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Effect of Bacterial Pneumonia on Lung SIV Replication in Alcohol Consuming SIV Infected Rhesus Macaques

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Abstract

Background—Opportunistic infections in HIV-infected persons have been shown to increase the rate of HIV replication. In populations where prophylaxis against *Pneumocystis* pneumonia is utilized, bacterial pneumonia is now the leading cause of lower respiratory tract infection in HIV+ patients. Our prior studies have shown that chronic alcohol consumption in simian demarcated immunodeficiency virus (SIV) infected rhesus macaques increases plasma viral load set point and accelerates progression to end-stage AIDS. While chronic alcohol abuse is well-known to increase the incidence and severity of bacterial pneumonia, the impact of alcohol consumption on local and systemic SIV/HIV burden during lung infection is unknown. Therefore, we utilized the macaque SIV infection model to examine the effect of chronic ethanol feeding on SIV burden during the course of pulmonary infection with *Streptococcus pneumoniae*, the most commonly identified etiology of bacterial pneumonia in HIV+ and HIV- persons in developed countries.

Methods—Alcohol was administered starting 3 months before SIV_{Mac251} inoculation to the end of the study via an indwelling intragastric catheter to achieve a plasma alcohol concentration of 50–60 mM. Control animals received isocaloric sucrose. Four months after SIV infection, the right lung was inoculated with 2×10^6 CFU *S. pneumoniae*.

Results—Leukocyte recruitment into the lung, pulmonary bacterial clearance, and clinical course were similar between ethanol and control groups. While plasma SIV viral load was similar between groups post-pneumonia, chronic ethanol-fed macaques showed a prolonged increase in SIV RNA in bronchoalveolar lavage (BAL) fluid. Alveolar macrophages isolated from ethanol-fed macaques one day post-pneumonia showed greater nuclear factor kappa beta (NF- κ B) activation.

Conclusions—This study indicates that chronic ethanol feeding results in enhanced local, but not systemic, SIV replication following pneumococcal pneumonia. Increased NF- κ B activity in the setting of chronic ethanol ingestion may play a mechanistic role in this observation.

Keywords

Human immunodeficiency virus; Simian immunodeficiency virus; AIDS; viral load; *Streptococcus pneumoniae*; pneumonia; Nuclear Factor–Kappa Beta

INTRODUCTION

Alcohol abuse and human immunodeficiency virus (HIV) infection are major public health problems in the United States and throughout the world (Karon et al., 2001; Stinson et al., 1997). Several studies have shown an association between alcohol consumption and the risk of becoming infected with HIV (Gilbart et al., 2000; Kalichman et al., 2003; Semple et al., 2003; Simbayi et al., 2004). The incidence of alcohol abuse among HIV-infected individuals is greater than the population as a whole (Lefevre et al., 1995; Miguez et al., 2003). Despite the high incidence of alcohol use among HIV-infected patients, the consequence of heavy alcohol consumption on HIV progression is poorly understood. A propensity for alcohol consumption to increase plasma viral loads has been reported in previous studies in which nonhuman primate were infected with simian immunodeficiency virus (SIV) or a combination of SIV and (simian/human immunodeficiency virus (SHIV) (Poonia et al., 2005). In a subsequent study, we found a higher plasma viral load “set point” following the primary stage of SIV infection and accelerated progression to acquired immune deficiency syndrome (AIDS) in ethanol-treated rhesus macaques (Bagby et al., 2006). This finding is consistent with other studies which associate high viral load set points with more rapid retroviral disease progression (Staprans et al., 1999; Watson et al., 1997).

