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Chronic binge alcohol administration increases intestinal T cell proliferation and turnover in rhesus macaques

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Abstract

Background—Alcohol use results in changes in intestinal epithelial cell turnover and microbial translocation, yet less is known about the consequences on intestinal lymphocytes in the gut. Here we compared T cell subsets in the intestine of macaques before and after 3 months of chronic alcohol administration to examine the effects of alcohol on intestinal T cell subsets.

Methods—Rhesus macaques received either alcohol or isocaloric sucrose as a control treatment daily over a 3 month period via indwelling gastric catheters. Intestinal lymphocyte subsets were identified in biopsy samples by flow cytometry. Twenty-four hours prior to sampling, animals were inoculated with BrdU to assess lymphocyte proliferation. Immunohistochemistry was performed on tissue samples to quantitate CD3+ cells.

Results—Animals receiving alcohol had increased rates of intestinal T cell turnover of both CD4+ and CD8+ T cells as reflected by increased BrdU incorporation. However, absolute numbers of T cells were decreased in intestinal tissues as evidenced by immunohistochemistry for total CD3 expression per mm² intestinal lamina propria in tissue sections. Combining immunohistochemistry and flow cytometry data showed that the absolute numbers of CD8+ T cells were significantly decreased, whereas total of CD4+ T cells were minimally decreased.

Conclusions—Collectively, these data indicate alcohol exposure to the small intestine results in marked loss of CD3+ T cells, accompanied by marked increases in CD4+ and CD8+ T cell proliferation and turnover, which we speculate is an attempt to maintain stable numbers of T cells in tissues. This suggests alcohol results in accelerated T cell turnover in the gut, which may contribute to premature T cell senescence. Further these data indicate that chronic alcohol administration results in increased levels of HIV target cells (proliferating CD4+ T cells) that may support higher levels of HIV replication in intestinal tissues.

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Keywords

AIDS; animal models; chronic binge drinking; ethanol; rhesus macaque; mucosal immunology; T cell

Introduction

The stomach and upper small intestine are the major sites of ingested alcohol absorption. Excess alcohol consumption is associated with a myriad of gastrointestinal disturbances including maldigestion, malabsorption, bacterial overgrowth, hypermotility, and even upper intestinal physical damage including ulcerations and inflammation (Chiang et al., 2012). It is also increasingly recognized that intestinal lymphocytes, especially T cells, are critical for regulating intestinal homeostasis along with mucosal architecture and epithelial barrier function (Hepworth et al., 2013, Barnes and Powrie, 2009). However, T cell dynamics in intestinal tissues are rarely examined in humans, or in relevant animal models of alcohol use. Intestinal lymphocytes represent the largest single pool of lymphocytes in the body, as they are distributed throughout the intestinal tract (Lackner and Veazey, 2007). They are found as highly activated T cells and terminally differentiated plasma cells scattered throughout the diffuse lamina propria of the gut where “effector” functions occur. In addition, they are also found in a vast reservoir of organized lymphoid follicles known as the gut associated lymphoid tissue (GALT) that increase in frequency in the terminal small intestine (ileum) and throughout the large intestine, including the colon, cecum, appendix, and rectum (Lackner et al., 2009). This design promotes effective activation or programming of immune responses to antigens encountered in both the proximal and distal intestinal tract, and provides a mechanism for immunologic memory and homing of antigen specific lymphocytes that can disperse throughout the intestinal tract after encountering their cognate antigen (von Andrian and Mempel, 2003). Intestinal lymphocyte homeostasis is thus highly regulated, and changes in these subsets or proportions may dramatically affect immune responses and disease processes.

The direct effects of alcohol are known to be involved in the pathology of various alcohol-related gastrointestinal diseases. Chronic or excessive alcohol use induces intestinal epithelial injuries that allow large molecules such as endotoxins and even intact microbes to pass through the mucosal barrier in the blood stream in a process known as bacterial translocation. This results in mucosal inflammation and fibrosis of tissues, such as the liver (Elamin et al., 2013). Accumulating evidence indicates bacterial translocation also has a deleterious effect on HIV infection, as it leads to chronic systemic immune activation, which drives local and systemic viral replication (Brenchley et al., 2006). A number of studies have focused on the effects of alcohol on intestinal epithelial cells in rodent models (Li et al., 2012, Draper et al., 1983, Fleming et al., 2001), but few have examined the dynamics of intestinal T cell subsets in relevant animal models of chronic alcohol administration. Thus, here we examined the dynamics, turnover, and changes in absolute numbers of T cell subsets in the small intestine of macaques receiving alcohol in a well-established chronic binge alcohol administration (CBA) model.

