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Differential susceptibility of macrophage cell lines to *Bacillus anthracis*–Vollum 1B

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Abstract

Bacillus anthracis (BA) is a spore forming bacterium and the causative agent of anthrax disease. Macrophages (Mϕs) play a central role in anthrax disease. An important step in disease progression is the ability of BA to secrete lethal toxin (LeTx) that kills Mϕs. LeTx is a heterodimer composed of protective antigen (PA) and lethal factor (LF). Researchers have shown that Mϕ cell lines demonstrate differential susceptibility to purified LeTx; for example RAW264.7 and J774A.1 Mϕs are sensitive to LeTx whereas IC-21 Mϕs are resistant. Research has also suggested that exogenous factors, including other BA proteins, can influence the activity of LeTx. For this reason, the objective of the current work was to examine if RAW264.7, J774A.1, and IC-21 Mϕs demonstrated differential susceptibility when cultured with a LeTx-producing strain of BA. Here, we co-cultured Mϕs with $LeTx^+$ Vollum 1B (V1B) spores for >15 h and assayed for Mϕ cell death by morphology, trypan blue (TB) staining, neutral red (NR) activity, and lactate dehydrogenase (LDH) activity in the culture media. Following the addition of V1B spores, necrosis (≈50% mortality) was observed in RAW264.7 and J774A.1 Mϕs at 7.5 and 10 h, respectively. By 15 h, both RAW264.7 and J774A.1 Mϕs demonstrated 100% mortality. In contrast, IC-21 Mϕs, under identical culture conditions, remained viable (98%) and activated throughout the course of the experiment (>24 h). The mechanism of RAW264.7 cell death appeared to involve LeTx because the V1B-induced cytotoxicity was dose-dependently reversed by the addition of anti-PA antibody to the culture media. These observations suggest there is differential susceptibility of Mϕ cell lines to the $LeTx^+$ V1B strain of BA. Further development of this in vitro model may be useful to further characterize the interactions between Mϕs and BA spores.
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Keywords: Macrophage; Vollum 1B; Anthrax; Toxicity; Lethal factor

1. Introduction

Bacillus anthracis (BA) is a large, gram-positive, spore-forming, non-motile bacterium that is the causative agent of anthrax disease (Takamatsu and Watabe, 2002). BA spores are the infective form of the bacteria and are characterized as dehydrated particles that lack measurable metabolic activity (Inglesby et al., 2002). Following infection, BA spores germinate into vegetative cells that are metabolically active and secrete several known toxins. Generally, clinical manifestations of anthrax disease reflect the bacteria's route of entry-cutaneous, gastrointestinal and inhalation, with more than 95% of natural occurring cases cutaneous (Dixon et al., 1999). However, there is a renewed interest in

Abbreviations: BA, *Bacillus anthracis*; LDH, lactate dehydrogenase; Mϕ, macrophage; NR, neutral red; TB, trypan blue; V1B, Vollum 1B; LeTx, lethal toxin; EdTx, edema toxin

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the inhalational form of anthrax disease due to the potential to use BA as an airborne biological weapon and a mortality rate approaching 100% (Matsumoto, 2003; Henry, 2001).

Macrophages (Mϕs) play key roles in inhalational anthrax. First, following deposition in the lungs, BA spores are phagocytosed by alveolar Mϕs and transported to the draining mediastinal and treacheobronchial lymph nodes (Henry, 2001; Shafa et al., 1966). Second, it is thought that BA germinates from its dormant spore state into its metabolically active vegetative state inside the Mϕ during migration (reviewed in Guidi-Rontani, 2002). Germination is closely followed by synthesis of several virulence factors that are required for disease progression (Guidi-Rontani et al., 2001; Dixon et al., 2000; Guidi-Rontani et al., 1999). Finally, the late stage of systemic anthrax disease is characterized by sudden shock caused by TNF-α and IL-1 secreted, in part, from Mϕs (Dixon et al., 1999).