Alcohol abuse impairs host immune defenses, resulting in a higher incidence and severity of infections, especially pneumonia (Szabo, 1999; Young and MacGregor, 1989; Zhang et al., 2002). Opportunistic infections, particularly of the lung, are a frequent complication of HIV infection (Murray and Mills, 1990b). Although not as widely appreciated, HIV infection is associated with an increased risk of bacterial pneumonia and bronchitis, and recurrent pneumonia is an AIDS-defining diagnosis (Anonymous, 1992). In one retrospective study, it was more common for HIV-infected individuals to be hospitalized for bacterial pneumonia than for *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) pneumonia (Magenat et al., 1991). In a prospective multicenter study of 1,353 HIV infected patients in the United States, bacterial pneumonia was the most common pulmonary complication diagnosed within 18 months of study entry (Wallace et al., 1993). With the widespread use of PCP prophylaxis in developed countries, the incidence of PCP pneumonia has fallen significantly (Morris et al., 2004). In the U.S., bacterial pneumonia is now the leading cause of community-acquired pneumonia in HIV-infected patients, and *Streptococcus pneumoniae* is the most commonly identified bacterial pathogen in this cohort (Morris et al., 2011; Siemieniuk et al., 2011). Furthermore, persons infected with HIV currently account for at least 40% of all adult cases of invasive pneumococcal disease in the United States (Nuorti et al., 2000). Pneumococcal disease can occur early in the course of HIV infection and is frequently recurrent. Alcohol consumption is a well-known risk factor for bacteremic pneumococcal pneumonia in the non-HIV population and was identified as a risk factor for invasive disease and death in HIV infected patients with pneumococcal bacteremia (Koziel et al., 1999; Nuorti et al., 2000).

HIV-associated opportunistic infections such as *Mycobacterium tuberculosis*, *Mycobacterium avium* complex (MAC), cytomegalovirus, *Cryptococcus neoformans*, and *P. jirovecii* increase HIV RNA levels and contribute to the pathogenesis of HIV disease and the associated immune dysfunction (Koziel et al., 1999). However, the effect of such infections on viral replication in physiologic compartments such as the lung in the context of alcohol abuse is unknown. Therefore, the current study was undertaken in SIV infected rhesus macaques to determine the effect of chronic binge alcohol consumption on the viral response during pulmonary *S. pneumoniae* infection.

MATERIALS AND METHODS

Animals

This study was conducted at the Tulane National Primate Research Center (TNPRC) in Covington, LA on 24 male rhesus macaques (*Macaca mulatta*) of Indian origin that were 4–6 years of age. Institutional Animal Care and Use Committees at both TNPRC and Louisiana State University Health Sciences Center in New Orleans (LSUHSC-NO) approved animal experimental procedures. Macaques selected were determined to be simian retrovirus and simian T-lymphotropic virus-1 free based on assays performed by the Pathogen Detection Laboratory (California National Primate Research Center, Davis, CA). In vitro infection of peripheral blood mononuclear cells (PBMC) by SIVmac251 was used to select animals that were slow progressors as previously described (Bagby et al., 2006). Macaques were fed a commercial primate chow supplemented with fruit and provided water ad libitum throughout the study. Animals received ethanol or sucrose via a permanent indwelling intragastric catheter that was attached to a cage mounted swivel via a tether as previously described (Bagby et al., 2003). Briefly, ethanol was administered via the gastric catheter to achieve plasma ethanol concentrations of 50 to 60 mM starting 3 months before SIV infection and continuing for the duration of the study. Some alcohol (N=8) or sucrose (N=8) treated macaques received alcohol or sucrose over a 5 h period 4 consecutive days each week for the duration of the study. Others received alcohol or sucrose daily (4 alcohol and 4 sucrose treated macaques). In this case, 30% ethanol was given as a 0.5 h intragastric infusion. Regardless, each ethanol-treated animal received 13–14 g ethanol per Kg/wk. A blood sample was obtained weekly in order to adjust infusion rates so that plasma alcohol concentrations were between 50 to 60 mM.

Three months after initiating ethanol or sucrose administration, all study animals received 10,000 times the dose expected to infect 50% of animals (ID₅₀) SIVmac251 i.v. at the conclusion of an ethanol or sucrose session.

