (PMPs). The results demonstrated that platelets were significantly activated in murine AIDS and chronic ethanol consumption in mice. Increased platelet CD62p expression and increased PMPs were most pronounced in advanced stages of murine AIDS. Chronic ethanol consumption persistently enhanced platelet activation and PMP formation. No obvious synergistic effects were observed in murine AIDS at the presence of chronic ethanol consumption. The mechanism of platelet activation and PMP formation may differ in murine AIDS with chronic consumption. Moreover, Platelet CD62p and PMPs may be related to important pathological consequences such as myocardial infarction and inappropriate inflammatory response.
2.2. INTRODUCTION

Immune related thrombocytopenia has been described extensively in AIDS patients [1,2,3]. The reduction in platelet counts may be due to antibodies against platelets and megakaryocytes [32]. However, platelet function, specifically adhesion molecule expression and platelet-derived factors in murine AIDS, have not been investigated. Platelets play a major role in hemostasis and wound healing. The increase in myocardial infarction [4] and high incidence of thrombosis [5,6] in AIDS patients may be related to chronically activated platelets. Recently, platelets have been identified as inflammatory and immunological mediators.Activated platelets express adhesion molecules to facilitate direct interaction with leukocytes. Platelet-leukocyte and endothelial cell conjugation may plug coronary capillaries, mechanically blocking blood flow [7]. Inflammatory mediators such as interleukin (IL)-1[8], IL-8 [9], RANTES, macrophage inflammatory protein (MIP)-1α [10], Platelet activating factor (PAF) released by activated platelet amplify the inflammatory response. Overall, progressive cardiovascular manifestations in AIDS may be related to mechanical or chemical injuries by activated platelets.

Platelets may be activated in murine AIDS. The reasons for activation are that, first, collagen may be exposed because the integrity of vascular endothelium is disrupted; second, thromboxane is elevated by retrovirus infection [11]; and third, PAF is released by retrovirus infected cells [12-14]. All above are potent platelet activating mediators. Upon platelet activation, CD62p (P-selectin), originally described in the α-granule of
platelets, can be rapidly translocated to the plasma membrane, responding to a variety of stimulators. CD62p expressed on activated platelets interacting with neutrophils and vascular endothelial cells through the carbohydrate ligand, sialyl Lewis X, is a crucial step for blood aggregation, neutrophil activation and the inflammatory processes.

PAF has been implicated in the pathogenesis of human AIDS. PAF is a bioactive phospholipid-derived mediator. A variety of cell types, including platelets, basophils, neutrophils, monocytes/macrophages, and endothelial cells can release PAF in both secreted and cell-bound forms. In addition to platelet stimulation, PAF causes vasoconstriction, increases venular permeability, induces neutrophil adhesion molecule expression, enhances TNF-α programmed apoptosis, neutrophil chemotaxis, degranulation, and the oxidative burst [14,15,16,17,18]. Platelets and neutrophils tend to mutually enhance their activation by PAF. Thus, PAF can elicit most of the myocardial features of inflammation. It has been reported that the brain level of PAF increased in AIDS [12,19], but the plasma level of PAF is unknown. Iwamoto et al [20] found that eighty percent of the PAF released from the platelets was recovered in the microparticle fraction. Thus, the number of platelet microparticles (PMP) could be related to the level of plasma PAF.

AIDS patients experience significant behavior changes, and 14% of HIV infected patients abuse alcohol [21]. Some [22,23] have proposed that moderate ethanol consumption is associated with a reduction in thromboembolic complications of coronary artery disease,
possibly partially attributable to modulation of platelet responses, but no direct evidence has verified that chronic ethanol consumption modulates platelet adhesion molecule expression or PMP formation. In our present study, both factors were investigated as influencing platelet CD62p expression and platelet-derived microparticle formation.

2.3. MATERIALS AND METHODS

**Animals.** Female C57BL/6N mice (National Cancer Institute) at 8-12 weeks of age and weighting about 20-22.5 g were randomly assigned to 4 different groups: control, murine AIDS, ethanol, and murine AIDS plus ethanol. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20° to 22° C with constant humidity and a 12:12 hour light dark cycle. Murine AIDS was induced by LP-BM5 murine leukemia retrovirus infection. The LP-BM5 viruses were administered intraperitoneally with 0.1 ml of an LP-BM5 innoculum with titer of 4.5 log 10 plaque forming units/ml. Infection leads to the rapid induction of clinical symptomatology that is similar to human AIDS. In the first week, 10%(v/v) of ethanol in autoclaved tap water was made available to the chronic ethanol group mice in a 300 ml plastic bottle with a stopper. The ethanol concentration increased to 20% (v/v) in the second week and kept 20% (v/v) for the rest of the treatment periods. The average of ethanol intake per mice was 2.8ml/day. Mice are active at night; therefore, we took blood samples in night (after lights turned off three and half hours) to estimate blood ethanol concentration (Sigma Diagnostics Alcohol Kit). The average of blood ethanol concentration was 66.9mg/dl (0.0669%).
Flow Cytometry for Platelet CD62p and PMP. Platelet CD62p and PMPs were assessed in whole blood using flow cytometry. CD61 is a beta subunit of platelet GPIIb/IIIa. It is expressed consecutively and specifically on platelets. We found that more than 99% of platelets were CD61 positive in control mice. Therefore, CD61 positive microparticles were derived from platelets. We named these CD61 positive microparticles as PMP. In this procedure, 1.4:10 citrated whole blood was collected and 20 ul were added to 1 ml of filtered PBS. 100ul of the mixture were incubated with a saturating concentration of FITC-conjugated anti-CD62p and anti-CD61 antibody for 10 minutes at room temperature. Samples were protected from light by using foil wrapped round bottom polypropylene tubes (Becton Dickinson) then fixed with 1% paraformaldehyde, until data acquisition in flow cytometer (Becton Dickinson, FACScan Clinical Flow Cytometry). To determine the capacity of platelets to upregulate P-selectin, whole blood samples were stimulated with 50um Adenosine diphosphate (ADP) for 10 minutes then stained with the antibodies described above. By using whole blood, harsh manipulation such as centrifugation, commonly used to separate platelets from whole blood, which may cause artifactual up-regulation of receptors on platelets, can be avoided. During FACScanning, using FSC and SSC sorted the population of platelets (figure 2.1), and FL1 channel determined the intensity of FITC. The data from the FACS processing was further analyzed using WinMDI 2.8. Data were expressed as the percentage of positive events.
**Analysis of Platelet CD62p and PMP.** Collected data from flow cytometry was reopened in WinMDI 2.8. Dotplots (figure 2.1, A) represented as side scatter (SSC) vs. forward scatter (FSC) was used to sort platelet and microparticle populations. Using a tool bar created platelet (ellipse) and microparticle (sortrect) regions. The same file was reopened as a histogram that represented total events. From this original histogram, individually gating ellipse and sortrect regions subsequently made two histograms, one represented as platelet population (figure 2.1, B) and another one represented as microparticle events (Figure 2.1, D). Background fluorescence for platelets (figure 2.1, B) and microparticles (figure 2.1, D) was measured from a sample containing only blood and was designated by marker 2 (M2). Platelets CD62p positive, and microparticle CD61 positive exhibited a shift in fluorescence intensity into the marker 1 (M1) region (Figure 2.1, C, E). Data were analyzed by built-in statistics and reported as the percentage of gate and mean fluorescence of gate in M1. We only used the percentage of gate for further statistical analysis. The reasons were (1) as soon as platelets were activated, platelet CD62p was expressed, and then shed into plasma. The percentage of CD62p positive was more useful than the mean fluorescence of gate. (2) PMP identification was dependent on CD61 expression only. Any microparticles carried on CD61 were platelet-derived microparticles.
**Platelet Counts.** Platelet counts were determined by an automated cell counter (Serono Diagnostics, Allentown, PA, Model 9018 CP) and then were verified manually on Wright stained blood smears.

**Statistical Analysis.** All statistics were calculated using Prism Statistical software (version 3.0). Comparison among groups was made using ANOVA with Newman-Keuls post-hoc testing when significant differences were observed. Comparing the same blood samples in the absence and presence of stimulators was made using paired t tests. A probability of less than 0.05 was considered statistically significant.

**2.4. RESULTS**

**Platelet CD62 Expression.** Platelet activation was studied in the basis of platelet CD62 expression. Figure 2.2-2.5 presents platelet CD62p expression in all groups and over a three-month period. The percentage of positive platelet CD62p in the control mice (25 ± 2.62) was considered as the basal level of CD62p expression. In murine AIDS, platelet CD62p expression significantly increased after three months of LP-BM5 infection with up to 78% of platelets expressing CD62p. There was no significant difference in the one and two month of murine retrovirus infection groups compared to control, it is slightly increased after one-month LP-BM5 infection thereafter decreased in two-month periods. In ethanol consumption mice, platelet CD62p expression was consistently increased and continued to increase as mice chronically consumed ethanol over three month periods.
After one month of ethanol consumption, more than half platelets (58%) were activated. Activation increased to 77% after three months of ethanol consumption. In retrovirus infected mice given chronic ethanol consumption, the pattern of platelet CD62 expression over three month periods was unchanged but higher than controls. Around 52-62% of the platelets expressed CD62p. Overall, platelet CD62p expression increased in murine AIDS, murine AIDS plus ethanol, and ethanol consumption mice. ADP in concentrations of 50 um maximally stimulated platelet CD62 expression in all groups over three-month periods.

**Measurement of PMPs.** We identified CD61 positive in microparticle regions as PMPs. Figure 2.6-2.9 presented the percentage of PMPs in total microparticles in the different groups over three month periods. The fraction of PMPs in control mice (15.48 ± 2.47) was regarded as a basal level of PMPs. In murine AIDS, the fraction of PMPs increased after two month of LP-BM5 infection, and then suddenly jumped from 24% up to 54% after three months. In chronic ethanol consumption mice, PMPs were elevated after one month of ethanol intake (P<0.05) compared to the basal levels. Thereafter, PMPs continually climbed up to 58% and reached a plateau at two and three month of ethanol consumption. In the murine AIDS with chronic ethanol consumption group, PMPs remained at consistently high levels over three month periods. In summary, the fraction of PMPs, that expressed as a percentage of CD61 positive in microparticle region was elevated in murine AIDS with and without ethanol as well as during ethanol consumption alone. PMPs reached maximal levels at three month of intervention in all three groups.
Platelet Counts. We counted platelets at three-month intervention. Platelets in all groups were in the normal ranges with no significant difference among the groups (table 2.1).

2.5. DISCUSSION

Our present study demonstrates platelet activation with normal platelet counts in murine AIDS and chronic ethanol consumption. An increased platelet CD62p expression and high amount of PMPs, reflecting enhanced platelet activation, were most pronounced in the advanced stages of murine AIDS in the presence or absence of chronic ethanol consumption.

