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Effects of binge alcohol exposure on *Burkholderia thailandensis*—alveolar macrophage interaction

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ABSTRACT

Alcohol consumption has diverse and well-documented effects on the human immune system and its ability to defend against infective agents. One example is melioidosis, a disease caused by infection with *Burkholderia pseudomallei*, which is of public health importance in Southeast Asia and Northern Australia, with an expanding global distribution. While *B. pseudomallei* infections can occur in healthy humans, binge alcohol use is progressively being recognized as a major risk factor. Although binge alcohol consumption has been considered as a risk factor for the development of melioidosis, no experimental studies have investigated the outcomes of alcohol exposure on *Burkholderia* spp. infection. Therefore, we proposed the use of non-pathogenic *B. thailandensis* E264 as a useful BSL-1 model system to study the effects of binge alcohol exposure on bacteria and alveolar macrophage interactions. The MH-S alveolar macrophage (AMs) cell line was used to characterize innate immune responses to infection *in vitro*. Our results showed that alcohol exposure significantly suppressed the uptake and killing of *B. thailandensis* by AMs. Alveolar macrophages incubated in alcohol (0.08%) for 3 h prior to infection showed significantly lower bacterial uptake at 2 and 8 h post infection. Activated AMs with IFN-γ and pre and post-incubation in alcohol when exposed to *B. thailandensis* released lower nitric oxide (NO) concentrations, compared to activated AMs with IFN-γ from non-alcoholic controls. As a result, *B. thailandensis* survival and replication increased ~2.5-fold compared to controls. The presence of alcohol (1%) also increased bacterial survival within AMs. Alcohol significantly decreased bacterial motility compared to non-alcoholic controls. Increased biofilm formation was observed at 3 and 6 h when bacteria were pre-incubated in (0.08%) alcohol. These results provide insights into binge alcohol consumption, a culturally prevalent risk factor, as a predisposing factor for melioidosis.

Introduction

The gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of the tropical disease melioidosis. Melioidosis is characterized by pneumonia, tissue abscess, and sepsis, with a mortality rate of up to 40%, with pneumonia being the presentation in half of all reported cases (Limmathurotsakul et al., 2013). Melioidosis is highly endemic in Thailand and Northern Australia. Increasing numbers of travelers, returning military personnel from the regions, and improved diagnostics in other tropical countries may reflect the expansion of the organism's known global distribution (Limmathurotsakul et al., 2016; Wiersinga, Currie, & Peacock, 2012). *B. pseudomallei* is a Tier 1 select agent and can only be manipulated under biosafety level 3 (BSL-3) conditions (Lu, Popov, Patel, & Eaves-Pyles, 2012).

Despite recent advancements in *Burkholderia* research, its intracellular survival strategies are still not well understood. Increasing epidemiological evidence suggests that risk factors for melioidosis may be of greater influence than infectious dose, route of infection, or bacterial virulence for developing melioidosis (Currie et al., 2000). The presence of one or more risk factors have been observed in 80% of confirmed melioidosis cases, with nearly 40% of Australian cases having binge alcohol use as a risk factor

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(Currie, 2015). Binge alcohol consumption was initially recognized as a primary risk factor for melioidosis in early Australian clinical studies (Guard, Khafagi, Brigden, & Ashdown, 1984; Rode & Webling, 1981). Alcohol is widely accepted as the most popular recreational drug with well-documented adverse effects associated across geographic and social boundaries (Jones & Holmgren, 2009; Pruett et al., 2010). Studies in both human and animal models indicate that acute alcohol or binge alcohol intake, which is characterized by consumption of alcohol reaching a blood alcohol concentration of at least 0.08% within 2 h, may be associated with increased health risks over the lifetime of the individual (Moreira, Smith, & Foxcroft, 2010; Wechsler & Nelson, 2008).

Excessive alcohol consumption has been linked to impairment of macrophage function and increased risk for pulmonary infections and sepsis as seen in melioidosis (Bhatty, Jan, Tan, Pruett, & Nanduri, 2011; Nelson et al., 1991). Alveolar macrophages are the first line of defense during pulmonary infections, typically located in the distal respiratory tract (Knapp et al., 2003), and are capable of detecting, capturing, and eliminating invading pathogens while being responsible for initiating the early host immune response (Qiu et al., 2012; Renwick, Donaldson, & Clouter, 2001). Moreover, binge alcohol toxicity suppressed bacterial phagocytosis and killing in J774.16 macrophages when infected with *Acinetobacter baumannii* (Asplund, Coelho, Cordero, & Martinez, 2013; Boé, Nelson, Zhang, & Bagby, 2001). Additionally, alcohol increased expression of virulence factors during infections and decreased pulmonary immune defenses in infections caused by *Mycobacterium avium*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Bermudez, Young, Martinelli, & Petrofsky, 1993; Camarena, Bruno, Euskirchen, Poggio, & Snyder, 2010; Goral, Karavitis, & Kovacs, 2008; Gordon, Irving, Lawson, Lee, & Read, 2000). These findings reflect the capacity of alcohol to alter the initial interaction between host immune mechanisms and pathogens during infection.

