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Clostridium difficile toxin B inhibits the secretory response of human mast cell line-1 (HMC-1) cells stimulated with high free-Ca^{2+} and GTPγS

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ABSTRACT

Clostridium difficile toxins A and B (TcdA and TcdB) belong to the class of large clostridial cytotoxins and inactivate by glucosylation some low molecular mass GTPases of the Rho-family (predominantly Rho, Rac and Cdc42), known as regulators of the actin cytoskeleton. TcdA and B also represent the main virulence factors of the anaerobic gram-positive bacterium that is the causal agent of pseudomembranous colitis. In our study, TcdB was chosen instead of TcdA for the well-known higher cytotoxic potency. Inactivation of Rho-family GTPases by this toxin in our experimental conditions induced morphological changes and reduction of electron-dense mast cell-specific granules in human mast cell line-1 (HMC-1) cells, but not cell death or permeabilisation of plasma-membranes. Previously reported patch-clamp dialysis experiments revealed that high intracellular free-Ca^{2+} and GTPγS concentrations are capable of inducing exocytosis as indicated by significant membrane capacitance (C_m) increases in HMC-1 cells. In this study, we investigated the direct effects of TcdB upon HMC-1 cell "stimulated" C_m increase, as well as on "constitutive" secretion of hexosaminidase and interleukin-16 (IL-16). Compared to untreated control cells, HMC-1 cells incubated with TcdB for 3–24 h exhibited a significant reduction of the mean absolute and relative C_m increase in response to free-Ca^{2+} and GTPγS suggesting an inhibition of secretory processes by TcdB. In conclusion, the HMC-1 cell line represents a suitable model for the study of direct effects of *C. difficile* toxins on human mast cell secretory activity.

1. Introduction

Clostridium difficile is an anaerobic, gram-positive bacterium and pathogenic agent of pseudomembranous colitis, a severe enteric disease, with intense colonic inflammation (Meyer et al., 2007). Its main virulence factors are two exotoxins, the enterotoxin A (TcdA) and the cytotoxin B (TcdB), belonging to the class of large clostridial toxins and inactivating some low molecular mass GTPases of the Rho-family (predominantly Rho, Rac and Cdc42) by glucosylation (Jank et al., 2007; Voth and Ballard, 2005). The Rho-family GTPases are critical regulators of the actin cytoskeleton and participate in several signalling events (Mackay and Hall, 1998; Van Aelst and D'Souza-Schorey, 1997).

Epithelial cells, monocytes–macrophages, neurons of the enteric nervous system and mast cells are the most significant actors in gut inflammation. These cell types can be directly or indirectly affected by TcdA and TcdB, and secrete mediators that regulate the inflammatory activity of the intestine (Flegel et al., 1991; He et al., 2002; Kim et al., 2002; Neunlist et al., 2003; Tixier et al., 2005; Voth and Ballard, 2005). Particularly, it is known that enteric mast cells play a crucial role in the intestinal defence mechanisms against pathogens (Abraham and Malaviya, 1997; Galli et al., 1999) and their involvement in *C. difficile*-induced inflammation has been amply verified (Calderón et al., 1998; Voth and Ballard, 2005; Wershil et al., 1998).

In our study, TcdB was chosen instead of TcdA, for the well-known higher cytotoxic potency. TcdB is ~2 times more potent than TcdA on HMC-1 (human mast cell line-1) cells

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(Meyer et al., 2007), even ~10 times on human colonic epithelial cells (Riegler et al., 1995) and up to 500–1000 times on other cell types, possibly depending on the enzymatic activity, cellular content of specific substrates and density of toxin-specific receptors on the cell surface, but also the type of cell preparation (Chaves-Olarte et al., 1997; Flegel et al., 1991). E.g., whereas on isolated strips of human colonic mucosa from patients with colorectal cancer TcdB was found to be ~10 times more potent than TcdA, on human colon carcinoma T84 cells TcdA proved to the contrary 10 times more potent than TcdB (Chaves-Olarte et al., 1997; Riegler et al., 1995).

TcdB is a monoglucosyltransferase which consists of 2366 residues (269.6 kDa) (Jank et al., 2007; Just et al., 1997). Specifically in RBL (rat basophilic leukaemia) cells it inactivates Rho-family GTPases by glucosylation at Thr^{37} (RhoA, B and C) and Thr^{35} (Rac and Cdc 42), respectively (Just et al., 1995).

A restructuring of actin cytoskeleton due to the TcdB-mediated inactivation of Rho-family GTPases has been well-documented in RBL cells, rat peritoneal mast cells and HMC-1 cells (Djouder et al., 2000, 2001; Meyer et al., 2007; Prepens et al., 1996; Wex et al., 1997) as well as in several cell types other than mast cells (Just et al., 1994; May et al., 2013; Mitchell et al., 1987; Siffert et al., 1993).

In RBL cells, TcdB has been recognised to prevent the "stimulated" secretory response of many different stimulators (Djouder et al., 2001, 2003; Prepens et al., 1996) but, on the other hand, facilitates "spontaneous" or "constitutive" secretion in HMC-1 cells (Meyer et al., 2007). On the contrary, depolymerisation of the cytoskeleton by actin inhibitors resulted in an increased release induced by the same stimulators, but this was ineffective if cells were co-treated with the toxin (Prepens et al., 1996). These data showed that TcdB inhibits the "stimulated" secretion of RBL cells by glucosylating the Rho-GTPases, independently of its effect on actin cytoskeleton.