Platelet CD62p expression associated with pathological blood aggregation, neutrophil activation and the inflammatory responses may contribute to the increased incidence of myocardial infarction in AIDS. The mechanism leading to enhanced platelet CD62p expression is not fully elucidated in murine AIDS, but several factors may be involved. (1) An increase in the plasma level of soluble agonists; (2) collagen induced contact activation; and (3) pathological antigen and antibody-mediated platelet activation. All these factors may activate platelets through the combination of protein kinase C (PKC), PKA and arachidonic acid pathways.
PAF is a potent platelet agonist with procoagulant activity [24,25]. The brain level of PAF is increased in AIDS [12,14,19], while the plasma level of PAF was not reported. Iwamoto et al [20] found that PMPs contained an abundance of soluble PAF. In the present study, we measured PMPs to estimate the level of PAF. Our data clearly demonstrated increased PMPs in murine AIDS and chronic ethanol consumption. The concentrated PAF on the PMPs may exert the response in the target cells such as platelets and neutrophils effectively. PAF binds with G protein coupled PAF receptors on platelets and then activates platelets through both PKA and PKC pathways. PAF in PMPs may contribute to platelet CD62p expression in murine AIDS and/or chronic ethanol consumption. PMP formation and platelet activation may mutually involve a positive feedback loop. Platelet activation results in formation of PMPs. The concentrated PAF in PMPs inversely activates platelets to form more PMPs, which are capable of binding factors Va, VIII, Xa and protein S [26-30]. The surface of PMPs is enriched by GPIIb/IIIa, which may bind with neutrophils, vascular endothelial cells and platelets through a fibrinogen bridge. The PMP-cell interactions may also involve pathological haemostatic and inflammatory events in murine AIDS and chronic ethanol consumption.

Collagen-induced contact platelet activation may occur in murine AIDS. Retrovirus may directly or indirectly disrupt the vascular endothelium [31,32]. Collagen could be exposed due to endothelial injury. Collagen exposure to platelets results in several intracellular signaling events that lead to rapid platelet activation and expressed CD62p on platelet membranes.
In addition to activation by soluble agonists and collagen exposure, platelets could also become activated by antiplatelet antibodies under pathological conditions. Abnormal antiplatelet antibodies bind to platelet surface glycoproteins or phospholipids via the Fab portion of the IgG molecule [33-35]. Antibodies could also bind to the platelet Fc receptor (Fc gamma RII) through their Fc portion and activate platelets [36,37]. The signal transduction pathway induced by antibodies may ultimately lead to platelet CD62 expression. Bettaieb et al [38] found significant antiplatelet antibodies in AIDS patients. Cross-reactive antibodies between HIV-GP160/120 and platelet GPIIb/IIIa may lead to platelet activation. Platelets expressed CD4 [39] along with the HIV co-receptor CXCR4 [40] is the response to retrovirus infection. It may contribute to platelet activation as well.

No matter how platelet activation is induced, Platelet CD62p expression and PMP formation may have pathologically relevant consequences in murine AIDS and chronic ethanol consumption. We found that almost 80% of platelets expressed CD62p in the late stage of murine AIDS in the presence or absence of ethanol consumption. Our data indicate that platelet activation may contribute to the progression of AIDS. Activated platelets release soluble factors that may act as hormones, IL-1, 8, RANTES, and PAF [8-10] which stimulate neutrophils and cause pathological inflammatory responses. Activated platelets may undergo apoptosis to form PMPs that enrich PAF. PAF, like an autocrine, further activate platelets and exhibit procoagulant activity to accelerate the coagulation cascade [24,25]. Besides their stimulatory effects, CD62p expressed on activated platelets serves as a ligand to facilitate platelet-leukocyte, platelet-endothelial
cells, and platelet-platelet interactions. Overall, platelet activation may contribute to vascular thrombosis formation and neutrophil dysfunction.

Moderate ethanol consumption has cardiac protective effects \cite{23,41} by causing vascular relaxation, reducing fibrinogen level, and modulating platelet function. However, the effects of ethanol on platelets are controversial \cite{42-44}. Serebruany et al \cite{23} reported that chronic ethanol consumption decreased platelet CD62p expression. Our study demonstrated that ethanol consumption persistently enhanced platelet CD62 expression and PMP formation over a three month period. An increased platelet CD62p expression was also observed in chronic ethanol consumption plus murine AIDS, although there were no apparent synergistic effects. The mechanism of ethanol-induced platelet CD62 expression is distinct from that of retrovirus infection. It is possible that ethanol enhances platelet CD62 expression by reactive oxygen species (ROS) formation, directly increasing production of ROS by dehydrogenase, microsomal oxidation system and catalase. An elevated ROS may induce platelet apoptosis to form PMPs. The high concentration of PAF in PMP activates platelets to express CD62P. Activated platelets produce more PMPs. As soon as this cycle is turned on by ethanol consumption, more platelets become activated and more PMPs are formed.

In summary, platelets were chronically activated and more PMP formation occurred in the late stages of murine AIDS. Chronic ethanol consumption caused persistent platelet activation and PMP formation. The mechanism of ethanol induced platelet activation may not be the same as with retrovirus infection. Platelet CD62p and PMPs may cause
important pathological consequences. Cardiovascular manifestations in AIDS may be related to previously unrecognized consequences via platelet CD62p expression and PMP formation.
2.6. TABLES AND FIGURES

Figure 2.1. Representative FACScans for platelet CD62p and PMP CD61 expression.

Dotplots (A) gives representative side scatter (SSC) vs. forward scatter (FSC). Regions in ellipse and sortrect are platelet and microparticle populations, respectively. Background fluorescence for platelets is given (B) by gating platelet region and is designated by marker 2 (M2). Platelets that are positive for CD62p exhibited a shift in fluorescence intensity into marker 1 (M1) region (C). Background fluorescence for microparticles is given (D) by gating microparticle region and is designated by marker 2 (M2). Microparticle CD61 positive exhibits shift in fluorescence intensity into marker (M1) region (E). Microparticle expressed CD61 is defined as PMPs. Data are expresses as the percentage of positive.
Figure 2.2. Platelet CD62p expression after one-month intervention

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.05, control vs. murine AIDS with ethanol consumption, control vs. ethanol group.

Paired test compared unstimulated to ADP (50 um) stimulated groups. P<0.001 in all paired groups.
Figure 2.3. Platelet CD62p expression after two-month intervention

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.001, murine AIDS vs. ethanol, murine AIDS vs. murine AIDS plus ethanol, control vs. ethanol and control vs. murine AIDS plus ethanol.

Paired test compared unstimulated to ADP (50 um) stimulated groups. P<0.001 in all paired groups.
Figure 2.4. Platelet CD62p expression after three-month intervention

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.01 control vs. murine AIDS, control vs. ethanol, control vs. Murine AIDS plus ethanol.

Paired test compared unstimulated to ADP (50 um) stimulated groups. P<0.001 in control and murine AIDS plus ethanol group. P<0.05 in murine AIDS and ethanol groups.
Figure 2.5. Platelet CD62p expression among three groups over three month periods.

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.001, three-month murine AIDS vs. one or two-month murine AIDS, P<0.05 one-month murine AIDS vs. two-month murine AIDS.
Figure 2.6. PMPs expressed as the percentage of CD61 Positive in one-month intervention.

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.05, control vs. ethanol consumption in the presence and absence of murine AIDS.
Figure 2.7. PMPs expressed as the percentage of CD61 positive in two-month intervention.

ANOVA with Newman-Keuls post-hoc testing among the groups, $P<0.001$, control vs. two-month ethanol consumption in the absence or presence of murine AIDS, two-month murine AIDS with ethanol consumption vs. ethanol or murine AIDS alone. $P<0.05$, control vs. two-month murine AIDS.
Figure 2.8. PMPs expressed as the percentage of CD61 positive in three-month intervention.

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.001, control vs. ethanol group, murine AIDS in the presence or absence of ethanol; murine AIDS with ethanol vs. ethanol or murine AIDS alone. P<0.01 murine AIDS vs. ethanol.
Figure 2.9. The fraction of PMP among three groups over three month periods.

ANOVA with Newman-Keuls post-hoc testing among the groups. In murine AIDS groups, P<0.001, three-month murine AIDS vs. one or two-month murine AIDS. In ethanol consumption groups, P<0.001, one-month ethanol vs. two or three-month ethanol consumption.

In murine AIDS with ethanol consumption groups, P<0.001, one-month vs. three-month murine AIDS with ethanol consumption. P<0.01, one-month vs. two-month murine AIDS with ethanol consumption. P<0.05 two-month vs. three-month murine AIDS with ethanol consumption.
Table 2.1. Blood platelet counts in different groups at three-month intervention

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Murine AIDS</th>
<th>Ethanol</th>
<th>MurineAIDS+Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet (K/ul)</td>
<td>807.8</td>
<td>705</td>
<td>766</td>
<td>575</td>
</tr>
<tr>
<td></td>
<td>± 133</td>
<td>± 89</td>
<td>± 90</td>
<td>± 123</td>
</tr>
</tbody>
</table>

Platelet counts were in the normal range among these four groups at three-month observation periods. No significant group difference was found.

ACKNOWLEDGEMENT

Supported by NIH HL 63667 and 59794
2.7. REFERENCE


21. Welch KJ. Correlates of alcohol and/or drug use among HIV-infected individual. AIDS patient Care and Stand. 14:317-23, 2000


CHAPTER 3. ETHANOL MODULATES CORONARY PERMEABILITY TO MACROMOLECULES IN MURINE AIDS

3.3. ABSTRACT

The cardiovascular complications of AIDS are serious. However, the underlying mechanisms are unclear. Less is known about how ethanol affects the coronary microcirculation in AIDS individuals. The aim of this study was to assess the function of coronary microcirculation in murine AIDS mice in the presence or absence of chronic ethanol consumption. After intervention for three months, mouse hearts were prepared for direct visualization of the coronary microcirculation and quantification of transcoronary macromolecular leakage. Hearts were isolated and perfused with diluted rat blood containing fluorescent albumin (FITC-BSA). Coronary microvascular fields were observed using intravital fluorescence microscopy after 5, 15, and 25 minutes of perfusion. The videotaped results were analyzed using Dazzle DVC and Adobe software. The O/I ratio was used to quantify FITC-BSA leakage. We found that the mean O/I ratio for the murine AIDS group was significantly greater than the control group and also significantly increased over a period of perfusion. Chronic ethanol consumption did not alter coronary permeability to macromolecules but improved the coronary hemodynamics in murine AIDS. These findings suggest that murine AIDS impairs the structural and functional coronary endothelium, and moderate ethanol modulates the function of the coronary microcirculation.
3.2. INTRODUCTION

AIDS is a health crisis affecting more than 47 million people worldwide [1]. HIV cardiomyopathy is the fourth leading cause of dilated cardiomyopathy in the United States [2]. Congestive heart failure has become the leading cause of death in pediatric patients with AIDS, and half of the children die within 6 to 12 months [3]. Cardiovascular involvement in AIDS may contribute to coronary microvascular endothelial dysfunction. Murine AIDS induced by LP-BM5 retrovirus in mice has been used as an excellent model for human AIDS research. We have not found reports that AIDS alters coronary microcirculation. Indirectly, several studies report that the retrovirus impairs the blood-brain barrier [4-6]. We hypothesize that endothelial function is altered in AIDS, causing an increased coronary microvascular permeability to macromolecules.