Intrapulmonary *S. pneumoniae* inoculation

Approximately 4 months after SIV inoculation animals received 2×10^6 colony forming units (cfu) of *S. pneumoniae* (serotype 19F) suspended in 2 ml saline into a subsegment of the right lower lobe using a pediatric bronchoscope as previously described (Philipp et al., 2006). Inoculations occurred 2 hours after starting alcohol or sucrose delivery. Blood samples and bronchoalveolar lavage (BAL) samples were obtained 1, 3, 7, 14, 30, 60 and 90 days after inoculation with *S. pneumoniae*. To perform BAL, the bronchoscope was passed orally into the trachea and wedged in a subsegment of the left lower lobe and then the right lower lobe. Each side was lavaged 3 times with 30cc PBS containing 0.1% dextrose. Cells were recovered for analysis by centrifugation. Fluid was divided into several aliquots and stored at -80 C for later analysis of SIV gagRNA.

Hematology, Lymphocyte Subset Analysis

Complete blood counts and differentials were determined using a Bayer Advia 120 optimized for blood from rhesus macaques. Blood lymphocyte subsets were determined as previously described using fluorochrome-conjugated monoclonal antibodies against human phenotypic cell surface antigens and analyzed on a Becton Dickinson FACSaria flow cytometer (Bagby et al., 2006).

Analytical Procedures

Plasma ethanol was measured using an AM1 Analyzer obtained from Analox Instruments USA (Lunenburg, MA). Plasma SIV gagRNA was measured quantitatively by reverse transcription and real time polymerase chain reaction (real time RT-PCR) using gene

specific primers and FRET probe as previously described (Bagby et al., 2003). Copy number was determined using a standard curve of previously cloned SIVgag cRNA. The lower limit of detection was 10 copies per reaction. Protein in BAL fluid was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Cytokine concentrations in BAL fluid were determined using a multiplexed cytokine bead array assay (Millipore, Billerica, MA) as analyzed on a BioPlex device (Bio-Rad, Hercules, CA). The cytokines that were assayed include TNF α , IL-1 β , -6, -8, -12, & -17a, MIP1 α , MIP1 β , IFN γ , and G-CSF.

Alveolar Macrophage NF- κ B Activation

BAL cells were obtained as described and plated at 37° C in RPMI 1640 + 10% FBS. After 30 minutes, non-adherent cells were removed by three vigorous washes with media. Nuclear extracts from the remaining alveolar macrophages were obtained and analyzed for NF- κ B binding activity by chemiluminescent ELISA (Pierce Biotechnology; Rockford, IL).

Statistical Analysis

Data are presented as the mean $\pm$ SEM unless otherwise stated. Statistical analysis was performed using SAS/STAT software (SAS Institute Inc. 2002. SAS OnlineDoc® 9. Cary, NC). With the exception of BAL fluid cytokine analysis, longitudinal data were tested for treatment and time differences using analysis of variance (ANOVA) for Mixed and Random Effect Models (AVMREM) with repeated measures and model fitting by restricted maximum likelihood. When differences were found, a Tukey-Kramer range test (Kramer, 1956) was used to identify differences for appropriate comparisons. For BAL fluid cytokines overall time effects were tested with the Friedman's test, pre vs day comparisons were assessed with the Signed Rank Test, and group differences were assessed with the Wilcoxon Rank Sum test.

RESULTS

With the exception of one alcohol treated animal that was euthanized for signs of end-stage SIV disease, 23 animals survived the first 4 months of SIV infection and therefore received an intrapulmonary inoculation with *S. pneumoniae*. The dose of *S. pneumoniae* chosen was expected to produce a meaningful infection but with low mortality. During the first week of the lung infection, one sucrose and 2 alcohol treated animals were euthanized because of the lung infection. Thereafter, 2 additional sucrose and 2 alcohol treated animals were euthanized either as a consequence of pneumonia or the SIV infection. Thus, 7 and 8 alcohol and sucrose treated animals, respectively, survived to the completion of the observation period (7 months post SIV inoculation and 3 months after *S. pneumoniae* challenge). In cases where animals were euthanized prior to study completion, the TNPRC end-stage criteria were followed as previously described (Bagby et al., 2006).

Fig. 1 summarizes plasma ethanol concentrations and body weight from pre-SIV inoculation. Differences between animals receiving alcohol 4 consecutive days each week or daily were not evident in any of the data so cohorts were combined. Plasma ethanol was maintained close to the target range of 50–60 mM. Body weight did not differ between alcohol and sucrose treated animals, and steadily increased (Bagby et al., 2006) throughout the observation period in both treatment groups.

