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Electrochemical detection of the binding of *Bacillus anthracis* protective antigen (PA) to the membrane receptor on macrophages through release of nitric oxide

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ABSTRACT

Anthrax is a serious bacterial disease of man and animals whose pathogenesis involves the secretion of lethal toxins in the host. The intracellular delivery of toxic complexes involves a complex structural rearrangement of sub-domains of the exotoxin protective antigen (PA). We have used a biocompatible microelectrode array, coated with J774 mouse macrophages, to detect PA binding and intracellular signaling resulting in nitric oxide (NO) release. We have found that exposure of macrophages to PA *in vitro* activates the inducible isoform of NO synthase (iNOS), thus increasing the extracellular concentration of NO and nitrite, in a dose- and time-dependent manner. However, the cell-binding domain 4 of PA (PA4) could substitute for full-length PA to achieve equivalent NO release, suggesting that the heptamerisation of PA, ultimately required to deliver toxic complexes into the cell, is not a requirement for the activation of an intracellular cascade through the ERK 1/2 and the PI-3 K/ Akt kinase pathways and that these events could be triggered by the binding of PA4 alone to its cell membrane receptor. Further, we have found that pre-incubation of the cells with azidothymidine, a pro-oxidant drug, significantly improves the limit of detection of rPA-induced NO release thus offering a sensitive tool for the analysis of the kinetics of anthrax intoxication and ultimately drug discovery.

1. Introduction

Anthrax is a serious bacterial disease, caused by exposure to the Gram-positive bacterium *Bacillus anthracis* (*B. anthracis*), a sporulating, rod-shaped bacterium found in the environment, particularly the soil, or as a zoonotic infection of herbivores. Man can become infected through environmental exposure to soil or contaminated products from infected animals, such as hides, hair, wool and excreta.

Once *in vivo*, *B. anthracis* spores germinate and release three proteins, termed Protective antigen (PA), lethal factor (LF) or edema factor (EF) and these latter two combine in pairs with PA to form the potent toxins, termed edema toxin (ET) and lethal toxin (LT). Much is now known about the process of host cell-binding by PA prior to endocytosis of either of these toxic complexes (reviewed by Abrami et al., 2005; Collier and Young, 2003; Leppla, 2000). The full-length (83 kDa) PA binds host cell receptors (capillary morphogenesis factor 2, CGM2 (Scobie et al., 2003) or trans-endothelial membrane receptor 8, TEM8 (Bradley et al., 2001)) and then undergoes furin cleavage to release a 20 kDa N-terminal fragment, leaving a 63 kDa truncated protein. PA_{63} then undergoes a structural rearrangement with heptamerisation, so that domain 4 is in contact with the host cell; in the process, binding sites for either LF or EF are exposed on the heptamerised PA and the latter associates with lipid rafts. Subsequently, the toxic complex undergoes clathrin-mediated endocytosis and enters early endosomes with subsequent microtubular transport through vesicles into late perinuclear endosomes. The acidification of the endosomes allows the unfolding and release of LF and EF (Kao et al., 2005; Koehler and Collier, 1991). LF is released into the cytoplasm, whereas EF remains bound to the late endosomal perinuclear membrane (Guidi-Rontana et al., 2000; Zornetta et al., 2010). In the cytoplasm, LF cleaves and inactivates mitogen-activated protein kinase kinases (MAPKK's) to disrupt phosphorylation and transcription in the nucleus, ultimately preventing protein synthesis and causing cell death (reviewed by Guichard et al., 2011), whilst EF, a calcium and calmodulin-dependent adenylate cyclase, causes a rapid increase in perinuclear cAMP resulting in cellular, tissue and ultimately

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organ edema. Both LF and EF also have the effect of suppressing pro-inflammatory cytokine secretion (Tournier et al., 2005) and weakening vascular endothelial barriers by down regulating vascular cadherin, important in cell–cell adhesion (Guichard et al., 2010). This effect is thought to contribute to the vascular leakage that is typical of systemic anthrax (Moayeri and Leppla, 2004).

The inhibition of binding of PA to its host cell receptors would prevent these downstream toxic sequelae and could save lives in the event of anthrax exposure. A small molecule inhibitor could potentially act to prevent PA binding to its receptor, but a critical pre-requisite of deploying such countermeasures is the ability to detect a biomarker of the initial binding of PA to host cells, to aid diagnosis of infection and intervention to prevent the downstream cytotoxicity.

Sensitive immunoassays are available for PA with limits of detection of 0.06 μg ml^{-1} and 97.6% specificity (Park et al., 2009). Although serum levels of PA in infected animals can reach levels of 100 mg ml^{-1}, these may take up to 12 days to develop, even in symptomatic individuals (Muller et al., 2004).

The purpose of our study is to investigate the relationships between PA exposure and nitric oxide (NO) generation by macrophages *in vitro*, to determine whether NO might provide a biomarker of infection.

