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Prevention and treatment of *Clostridium perfringens* epsilon toxin intoxication in mice with a neutralizing monoclonal antibody (c4D7) produced in *Nicotiana benthamiana*

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ABSTRACT

Epsilon toxin (ETX), produced by *Clostridium perfringens* types B and D, is among the most lethal toxins known. ETX is a potential bioterrorism threat that was listed as a Category B agent by the U.S. Centers for Disease Control until 2012 and it still remains a toxin of interest for several government agencies. We produced a monoclonal antibody (MAb) against ETX (ETX MAb c4D7) in *Nicotiana benthamiana* and characterized its preventive and therapeutic efficacy in mice. The ETX preparation used was highly lethal for mice (LD_{50} = 1.6 μg/kg) and resulted in a mean time from inoculation to death of 18 and 180 min when administered intravenously or intraperitoneally, respectively. High lethal challenge resulted in dramatic increases of a variety of pro-inflammatory cytokines in serum, while lower, but still lethal doses, did not elicit such responses. ETX MAb c4D7 was highly effective prophylactically (ED_{50} = 0.3 mg/kg; ED_{100} = 0.8 mg/kg) and also provided protection when delivered 15–30 min post-ETX intoxication. These data suggest that ETX MAb c4D7 may have use as a pre- and post-exposure treatment for ETX intoxication.

1. Introduction

Epsilon toxin (ETX) is a 33 kDa protein produced by *Clostridium perfringens* types B and D. ETX is considered the third most potent of all clostridial toxins after botulinum and tetanus toxins (Lonchamp et al., 2010; Robertson et al., 2011; Stiles et al., 2013). Accordingly, ETX has been of concern as a potential bioterrorism agent, which was listed by the U.S. Centers for Disease Control as a Category B agent until 2012 and still remains a toxin of interest to many government agencies throughout the world. Category B agents are considered to be moderately easy to disseminate and would result in significant morbidity if human populations were to be exposed.

C. perfringens type D naturally affects several domestic animal species, including sheep, goats and cattle, causing enterotoxemia, when ETX is produced in the intestine and absorbed into the systemic circulation targeting several internal organs (Garcia et al., 2012). The action of ETX on the gastrointestinal tract is usually minimal, except in goats where it may produce enterocolitis (Garcia et al., 2012).

ETX is produced during the vegetative growth of *C. perfringens* and secreted as a relatively inactive prototoxin of 32.9 kDa that can be fully activated by removal of 11–13 N-terminal and/or 22–29 °C-terminal amino acids (Minami et al., 1997; Miyata et al., 2001). Proteases capable

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of activating ETX include trypsin, chymotrypsin, *C. perfringens* lambda toxin and others (Bokori-Brown et al., 2011). ETX appears to form heptameric pores in target cells including endothelial cells; the action of this toxin on the latter causes increased vascular permeability that leads to vasogenic edema mainly in the brain and lungs, that often produces fatal neurologic and respiratory disturbances (Bokori-Brown et al., 2011). ETX may also have a direct effect on neurons by stimulating the release of dopamine from dopaminergic nerve endings and glutamate within the rat and mouse hippocampus (Finnie et al., 1999; Bokori-Brown et al., 2011).

Passive immunization with monoclonal antibodies (MAbs) has been used to neutralize the action of a wide variety of toxins and microorganisms, including ETX (Zeitlin et al., 2000; Chow and Casadevall, 2012). 4D7 is a murine anti-ETX MAb developed as an ELISA reagent with known neutralizing activity (Hauer and Clough, 1999). To make this MAb more appropriate for potential human use, we chimerized the murine variable regions of 4D7 with human constant regions (ETX MAb c4D7). This antibody was produced in a rapid low-cost *Nicotiana benthamiana* manufacturing system (magnICON) previously used for production of other MAbs (Pogue et al., 2010) and vaccines (Bendandi et al., 2010) under Good Manufacturing Practices (GMP). We present here a study of the preventive and therapeutic use of ETX MAb c4D7 against ETX in mice.

