Production and Characterization of *C. difficile* Binary Toxin Monoclonal Antibodies

S. X. Su, Y. Li, C. Dunlap

Thermo Fisher Scientific, Microbiology, Fremont, CA

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Abstract

Background: Many pathogenic *Clostridium difficile* strains produce not only toxin A and toxin B but also binary toxin (CDT). Recent studies indicated that *C. difficile* strains containing *cdt* gene together with toxin A and toxin B play an important role in the severity of human *C. difficile* Infection (CDI), with higher recurrent CDI and CDI related fatality rates, compared to the strains with toxin A and B genes only. To date, there has not been a CDT reacting monoclonal antibody produced. **Methods:** A *C. difficile* strain expressing binary toxin was cultured to produce the immunogens. Culture supernatant fractions enriched for the CDTa and CDTb subunits were separated using multistep biochemical and immunological methods, including size exclusion chromatography and immunoabsorptions. Protein bands corresponding to the molecular weights of CDTa and CDTb subunits were analyzed by mass spectrometric (MS) peptide mapping to identify the two subunits. Standard procedures were then applied to produce monoclonal antibodies (mAbs) from the purified subunits. **Results:** Out of 86 clones initially screened several were selected reacting strongly to the CDT immunogens by ELISA. Evaluation and characterization by Western blot analysis identified at least six clones that were specific to CDT subunit B. SEC HPLC analysis of the Protein G column purified mAbs demonstrated over 95% purity. Isoelectric focusing gel electrophoresis was performed to determine the pIs of selected mAbs. Immuno-inhibition study indicated that clone 18D12 may recognize the same epitope as clone 7A1 but not the other mAbs. Affinity columns made from two of the mAbs were able to purify CDT subunit B as confirmed by MS peptide mapping and sequencing analysis. Preliminary qualitative microplate EIA and lateral flow immuno-chromatography testing using two of the mAbs detected binary toxin expression from all 11 culture supernatants of *C. difficile* clinical isolates containing the *cdt* gene. **Conclusions:** Monoclonal antibodies that react to *C. difficile* binary toxin subunit B were produced. These monoclonal antibodies should be very useful tools in studying the effects of expressed CDT protein on the mechanisms of human CDI related disease and in developing immunoassay to detect CDT expression.

Introduction

Clostridium difficile binary toxin (CDT) is an actin specific ADP-ribosyltransferase that contains two subunits, CDTa and CDTb. CDTa is the enzymatic component and CDTb is believed to be the host cell binding component. *C. difficile* CDTa was first described from the *C. difficile* strain CD196 in 1988 (1) and the first set of CDT genes encoding a CDTa polypeptide with 463 deduced amino acid (aa) sequence and CDTb polypeptide with 863 deduced aa sequence (designated *cdtA* and *cdtB)* was published 1997 (2). There is 81% aa identity between CDTa and the corresponding component of *C. perfringens* iota toxin a (Ia), and 84% aa identity between the CDTb and iota toxin b (Ib). The role of CDT in the pathogenesis of *C. difficile*-associated diarrhea (CDAD) is yet to be determined since many human *C. difficile* strains do not produce functional CDT. The emergence of binary toxin containing hypervirulence *C. difficile* strains since 2005 indicated that CDT could be an additional virulence factor associated with severity of human *C. difficile* Infection (CDI) and the increased morbidity and mortality (3). Recently published data indicated that *C. difficile* strains containing *cdt* gene may be directly related to the higher recurrent CDI (4), and the coexistence of *cdt* gene with toxin A (*tcdA*) and toxin B (*tcdB*) genes increased CDI related fatality rates, compared to the strains with toxin A and B genes only (5). To date, there has not been a CDT reacting monoclonal antibody produced from CDT expressing strains. We report here the production and characterization of monoclonal antibodies produced against CDTb polypeptide isolated from *C. difficile* strain CCUG 20309.

Methods

❑*C. difficile* strain CCUG 20309 was grown in Brain Heart Infusion (BHI) medium at 37˚ C for 48 hours. The culture supernatant was treated with ammonia sulfate (AS) to concentrate the proteins. The concentrated proteins were passed through a series of immunoabsorption columns to remove some major non-CDT proteins. Size-exclusion (SEC) and DEAE ion-exchange chromatography methods or electro-elution were used to further separate CDTa and CDTb polypeptides.

❑Fractions containing the enriched CDTa and CDTb were separated by SDS-PAGE and stained with Coomassie Blue (Figure 1A). A band corresponding to CDTa at around 50 kDa and a band corresponding to CDTb at around 110 kDa molecular weight markers area were sliced and sent for protein ID by mass spectrometric (MS) peptide mapping at Alphalyse. Isoelectric focusing gel (IEF) was used to characterize the pIs of immunogens.