Similar to our previous study (Bagby et al., 2006), plasma viral loads were higher in our alcohol treated animals compared to the sucrose treated animals prior to *S. pneumoniae* (Fig. 2). An alcohol treatment effect was observed when day 7 through 120 data were analyzed ($p < 0.05$). Time-matched differences based on the analysis of variance and the Tukey Kramer Range Test or t-test did not differ. Total leukocyte, PMN leukocytes and

lymphocyte counts remained in the normal range during the first 4 months of SIV infection and did not differ between sucrose and alcohol treatment groups (data not shown). During the first 120 days of infection CD4⁺ T cells decreased as predicted (Fig. 3). Decreases were similar between sucrose and alcohol treated animals.

In response to *S. pneumoniae* lung infection, total blood leukocyte counts increased similarly in both sucrose and alcohol treated animals (data not shown), largely as a result of neutrophilia (Fig. 4). Whereas no difference was evident between sucrose and alcohol treated animals in blood PMN counts, the response was quite variable with 6 of 12 sucrose and 3 of 10 alcohol treated animals having values greater than 10⁴ counts/ μ l one day after receiving *S. pneumoniae*.

To minimize the lethal consequence of bacterial lung infection and to assess the local consequence of lung infection on viral production, bacteria were only delivered to the right lung. As expected, the increase in PMN leukocytes recovered by BAL was greater from the infected right lung than the left lung (Fig. 4). In the right lung, neutrophil recruitment was a significant component of the increase in total cells recovered, but macrophage numbers were also significantly increased (data not shown). The increase in BAL cells was transient and typically returned to pre-infection levels within 7 days after the bacterial challenge. Statistical differences were not evident between alcohol and sucrose treated animals. The increase in neutrophil numbers tended to be attenuated one day after *S. pneumoniae* inoculation in the alcohol treated animals, but this proved to be insignificant owing to the variable response. Bacterial CFU in BAL fluid were variable among individual animals, such that no significant differences in CFU were found between sucrose and alcohol groups. One day after inoculation, *S. pneumoniae* CFU were higher in BAL collected from the right lung (3.9 ± 0.7 and $4.9 \pm 0.6 \log_{10}$ CFU/ml) than the left lung (2.6 ± 0.6 and $2.6 \pm 0.6 \log_{10}$ CFU/ml) for sucrose and alcohol treatment groups, respectively. These differences were less evident by day 3. Whereas infection was typically controlled within 7 days, low levels of *S. pneumoniae* ($\sim 10^2$ cfu/ml) remained detectable in BAL fluid from both the right and left lungs of some animals 4 weeks after initiating the infection.

Based on previous studies (Bush et al., 1996; Israel-Biet et al., 2004; Kohli et al., 2006; Zhang et al., 1995), we expected and found pulmonary SIV viral load to be increased in response to the *S. pneumoniae* challenge. Immediately prior to bacterial challenge no differences existed in BAL SIV RNA concentrations between sucrose and alcohol treated animals though alcohol treated animals tended to have higher values. The bacterial challenge increased BAL SIV gagRNA copies in the right lung, while the increase in the left lung was not statistically significant (Fig. 5). Increases were evident one day after *S. pneumoniae* challenge in both sucrose and alcohol treated animals. Data analysis showed that alcohol consumption significantly prolonged the increase in BAL SIV gagRNA compared to the response in sucrose treated animals. When right lung data were analyzed from pre-pneumonia through day 14 post-pneumonia, there was a significant difference between alcohol and sucrose treated animals in that BAL SIV gagRNA in the alcohol-treated animals was increased compared to pre-bacterial challenge. In contrast, right lung BAL viral load in the sucrose treated animals had statistically returned to pre-bacterial values by day 3. The observed increase in BAL fluid SIV RNA in response to lung infection did not correspond with an increase in plasma viral load in either sucrose or alcohol treated animals (Fig. 2). However, plasma viral load was increased in alcohol treated animals compared to sucrose treated animals from pre-*S. pneumoniae* inoculation through day 14 of lung infection.