2. Material and methods

2.1. Animals, reagents and general experimental procedures

Male and female Balb/C mice (17–21 g) housed in a temperature and light cycle controlled room were used. All procedures involving animals were reviewed and approved by the University of California, Davis Committee for Animal Care and Use (Permit 16,940). Intravenous (iv) and intraperitoneal (ip) injections were performed by inserting a 0.5-inch, 27-gauge needle into the coccygeal vein or into the caudal part of the abdominal cavity, respectively. Total injection volume (iv or ip) was always 0.5 ml. Trypsin-activated purified ETX was obtained from BEI (ATCC 3626). This toxin preparation was found to be >95% pure. ETX was diluted in 1% peptone water as needed.

The assay endpoints for each experiment were defined as spontaneous death, development of severe clinical signs necessitating euthanasia, or survival without clinical alterations during a set period of time (see below). Euthanasia was performed by CO_2 asphyxiation.

2.2. Determination of the LD_{50} for ETX

In order to determine the iv and ip lethal dose fifty (LD_{50}) of the ETX preparation used, serial dilutions of this toxin were prepared to obtain concentrations of 2000 ng/ml, 200 ng/ml, 20 ng/ml, 2 ng/ml, 0.2 ng/ml and 0.02 ng/ml. Groups of 8 mice received 0.5 ml iv or ip of each dilution. The LD_{50} was calculated as previously described (Sayeed et al., 2005). Negative control mice were treated iv with 1% peptone water. Maximum assay duration was 48 h.

2.3. Route comparison of the effect of ETX

Two groups ($n = 8$/group) of mice were inoculated with 30 LD_{50} ip and iv, respectively, and the time from inoculation to death was recorded to compare the effect of ETX administered ip versus iv. Maximum assay duration was 48 h.

2.4. Cytokine analysis

Cytokine levels in pooled serum of mice inoculated with variable doses of ETX were assayed using the Milliplex® MAP Mouse Cytokine/Chemokine Polystyrene Bead Panel (#MPXMCYTO-70K, Millipore Corp., Billerica, MA 01821). For this, groups of four mice were inoculated iv with 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 μg/kg of ETX, The mice were bled by cardiac puncture under anesthesia as soon as possible after onset of clinical signs (2–6 h after inoculation) or, in those that did not show clinical alterations, at periods between 2 and 36 h after inoculation. The serum samples from all the mice in each group were pooled and stored at −80 °C until tested. The protocol established by the manufacturer was used. Briefly, mouse serum samples were thawed, mixed by vortexing, and then clarified through filter spin columns (#UFC30DV00, Millipore Corp.) by room temperature centrifugation at 12,000 × g for 4 min. 25 μl of each standard, control, or undiluted sample were added in duplicate to antibody-conjugated beads and incubated in a 96-well filter plate overnight at 2–8 °C with shaking at 650 rpm. After 16–18 h, wells were washed and 25 μl of detection antibody was added to each well. After 1 h of incubation (20 °C/650 rpm), 25 μl of Streptavidin-Phycoerythrin was added to each well and incubated for 30 min (20 °C/650 rpm). Final washes were completed following which 150 μl of sheath fluid was added to each well. The plate was analyzed using a Bio-Plex® 200 Suspension Array System (Bio-Rad Laboratories, Hercules, CA). The instrument settings were as follows: 50 events per bead, 100 μl sample size, and gate settings at 8000–15,000. The software used to perform the assay and analyze data was the Bio-Plex Manager™ Software 6.0, which calculated concentrations in pg/ml based on the respective standard curve for each cytokine. Cytokines measured included GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, MCP-1, and TNFα. Only cytokines at detectable levels were reported for the purposes of this analysis. Dosage groups were combined into two major 'low' and 'high' (5–20 and 25–50 μg/kg, respectively) groupings for ease of interpretation of data and to compensate for lack of group size to support statistical comparison. Group concentrations for individual cytokines were statistically compared using Wilcoxon Signed rank test; $P < 0.05$ was considered significant.

2.5. Production of anti-ETX MAb c4D7

Genes containing the variable region sequences of 4D7 were synthesized (Life Technologies; San Diego, CA) and subsequently cloned into plant expression vectors (TMV and PVX, Icon Genetics, GmbH; Giritch et al., 2006) containing codon-optimized human kappa and human IgG_1