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A *Toxoplasma gondii* mutant defective in responding to calcium fluxes shows reduced *in vivo* pathogenicity

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Abstract

Toxoplasma gondii is an important opportunistic pathogen in immunocompromised individuals. Successful propagation in an infected host by this obligate intracellular parasite depends on its ability to enter and exit host cells. Egress from the cell can be artificially induced by causing fluxes of calcium within the parasite with the use of calcium ionophores. While this ionophore-induced egress (IIE) has been characterized in detail, it is not known whether it mimics a normal physiological process of the parasite. This is underscored by the fact that mutants in IIE do not exhibit strong defects in any of the normal growth characteristics of the parasite in tissue culture. We have isolated and characterized a *T. gondii* mutant that along with a delay in IIE exhibits a severe defect in establishing a successful infection *in vivo*. In tissue culture this mutant displays normal ability to invade, divide within cells and convert into the latent encysted bradyzoite form. Nevertheless, mice infected with this mutant are less likely to die and carry less brain cysts than those infected with wild type parasites. Thus, our results suggest that normal response to calcium fluxes plays an important role during *in vivo* development of *T. gondii*.
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Keywords: Toxoplasma; Egress; Calcium; Virulence; Bradyzoite

1. Introduction

Toxoplasma gondii is one of the most widespread and successful protozoan pathogens and is a common parasite in humans, where it has become one of the main opportunistic pathogens in AIDS patients [1]. The life cycle of *T. gondii* is complex and includes both sexual and asexual stages. While the sexual cycle is limited to the gut of felines, the asexual cycle can occur in a wide range of hosts and has two major forms: the rapidly growing tachyzoite and the slow growing bradyzoite. Tachyzoites can invade virtually any nucleated cell, replicate, and exit quickly, thereby disseminating throughout the infected host. In most cases the infection is controlled by the immune system, although some tachyzoites can evade elimination by switching to the dormant bradyzoite form contained within a walled cyst. Bradyzoite cysts can persist within the infected tissue for the life of the host, hidden from the immune system and anti-parasitic drugs. In immunocompromised individuals such as AIDS, leukemia and lymphoma patients, new infections or rupture of pre-existing cysts can lead to toxoplasmic encephalitis [1–3]. Additionally, in congenital infections, the disease can lead to severe neurological problems or even death [4].

A devastating consequence of the uncontrolled growth of *T. gondii*, and a cause of much of its associated disease, is the lethal lysis of the host cell as tachyzoites exit the parasitophorous vacuole in which they replicate. Egress from the vacuole and host cell is a rapid process, and *in vitro* (and presumably *in vivo*) is synchronous for all parasites within a given cell but asynchronous between different cells. What signals the parasite to undergo egress remains unknown, as are the molecular mechanisms by which the parasites exit their host cells. Nevertheless, it has become evident that egress can be influenced by changes in ion homeostasis inside and outside the parasite. For instance, it has been shown that a loss of potassium (K^+) from

Abbreviations: IIE, ionophore-induced egress; IID, ionophore-induced death; HFF, human foreskin fibroblast

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the host cell upon artificial permeabilization can trigger *T. gondii* egress and that this induction depends on intraparasitic calcium (Ca^{2+}) fluxes [5]. The relation between Ca^{2+} fluxes and egress is particularly evident in experiments with the calcium ionophore A23187, which induces the parasites to quickly exit their host cell in a process called ionophore-induced egress (IIE) [6]. Similarly, when exposed extracellularly to A23187, parasites activate the secretory and cytoskeletal responses required for invasion [7,8]. The prolonged exposure to the ionophore while outside the host cell causes *T. gondii* to irreversibly lose its ability to invade cells, presumably due to the exhaustion of essential invasion factors. The result of this inhibition for the obligate intracellular parasite is death, and thus this phenomenon is known as ionophore-induced death (IID) [8].