To address the possibility that the increase in lung SIV during pneumonia was caused by translocation of plasma virus, protein in BAL fluid was determined to assess lung permeability. Whereas an increase in protein was evident in some BAL samples, the

majority of values were less than 2 mg/ml (86%), and of those above 2 mg/ml only a single sucrose animal also had a BALF SIV RNA value in the upper 1/3 of values detected. Correlation analysis failed to identify a relationship between BAL protein and SIVgag RNA ($r = 0.28$ and 0.30 in right and left lung BALF, respectively, $p > 0.05$).

Because immune activation promotes SIV replication, we assessed the lung host response to the *S. pneumoniae* infection by measuring NF- κ B and 10 different cytokines in BAL cells and fluid, respectively, collected prior to and at different times after challenge. Activation of NF- κ B has been shown to drive HIV replication (Rennard et al., 2000), and bacterial pneumonia is a strong inducer of this classic pro-inflammatory transcription factor (Sha et al., 1995). We therefore assessed the activation of NF- κ B in alveolar macrophages from animals before and following bacterial pneumonia. Prior to pneumococcal infection, there were no differences in NF- κ B activation between alcohol and sucrose groups (Fig. 6). One day following inoculation, increases in NF- κ B activity were seen in the right (infected) lung for both alcohol and sucrose animals, with a trend toward a greater response in the alcohol animals. Alveolar macrophage NF- κ B activity returned to near baseline levels by day 3, with no further apparent differences between alcohol vs. sucrose groups or between infected vs. non-infected lung.

To determine if chronic alcohol feeding affects the local cytokine response to bacterial lung infection in SIV-infected macaques, we measured BAL fluid concentration of 10 cytokines before and during the course of pneumonia. The six cytokines with the highest response to lung infection in SIV infected animals are shown in Fig. 7. The other cytokines (IL-1 β , IL-17a, MIP-1 α , IFN γ) mostly remained below the limit of detection during the observation period. TNF α , a known activator of NF- κ B, was transiently increased in alcohol treated animals, but as with NF- κ B, the difference was not significant ($p < 0.05$). Likewise, differences between sucrose treated and alcohol treated animals for other cytokines that increased with the lung infection were not statistically significant ($p < 0.05$).

DISCUSSION

The lung, in order to perform its primary function of gas exchange, represents the largest mucosal/epithelial surface area of the body in direct contact with the external environment. Hence, the lung is repeatedly challenged with a multitude of potential pathogens on a regular basis. Considerable work has detailed the deleterious effects of ethanol intoxication on multiple facets of pulmonary host defenses (Happel et al., 2004; Happel and Nelson, 2005; Zhang et al., 2002). As alcohol use disorders are prevalent in HIV+ persons, it is critical to understand the consequences of alcohol abuse on common lower respiratory tract infections, such as bacterial pneumonia, in the setting of HIV infection.

SIV infection of the rhesus macaque is an excellent animal model of HIV infection, with clinical features of opportunistic infection, wasting, and end-organ dysfunction closely resembling that of HIV infection, as well as the advantage of more rapid disease progression. Previously, we have shown that chronic ethanol feeding in rhesus macaques increases the plasma viral load “set point,” and these animals also progress to end-stage AIDS more rapidly than sucrose control subjects (Bagby et al., 2006). Prior studies in the rhesus macaque model performed by our group and others have also observed an increased plasma viral load during the early course of infection (Poonia et al., 2005). The current study is consistent with these prior results in that SIV viral load set-point (prior to pneumococcal infection) was significantly higher in the alcohol-fed group (Fig. 2). Also similar to previous studies, alcohol animals showed similar declines in peripheral CD4+ T cells, and CD4+ T cell subsets (e.g. central and effector memory T cells, data not shown) compared to control animals (Fig. 3). Despite several experimental differences, these studies are concordant in

their finding of increased retroviral load in chronic alcohol-fed macaques. These studies further identify that this effect of alcohol occurs early following SIV infection, an observation which would be difficult to study in the early, asymptomatic stage of HIV disease.