Alcohol abuse has a negative impact on human health. However, the relation of alcohol intake to mortality resembles a J-shape curve. The higher the intake, the higher the mortality, excepting abstainers have higher mortality than moderate drinkers [7,8]. Epidemiological studies [7-12] reported that moderate consumption of ethanol reduces the risk of coronary heart disease, sudden cardiac death, and ischemic stroke. It is seen that regular moderate drinking tends to have a beneficial effect on the heart. The
mechanisms for cardiovascular protective effects are not well established. We propose that moderate ethanol could modulate coronary microvascular function.

HIV infected individuals experience behavioral changes. 14% of HIV infected patients abuse alcohol [13]. Moderate ethanol intake may modulate cardiovascular system in AIDS individuals. There are no reports of the effects of AIDS plus ethanol consumption on coronary vascular function.

3.3 MATERIALS AND METHODS

Animals. Female C57BL/6N mice (National Cancer Institute) at 8-12 weeks of age and weighting about 20-22.5 g were randomly assigned to 4 different groups: control, murine AIDS, ethanol, and murine AIDS plus ethanol. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20° to 22° C with constant humidity and a 12:12 hour light dark cycle. Murine AIDS was induced by LP-BM5 murine leukemia retrovirus infection. The LP-BM5 viruses were administered intraperitoneally with 0.1 ml of an LP-BM5 inoculum with titer of 4.5 log 10 plaque forming units/ml. Infection leads to the rapid induction of clinical symptomatology that is similar to human AIDS. In the first week, 10%(v/v) of ethanol in autoclaved tap water was made available to the chronic ethanol group mice in a 300 ml plastic bottle with a stopper. The ethanol concentration increased to 20% (v/v) in the second week and kept 20% (v/v) for the rest of the treatment periods. The average of ethanol intake per mice was 2.8ml/day. Mice are active
at night; therefore, we took blood samples in night (after lights turned off three and half hours) to estimate blood ethanol concentration (Sigma Diagnostics Alcohol Kit). The average of blood ethanol concentration was 66.9mg/dl (0.0669%).

**Heart Isolation.** The animal model used to directly visualize coronary microcirculation was a modified Langendroff heart preparation [14]. The procedure was as follows. After three months of intervention, mice were anesthetized with sodium pentobarbital (55mg/kg ip). The abdomen was opened to expose the abdominal aorta. Immediately a PE 10 catheter was inserted and advanced to the aortic arch. The vena cava was then cut and cold cardioplegic solution (Abbott's Cardioplegic Solution for Cardiac Perfusion) was injected immediately through the catheter to arrest and protect the heart. After the heart stopped beating, a medial sternotomy was rapidly performed to expose the heart. Loose ligatures were placed around the right innominate artery and ascending aorta. Heparin (150U) was injected into the right atrium. The ligatures around the subclavian and common carotid arteries were tied, and a PE 50 catheter was inserted into the innominate artery. The catheter was advanced toward the heart, until the tip extended just into the aorta. The catheter was secured and connected it to the extracorporeal perfusion system. A small hole was cut in the right atrium. The aortic ligature was tied quickly, ensuring that all perfusate flow was retrograde to the coronary circulation. Then the heart was removed from the thoracic cavity and placed on a heated Lucite stage for intravital fluorescence microscopy of the left ventricular epicardial microcirculation [14]. The
isolated hearts were perfused with a physiologic solution that maintains normal cardiac and normal coronary microvascular functions [14].

**Preparation of Diluted Whole Blood for Perfusate.** Donor rats (450-500g) were anesthetized with ether, and 6 ml of arterial blood was withdrawn immediately into a heparinized syringe via cardiac puncture. The rat blood was then diluted 1:1 with Krebs-bicarbonate solution. A small aliquot of diluted whole blood was used to measure pH, PO$_2$, PCO$_2$, hematocrit (Hct), leukocyte and platelet counts. Typical values obtained from the diluted whole blood were: pH 7.37-7.45, PO$_2$ 100-125 mmHg, PCO$_2$ 30-40 mmHg, Hct 21%, leukocyte counts $5.2 \times 10^3$/ul, platelet counts $2.0 \times 10^5$/ul.

**Preparation of FITC-Albumin.** The preparation was a two-day process [15]. The FITC was conjugated to albumin the first day, as follows: 1.25 g of albumin was added to 18.75 ml of carbonate buffer (CBB pH=9.0) in a small beaker and stirred until albumin was completely dissolved. In a glass beaker, 0.0625 g of FITC was added to 6.25ml of CBB and stirred until FITC was completely dissolved. The albumin /CBB and the FITC/CBB were then mixed and covered with foil. The mixture was stirred at a slow speed overnight at 4$^\circ$C. The next day, 25ml of FITC-albumin was gently pipetted onto the top of Sephadex Column (Sephadex G-25, medium, Amersham Pharmeria Biotech; 100ml Cap. Aldrich Flash-Chromatography Columns), and PBS was continuously added to the top of the column once all of the FITC-albumin had passed the top layer in order to prevent from running dry. The FITC-albumin (MW $389+67,000=67,389$ Dalton) was separated in
the column by different sizes; the FITC bound to the albumin will pass though the column first, and the unbound FITC remained in a band toward the top of the column. Once the FITC-albumin conjugate began to drip from the column, a total of 15 ml were collected into a 15ml tube and poured into the outer tube of an Amicon Centriprep concentrator and centrifuge at 1500g for 10 minutes at 15°C (90% retentive recovery/molecular weight cut-offs 10 KD). The conjugated FITC-albumin (approximate 10 ml) was poured into a sterile 15ml tube, bringing the total volume up to 10ml with sterile PBS.

**Measurement of Coronary Microvascular Permeability to Macromolecules.** The isolated perfused mouse heart was oriented on the microscope stage with the free wall of the left ventricle facing up. A large coronary vein that courses along the left ventricle from apex to base was used to orient the heart on the stage. The aortic catheter was then connected to the syringe pump containing the perfusate. The coronary perfusate consisted of a mixture of 47.5% fresh whole rat blood obtained by cardio puncture from a donor rat, 47.5% Krebs-BSA and 5% FITC-BSA. The K⁺ of the final perfusate solution was 30mM to insure cardiac arrest while observing the coronary microcirculation. The perfusate blood gas was measured before perfusion. During constant flow perfusion at a flow rate of 0.3ml/min, the coronary perfusion pressure was monitored (Pressure Monitor BP-1) via a sideline.
Five coronary microvascular fields were observed after 5, 15, and 25 minutes of perfusion using intravital fluorescence microscopy (Hamamatsu). Venular fields were brought into clear focus and videotaped at each time point. After 30 minutes of observation, perfusion was stopped. The videotaped results were analyzed using Dazzle DVC and Adobe software (Adobe 5.5). Five to six fields/heart/time points were determined for O/I ratio (the ratio of the density of outside/inside venular). The O/I ratio was used to quantify FITC-BSA leakage (figure 3.1) [14].

**Statistical Analysis.** Data are collected and tabulated on spreadsheets (Microsoft Excel 7.0). All values were expressed as mean ± standard error. Statistical Analysis was performed by SPSS Statistical software (SPSS Windows 10.0). Comparisons among the groups were made by Analysis of Variance (ANOVA). If significant differences were observed, Newman-Keuls post-hoc testing was performed. To compare data as a function of time, we used repeated ANOVA.

3.4. RESULTS

**Coronary Vascular Resistance.** Table 1 summarizes the coronary vascular resistance through the experiment. The initial coronary resistance was quite similar among the control, ethanol and ethanol plus murine AIDS groups. Coronary resistance was relatively consistent over a period of perfusion in all three groups. In murine AIDS, the
initial value was slightly increased, but no significant difference was found compared to the other three experimental groups. Coronary resistance increased with perfusion in the murine AIDS group. We also observed that, in two of six hearts from this group, coronary perfusion pressure suddenly climbed up to 200mmHg at the end of the perfusion period. A few minutes later, a marked increase ($O/I \geq 1$) in transcoronary protein extravasation was found.

**Coronary Permeability to Macromolecules.** All four groups of hearts were perfused at the same flow rate of 0.3ml/min and the same FITC-BSA delivery rate. The duration of exposure to the excitation light was the same for all groups. Five fixed fields for each perfusion time were observed to limit the differences in fluorescence background. As figure 3.2 demonstrated, in control group the $O:I$ values were relatively constant over the period of perfusion. A repeated ANOVA indicated that the $O:I$ ratio was not significantly increased with time. In the murine AIDS mice, we observed some extravasation of FITC-BSA in the first 5 minute period of perfusion ($O/I=0.77\pm0.03$). Compared to the control group at the same time point, the increase was significant ($P<0.05$). In addition, one or two severe, more localized "leaky spots" of FITC-BSA were observed in three out of six murine AIDS hearts. This phenomenon was not observed in the control mice. Upon perfusion, resolution of the microvessels was reduced markedly as the FITC-BSA diffused in the extravascular space. Extravasation continued with time, and, after 25 minutes of perfusion, the $O/I$ reached $0.95\pm0.02$. We assume that $O/I = 1$ was the equilibrium status for albumin leakage, although the $O/I$ ratio in some of fields actually
exceeded 1.0. Because FITC-BSA accumulated in the extravascular space at this time, it was occasionally difficult to find some of the capillary-venular fields chosen at the initial reading. Overall, the O/I ratio in the murine AIDS mice (figure 3.3) was significantly increased compared to the control group (figure 3.4) at each perfusion period (P<0.001) and significantly increased as a function of time.

Chronic ethanol consumption did not alter coronary microvascular permeability to albumin compared to control mice. The O/I ratio was not significantly changed during the perfusion periods. The coronary microvascular barrier to albumin was maintained over perfusion time. In the murine AIDS plus ethanol consumption group, an increased transcoronary protein extravasation was observed in the initial perfusion time (P<0.05) and maintained no significant change over the perfusion periods. Chronic ethanol consumption did not reverse the effect of murine AIDS on coronary permeability to macromolecules but modulated coronary haemodynamic parameters.