We have used the rhesus macaque model of CBA to examine the effects of alcohol and simian immunodeficiency virus SIV infection as a model for human alcohol use disorder (AUD) and HIV infection for several years (Bagby et al., 1998). Using this model, we previously showed that alcohol use increases early peak SIV viral loads and set points, and accelerates progression to end-stage disease (AIDS) (Molina et al., 2008, Bagby et al., 2006, Poonia et al., 2005, Bagby et al., 2003). However, the mechanisms by which alcohol induces immunologic changes in the intestinal tract are largely unexplored. Further, prior studies of mucosal immunology rely on flow cytometry of cell suspensions prepared from mucosal tissues, and quantifying changes in absolute numbers of cell subsets in intestinal tissues are difficult to perform. In previous studies, we showed that alcohol increases percentages of central memory CD4+ T cells in the small intestine, which may explain higher viral loads in animals receiving alcohol (Poonia et al., 2006). Here, using a novel method for assessing S-phase cell division, and quantitative image analysis of T cells in tissues sections, we examined the proliferation rate and phenotype of small intestinal T cells of macaques chronically receiving alcohol and compared them to those receiving isocaloric sucrose as controls. By combining multicolor flow cytometry with immunohistochemistry and morphometric analysis of intestinal tissues, we determined changes in absolute numbers of T cell subsets in intestinal tissues of macaques receiving alcohol or sucrose. Surprisingly, we found decreases in absolute numbers of T cell subsets in intestinal tissues of macaques receiving alcohol, accompanied by markedly increased cell proliferation and turnover of both CD4+ and CD8+ T cell subsets in tissues. Marked losses of T cells, accompanied by accelerated turnover of these T cell subsets in mucosal tissues may be linked to the intestinal damage attributed to chronic or excessive alcohol use, and may partially explain some of the co-morbidities associated with alcohol use and HIV infection.

Materials and Methods

Animals and study design

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at both the Tulane National Primate Research Center (TNPRC) in Covington, LA, and Louisiana State University Health Sciences Center in New Orleans, LA and adhered to the NIH guidelines for the use of experimental animals. Three to six year old male rhesus monkeys (*Macaca mulatta*) obtained from the breeding colonies at the TNPRC were studied as previously described (Bagby et al., 2003).

Age and body weight-matched animals were randomized to either chronic sucrose-fed or alcohol-fed macaques. Alcohol was administered daily for the three-month duration of the study by indwelling gastric catheter as previously described (Bagby et al., 2003). Briefly, animals were fitted with a gastric catheter and catheter-protecting jacket and tether attached to a swivel that allowed animals to move about their cages during alcohol administration. Animals were administered alcohol daily over a 30-minute period via the intra-gastric catheter. Animals received 13 to 14 g ethanol (30% w/v water) per Kg body weight/wk per week for 3 months. Blood alcohol concentrations were monitored weekly 2 hrs after initiating the 30-minute delivery, and adjustments were made to keep BAL between 50-60 mM. Infusion rates (g ethanol/Kg body weight) did not change throughout the experimental

period, suggesting no significant alteration in rate of alcohol metabolism during the study period. Time-matched control monkeys were subjected to the same surgical procedures, but received an isocaloric sucrose infusion. A total of 24 animals in 2 groups consisting of animals regularly receiving alcohol (n=12) or isocaloric sucrose controls (n=12), were studied.

Blood samples were collected throughout the study and endoscopic small intestinal biopsies (jejunum) and lymph nodes were collected prior to start of alcohol administration (baseline) and after 3 months of daily alcohol / sucrose administration. One day (24 hrs) prior to each sampling, animals received 20mg/kg of an intravenous bromo-deoxyuridine (BrdU) solution. BrdU is a thymidine analog, which is incorporated into DNA of actively dividing cells. Thus, only cells in S phase division take up the BrdU, making this a definitive marker of S-phase cell division. We routinely use this technique to definitively identify and quantify S-phase dividing cells in tissues of SIV-infected macaques (Wang et al., 2010, Wang et al., 2013).