Our current work adds to these previous studies by identifying that chronic ethanol consumption enhances SIV production in the lung during pneumococcal pneumonia, an effect which persists well beyond the resolution of bacterial infection (Fig. 5). Prior to inoculating animals with *S. pneumoniae* BAL fluid SIV RNA did not differ ($p > 0.05$) between sucrose and alcohol SIV infected animals. This differed from plasma SIV RNA during the 3+ months prior to *S. pneumoniae* inoculation although a time-matched significant difference in plasma SIV RNA was also not present just prior to lung infection (Fig. 2). This also differs from previous reports that identified an increase in SIV RNA in gut and lymphoid tissues, and cerebral spinal fluid (Kumar et al., 2005; Poonia et al., 2006). During the early period of lung infection, the increase in plasma viral load seen in the alcohol compared to the sucrose treated animals during the first 4 months of SIV infection continued during the first 14 days after the lung *S. pneumoniae* challenge. However, plasma viral load did not increase as a result of the lung infection in either alcohol or sucrose treated animals ($p > 0.05$; Fig. 2). This finding is consistent with findings in HIV+ patients (Koziel et al., 1999).

Opportunistic infections, particularly of the lung, are frequent complications of HIV infection (Morris et al., 2011; Murray and Mills, 1990a). Although pneumonia with *Pneumocystis jirovecii* was at one time the predominant lung pathogen in HIV infected individuals, the widespread use of PCP prophylaxis has greatly reduced case infection rates among HIV+ populations. *S. pneumoniae* is the most commonly identified bacterial pathogen causing pneumonia in PCP-prophylaxed HIV+ populations, such that recurrent bacterial pneumonia is now an AIDS-defining illness (Madeddu et al., 2010; Schneider et al., 2008; Siemieniuk et al., 2011). Pneumococcal disease can occur early in the course of HIV infection and is frequently recurrent. Alcohol abuse is a well-known risk factor for bacteremic pneumococcal pneumonia in the non-HIV population and is a risk factor for invasive disease and death in HIV infected patients with pneumococcal bacteremia (Nuorti et al., 2000). Alcohol abuse is common in the HIV population (Lefevre et al., 1995; Miguez et al., 2003), and some of the mechanisms by which alcohol impairs pulmonary host defenses include inhibition of proinflammatory cytokine and chemokine production by macrophages, migration of neutrophils to sites of infection, and pathogen killing (MacGregor and Louria, 1997; Zhang et al., 2002). Thus, it is plausible that the immunocompromised state caused by alcohol abuse in the HIV+ host would predispose to earlier and more frequent opportunistic infections.

Although the long-term consequences of secondary pulmonary infection on HIV progression remain under active investigation, several studies have demonstrated that HIV-associated pulmonary infections including *Mycobacterium tuberculosis*, *Mycobacterium avium* complex, cytomegalovirus, *Cryptococcus neoformans*, and *Pneumocystis jirovecii* pneumonia, increase HIV RNA levels and contribute to disease progression and immune dysfunction (Bush et al., 1996; Day et al., 2004; Kohli et al., 2006; Koziel et al., 1999; Orenstein et al., 1997; Shaunak et al., 2001; Sogaard et al., 2009; Sulkowski et al., 1998; Zhang et al., 1995). In SIV infected macaques, coinfection with *Mycobacterium bovis* bacilli Calmette-Guerin (BCG), significantly accelerates the course of SIV disease (Young and MacGregor, 1989). Furthermore, a recent review of studies investigating the effect of treating co-infections on HIV progression identified a consistently beneficial effect on HIV disease course (Modjarrad and Vermund, 2010). Taken together, these results highlight the importance of secondary infection in the context of HIV infection, as well as the need to

better understand the ramifications of alcohol abuse in HIV, given ethanol's well-characterized effects on host defenses.