3.5. DISCUSSION

Cardiovascular complications of in AIDS may contribute to the coronary microvascular endothelial dysfunction. We were interested in observing significant increases in coronary vascular permeability to macromolecules in murine AIDS mice compared to control mice. These findings suggest that murine AIDS impairs the normal function of the coronary microcirculation. The possible mechanisms conclude that 1) retroviruses
directly attack cardiovascular endothelial cells; 2) elevated TNFα and platelet activation factor (PAF) released by virus infected cells are cytotoxic to endothelial cells; and 3) overproduced reactive oxygen and nitrogen species by oxidative stress amplifies the endothelial cell dysfunction [16,17]. Certain endothelial cells, such as those lining liver sinusoids, human umbilical vein endothelial cells, bone marrow stromal endothelial cells or brain microvascular endothelial cells, have been found to be variably permissive for HIV infection [18]. Zietc et al [19] also reported that the aortic endothelial cell pattern in HIV-1-infected patients was clearly disturbed. Overall, these observations indicate that retrovirus may directly attack to the vascular endothelium. Our laboratory [20-22] and others [23,24] found that retrovirus infection stimulated TNF-α production. TNFα might unzip tight conjunction between endothelial cells, causing macromolecular movement into the extravascular space. TNFα may disrupt the integrity of the coronary vascular endothelium by its cytotoxic mechanism. Recently, in-vitro data suggests that TNF-α-programmed apoptosis is enhanced by PAF receptor activation [25,26]. PAF, a versatile lipid inflammatory mediator, is also elevated by retrovirus infection [25-29]. PAF may not only act as an apoptotic cofactor but may also stimulate neutrophils and platelets to form aggregating plugs in the microcirculation. Mechanical obstruction would increase the coronary resistance and further impair cardiovascular performance. Cardiovascular endothelial damage in murine AIDS might also be affected by free radicals. Cell death in culture paralleled increased nitrogen oxide (NO) synthesis [30] and reactive oxygen species (ROS) [31]. Viral replication and cytokine stimulation in AIDS are oxidative signals [32,33]. No direct evidence exists to verify increased NO and ROS production by
cardiovascular endothelial cells in LP-MB5 infected mice, but overproduced NO by induced nitrogen oxide synthase [16] and ROS by circulating neutrophils [our unpublished data] may rapidly diffuse into coronary vascular endothelial cells to cause endothelial dysfunction. Therefore, retrovirus induced cytokine dysregulation may trigger cardiovascular endothelial cell apoptosis and exaggerate coronary hemodynamics.

Epidemiological studies demonstrate a significant protective effect of moderate alcohol consumption on the incidence of cardiovascular diseases. Possible mechanisms to explain the cardioprotective effect of ethanol are [34,35]: vascular relaxation, HDL elevation, lowering fibrinogen level, modulating platelet function, and antithrombotic properties. Recently, Arbabi et al [36] found that ethanol inhibited proinflammatory cytokine, TNF and IL-8, secretion. A lower level of proinflammatory cytokines reduces their stimulating effect on neutrophils and platelets. Keeping neutrophils and platelets in a resting condition benefits the coronary microcirculation. Ethanol also suppresses cytokine induced iNOS expression [37], which may involve a mechanism of cardiovascular endothelial cell apoptosis. In addition, ethanol is a vasorelaxant [38]. This effect may be related to ethanol increase in both vascular endothelial growth factor mRNA expression and protein expression. [39]. Overall, the cardiac protective effect of moderate ethanol consumption may be mediated by cytokine modulation and vasorelaxation. In our study, chronic ethanol consumption alone did not alter coronary permeability to macromolecules. When murine AIDS mice were exposed to ethanol, coronary resistance was consistently maintained over the perfusion periods even though coronary
permeability increased. These results suggest that chronic ethanol consumption may modulate the coronary vascular function. However, ethanol did not improve the damage to the coronary microvascular barrier to macromolecules caused by retrovirus infection.

In summary, altering the coronary microcirculation may contribute to cardiovascular complications of AIDS. The structural and functional changes in the cardiovascular endothelium lead to increased coronary permeability to macromolecules. The retrovirus could directly or indirectly perturb the integrity of coronary endothelium. Chronic moderate ethanol consumption may preserve endothelial function. However, the structural destruction by the retrovirus is irreversible.
### Table 3.1. Coronary vascular resistance over periods of perfusion time.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Coronary resistance mmHg .ml⁻¹.min.g⁻¹</th>
<th>Perfusion Periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Middle</td>
</tr>
<tr>
<td>Control</td>
<td>32.21 ± 3.85</td>
<td>37.92 ± 3.71</td>
</tr>
<tr>
<td>Murine AIDS#</td>
<td>43.25 ± 4.58</td>
<td>51.05 ± 5.13</td>
</tr>
<tr>
<td>Murine AIDS+Ethanol</td>
<td>33.04 ± 2.78</td>
<td>40.41 ± 3.21</td>
</tr>
<tr>
<td>Ethanol</td>
<td>31.29 ± 2.33</td>
<td>40.02 ± 3.75</td>
</tr>
</tbody>
</table>

Values are means ± SD for n=6 hearts for each group.

# Coronary resistance increased over a period of perfusion in murine AIDS group.

** P<0.01, comparison between different groups, murine AIDS verse control. P<0.001 murine AIDS verse ethanol and murine AIDS plus ethanol.
Figure 3.1. Direct visualization of a coronary vascular fields (×320) with 5% FITC-BSA in the perfusate.

Due to the large size of BSA, the fluorescent marker is retained within the coronary vessel by an intact endothelial barrier, but leaks across the vessel wall in situation of endothelial layer dysfunction. The mean luminosity in coronary vessel (I) and directly over adjacent to the coronary vessel (O) are quantified. O:I ratio is calculated as the function of coronary vascular leakage.
Figure 3.2. The measurement of transcoronary extravasation of the fluorescent protein (O:I).

Data were expressed as mean ± SEM in 6 mice for each group. An increased O/I ratio as a function of perfusion time (P<0.001) was in murine AIDS only. In other three groups, control, ethanol, and murine AIDS plus ethanol, the O:I values were relatively consistent over a period of perfusion. ANOVA with Newman-Keuls post-hoc testing in comparison groups at each perfusion time. P<0.05, control verse murine AIDS and murine AIDS plus ethanol, Ethanol verse murine AIDS and murine AIDS plus ethanol during 5-10 minute perfusion. P<0.05, control verse murine AIDS during 15-20 minute perfusion, control verse murine AIDS plus ethanol, and ethanol verse murine AIDS during 25-30 minute perfusion. P<0.001, control verse murine AIDS during 25-30 minute perfusion.
Figure 3.3. Representative images of coronary permeability to FITC-albumin in murine AIDS heart.

Coronary microvascular fields A, B, C (×320) were observed after 5, 15, and 25 minutes of perfusion in murine AIDS, respectively. Leaky spots" of FITC-BSA (D) was found after 25 minutes of perfusion (×50).
Figure 3.4. Representative images of coronary permeability to FITC-albumin in the control heart.

Coronary microvascular fields (A, B, C) were observed after 5, 15, and 25 minutes of perfusion, respectively. No significant leakage was found over a perfusion period.

ACKNOWLEDGMENTS.

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CHAPTER 4. CHRONIC ETHANOL CONSUMPTION MODULATES ISCHEMIA-REPERFUSION INJURY IN MURINE AIDS

4.1. ABSTRACT

The cardiovascular manifestations of AIDS are very complicated. The high incidence of cardiovascular complications may be associated with acute ischemia-reperfusion injury in AIDS. Epidemiological studies suggest that moderate ethanol consumption have myocardial protective effects. However, it is unknown if chronic ethanol consumption has benefits on acute ischemia-reperfusion injury in the presence or absence of murine AIDS. The aim of this study was to determine if chronic ethanol consumption modulates ischemia-reperfusion injury in murine AIDS. Four groups were studied: control, murine AIDS, ethanol, and ethanol plus murine AIDS. We found that survival from an acute myocardial infarction (AMI) was reduced in the advanced stage murine AIDS mice. Although early stage murine AIDS hearts did survive the AMI, the infarct size was larger. Chronic ethanol consumption was cardioprotective, improving the survival of murine AIDS mice. However, chronic ethanol consumption did not significantly reduce infarct size in murine AIDS. Our results indicate that multiple deleterious effects of murine AIDS may cause cardiovascular dysfunction. The beneficial effects of chronic ethanol consumption in ischemia-reperfusion injury may be mediated by neutrophil adhesion molecule down-regulation and cytokine modulation.
AIDS is a health crisis affecting more than 47 million people worldwide. Since the epidemic began, 21.8 million people have died from AIDS. Cardiovascular involvement in AIDS has been recognized recently. The prevalence of cardiac involvement in patients with HIV, both asymptomatic and symptomatic, ranges from 28% to 73% [1]. The cardiovascular mortality rate associated with HIV infection in the United States is estimated to be 1-6% [2]. After the introduction of highly active antiretroviral therapy (HAART), the survival rate of HIV infected patients is significantly enhanced; however, cardiovascular involvement in AIDS has become more apparent. HIV cardiomyopathy was reported to be the fourth leading cause of dilated cardiomyopathy in the United States [3]. Congestive heart failure has become the leading cause of death in pediatric patients with AIDS, and half of them die of within 6 to 12 months [4]. The incidence of myocardial infarction (MI) in HIV infected patients has increased from 0.86 in year 1983-86 to 3.41 in year 1995-98 respectively per 1000 patients [5,6]. The pathogenesis underlying cardiovascular manifestations in HIV infected patients is complicated. The high incidence of cardiovascular complications may be related to ischemia-reperfusion injury in AIDS.

Recent epidemiological studies have suggested that moderate ethanol consumption has been associated with a reduced risk for developing coronary artery disease, angina pectoris, acute myocardial infarction and sudden death [7-12]. It has been observed that
regular moderate drinking has cardioprotective effects. However, it is unknown if chronic ethanol would attenuate ischemia-reperfusion injury during retrovirus infection.

Neutrophils are a principal mediator of ischemia-reperfusion injury [13]. Upon neutrophil activation, they express a higher affinity CD11/CD18 to mediate firm adhesion to vascular endothelial cells and eventually transmigrate to heart tissue. Accumulated neutrophils in hearts release the proteolytic enzymes, and cytotoxic H₂O₂ result in severe ischemia-reperfusion injury. We found that neutrophils in murine AIDS were highly activated and down-regulated during chronic ethanol consumption (chapter 1). Therefore, we hypothesized that murine AIDS mice are susceptible to severe ischemia-reperfusion injury, and that chronic ethanol consumption attenuates ischemia-reperfusion injury.