Sampling and assays

At least 12 jejunal pinch biopsies were collected by endoscopy as previously described (Poonia et al., 2006). Two biopsies were preserved in formalin for immunohistochemistry and the rest processed to prepare single cell suspensions for flow cytometry. Briefly, biopsies were incubated with 1 mM EDTA in Hanks balanced salt solution for 30 min, followed by 1 hour in RPMI containing 20 U/mL of collagenase while shaking at 37°C. Single cells suspensions were prepared by slowly pipetting 5-10 times with a 16-g feeding needle. Peripheral lymph node biopsies and blood were also collected prior to alcohol and after alcohol administration. Blood samples were stained by a whole-blood staining procedure, and lymph node samples were minced and pressed through nylon screens to produce single cell suspensions for flow cytometry. All cells were stained for flow cytometry with the monoclonal antibodies CD3, CD4, CD8, and anti-BrdU. All antibodies and reagents were purchased from BD Biosciences Pharmingen (San Diego, CA) unless otherwise noted. In a separate stain, CD3, CD4 and CD8 cells were also stained for necrosis and apoptosis using Annexin V and LIVE/DEAD (LD) Fixable Aqua Dead Cell Stain Kit (Invitrogen, Grand Island, NY).

For immunohistochemistry, intestinal biopsies were fixed in formalin, sectioned, and stained with anti-CD3 (Dako, Glostrup, Denmark) to quantify the absolute number of T cells in intestinal sections by image analysis as previously described (Veazey et al., 2000b). In brief, formalin-fixed, paraffin embedded sections were de-paraffinized and antigens “unmasked” using high temperature antigen retrieval consisting of heating slides in a steam bath chamber (Black and Decker Flavor Center Steamer Plus) with 0.01 M citrate buffer, pH 6.0 for 20 min, cooled, and washed twice in PBS. Slides were then washed with PBS and incubated with appropriately diluted CD3 (rabbit polyclonal, Dako Inc.) for 1 hour at room temperature, then washed and then incubated with fast red labeled anti-rabbit IgG (Invitrogen, Carlsbad, CA). Sections incubated with mouse serum alone and with and without primary and secondary antibodies were included as negative controls on paraffin-embedded slides, and no non-specific background staining was detected in controls. Slides

were mounted with fluorescent mounting medium (Dako, Inc.) and imaged using Inform software. At least 10 images (200X) were generated from each biopsy and absolute numbers of positive (CD3+) T cells per mm² intestinal lamina propria were automatically counted. To determine absolute numbers of CD4+ and CD8+ T cell subsets in tissues, absolute numbers of total CD3+ T cells determined in tissues by immunohistochemistry were multiplied by the percentage of the T cell subset of interest by flow cytometry after gating through CD3+ cells.

Statistical analysis

Graphical presentation and statistical analysis of the data were performed using GraphPad Prism v5.0b (GraphPad Software Inc., San Diego, CA). Comparisons between groups in each figure were performed with a Two-way ANOVA with repeated measures for the sucrose and alcohol groups, and P values <0.05 were considered significant.

Results

Flow cytometry revealed striking differences in rates of intestinal T cell proliferation between animals receiving alcohol compared to the sucrose-treated controls. By flow cytometry, we first found significantly higher percentages of proliferating (BrdU+) CD4+ T cells (P<0.05) and CD8+ T cells (P<0.001) in cell suspensions of animals receiving alcohol (**Fig. 1**). Since BrdU incorporation is reflective of S-phase cell division, this indicates that chronic alcohol administration is associated with a significant increase in the *rates* of both CD4+ and CD8+ T cell proliferation.