Although it displays some limitations due to its neoplastic nature (Butterfield et al., 1988), the HMC-1 cell line represents a suitable model for the study of human mast cell degranulation since it is capable of expressing several cytokines (Grützkau et al., 1997; Möller et al., 1998 Xia et al., 2011) and shows secretory activation in response to classic mast cell stimulators (Balletta et al., 2013).

All previous studies in HMC-1 cells have investigated the effects of TcdB on the "constitutive" exocytosis of this cell line. By applying TcdB directly to HMC-1 cells, stimulatory effects were observed on "constitutive" secretion of hexosaminidase, interleukin-8 (IL-8) and -16 (IL-16), as well as on prostaglandin synthesis (Gerhard et al., 2011; Meyer et al., 2007). An exploration of the effects of TcdB on the "stimulated" secretion in HMC-1 cells has not been reported so far.

Membrane capacitance (C_m) measurement technique (Gillis, 1995, 2000), applied in the patch-clamp whole-cell mode (Hamill et al., 1981), showed that HMC-1 cells exhibit a C_m increase when dialysed with high free-Ca^{2+} and GTPγS containing intracellular solution (Balletta et al., 2013).

In the present study, the direct effect of TcdB on "stimulated" secretion in the human mast cell line HMC-1 was investigated using the patch-clamp technique. The HMC-1 cells were incubated for different time periods with TcdB diluted in the cell culture medium. Intracellular activity of the toxin was revealed by an immunoblot analysis of glucosylated Rac1. After undergoing the TcdB treatment, HMC-1 cells were stimulated by intracellular application of high free-Ca^{2+} and GTPγS. Cells incubated with TcdB exhibited a significant reduction of the mean C_m increase compared to control cells not treated with the toxin, suggesting a significant inhibition of the "stimulated" secretion.

2. Material and methods

2.1. Cell culture

HMC-1 cells were kindly supplied by Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, MN, USA) and were cultured in Iscove's basal medium plus stable glutamine (Biochrom AG, Berlin, Germany) supplemented with 10% v/v FBS (Biochrom AG), 100 μM penicillin (Biochrom AG), 100 μg/ml streptomycin (Biochrom AG) and 0.01% v/v monothioglycerol (Sigma, St. Louis, MO, USA), as previously described (Balletta et al., 2013).

2.2. Application of TcdB

2.2.1. Purification of TcdB

TcdB from the culture supernatant of *C. difficile* was purified by ion-exchange chromatography using a MonoQ column (Amersham Biosciences, Freiburg, Germany), as previously described (Just et al., 1997).

2.2.2. Cell incubation with TcdB

TcdB was added to the cell culture medium (2×10^6 cells/ml) at a 4 μg/ml (12 nM) concentration. This toxin enters the cells by endocytosis after binding to specific cell receptors (Chaves-Olarte et al., 1997; Florin and Thelestam, 1983; Voth and Ballard, 2005). After an incubation time period of 3, 7, 11, 15 or 24 h (groups TcdB3h–24h in Table 1), HMC-1 cells underwent patch-clamp measurements.

Table 1
Effects of *C. difficile* toxin B (TcdB) treatments on membrane capacitance (C_m) in HMC-1 cells stimulated with intracellular free-Ca^{2+} and GTPγS. Treatment groups differ according to the time period of incubation with TcdB (from 3 to 24 h). After undergoing the toxin treatment, cells were stimulated by intracellular application of 14.5 μM free-Ca^{2+} and 300 μM GTPγS. Cells of the control group Ctr (Balletta et al., 2013) were directly stimulated without any toxin pre-treatments. Data are expressed as means ± S.E.M.; $^{*}P < 0.05$ of group TcdB24h vs. groups Ctr TcdB3h–11h (resting C_m) and $^{**}P < 0.01$ of group Ctr vs. all the other treatment groups (TcdB3h–24h) in a Newman–Keuls multiple comparison test post single-factor ANOVA. There was no significant difference among TcdB-treatment groups (TcdB3h–24h).

Treatment groups	Resting C_m (pF)[a]	Maximum C_m (pF)[b]	Response absolute C_m increase (pF)[c]	Response relative C_m increase (%)[d]	n[e]
Ctr	18.64 ± 1.99	21.90 ± 1.84	3.26 ± 0.33**	21.14 ± 3.40**	15
TcdB3h	12.82 ± 1.90	13.38 ± 1.87	0.56 ± 0.19	5.29 ± 1.94	8
TcdB7h	15.02 ± 2.97	15.69 ± 3.05	0.67 ± 0.13	4.99 ± 0.98	8
TcdB11h	15.67 ± 1.38	16.38 ± 1.32	0.71 ± 0.25	5.27 ± 2.09	8
TcdB15h	20.58 ± 1.44	21.71 ± 1.74	1.13 ± 0.37	5.25 ± 1.32	5
TcdB24h	27.41 ± 4.08*	28.85 ± 4.07	1.44 ± 0.39	6.47 ± 2.03	7

[a] Initial value of C_m.
[b] C_m value reached after cell dialysis with the intracellular solution containing 14.5 μM free-Ca^{2+} and 300 μM GTPγS.
[c] Difference between maximum and resting C_m.
[d] For each sample cell, the value of absolute C_m increase in pF was normalised to the individual value of the resting C_m (100%) and the response of all treatment groups was expressed as mean relative (in %) C_m increase. Note, that the mean absolute and relative C_m values may therefore not be completely identical.
[e] Number of cells in each group.