B. thailandensis E264, a genetically similar and non-virulent strain, has been used as an acceptable model to study *B. pseudomallei* infections (Haraga, West, Brittnacher, Skerrett, & Miller, 2008; Rainbow, Hart, & Winstanley, 2002). Furthermore, *B. thailandensis* can be found together in soil with *B. pseudomallei* in melioidosis endemic regions (Brett, DeShazer, & Woods, 1998; Yu et al., 2006). The present study was designed to investigate the role of binge alcohol exposure on the interaction between the non-virulent *B. thailandensis* and alveolar macrophages. Our results indicate that binge alcohol toxicity can increase non-pathogenic *B. thailandensis* bacterial infectivity while decreasing host innate immune responses to infection.

Materials and methods

In vitro alveolar macrophage cell culture and alcohol exposure

The mouse alveolar macrophages (AMs) cell line (MH-S ATCC # CRL-2019) was used in these studies. Cells were regularly grown in T-75 cell culture flasks in phenol red-free RMPI-1640 medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells were incubated at 37 °C and 5.5% CO_2. Cells were seeded in 24-well cell culture plates at 4×10^5 cells/well and incubated in RPMI-1640 media supplemented with 0.08% or 0% (v/v) alcohol for 3 h. Low evaporative cell culture plates, a compensating system described by Eysseric et al. (1997), and alcoholic media changes were used to ensure consistent alcohol concentration. Alcohol concentration was selected based on ≥90% cell viability utilizing the Trypan blue exclusion test of cell viability. AMs were primed with or without IFN-γ prior to alcohol exposure. After washing AMs with pre-warmed Dulbecco's phosphate-buffered saline (PBS), cells were incubated at 37 °C in RPMI alcohol-free media containing 400–600 U/mL mouse IFN-γ (PROSPEC # CYT-358) for 24 h. All assays were run in duplicate and at least two independent experiments were performed with similar results.

Growth curves and alcohol exposure

Growth curves were performed on *B. thailandensis* to determine the effects of different concentrations of alcohol on growth. Bacterial cultures in Luria Bertani (LB) broth were supplemented with 0%, 0.08%, 1%, 2%, and 3% (v/v) alcohol in an orbital shaker incubator (200 rpm) (New Brunswick C25, Edison, NJ, USA). Every 60 min, 500-μL samples were tested for OD_{600} absorbance in a spectrophotometer (Eppendorf Bio Photometer AG2233, Hamburg, Germany). Growth curves were plotted and analyzed using Prism 5.0 software. Growth curve assay was replicated in at least three independent experiments.

Alveolar macrophages: live B. thailandensis uptake assays

Bacterial uptake assays were conducted to determine the effects of alcohol on macrophage initiation of phagocytosis, which is defined as contact, uptake, or engulfing of foreign material (Qiu et al., 2012). Alveolar macrophages were cultured as previously described in RPMI media containing 0.0% or 0.08% alcohol. Bacteria were grown overnight (12–18 h) at 37 °C in a shaking incubator (200 rpm) (New Brunswick C25, Edison, NJ, USA), using LB agar or broth. Bacteria were diluted 1:10 and grown to late-logarithmic phase. Bacteria were centrifuged, washed twice with PBS, and resuspended with RPMI-1640 medium without antibiotics. Bacteria were diluted to provide a multiplicity of infection (MOI) of 1:1. After 2- and 8-h incubations, extracellular bacteria were removed from cultures by treatment with 250-μg/mL kanamycin for 2.5 h. Viable bacteria were recovered by lysing AMs with PBS containing 0.1% TX-100. Homogenates were diluted and grown on LB agar plates, incubated at 37 °C, and colony-forming units (CFUs) were determined after 24–48 h. Bacterial uptake assays were replicated in two independent experiments with similar results.

Nitric oxide assays and alcohol toxicity during infection

Alveolar macrophages were grown as previously described. Prior to infection, AMs were primed with 400-U/mL IFN-γ for 24 h. In order to determine the effects of alcohol on nitric oxide (NO) produced by AMs, two groups of AMs were incubated in 0.0% alcohol, while another group was incubated in 0.08% alcohol for 3 h prior to bacterial challenge. A MOI of 0.5:1 was administered and cell viability was ≥90%. For post-infection challenge, two groups were washed and supplemented with non-alcoholic media, and the additional group was washed and supplemented with 0.08% alcohol and RPMI media. Levels of nitrites in the media were assayed at 4, 8, and 12 h post *B. thailandensis* challenge. The nitrite concentrations were determined using the Griess reagent (Promega, Madison, WI, USA). Dilutions of sodium nitrite in RPMI culture media supplemented with 0.08% or 0% alcohol were used as standards. Absorbance at 550 nm was read for each treatment sample in triplicate, and replicated independently at least twice.

Alcohol and macrophages: live B. thailandensis suppression and killing assays

Confluent AMs were grown in media supplemented with 0% or 0.08% alcohol for 3 h, followed by a non-alcoholic media change.