4.3. MATERIALS AND METHODS

Animals. Female C57BL/6N mice (National Cancer Institute) at 8-12 weeks of age and weighting about 20-22.5 g were randomly assigned to 4 different groups: control, murine AIDS, ethanol, and murine AIDS plus ethanol. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20° to 22° C with constant humidity and a 12:12 hour light dark cycle. Murine AIDS was induced by LP-BM5 murine leukemia retrovirus infection. The LP-BM5 viruses were administered intraperitoneally with 0.1 ml of an LP-BM5 inoculum with titer of 4.5 log 10 plaque forming units/ml, as done previously by our lab [14,15,16]. Infection leads to the rapid induction of clinical
symptomatology that is similar to human AIDS. In the first week, 10%(v/v) of ethanol in autoclaved tap water was made available to the chronic ethanol group mice in a 300 ml plastic bottle with a stopper. The ethanol concentration increased to 20% (v/v) in the second week and kept 20% (v/v) for the rest of the treatment periods. The average of ethanol intake per mice was 2.8ml/day. Mice are active at night; therefore, we took blood samples in night (after lights turned off three and half hours) to estimate blood ethanol concentration (Sigma Diagnostics Alcohol Kit). The average of blood ethanol concentration was 66.9mg/dl (0.0669%).

**Murine Heart Model for Myocardial Ischemia and Reperfusion.** We modified a murine model for myocardial ischemia and reperfusion based on the protocol of Michael, et al [17]. Female C57BL/6 mice were anesthetized with sodium pentobarbital (55mg/kg ip). Each mouse was placed in a supine position with paws taped to the operating table. A 5-0 ligature was placed behind the front lower incisors and pulled taut so that the neck was slightly extended. A midline cervical skin incision was made. The salivary glands were separated from the midline to allow access to the right carotid artery and trachea. A tracheotomy was then performed to facilitate breathing. A section of polyethylene tubing (PE) 90 tubing was inserted into the mouse’s trachea and connected via a loose PE 160 junction to a respirator (Harvard Rodent Ventilator Model 683). The respirator’s tidal volume was set at around 1.0ml/min, and the rate was set at 120 strokes/min; supplementation was with 100% oxygen. Normal chest expansion was monitored for adjusting optimal tidal volume. The right carotid artery was then cannulated with PE-10
tubing to monitor arterial blood pressure and heart rate. The arterial cannula was filled with heparinized PBS (2U/ml) and connected to a blood pressure transducer and a blood pressure monitor (Gould WindograQ). After an equilibration period of 10 minutes, a thoracotomy was performed. With an electrocautery, an incision was made to the left of the sternum. The pericardial sac was then removed. Ligation of the left anterior descending branch (LAD) was performed using a 7-0 silk suture attached to a needle. A small piece of PE 50 was used to secure the ligature without damaging the artery. The animals were subjected to 30 minutes of LAD occlusion and 120 minutes of reflow. At the end of the 2-hour period of reperfusion, the LAD was religated with a 7-0 silk suture. Blood samples were taken from the carotid catheter for monitoring blood gas. Trypan blue (1.2 ml, 0.4%; Sigma Chemical) was injected retrogradely into the carotid artery catheter to delineate the in vivo area at risk [17]. At the end of the protocol, the heart was excised and sectioned transversely into four 1-mm sections with one section made at the site of the ligature. Each section was scanned with a high-resolution scanner (1200 dpi Hewlett-Packard-model 5370C). Each slice was counterstained with 1.0% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma Chemical) solution for 5 minutes at 37°C. Each section was then placed in a 10% of buffered formalin solution. Next day, each slice was scanned again to determine infarct size.

**Measurement of the Area at Risk and Infarct Size.** A scanned transverse section from 1 mm distal to ligature was opened in Adobe software (Adobe Photoshop 5.5) and analyzed for the area at risk and the infarction size (figure 4.1). Using a toolbar, a 1 mm² area was converted the number of pixels. A converting factor of expression area as mm²
was obtained by dividing total pixels of outlined areas by the number of pixels in 1 mm$^2$. The total area of the myocardial section and the trypan blue un-stained area (area at risk) were outlined and computed into mm$^2$. The area at risk in this section was calculated by dividing the area at risk by the total area. To measure the infarct size, the TTC stained same section was used to determine the total and infarct areas. The same calculation was made for the infarct areas. The final infarct / area at risk fraction was calculated by dividing the infarct size by the area at risk. After formalin fixation, we accounted for shrinkage. To correct this effect, we measured total area of the same section before and after the formalin fixation and then calculated final infarct/area at risk (see appendix 1)

$= \text{infarct area} - \text{total area (before formalin fixation)} / \text{area at risk} - \text{total area (after formalin fixation)}$

**HE Stain for Assessment of Neutrophil Trafficking in the Hearts.** The hearts for each group were removed after the same ischemia-reperfusion protocol except sham control. The hearts was stored in 4.0% paraformaldehyde until heart sections were embedded in the plastic cartridge (Tissue Embedding Center, Tissue-TEK II). After a series of dehydration and rehydration in a tissue processor (LX120 Tissue Processor, OXIS Instruments), 5-μm-thick sections were cut (Microtome, LEICA RM 2155) and transferred to slides. The slides were soaked in 95% ethanol for 10 minutes to remove some of the plastic embedding and to allow the tissue to stain. After the 10-minute ethanol wash, the heart sections were stained with hematoxylin solution for 10 minutes and eosin for 3 minutes. Three slides for each heart were then observed microscopically.
(× 400 using Zeiss microscope), and the average number of PMNs per section of the left ventricle was reported.

Statistical Analysis. Statistical analysis was performed using Prism Statistical Software (version 3.0). Comparisons among groups were made using ANOVA with Newman-Keuls post-hoc testing when significant differences were observed. A probability of less than 0.05 was considered statistically significant. Survival study was performed by SPSS (SPSS Windows 10.0).

4.4. RESULTS

Survival rate during ischemia and perfusion surgery. Figure 4.2. A, B presents the percentage of survival mice the 30-minute ischemia and 120-minute reperfusion protocol. Two-month murine AIDS mice did not survive the I/R protocol. Of a total of 10 mice in the two-month murine AIDS group, only one mouse survived the entire period of ischemia-reperfusion. Nine of 10 mice died during ischemia-reperfusion. Seventy % them died during ischemia, especially at 10 minutes of ischemia (40% of total). To gain some index of infarction we used one-month murine AIDS mice for ischemia-reperfusion study. No significant difference in survival was observed in control verse one-month murine AIDS (A) or two- month ethanol consumption mice (B). Two of 12 mice died in control, respectively, at 20-minute ischemia and 75-minute reperfusion. One of 11 mice died at 20-minute ischemia periods in one-month murine AIDS (A) and two-month ethanol consumption groups (B). Chronic ethanol consumption improved the survival
rate in two-month murine AIDS mice (P<0.05) compared to two-month murine AIDS in the absence of ethanol consumption. In this group, eight of 16 mice (50%) went though 30-minute ischemia and 120-minute reperfusion periods. Among the mice that died, half of them died during the ischemic period and the other half died during the reperfusion.

**Hemodynamic parameters.** Blood pressures and heart rates were recorded for all groups of mice throughout the myocardial ischemia-reperfusion experiments and were given in figure 4.3. Heart rate, systolic and diastolic blood pressure significantly decreased from the initial LAD ligation to 10-minute occlusion in each group. Thereafter, if mice endured the ischemic attack, all three hemodynamic parameters recovered somewhat 20 minutes of occlusion and held constantly throughout the remainder of the experiment. Since only one of ten mice went through the entire period of ischemia-reperfusion in the two months of murine AIDS group, we could not include hemodynamic data during reperfusion for this group. We also found that two-month murine AIDS mice suffered very low blood pressure (<25/15 mmHg) with slow heart rates (<360/min) immediately after LAD ligation, compared to the other groups of mice. No significant group differences in heart rate or systolic and diastolic blood pressure were observed at any point in the experiments.

**Myocardial area at risk and infarct size.** The infarct size and area at risk were measured after 30-minute occlusion and 120-minute reperfusion (figure 4.4). Despite similar sizes of areas at risk in each group, one-month murine AIDS hearts suffered from
a larger area of infarction after ischemia-reperfusion (figure 4.5). Two months of ethanol consumption significantly reduced the infarct size/area at risk compared to the control mice (P<0.01).

**Myocardial PMN Accumulation.** PMNs within the ischemic-reperfused hearts after 30 min of myocardial ischemia and 120 min of reperfusion were counted and reported the number of PMNs in each section of the left ventricle wall (figure 4.6). Variable numbers of PMNs were mainly marginated (adjacent to endothelium) and usually clustered within epicardial blood vessels in the one month of murine AIDS hearts. No significant difference existed in PMN trafficking between control and chronic ethanol consumption hearts. However, chronic ethanol consumption significantly decreased PMN infiltration in the murine AIDS hearts (P<0.05). No PMNs were found in sham group hearts.

### 4.5. DISCUSSION

The incidence of myocardial infarction in AIDS is increasing. Ischemic attacks may contribute to sudden death in AIDS patients. Our present data strongly support the idea that retrovirus infected hearts are more vulnerable to heart attack. When cardiovascular ischemic events occur in the late stage of AIDS, hearts have no protective ability against this attack. This may be due to underlying cardiovascular involvement by retrovirus infection. Cardiac manifestations such as myocarditis, dilated cardiomyopathy, endocarditis, pericardial effusion, arteriopathy and cardiac malignant neoplasms have
been found in AIDS patients [19-22]. Ischemic attack could exaggerate the cardiovascular complications and rigorously affect heart performance.

The pathogenesis underlying cardiovascular complications of AIDS may be related to ischemia-reperfusion injury. Several causative factors contributing to cardiovascular complications of AIDS may amplify ischemia-reperfusion injury. Evidence suggests that the retrovirus itself causes the cardiovascular complications. Researchers found the presence of HIV-1 in the AIDS patient’s myocardium [23-26]. Barbaro et al [19] found that cardiac myocytes were infected with HIV-1 in 58 patients and nearly two thirds of those samples had myocarditis. These findings suggest that the myocarditis is related to a direct action of the retrovirus. Endothelial cells infected by retrovirus are possibly responsible to cardiovascular dysfunction in AIDS. Altered function of vascular endothelial cells is associated with hyperactivity of the microcirculation and with coronary vasospasm, resembling the changes seen in cocaine abuse. Coronary artery spasm may lead to ischemic attack and myocellular necrosis, subsequently causing hypertrophy [27]. A number of opportunistic infections in AIDS were reported involving the heart [28-32]. Multiple infections may trigger cellular and humoral-mediated cardiac injury as well.

Autoimmune reaction may disrupt heart function. Acierno [33] suggested that myocardial damage could relate to uncontrolled hypergammaglobulinemia. Murine AIDS characteristically develops hypergammaglobulinemia. Many researchers [34-40] have
proposed an autoimmune mechanism for HIV-related myocardial disease similar to those described with antimyosin antibodies. Viral genes may alter the cell surface of the muscle fiber. Some of these cell surface proteins become immunogenic and elicit a progressive autoimmune reaction. A series of experiments revealed the presence of circulating cardiac autoantibodies to heavy chain myosin in AIDS patients having cardiovascular complication.