Nitric oxide (or its by-products nitrite and peroxynitrite) are released from immune (Amatore et al., 2006b; Amatore et al., 2001) or other cells (Lantoine et al., 1995; Privat et al., 1997; Arbault et al., 2004; Amatore et al., 2006a; Patel et al., 2006, 2008a, 2008b) following the induction of nitric oxide synthase (NOS) during activation of intracellular kinase cascades and this has been frequently studied with electrochemical methods which allows real-time and *in situ* measurements of the released reactive species. Here we have taken this one step further by using quantitative electrochemical sensing of NO release as a functional assay for cell-toxin interactions. Previously, we have reported the use of a fibronectin-coated microfabricated multiple microelectrode array (MMA) to measure eNOS activity and NO release and to investigate the intracellular signaling induced by stimulation of endothelial cells by angiogenic factors (Patel et al., 2008b; Trouillon et al., 2010a, 2010b, 2011). The fibronectin coating improved the biocompatibility of the sensor and its resistance to biological fouling (Trouillon et al., 2009a, 2009b). Here, we have applied this technology to investigate the significance of PA cell-binding on iNOS activation. In the absence of LF or EF, little is known about the effect of PA on macrophages and an ability to differentiate the cell binding from the endocytotic event could be useful. Ultimately our objective is to achieve *in situ* real time sensing of functional activity in living cells, for application in drug discovery and the diagnosis of infection.

2. Experimental procedures

2.1. Chemical

All the chemicals used in this study were purchased from Sigma, unless stated otherwise, and were used without further purification. Deionized water purified through a Millipore system was used throughout all the experiments (Resistivity: 18 MΩ cm).

2.2. Preparation of the recombinant antigens and PA antibody

Recombinant PA (rPA) was used in this study and was expressed and purified from *E. coli*, as previously described (Williamson et al., 2005). The recombinant domains 1 and 4 of PA (rPA1 and rPA4) were expressed from *E. coli* as fusion proteins with glutathione-*s*-transferase (GST), cleaved and purified, as previously described (Flick-Smith et al., 2002). Briefly, the different DNA sub-units, coding for the different proteins, were amplified by PCR, cloned into the expression vector pGEX-6-P3 (Amersham–Pharmacia) and expressed as fusion proteins with an N-terminal GST protein. The resulting proteins were then purified by a glutathione Sepharose CL-4B affinity column (Amersham–Pharmacia) or by ion-exchange chromatography.

Antisera to rPA were produced by immunization of A/J mice with rPA in alum (i.m.) using a standard immunization regimen (Williamson et al., 2005).

2.3. Cell culture

Mouse macrophages J774 (obtained from ECACC, European Collection of Animal Cell Cultures, Health Protection Agency, Porton Down, UK) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% v/v fetal calf serum and 5 mg ml^{-1} L-glutamine. This mixture is then referred to as culture medium. The flasks containing the cells were stored in a humid incubator (37 °C, 5% CO_2).

2.4. Modification and preparation of the multiple microelectrode arrays (MMAs)

The sensor arrays featured 6 independent, 2 μm recessed, working electrodes (the working electrodes are 35 μm in diameter and are 230 μm apart, *cf.* Patel et al., 2008b), as shown on Fig. 1A. A counter electrode is included in the device, and the setup was completed with an Ag|AgCl (3 M KCl) reference electrode. The MMA were prepared as previously described (Trouillon et al., 2010a, 2010b). The MMAs were modified using a Sylgard (Dow Corning) custom-made reaction cell to allow deposition of a 500 μl volume on the sensor. A lid made from a Petri dish was also fitted on the cell to minimize evaporation and avoid contamination when the sensor had to be placed in the humid incubator. Prior to any experiment, biological debris was removed using trypsin solution. Trypsin was deposited into the cell and incubated at 37 °C for 1 h. They were then rinsed with 70% aqueous ethanol followed by water. The gold electrodes were electrochemically cleaned by performing cyclic voltammograms between 1.6 and −0.3 V vs Ag|AgCl at 0.5 V s^{-1} in 0.1 M sulfuric acid until the current–voltage curves were reproducible. The electrode potential was then held at −0.2 V vs Ag|AgCl to reduce gold oxides. The sensor was sterilized with 70% ethanol, placed into the sterile safety hood and rinsed with PBS (pH=7.4). 50 ml of bovine fibronectin (20 mg ml^{-1} in DMEM) was deposited on the sensor and allowed to dry. Excess of fibronectin was rinsed away with PBS.

2.5. Electrochemical measurements

Cells were harvested and counted with a cytometer and Trypan blue. 10,000 cells were suspended in 500 μl of culture medium and deposited on the sensor. The chip was incubated overnight (37 °C, 5% CO_2). For some experiments, the cells were pre-incubated with inhibitors or pro-oxidant drugs, as detailed below. The following day, a set of three successive differential pulse voltammograms (DPVs) was recorded between 0.4 V and 1.2 V vs Ag|AgCl. rPA, rPA1 or rPA4 (dissolved in DMEM, stock concentration: 5 mg ml^{-1}) were added at relevant concentrations (50 μg ml^{-1} for most of the tests). The MMA was placed in the incubator and another set of two DPV was recorded after 4 h. In the case of the time dependence tests, the second set of DPV was performed 1, 2, 4 or 6 h after addition of rPA. In some experiments, the pro-oxidant drug azidothymidine (AZT) was used at a