The virulence of BA is due to three known factors: lethal toxin (LeTx), edema toxin (EdTx), and an antiphagocytic capsule (Ascenzi et al., 2002). Like many bacterial toxins, LeTx and EdTx each possess two subunits, a B-domain (cell binding) and an A-domain (active or enzymatic). The A-domain for LeTx is a 90 kDa protein called lethal factor (LF), which is a highly specific metalloprotease that cleaves mitogen-activated protein kinase kinase (MAPKK) leading to interruption of cellular signaling pathways in susceptible cells (Pannifer et al., 2001; Hanna, 1999). The A-domain for EdTx is an 89 kDa protein called edema factor (EF), which is a calmodulin-dependent adenylate cyclase that interferes with cellular signaling events by increasing levels of cyclic AMP (cAMP) in susceptible cells (Kumar et al., 2002; Leppla, 1982). The B-domain for both LeTx and EdTx is an 83 kDa protein called protective antigen (PA), which binds cell surfaces and delivers either LF or EF to the cytosol. LF, EF and PA are non-toxic individually and are coded by separate genes located on a single plasmid [pXO1; 184.5 kilobase]. The genes for the third virulence factor, an antiphagocytic capsule, are coded on a separate plasmid [pXO2; 95.3 kilobase] and are involved in synthesis of a poly-D-glutamic acid capsule that inhibits phagocytosis of vegetative BA (Makino et al., 2002).

LeTx is considered the major virulence factor in BA. Indeed, anthrax disease can be mimicked in animals by exposure to purified LeTx (Hanna et al., 1993). LeTx is also highly cytotoxic for Mϕs and can induce necrosis and apoptosis (Popov et al., 2002; Parker et al., 2002; Hanna et al., 1992). Additionally, Mϕ cell lines, as well as primary cultures, demonstrate differential susceptibility to purified LeTx (Friedlander et al., 1993; Singh et al., 1989). Specifically, RAW264.7 and J774A.1 cell lines are sensitive to LeTx whereas IC-21 Mϕs appear resistant. The mechanisms of action of LeTx in susceptible Mϕs is complex and remains to be clearly defined; however, studies suggest toxicity may involve disruption of second messenger systems (Bhatnagar et al., 1999; Vitale et al., 1998), cytokine production (Kim et al., 2003), proteasome activity (Tang and Leppla, 1999), and/or oxidative stress (Hanna et al., 1994).

Although studies that use purified LeTx and sensitive or resistant Mϕs advance our understanding of anthrax disease, it is important to examine the more complex interactions between Mϕs and live LeTx-producing bacteria for several reasons. For example, other proteins expressed by the bacteria may affect the regulation and activity of LeTx. Indeed, it has been shown that EdTx acts as an enhancer that increases the activity of LeTx in Mϕs (Kumar et al., 2002; Pezard et al., 1991). Thus, studies that examine the affect of purified LeTx on Mϕs may not fully describe the activity of LeTx from bacteria that are concomitantly synthesizing EdTx and/or other proteins. Additionally, BA spores germinate and escape destruction by the Mϕ following phagocytosis, which is thought to be a critical step in the initial stages of infection. The mechanisms used by BA during escape remain to be fully defined but may be closely linked with LeTx gene activation inside the Mϕ (Guidi-Rontani et al., 1999) as well as activation of other undefined 'escape' genes on pXO1 (Dixon et al., 2000). Therefore, examination of Mϕ sensitivity or resistance to purified LeTx cannot address this critical aspect of anthrax disease; live $pXO1^+$ bacteria are required. Finally, it is widely recognized that another important step in anthrax progression involves pro-inflammatory cytokine production from Mϕs and studies suggest that the ability of Mϕs to produce TNF-α in response to LeTx depends on whether LeTx is used in purified form (Erwin et al., 2001) or generated from phagocytosed $LeTx^+$ BA spores (Pickering and Merkel, 2004). Therefore, collectively, examining sensitivity and resistance of Mϕ cell lines to $LeTx^+$ BA spores will not only help to define the actions of LeTx in a more physiologically relevant context (i.e., derived from a live bacteria), but may also help to define the role of other bacterial toxins that can act alone or synergistically with LeTx.

The objective of the present work was to examine the susceptibility of Mϕ cell lines to the Vollum 1B (V1B) strain of BA. V1B has both pXO1 and pXO2 plasmids and therefore contains all known virulence factors ($LeTx^+$, $EdTx^+$, and $capsule^+$). Our experimental design consisted of exposing equal numbers of Mϕs and V1B spores, then measuring Mϕ death over time (based on Guidi-Rontani et al., 2001, 1999). Cytotoxicity was determined using Mϕ morphology, trypan blue (TB) staining, neutral red (NR) activity, and lactate dehydrogenase (LDH) activity. Finally, to begin addressing the mechanism of V1B-induced Mϕ death, the activity of PA was blocked by the addition of anti-PA monoclonal antibody to the cultures.