Specific and nonspecific myocardial inflammatory responses may involve cardiovascular complications. The prevalence of myocardial inflammatory infiltration is increased in AIDS [41-44]. We also found neutrophils were significantly activated in murine AIDS. Myeloperoxidase (MPO) activity as a neutrophil infiltration marker is increased in murine AIDS hearts compared to control hearts (Chapter 1). Inflamed cells release a variety of cytokines, including TNF-α, IL-1, IL-6, and platelet activating factor (PAF) in AIDS (45-51). All of them affect heart performance to various degrees. The cytokine IL-1 has a suppressive effect on adrenergic agonist-mediated increase in cyclic adenosine monophosphate (cAMP) in cardiomyocytes. IL-2 and IL-6 have reversible myocardial depressant effects, in-vivo [52,53]. Long-term treatment of cardiomyocytes with IL-1 and TNFα reduced contractility. Myocardial depression effects of TNFα infusion cause left ventricle dysfunction [54]. TNF-α induces cell apoptosis and PAF acts as a cofactor in accelerating apoptosis [50]. Pinsky et al [55] proposed that cytokine effects may be regulated by nitric oxide (NO). Increased expression of inducible nitric oxide synthases (iNOS) was found, in-vitro, in cardiac myocytes treated with TNFα, IL-1 and interferon
Therefore, increased cytokines may contribute to the initiation and perpetuation of activated vascular cells causing heart dysfunction. Upon ischemia-reperfusion, hypersensitive neutrophils may become more activated. iNOS may overexpress and more cytokines may release in response the ischemia-reperfusion injury. Overall, activated neutrophils release ROS and the proteolytic enzymes that directly destroy heart tissue. Overproduced cytokines and overexpressed iNOS amplify the local pathological inflammatory reaction and perturb myocardial function. Our data strongly support this notion because a larger infarct size is observed in murine AIDS hearts.

The cardioprotective effect of moderate ethanol consumption has been observed. Some investigators [56,57] proposed that ethanol induces vascular relaxation, elevates HDL, lowers fibrinogen level, and modulates platelet function. Besides these beneficial factors, we found that neutrophil CD11b expression was down-regulated (chapter 1). The down-regulation of CD11b expression by ethanol could decrease the adherence of PMNs to endothelial cells and ultimately diminish neutrophil sequestration in the heart tissue [58,59]. Recently, Arbabi et al [60] found that ethanol inhibited inflammatory cytokines including TNF-α and IL-8 production. Ethanol also suppresses cytokine-induced iNOS expression [61]. In addition, moderate levels of ethanol induce expression of vascular endothelial growth factor and stimulate angiogenesis [62]. Thus, in our present study, we found that chronic ethanol consumption attenuated ischemia-reperfusion injury.
In summary, the present study clearly demonstrated that two months exposure to murine AIDS increases the vulnerability to acute ischemic attack. Even though early stage murine AIDS hearts survived during ischemia-reperfusion injury, infarction of the myocardium was severe. Chronic ethanol consumption improved survival but did not completely reverse the deleterious effects on hearts due to retrovirus infection. These results confirm the beneficial effects reported by others on hearts by moderate ethanol consumption.
4.6. LIST OF FIGURES
Figure 4.1. Representative images of area at risk and infarct size.

A trypan blue stained transverse section from 1 mm distal to ligature was scanned and reopened in Adobe Photoshop (5.5). This section was for analyzing the area at risk. Using a toolbar, a 1 mm² area (A) was converted the number of pixels. A converting factor of expression area as mm² was obtained by dividing total pixels of outlined areas by the number of pixels in 1 mm². The total area of the myocardial section (B) and the trypan blue un-stained area (area at risk) (C) were outlined and computed into mm². The area at risk in this section was calculated by dividing the area at risk by the total areas. To measure the infarct size, TTC counterstained same section was used to determine the total and infarct areas. The same calculation was made for the infarct areas. The final infarct / area at risk fraction was calculated by dividing the infarct size by the area at risk.
Figure 4.2. The percentage of survival mice during 30-minute ischemia and 120-minute perfusion.

No significant difference was observed in the control verse one-month murine AIDS (A) or two-month ethanol consumption (B). 2 of 12 mice died in the control, respectively, at 20-minute ischemia and 75-minute reperfusion. 1 of 11 mice died at 20-minute ischemia periods in one-month murine AIDS (A) and two-month ethanol consumption (B). From a total of 10 mice in two-month murine AIDS group, only one mouse went through whole periods of ischemia-reperfusion. 9 of 10 mice die during ischemia-reperfusion. 70% of them died at ischemia periods, especially, in 10-minute ischemia periods (40% of total). In two-month murine AIDS with chronic ethanol consumption group, 8 of 16 died during the different ischemia-reperfusion periods. Among the non-survival mice, half of them died during ischemia periods and another half died during reperfusion periods.
Figure 4.3. Heart rate, systolic and diastolic blood pressure during ischemia-reperfusion periods.

X-axis represented as ischemia-reperfusion times. O-0 was initial occlusion time. O-1 was 10-minute occlusion, O-2 was 20-minute occlusion, and O-3 was 30-minute occlusion. R represented reperfusion. R-0 was an initial reperfusion time, R-15 was 15-minute reperfusion. Record these parameters every 15 minutes until the total 120-minute reperfusion finished.

Four groups were included: control, one-month murine AIDS, two-month murine AIDS plus ethanol, two-month ethanol. A. Heart rate per minute, B. Systolic blood pressure, C. diastolic blood.
Figure 4.4. Infarct size and area at risk after 30-minute occlusion and 120-minute reperfusion.

For infarct/area at risk, $P<0.001$, two-month ethanol verse one-month murine AIDS and two-month murine AIDS plus ethanol. $P<0.01$, two-month ethanol verse control. For infarct size/total area, $P<0.001$, two-month ethanol verse one-month murine AIDS, $P<0.01$, two-month ethanol verse two-month murine AIDS plus ethanol, $P<0.05$, two-month ethanol verse control.
Figure 4.5. Representative infarct size images after 30-minute ischemia and 120-minute reperfusion.

Up left image was represented control hearts. Up right image was represented ethanol hearts. Low left image was represented murine AIDS with ethanol consumption hearts. Low right image was represented murine AIDS hearts.
Figure 4.6. PMN trafficking in hearts after 30 min of myocardial ischemia and 120 min of reperfusion.

No PMNs were found in the hearts of sham group. P<0.05, ethanol verse murine AIDS, murine AIDS plus ethanol verse murine AIDS.

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CHAPTER 5. VITAMIN E ATTENUATES MYOCARDIAL ISCHEMIA-REPERFUSION INJURY IN MURINE AIDS

5.1. ABSTRACT

The incidence of myocardial infarction in AIDS is increasing; however, its mechanism is unclear. No effective therapeutic agents have been discovered to reduce ischemia-reperfusion (I/R) injury in AIDS. The aim of this study was to determine if infarct size is increased in a murine model of AIDS and if I/R injury could be attenuated with vitamin E treatment. Three groups were studied: control, murine AIDS, and murine AIDS with vitamin E treatment. Anesthetized mice were subjected to 30 min of left anterior descending coronary artery occlusion and 2 h of reperfusion. We found that murine AIDS hearts had severe I/R injury compared to controls. Vitamin E treatment significantly reduced infarct size and inhibited neutrophil (PMN) CD11b expression (P<0.05). However, vitamin E treatment did not affect PMN reactive oxygen species (ROS) production and platelet CD62p expression. These results suggest that the reduction of I/R injury with vitamin E may be due to inhibition of PMN CD11b expression and PMN accumulation in the ischemic heart. Vitamin E may be a promising therapeutic agent.
5.2. INTRODUCTION

AIDS is a health crisis affecting more than 47 million people worldwide. 21.8 million people have died from AIDS since the epidemic began. One estimate of the prevalence of cardiac involvement in patients with HIV, both asymptomatic and symptomatic, is from 28% to 73% [1]. The cardiovascular mortality rate associated with HIV infection in the United States is estimated as 1-6% [2]. After the introduction of highly active antiretroviral therapy (HAART), the survival rate of HIV infected patients was significantly enhanced. However, cardiovascular involvement in AIDS becomes more apparent. HIV cardiomyopathy was reported to be the fourth leading cause of dilated cardiomyopathy in the United States [3]. Congestive heart failure has become the leading cause of death in pediatric patients with AIDS, and half of the children die within 6 to 12 months [4]. The incidence of myocardial infarction (MI) in HIV infected patients has increased dramatically, from 0.86 in year 1983-86 to 3.41 in year 1995-98 respectively per 1000 patients [5,6]. The pathogenesis underlying cardiovascular complications in HIV infected patients is unclear, but likely involves an interplay of several pathologic factors, such as cytokine dysregulation, cardiovascular endothelial cell dysfunction, platelet activation associated with hypercoagulation and thrombosis formation, and excessive neutrophil (PMN) activation amplifying inflammatory response in AIDS hearts.
I/R may profoundly alter cardiac function. A principal mediator of this phenomenon is the PMN. PMNs may plug coronary capillaries, mechanically blocking blood flow [7]. Upon reflow, adhesion of PMNs is caused by their adhesion molecule expression. A higher affinity β2-integrin (CD11/CD18) expresses on activated PMNs and ICAM-1 serves as a major endothelial ligand to CD11/CD18. Therefore, CD11b/CD18 plays a key role in mediating firm adhesion of PMNs to vascular endothelial cells prior to transmigration into ischemic heart tissues [8]. Activated PMNs release the proteolytic enzymes that directly induce heart tissue damage and proinflammatory mediators that amplify the local inflammatory reaction. The oxidative burst of PMNs releases cytotoxic H₂O₂. Overall, PMN recruitment in heart tissue results in severe I/R injury. PMN activation is enhanced in murine AIDS [9,10]. Platelet activation also may involve in I/R injury in murine AIDS. CD62p expressed on activated platelets interacts with the PMNs through the carbohydrate ligand, sialyl Lewis X. This interaction is a crucial step for PMN activation, which is actively involved in the inflammatory processes [14]. Activated platelets released soluble factors that modulate the activity of PMNs [11,12,13]. In addition, activated platelets induce the production of reactive oxygen specie (ROS) by PMNs [14,15]. Thus, platelet-PMN interactions may amplify several inflammatory responses to I/R injury in murine AIDS.