Surprisingly however, when counting absolute numbers of CD3+ T cells in the proximal small intestine (the same site sampled for flow cytometry) we found a significant decrease in absolute numbers of total T cells per mm² of gut (**Figs. 2, 3A**). Although both sucrose and alcohol groups had identical numbers of T cells/mm² in the gut prior to alcohol/sucrose administration, animals receiving alcohol had significantly lower absolute numbers of total T cells in the gut after 3 months of CBA (**Figs. 2, 3A**). Since CD3 was also included in the flow cytometry staining panels, we were able to extrapolate absolute numbers of CD4+ and CD8+ T cells in the gut by multiplying the percentages of these cell subsets within the CD3+ lymphocyte gate, with the total number of CD3+ T cells per mm² of intestine obtained by quantitative immunohistochemistry. Interestingly, this alcohol effect was primarily attributed to a loss of CD8+ T cells (p<0.001) as the total CD4+ T cells per mm² in the gut were only slightly decreased (**Fig. 3**). These differences were also reflected in the percentages of total CD3+CD8+ and CD3+CD4+ cells detected by flow cytometry. Percentages of CD8+ T cells (of total CD3+ T cells) were markedly decreased (P<0.001) whereas percentages of CD4+ T cells were slightly increased in alcohol treated animals (**Fig. 4**), indicating differential effects of alcohol on these two T cell subsets within intestinal gut tissues. Live/dead (LD) stains also revealed significant differences in rates of T cell subset death. The total percentages of CD4+ and CD8+ T cells that took up the LD stain (total necrotic cells) were markedly and significantly higher after alcohol administration (**Fig. 5A**). However, CD8+ T cells from animals receiving alcohol had significantly higher percentages (8.7% $\pm$ 1.7) of cells undergoing apoptosis (AnnexinV+, LD negative)

compared to rates of apoptotic CD4⁺ T cells (4.9% $\pm$ 1.1) from the same animals ($P < 0.001$). Similarly, the total proportion of necrotic cells (LD⁺) within the CD8⁺ T cell population (7.8% $\pm$ 1.2) was significantly higher ($P < 0.02$) than that of CD4⁺ T cells (6.0% $\pm$ 0.8) in animals receiving alcohol (Fig. 5B). In contrast, no significant changes in T cell turnover were detected in peripheral blood (data not shown).

Discussion

Using a combination of flow cytometry and immunohistochemistry, we found that alcohol decreases absolute numbers of intestinal T cells in the proximal small intestine, the region where ingested alcohol is being absorbed. This is accompanied by marked increases in both CD4⁺ and CD8⁺ T cell S-phase cell division as reflected by increased BrdU incorporation (Fig. 1), and increased T cell death, especially in the CD8⁺ T cell population (Fig. 5) indicating there is accelerated intestinal T cell death and turnover in animals receiving alcohol. These observations of T cell loss and turnover were mostly restricted to the intestine, where alcohol has been demonstrated to adversely affect mucosal barrier function.

Alcohol exposure of intestinal mucosal tissues in the upper GI tract (esophagus, stomach, and duodenum/jejunum) can induce numerous metabolic and functional changes in the intestine (Bode and Bode, 1997). Alcohol use also results in malabsorption, bacterial overgrowth, hypermotility, and even destruction of small intestinal villi (Chiang et al., 2012). Further, studies of alcohol dehydrogenase in human small intestine show abundant activity of multiple isoforms in the duodenum and jejunum, indicating that alcohol is metabolized into its more toxic metabolites, some of which are known to alter functions of host defense cells. It has been suggested the proximal small intestine substantively contributes to first-pass metabolism of alcohol, and that cytotoxic acetaldehyde byproducts of metabolism may play an etiological role in the pathogenesis of small bowel diseases associated with alcohol. Although we could not directly confirm the molecular etiology of the loss of T cells in the intestine, we did note that significantly higher percentages of CD4⁺ and CD8⁺ T cells were undergoing necrosis and apoptosis after alcohol (Fig. 5), and significantly higher percentages of CD8⁺ T cells were undergoing cell death than CD4⁺ T cells (Fig. 5B) which is consistent with the selective loss of CD8⁺ T cells shown in Figs. 3 and 4. Conceivably, the metabolism of alcohol in the small intestine has direct toxic effects on local intestinal T cells, resulting a compensatory increase in proliferation to restore intestinal homeostasis. Further, cells residing in the intestinal epithelium (intestinal epithelial lymphocytes or IEL's) are almost exclusively CD8⁺ T cells, so they may be in more direct contact with higher concentrations of ethanol than CD4⁺ T cells mostly residing in the deeper lamina propria, which may explain their selective loss.

Recent studies suggest that HIV infected patients even on ART show signs of premature immune senescence, despite undetectable viremia, suggesting T cell destruction and turnover still occur in inaccessible viral tissue reservoirs. Similarly, chronic alcohol users have been suggested to have premature aging and senescence (Pavanello et al., 2011). Thus, we hypothesize that chronic alcohol use and HIV combined, result in even greater T cell turnover in tissues, contributing to premature exhaustion of the T cell repertoire, and

premature senescence of the immune system, which may partially explain the higher rates of co-morbidity and mortality in HIV patients who drink alcohol (Braithwaite et al., 2007).