It is known that alveolar macrophages are viral targets and are an important reservoir of HIV in the lung (Segal et al., 2011; Sierra-Madero et al., 1994). We also found AM recovered from SIV infected rhesus macaques to be infected with the virus (data not shown). Although antiretroviral therapy suppresses HIV replication in blood cells, it is less successful in eliminating HIV in reservoir cells such as AM (White et al., 1999). Not only do AM serve as a source of HIV to infect other cells in the local milieu, but these infected cells are also likely to be involved in the pathogenesis of HIV induced pneumonitis (Denis and Ghadirian, 1994; Segal et al., 2011). In this regard, alcohol consumption may also alter host defense against SIV/HIV-infected cells resulting in higher viral expression. Although additional work is required in order to identify the specific mechanisms underlying our current observations, to our knowledge this is the only study that has investigated the effect of an opportunistic infection on viral replication in a physiologic compartment (i.e. the lung) in the context of alcohol abuse.

While alcohol consumption significantly enhanced the impact of pneumococcal infection on SIV viral load in the lung, we also found that this increase in SIV viral load was compartmentalized to the infected lung in that changes in plasma viral load as a consequence of lung infection were not evident in either sucrose or alcohol treated animals (Fig. 2). In this study, we chose a bacterial challenge that produced a substantial host response (Fig. 4), but that was effectively contained to the inoculation site and was largely sublethal. Perhaps the limited and transient nature of the infection minimized systemic changes in virus production; whereas in immunodeficient patients recurring infection of the lung may increase viral spread. Similar findings have been reported in HIV infected patients with opportunistic infections (White et al., 1999). This is not surprising as we, and others, have shown that the innate immune response to an intrapulmonary bacterial challenge is largely localized to the infected lung.

Many components of this response to bacterial lung infection (cytokines, chemokines, reactive oxygen species, inflammatory cells) have been shown to promote HIV/SIV replication by activation of NF- κ B. In this regard, it is known that tumor necrosis factor (TNF) stimulates HIV/SIV replication directly through activation of NF- κ B and its binding to the long terminal repeat of the virus (Hoshino et al., 2002; Nabel and Baltimore, 1987; Nakata et al., 1997; Osborn et al., 1989; Poli et al., 1994). HIV viral load and TNF production are strongly correlated ($r^2 > 0.95$) in involved lung segments of AIDS patients with tuberculosis (Nakata et al., 1997). Given our findings of enhanced viral replication in the lungs of alcohol-fed macaques during bacterial pneumonia, it is interesting that we observed greater NF- κ B activation in alveolar macrophages lavaged from this group at day 1 post-pneumonia (Fig. 6). These results are consistent with earlier work showing chronic ethanol exposure enhances nuclear translocation of NF- κ B in hepatic Kupffer cells (Bautista, 2002). More recently, *in vitro* studies showed increased NF- κ B in peripheral blood mononuclear cells chronically incubated in ethanol, likely due to ethanol-mediated impairment in IL-1R-associated kinase-monocyte (IRAK-M) signaling (Mandrekar et al., 2009). Because we were only able to measure NF- κ B in a subset of study animals it is not possible to make a definitive statement about the observed differences. Notwithstanding, the available data support the concept of NF- κ B facilitating the enhanced viral replication in the lungs of alcohol fed animals. Since NF- κ B activation in alveolar macrophages appeared limited to 1 day post-infection, it is possible that early but transient activation of NF- κ B in alveolar macrophages is sufficient to drive sustained viral replication in the lung, as these cells are long-lived viral reservoirs (Blankson et al., 2002). It is also plausible that enhanced replication in ethanol-fed macaques occurs in other cells in the lung or that NF- κ B

independent mechanisms may be operant in pulmonary SIV replication during bacterial infection.

Consistent with our NF- κ B data, the pro-inflammatory cytokine TNF α recovered in the BAL fluid showed a similar though not significantly different response in alcohol compared to sucrose animals one day post-pneumonia. Likewise, high variability of the other cytokines that responded to the lung infection impaired our ability to see any association between the host and viral response. Perhaps the timing of measures or the site (BAL vs tissue) also prevented us from showing an association. Because pre-pneumonia levels of cytokines were below the limit of detection and NF- κ B activity in AM were similar between sucrose and alcohol treated animals, we speculate that chronic alcohol feeding more likely increases viral replication as a result of enhanced cellular inflammation rather than through impairment of the host response to the bacterial pneumonia, since no differences were detected in cytokine expression or neutrophil recruitment following pneumococcal infection.