Vitamin E has been found to inhibit adhesion molecule expression by PMNs and vascular endothelial cells stimulated by a number of mediators [16-20]. Thus, vitamin E may decrease the interaction of PMNs and endothelial cells, block PMN migration into
extravascular spaces by normalizing cytokine production. Platelet adhesion, the principal event of platelet activation, provides a crucial step in the development of cardiovascular events such as myocardial infarction and ischemic stroke. Vitamin E is capable of inhibiting platelet aggregation by a variety of agonists [21-23]. Overall, vitamin E may be a promising candidate for attenuating I/R injury, due to its multi-beneficial effects on cardiovascular events in murine AIDS. In addition, HIV infected people lose vitamin E as the progression of AIDS continues. The loss of vitamin E also occurs in murine AIDS [24]. Supplementation with vitamin E slowed this process in murine AIDS [25]. Vitamin E supplements at extremely high levels (15-450 times the normal intake) significantly normalized cytokines produced by splenocytes without toxicity [26,27]. Therefore, the high degree of safety and tolerability of vitamin E warrant a test to determine if treatment would decrease myocardial ischemic injury in murine AIDS.

5.3. MATERIALS AND METHODS

**Animals.** Female C57BL/6N mice (National Cancer Institute) at 8-12 weeks of age and weighted about 20-22.5 were randomly assigned to 3 different groups: control, murine AIDS, and murine AIDS with vitamin E supplementation. Mice were housed in transparent plastic cages with a stainless wire lid in a room at 20° to 22° C with constant humidity and a 12:12 hour light dark cycle. Murine AIDS was induced by LP-BM5 murine leukemia retrovirus infection. The LP-BM5 viruses were administered intraperitoneally with 0.1 ml of an LP-BM5 innoculum with a titer of 4.5 log 10 plaque
forming units/ml, as done previously by our lab [24,26,27]. In the same day of LP-BM5 infection, mice in the vitamin E treated group were fed a vitamin E rich pellet diet (AIN-76 Purified Rodent Diet with 3.2 g/kg to add 1600 IU/kg of Vitamin E, Dyets). The amount of vitamin E in the diet is 50-fold higher than recommended requirements for mice [28]. The total experimental period was one month.

**PMN CD11b and ROS Production.** Flow cytometry was used to identify PMNs and their activity in whole blood. Freshly drawn citrated whole blood was divided into three aliquots. 40 ul of blood was for blood cell counts (Serono Diagnostics, Allentown, PA, Model 9018 CP). 20 ul was for platelet CD62p measurement. The rest (300 ul) was mixed with the vital nucleic acid stain, LDS-751 (1 ug/ml) (Molecular Probes, Eugene, OR). This mixture of whole blood and LDS-751 was used for all subsequent leukocyte experiments [29]. PMN CD11b and ROS measurements were performed by incubating saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated anti-CD11b mAb (Pharmingen, San Diego, CA), and 80 mM 2'7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) with the whole blood/LDS-751 mixture. Samples were protected from light by using foil wrapped round bottom polypropylene tubes (Becton Dickinson) and incubated in a 37°C water bath for 15 min, then diluted with 0.5 ml of cold PBS and placed on ice until data acquisition in flow cytometer. During FACScanning (Becton Dickinson, FACScan Clinical Flow Cytometry), a 488nm argon laser light was used for excitation, and fluorescence emission was detected as forward scatter (FSC), which was a measure of cell size, and side scatter (SSC), which
was a measure of cell granularity. In addition, a threshold fluorescence was set on the LDS-751 signal that allowed list-mode data collection on leukocytes in whole blood without interference from erythrocytes. Thus, PMN subpopulations could be separated on the basis of their dot plots pattern on FSC, SSC, and LDS–751 in the FL3 channel. The fluorescence intensity due to bound FITC-labeled CD11b antibody was monitored in FL1 channel. For measurement of ROS, this method [30] used the properties of DCFH-DA, which rapidly diffused across cell membranes and was then trapped within the cells by a deacetylation reaction. In the presence of hydrogen peroxide, this compound was oxidized to DCF, which was highly fluorescent in FL1 channel. To determine the capacity of PMNs to upregulate the CD11b adhesion molecule surface expression or ROS generation, inflammatory mediators f-Met-Leu-Phe (fMLP, 10^{-6}M of final concentration) were added to the samples for an additional 10-minute incubation. After 25 minutes of total incubation, samples were diluted with ice-cold PBS and kept on ice until analysis by flow cytometry.

**Platelet CD62 Expression.** Platelet CD62 (P-selectin) was assessed in whole blood using flow cytometry. In this procedure, citrated whole blood (1:4:10) was collected and 20 ul were added to 1 ml of filtered PBS. 100ul of the mixture were incubated with a saturating concentration of FITC-conjugated anti-P-selectin antibody (Pharmingen) for 10 minutes at room temperature. By using this technique, harsh manipulation such as centrifugation, commonly used to separate platelets from whole blood, which might cause artifactual up-regulation of receptors on platelets, was avoided. To determine the capacity of platelets to
upregulate P-selectin, whole blood samples were stimulated with 50um ADP for 10 minutes then stained with the antibodies described above. During FACScanning, using FSC and SSC sorted the population of platelets and FL1 channel determined the intensity of FITC. The data from the FACS processing was further analyzed using WinMDI 2.8.

**Murine Heart Model for Myocardial Ischemia and Reperfusion.** Murine model for myocardial ischemia and reperfusion of Micheal, et al [31] was modified. Female C57BL/6 mice were anesthetized with sodium pentobarbital (55mg/kg ip). Each mouse was placed in a supine position with paws taped to the operating table. A 5-0 ligature was placed behind the front lower incisors and pulled taut so that the neck was slightly extended. A midline cervical skin incision was made. The salivary glands were separated from the midline to allow access to the right carotid artery and trachea. A tracheotomy was then performed to facilitate breathing. A section of polyethylene tubing (PE) 90 tubing was inserted into the mouse’s trachea and connected via a loose PE 160 junction to a respirator (Harvard Rodent Ventilator Model 683). The respirator’s tidal volume was set around 1.0 ml/min, and the rate was set at 120 strokes/min; supplementation was with 100% oxygen. Normal chest expansion was monitored for adjusting optimal tidal volume. The right carotid artery was then cannulated with PE-10 tubing to monitor mean arterial pressure and heart rate. The arterial catheter was filled with heparinized PBS (2U/ml) and connected to a blood pressure transducer and blood pressure monitor (Gould Windograf). After an equilibration period of 10 minutes, a thoracotomy was performed. With an electrocautery, an incision was made to the left of the sternum. The pericardial sac was then removed. Ligation of the left anterior descending branch (LAD) was
performed using a 7-0 silk suture attached to a needle. A small piece of PE 50 was used to secure the ligature without damaging the artery. The animals were subjected to 30 minutes of LAD occlusion and 120 minutes of reflow. At the conclusion of the 2-hour period of reperfusion, the LAD was religated with a 7-0 silk suture. A blood sample was taken from the carotid catheter for monitoring blood gas. Trypan blue (1.2 ml, 1.0%; Sigma Chemical) was injected retrogradely into the carotid artery catheter to delineate the in vivo area at risk. At the end of the protocol, the heart was excised and sectioned transversely into four 1-mm sections with one section made at the site of the ligature. Each section was scanned with a high-resolution scanner (1200 dpi Hewlett-Packard-model 5370C). Each slice was counterstained with a 1.0% 2,3,5-triphenyltetrazolium chloride (TTC)(Sigma Chemical) solution for 5 minutes at 37°C. Each section was then placed in a 10% buffered formalin solution. The next day, each slice was scanned again to determine infarct size.

**Measurement of the Area at Risk and Infarct Size.** A scanned transverse section from 1 mm distal to the ligature was opened in Adobe software (Adobe Photoshop 5.5) and analyzed for the area at risk and the infarction size. Using a toolbar, a 1 mm² area was converted the number of pixels. A converting factor of expression area as mm² was obtained by dividing total pixels of outlined areas by the number of pixels in 1 mm². The total area of the myocardial section and the trypan blue un-stained area (area at risk) were outlined and computed in mm². The area at risk in this section was calculated by dividing the area at risk by the total area. To measure the infarct size, the TTC stained same section was used to determine the total and infarct areas. The same calculation was made
for the infarct areas. The final infarct / area at risk fraction was calculated by dividing the infarct size by the area at risk. After formalin fixation, we accounted for shrinkage. To correct this effect, we measured total area of the same section before and after the formalin fixation and then calculated final infarct/area at risk (see appendix 1)=infarct area ÷ total area (before formalin fixation)/area at risk ÷ total area (after formalin fixation).

**HE Stain for Assessment of Neutrophil Trafficking in the Hearts.** The hearts for each group were removed after the same I/R protocol except sham control. The hearts was stored in 4.0% paraformaldehyde until heart sections were embedded in the plastic cartridge (Tissue Embedding Center, Tissue-TEK II). After a series of dehydration and rehydration in a tissue processor (LX120 Tissue Processor, OXIS Instruments), 5-μm-thick sections were cut (Microtome, LEICA RM 2155) and transferred to slides. The slides were soaked in 95% ethanol for 10 minutes to remove some of the plastic embedding and to allow the tissue to stain. After the 10-minute ethanol wash, the heart sections were stained with hematoxylin solution for 10 minutes and eosin for 3 minutes. Three slides for each heart were then observed microscopically (× 400 using Zeiss microscope), and the average number of PMNs per section of the left ventricle wall was reported.

**Statistical Analysis.** Statistical analysis was performed using Prism Statistical Software (version 3.0). Comparisons among groups were made using ANOVA with Newman-Keuls post-hoc testing when significant difference was observed. Comparing same
samples in the absence and presence of stimulators was used paired test. A probability of less than 0.05 was considered statistically significant.

4.4. RESULTS

**PMN Activation.** PMN CD11b expression, a marker of PMN activation, was upregulated in murine AIDS (figure 5.1). After Vitamin E supplementation in murine AIDS, PMN CD11b expression was down-regulated (figure 5.1) \((P<0.01)\). The total fluorescence intensity for PMN CD11b was even slightly lower than a baseline level of control. In addition, the PMN capability of response to fMLP stimulation was recovered after vitamin E treatment. PMN ROS production also increased in murine AIDS. However, vitamin E supplement did not affect PMN ROS production (figure 5.2).

**Platelet Activation.** CD62p (P-selectin) is a component of the \(\alpha\) granule membrane of resting platelets expressed on the platelet surface after platelet activation. After one month of LP-BM5 infection, CD62p expression on platelets increased compared to controls \((P<0.01)\) (figure 5.3). Vitamin E treatment did not attenuate platelet CD62p expression. ADP releases endogenously by initial platelet activation and then further stimulates platelet aggregation in later phases of platelet activation. We added ADP ex-vivo in blood to determine the ability of platelet activation. Platelet CD62p was almost fully expressed after ADP (50um) stimulation in each group. No significant group differences were observed in ADP-stimulated platelet CD62p expression.
**Hemodynamic Data.** Blood pressure and heart rates were recorded for all groups of mice throughout the myocardial I/R experiments and reported in figure 5.4. Heart rate, systolic and diastolic blood pressure significantly dropped due to the LAD ligation to 10-minute occlusion in each group. Thereafter, if mice could endure ischemic attack, all three hemodynamic parameters came back in 20-minute occlusion periods and held constant throughout the entire experiments. Only one of 12 mice in the controls, one of 11 mice with the murine AIDS, and one of 7 mice with the murine AIDS and vitamin E supplementation died at 10-minute occlusion times. No significant group differences in heart rate, systolic and diastolic blood pressure were observed through the experiments.