Mutants with a pronounced delay in IIE (Iie^-) have been isolated and characterized [9,10]. The analysis of these mutants led us to propose a stepwise model for IIE. First, the ionophore induces the release of intracellular Ca^{2+} stores within the host cell and the parasites. The parasites respond to these changes in Ca^{2+} concentrations by first extending their conoid, and then permeabilizing the host plasma membrane and the parasitophorous vacuole by an unknown mechanism. The permeabilization event was shown to be parasite and Ca^{2+} dependent [9]. Chromium-release assays revealed a delay in the permeabilization of the host cell in the Iie^- mutants. Furthermore, the Iie^- phenotype of the mutants was rescued by treating the cells with a permeabilizing detergent such as saponin [9]. This important permeabilization step then allows a proposed final egress signal to reach the parasite, inducing them to leave. In addition, Iie^- mutants are also defective in early stages of the lytic cycle and some of them exhibit a resistance to IID (Iie^-Iid^- mutants), suggesting a commonality between IIE, IID and normal physiological processes of the parasite [9]. All but one of the Iie^- mutants isolated so far were generated by chemical mutagenesis, and efforts to identify the affected genes in those mutants have failed. Thus, the nature of the genetic disruption in all of the chemical Iie^- mutants is still unknown. Recently, an insertional mutant with a delay in IIE and a resistance to IID was shown to lack the product of a sodium hydrogen exchanger gene, *TgNHE1* [10]. The reason for the Iie^- phenotype in this particular mutant was determined to be due to an inability to maintain normal intraparasitic levels of calcium [10].

Interestingly, none of the previously reported mutants with a delay in IIE exhibit a delay in natural (i.e. non-induced) egress [9,10]. A possible explanation for this is that the difference in timing of IIE between mutant and parental strains is only on the order of minutes. Such a difference would be undetectable during natural egress, which is not synchronized and occurs about 40–55 h after infection, with a variability of many hours from cell to cell. It is also possible that while sufficient to induce egress, calcium fluxes are not necessary for this process in tissue culture and might play a more critical role in specific tissues or cell types. Indeed, it is not known whether any of the mutants deficient in responding to calcium ionophores have a defect in establishing and propagating an infection *in vivo*. This is due largely to the fact that all Iie^- mutants were established using a particular *T. gondii* strain, RH, which is not suitable for *in vivo* experiments given its extreme virulence in mice. Although its rapid growth characteristics make RH a favorite model for *in vitro* studies, *in vivo* a single parasite of this strain proves fatal to the mouse host, and the strain is not efficient at differentiating into the encysted form *in vivo* or *in vitro*. Consequently, it is not known what role, if any, the response to calcium fluxes plays during infection. Here we describe the isolation and characterization of a novel mutant with a defective response to calcium fluxes. This novel mutant is a derivative of the Pru parasite strain, one of the less virulent *T. gondii* strains, and therefore better suited for *in vivo* work. This has allowed us to investigate the effects of Iie^- and Iid^- phenotypes on virulence. In this study, we provide evidence that normal response to calcium fluxes is important in the establishment of a successful infection.

2. Materials and methods

2.1. Parasite and host cell maintenance and reagents

Parasites were maintained by passage through human foreskin fibroblasts (HFFs) at 37 °C and 5% CO_2. Normal culture medium was Dubelco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and 100 units penicillin/100 μg streptomycin/ml. Ionophore assays were performed using Hanks Balanced Salts Solution (HBSS) supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 20 mM Hepes, pH 7.2 (HBSSc). The calcium ionophore A23187 (Sigma) was dissolved in DMSO at 1 mM to make a stock solution.

2.2. Screen for mutants with delayed IIE

The Pru strain of *T. gondii* [11] deleted in hypoxanthine–xanthine–guanosine phosphoribosyl transferase (PruΔ*hpt*) and containing a signature-tag was used for creation of the mutant library [12]. For this IIE screen, we used all 4900 mutants previously generated which are maintained in 96-well plates [12]. In brief, 30 μl from each clone was passed to new HFF monolayers grown in 96-well plates. The parasites were then allowed to grow for 30 h at which time point all clones were exposed to 1 μM A23187 in pre-warmed HBSSc at 37 °C for 4 min. At this point monolayers were fixed in 100% methanol and stained with Diff-Quik (Dade-Behring). After quick visual inspection of all tested clones we selected a total of 20 clones that showed a higher number of intact vacuoles than expected for this time point. These 20 clones were tested again by performing the detailed IIE assay described below (see Section 2.3). Out of the 20 clones tested only 1, 52F11, exhibited a reproducible delay in IIE.

2.3. Quantitation of IIE

The efficiency of egress after calcium ionophore exposure was determined using established protocols [10] with some modifications. In brief, 1×10^5 parasites were added to each well of a 24-well tissue culture plate containing confluent HFFs. After 40 h of growth, the parasites were incubated at 37 °C in HBSSc containing 1 μM A23187 calcium ionophore or an equivalent