Based on these data, we propose a new, unifying hypothesis to explain the pathogenesis of alcohol use and SIV infection. Previously we showed animals on alcohol had consistently higher viremia and more rapid progression to disease (Amedee et al., 2014, Poonia et al., 2005). We also previously described increased proportions of target cells (memory CD4+ T cells) in the gut of animals receiving alcohol (Poonia et al., 2006). Here, using a BrdU pulse-labeling strategy, we found increased rates of CD4+ T cell turnover in the intestine due to alcohol, yet decreased absolute numbers of T cells in the gut. Dividing cells are by definition in a state of activation, and we have previously shown that both activated (Veazey et al., 2000b) and dividing cells (Wang et al., 2013) are major targets for SIV infection *in vivo*. Further, CCR5, the major co-receptor for HIV/SIV, is known to be expressed on activated CD4+ T cells (Veazey et al., 2000a, Ebert and McColl, 2002). Thus, while there is an absolute decrease in the number/percentage of CD4+ T cells, those present are highly activated, and thus have the receptors making them better targets for SIV.

We hypothesize alcohol use results in increased levels of T cell destruction in the upper small intestine, possibly as a direct result of toxic effects of alcohol byproducts. Concurrently there is a marked increase in CD4+ and CD8+ T cell proliferation in an attempt to re-establish tissue homeostasis. Further, alcohol use in HIV patients may exacerbate microbial translocation through similar mechanisms, to accelerate T cell destruction and turnover, resulting in higher viral loads and more rapid progression to disease. This unifying theory may open the door to new treatment strategies for patients who drink alcohol to lessen the comorbidities associated with alcohol use and infectious disease.

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Figure 1. Increased percentages of proliferating (BrdU+) CD4+ (A) and CD8+ (B) T cell subsets in small intestinal tissues of CBA administered-macaques compared to isocaloric sucrose (controls). Note marked and significant increases in the proportion of both CD4+ and CD8+ T cells are detected in cell suspensions prepared from intestinal biopsies before (pre) and after 3 months of alcohol consumption(post), whereas there are minimal changes in time-matched samples from sucrose controls. Data were generated by gating through intestinal CD3+ T lymphocytes. Error bars reflect standard error of the means (S.E.M.)

Figure 2.

Immunohistochemistry for CD3+ T cells (yellow) in intestinal biopsies from representative animals receiving alcohol. (A-D): Images on the left are from biopsies of 4 animals prior to alcohol/sucrose consumption as compared to biopsies from the same animal after 3 months of either chronic alcohol administration (B,D) or sucrose administration (controls; F, G). All images were collected at 200x magnification.

Figure 3.

(A) Absolute numbers of CD3+ T cells per mm² of intestinal lamina propria from CBA administered animals as compared to sucrose controls. Note a significant decrease in CD3+ T cells is confirmed in biopsies obtained after 3 months of CBA administration. Absolute numbers of total CD4+ (B) and CD8+ (C) T cells per mm² intestinal lamina propria as determined by multiplying the percentage of CD3+ T cell subsets detected by flow cytometry to the absolute counts of CD3+ cells per mm² tissue in the same animals. Note there are minimal changes in total numbers of CD4+ T cells in the intestine (B) but there is a highly significant decrease in total CD8+ T cells. Error bars reflect S.E.M.

Figure 4.

Percentages of total CD4+ (A) and CD8+ (B) T cells in the intestine of animals receiving alcohol compared to sucrose controls. There are slightly increased percentages of CD4+ T cells in cell suspensions from the gut, which is likely a reflection of the marked decrease in overall CD8+ T cells. Data were generated by gating through lymphocytes and then CD3+ T cells. Error bars reflect S.E.M.

Figure 5.

Percentages of total dead or dying T cell subsets in the intestine before and after 3 months of alcohol administration (A). Note markedly increased percentages of both CD4+ and CD8+ T cells are dying as indicated by uptake of the live dead (LD) viability dye. (B) Percentages of CD4+ and CD8+ T cell subsets undergoing apoptosis (AnnexinV+, LD neg) or total dead cells (necrosis = LD+/AnnexinV+/-) after 3 months of alcohol administration. Note significantly higher percentages of CD8+ T cells are dying as compared to CD4+ T cell subsets.