**Myocardial Area at Risk and Infarct Size.** The infarct size and area at risk were measured after 30-minute occlusion and 120-minute reperfusion (figure 5.5). Despite similar-sized areas at risk, the murine AIDS hearts suffered from a significantly larger area of infarction after I/R compared to control hearts (P<0.05). In general, the untreated murine AIDS and control hearts suffered infarcts that were nearly transmural in appearance. After one month of vitamin E treatment in murine AIDS, the infarct area of the area at risk decreased compared to murine AIDS without vitamin E treatment (P<0.01). In addition, infarct area in three of six hearts was characterized by epicardial necrosis in vitamin E treated murine AIDS (figure 5.6).

**Myocardial PMN Accumulation.** PMNs within I/R hearts after 30 min of myocardial ischemia and 120 min of reperfusion were counted and reported the number of PMNs per
section of left ventricle wall (figure 5. 7). No significant group differences existed in PMN trafficking in hearts. Variable numbers of PMNs were mainly marginated (adjacent to vessels) and usually clustered within epicardial blood vessels in the murine AIDS hearts. No PMNs were found in sham group hearts.

5.5. DISCUSSION

The major finding in the present study, vitamin E -decreased infarct size after I/R injury in murine AIDS, strongly supports the hypothesis that vitamin E could reduce the PMN-endothelial cell interaction, block of PMN migration, and PMN sequestration which contributes to I/R injury. Vitamin E may exert its benefits on I/R injury through several different ways: (1) an increased resistance of LDL to oxidation [20]; (2) a decreased production of several chemokines IL-6, 8, monocyte chemoattractant protein-1 (MCP-1) that are involved in the recruitment of PMNs to the site of stimulation [16,17]; (3) inhibition of CD11b/CD18 adhesion molecule expression on leukocytes, ICAM-1, vascular cell adhesion molecule—1 (VCAM-1), E-selectin adhesion molecules on endothelial cells [18-20]; and (4) influence platelet activation via arachidonic acid metabolism and PLC pathway [22,23].

PMNs play a pivotal role in cardiovascular I/R injury [8, 32]. CD11b, a PMN activating marker, is a key molecule involved in the adhesion of PMNs to platelets and vascular endothelial cells. PMN CD11b expression was upregulated in murine AIDS. A higher affinity CD11b expressed on activated PMNs interacts with ICAM-1 on cardiovascular
endothelial cells to facilitate PMN accumulation in the heart. Accumulated PMNs may release the ROS and proteolytic enzymes that directly destroy heart tissue. Destruction can occur while the PMNs are intravascular. Elevated proinflammatory mediators such as TNF-α, IL-1, IL-6, PAF [32-39] in murine AIDS may further activate PMNs and amplify myocardial injury. TNF-α not only reduced heart contractility but also induced myocardial apoptosis with PAF as a cofactor [40,41]. IL-1 and IL-6 have a myocardial suppressive effect [42,43]. ROS produced by activated PMNs is a harmful mediator to induce myocardial apoptosis. Overall, murine AIDS hearts are at increased risk for suffering severe I/R injury. In addition, PMNs may become more reactive during an I/R injury in murine AIDS. There are no direct reports of up-regulated ICAM-1 of endothelial cells in AIDS, but several investigators demonstrate an increased ICAM-1 expression on B cells in murine AIDS [44] and T cells in HIV-infected individuals [45]. PMN marginated adjacent to the endothelium after I/R. These results imply that LP-BM5 infected mice may also up-regulate ICAM-1 on endothelial cells. Thus, in our study, highly activated PMNs with CD11b expression and ROS production could be related to larger infarct size during I/R in murine AIDS. However, PMN ROS production is also necessary for its killing activity. After one month of vitamin E supplementation in murine AIDS, the I/R injury significantly decreased with the inhibition of PMN CD11b expression. PMN ROS production was not affected by vitamin E supplementation. This result indicates that PMN CD11b expression and ROS production may respond to different dosages of vitamin E. Vitamin E (1600IU/kg) in the diet is sufficient to attenuate I/R injury with minimum effect on PMN killing activity. PMN CD11b down-
regulation by vitamin E treatment may be due to normalizing the production of proinflammatory mediators.

Platelets also may be involved in I/R injury in murine AIDS. Their deleterious effects on I/R injury may mainly focus on the first stage of ischemia injury. Upon platelet activation, CD62p is translocated from α granule membranes to the surface of plasma membranes and mediates platelet adhesion to vascular endothelial cells and PMNs. Platelet-PMN and endothelial cell conjugation may plug coronary capillaries, mechanically blocking blood flow. Activated platelets may release soluble mediators that induce further PMN activation. Indeed, several investigators [10-15] found that activated platelet released IL-1, IL-8, macrophage inflammatory protein (MIP)-1α, RANTES, PAF. All these mediators are potent PMN activators. They may modulate PMN adhesion molecule expression and amplify PMN inflammatory response to the late stage of reperfusion injury. Our data demonstrated that platelet CD62p was up-regulated in murine AIDS. Vitamin E treatment did not affect platelet CD62p expression but decreased the infarct size after I/R injury. Here are several explanations. First, vitamin E may attenuate platelet releasing inflammatory mediators without effecting platelet CD62p expression. Platelet-induced I/R injury may mainly act on the first stage of ischemia injury. Second, platelet CD62p may be not involved in the late stages of reperfusion injury [46], and vitamin E reduced I/R injury mainly may mediate the inhibition of PMN CD11b expression.
In summary, the present study clearly demonstrated that the myocardium in murine AIDS exhibited an exaggerated response to acute myocardial I/R injury. Vitamin E, an adjuvant therapeutic agent, attenuates myocardial I/R injury in murine AIDS due to modified PMN adhesion molecule expression.
5.6. LIST OF FIGURES

Figure 5.1. PMN CD11b expression with or without fMLP stimulation.

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.05 in murine AIDS compared vitamin E treated to untreated groups.

Paired test compared unstimulated to fMLP stimulated groups. P<0.05 in the control, vitamin E treated murine AIDS groups.
Figure 5.2. PMN ROS production with or without fMLP stimulation.

ANOVA with Newman-Keuls post-hoc testing among the groups, $P<0.05$ in the control versus murine AIDS in the absence or presence of vitamin E treatment.
Figure 5.3. Platelet CD62p expression with or without ADP stimulation.

ANOVA with Newman-Keuls post-hoc testing among the groups, P<0.01 in the control verse murine AIDS and murine AIDS with vitamin E treatment. No difference in murine AIDS with or without vitamin E treatment. Paired test in the same groups between ADP stimulated and unstimulated platelets. P<0.001 in all three groups.
Figure 5.4. Heart rate (A), systolic (B) and diastolic blood pressure (C) during ischemia-reperfusion periods.

X-axis represented as I/R times. O-0 was initial occlusion time. O-1 was 10-minute occlusion, O-2 was 20-minute occlusion, and O-3 was 30-minute occlusion. R represented reperfusion. R-0 was initial reperfusion time, R-15 was 15-minute reperfusion. These parameters were recorded every 15 minutes until the total 120-minute reperfusion finished.
Figure 5.5. Infarct size and area at risk after 30-minute occlusion and 120 minute reperfusion.

Control n=9, murine AIDS n=8, murine AIDS treated Vitamin E n=6. For infarct/area at risk, P<0.01 in vitamin E treated murine AIDS verse murine AIDS. P<0.05 in the control verse murine AIDS. No significant difference in the area at risk of heart among the all three groups.
Figure 5.6. Representative infarct size images after 30-minute ischemia and 120-minute reperfusion.

Up left image was represented the control heart. Up and low right images were represented the heart from one month of murine AIDS with vitamin E treatment. Low left image was represented the heart from one month of murine AIDS mice.
Figure 5.7. PMN trafficking in hearts after 30 min of myocardial ischemia and 120 min of reperfusion.

No PMNs were found in the heart of sham group. No significant group difference existed in PMN trafficking per section of the left ventricle. Clustered PMNs within epicardial blood vessels were observed in murine AIDS hearts.

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SUMMARY AND CONCLUSION

Our present study demonstrated that PMNs were chronically activated in murine AIDS and the PMNs response to bacterial infection was decreased in the later stages of AIDS in the presence or absence of chronic ethanol consumption, or chronic ethanol consumption alone. PMN CD11b expression and ROS production may become promising prognostic markers to predict progressive murine AIDS. Hyperactivated PMNs accumulating in hearts may contribute to high incident cardiomyopathy in AIDS.

Circulating platelets were activated and more PMP formation was observed in the late stage of murine AIDS. Chronic ethanol consumption caused persistent platelet activation and PMP formation. The mechanism of ethanol-induced platelet activation may differ from retrovirus infection. Platelet CD62p and PMPs could bring important pathological consequences. Cardiovascular manifestation in AIDS may be related to previously unrecognized consequences via platelet CD62p expression and PMP formation.

Retrovirus could directly or indirectly perturb the integrity of coronary endothelium. Altering coronary microcirculation may contribute to cardiovascular manifestation in AIDS. The structural and functional changes in cardiovascular endothelium lead an increase of coronary permeability to macromolecules. Chronic moderate ethanol consumption may preserve the endothelial cellular function. However, the structural destruction by retrovirus is irreversible.
Murine AIDS mice could not endure an acute ischemic attack when the disease was in the progressive stage. Even though early stage murine AIDS hearts survived during ischemia-reperfusion injury, myocardium in murine AIDS exhibited an exaggerated response to acute ischemia-reperfusion injury. Chronic ethanol consumption attenuated acute ischemia-reperfusion injury but did not totally remove the deleterious effects on hearts due to retrovirus infection. Vitamin E also attenuated ischemia-reperfusion injury in murine AIDS likely due to modified PMN adhesion molecules expression and reduced tissue accumulation. Vitamin E may become an adjuvant therapeutic agent for AIDS-induced myocardial injury because of its high degree of safety and tolerability.
APPENDIX.

CALCULATION OF INFARCT SIZE AND AREA AT RISK
1 mm² = 2254 pixels

Area at risk = 19529 pixels = 19529/2254 = 8.66 mm²

Total area = 40526 - 1323 = 39203 pixels = 39203/2254 = 17.39 mm²

Area at risk (%) = 8.66/17.39 × 100% = 49.7%

Infarct area = 6388/2254 = 2.83 mm²

Total area = 38206 - 438/2254 = 16.76 mm²

Infarct area/total area = 2.83/16.76 × 100% = 16.9%

Infarct area/area at risk = 16.9/49.7 × 100% = 34.04%