In summary, our results show that chronic alcohol feeding results in greater SIV replication in the lung during intrapulmonary challenge with *S. pneumoniae* in SIV-infected rhesus macaques. Since alcohol abuse is common in HIV-infected persons, further study of the mechanisms by which alcohol modulates host viral responses during disease progression, including secondary infections, is warranted.

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Fig. 1. End-session plasma ethanol concentrations and body weight after inoculation with 10,000 times the ID₅₀ of SIVmac251. “P” refers to time prior to i.v. SIV inoculation or 2×10^6 CFU *S. pneumoniae* inoculation into the right lung which was administered at 4 months after SIV. Data are expressed as mean $\pm$ SEM (N = 7–11). Analysis of variance (ANOVA) for Mixed and Random Effect Models (AVMREM) did not detect differences in body weight among groups before or after *S. pneumoniae*.

Fig. 2.

Plasma SIV viral load after inoculation with SIVmac251. “P” refers to time prior to i.v. SIV inoculation or *S. pneumoniae* inoculation into the right lung which was administered at 4 months after SIV. Data are expressed as mean $\pm$ SEM (N = 7–12). * Analysis revealed an alcohol effect during the first 120 days ($p < 0.05$). Differences between groups were not statistically significant at any individual time point. † Analysis revealed an alcohol effect during first 14 days of lung bacterial infection ($p < 0.05$). No difference existed between pre- and post-lung infection ($p > 0.05$) in sucrose or alcohol treatment groups.

Fig. 3.

Blood CD4+ and CD8+ T cell numbers and CD4+/CD8+ ratio after inoculation with SIVmac251. "P" refers to time prior to i.v. SIV inoculation or *S. pneumoniae* inoculation into the right lung which was administered at 4 months after SIV. Data are expressed as mean $\pm$ SEM (N = 7–12). ¥ CD4+ cell numbers and CD4+/CD8+ ratio were persistently decreased after SIV inoculation to include period after lung infection (p < 0.05).

Fig. 4.

Blood PMN counts and lung PMN counts recovered by bronchoalveolar lavage in SIV infected macaques prior to and after *S. pneumoniae* inoculation. “P” refers to values obtained 1 week prior to *S. pneumoniae* inoculation. Data are expressed as mean $\pm$ SEM (N = 7–12). * refers to statistical difference compared to pre-pneumonia ($p < 0.05$). ‡ refers to statistical difference compared to left lung ($p < 0.05$). No significant differences existed between ethanol and sucrose treated animals.

Fig. 5.

Left and right lung BALF SIV RNA prior to and after right lung inoculation with *S. pneumoniae*. "P" refers to values obtained 1 week prior to *S. pneumoniae* inoculation. Data are expressed as mean $\pm$ SEM (N = 7–12). * refers to statistical difference compared to pre-pneumonia (*, $p < 0.05$). † refers to statistical difference between time-matched analysis values between sucrose and alcohol treated animals ($p < 0.05$).

Fig. 6. NF-κB binding activity in nuclear preparations from alveolar macrophages obtained by BAL of left and right lungs prior to and after *S. pneumoniae* inoculation into the right lung. Differences were not statistically significant (N = 3 to 4 per group per time point), but trended toward a greater increase in the ethanol group one day after infection.

Fig. 7.

Cytokine concentrations in BAL fluid collected from the right lung (site infection) prior to and after *S. pneumoniae* inoculation into the right lung. Data expressed as mean only (N = 7–12), BAL fluid samples with undetectable concentrations of cytokines were given a value of zero. * and † signifies statistical increase compared to pre-*S. pneumoniae* inoculation ($p < 0.0167$, Bonferroni correction) within the sucrose and alcohol treatment groups, respectively. No time-matched differences were detected between sucrose and alcohol treatment groups.