❑Enriched CDTa and CDTb fractions were used to immunize the mice for mAb production with standard mAb production method by a Thermo Fisher Monoclonal Antibody Group. Microplate EIA was used for initial antibody clone screening and later for mAb activity evaluation. Western blotting was also used for mAb screening.

❑MAbs were purified by Protein G chromatography and SEC-HPLC was used for mAb purity assessment. IEF was also used to characterize the pIs of monoclonal antibodies (mAbs).

❑Lateral Flow Chromatography method was used to evaluate the functionality of the selected mAbs in reacting to *cdt* gene containing *C. difficile* bacteria isolates and clinical samples.

Results

Isolation and analysis of the CDTa and CDTb immunogens: Figure 1A is the SDS PAGE showing the CDT immunogen enrichment/purification process and Figure 1B is the IEF gel showing the pIs isolated CDTa and CDTb fractions. It demonstrated that CDTa isolated had higher purity than the CDTb polypeptide. CDTa on the gels showed the molecular weight (MW) of around 50 kDa and pI of around 8 which matched its theoretical MW and pI closely; CDTb on the gels showed the MW of around 110 kDa and pI of 4.7-4.8 that matched the theoretical MW and pI. The two bands corresponding to 50 kDa and 110 kDa were sent to Alphalyse and analyzed by MS peptide mapping which confirmed that the 50 kDa band matched the CDTa sequence and the 110 kDa band matched the CDTb sequence.

Monoclonal ab production and evaluation: Immunogen CDTa on lane 5 was used to immunize 3 mice and immunogen CDTb was accidently mixed with small amount (~15%) of the purified CDTa and was used to immunize another 3 mice. The 3 mice immunized with the mixture of CDTb and CDTa died after first immunization. Fresh immunogenof CDTb without CDTa was prepared and immunized 3 new mice. No mouse died of separated immunization of CDTa and CDTb.

➢Initial microplate (MP) EIA screening selected more than 86 clones for further screening. Over a dozen were selected for ascites production due to strong reaction to CDTb or CDTa after screened by MP EIA. Some of the high titer ones were purified by Protein G column chromatography. Western Blotting was carried out for evaluation of each mAb. Figure 2 shows the Western blot of immuno-absorbed culture supernatant fraction reacting with some of the purified mAbs. Clones 7B5, 7A1, 1A1, 4G10, 18D12, 8C9, 14H9 were shown to react with the bands with MW of about 110 kDa and nearly 80 kDa. The higher MW band corresponded well to the precursor of the CDTb and the lower MW band may correspond to the mature form of CDTb. Unfortunately the two clones (1A1 and 4G10) reacting strongly in CDTa screening by ELISA reacted to the CDTb band on Western blot. The clone 1A1 also reacted to another band at the MW of 40 kDa area indicating that this mAb was either not from a single-cell clone or there is cross-reactivity with a polypeptide other than CDTb.

➢Affinity columns made from purified 7B5 and 7A1 were used to purify a protein band corresponding to 110 kDa on SDS PAGE and were sent to Alphalyse for ID. The MS peptide mapped sequences matched the CDTb polypeptide sequence.

➢Some of the mAbs were coated on microplate wells and incubated with isolated CDTb antigen. HRP labeled mAb 7A1 was used as detection antibody and result showed that 18D12 and 7A1 could not form a sandwich indicating that they might compete with the same epitope on CDTb (Table 1).

Table 1. Sandwich EIA Assay

Coated IgG @ 1ug/mL	8C9	14H9	18D12
Microplate reading @ 450 nm	1.6	over	0.057
Blank	0.056	0.052	0.054

➢Microplate EIA and Lateral flow chromatography (LF) were used with 2 of the mAbs to test the culture supernatants of 11 *C. difficile* clinical isolates containing *cdtA* and *cdtB* and Toxin (*tcd*) A and B genes. Results demonstrated that the mAbs detected CDTb expressed in all the 11 clinical isolates strongly as shown by high peak height units (phu) with an image analyzer or by visual scores (scaled from 1- to 4+) (Table 2).

Table 2. EIA and LF testing for *C. difficile* clinical isolates

	cdt A	*cdt* B	*tcd* A	*tcd* B	ELISA	Lateral Flow Immuno-Assay
CdtB Immunogen	N/A	N/A	N/A	N/A	over	N/A
CCUG 20309	Pos.	Pos.	Neg.	Pos,	0.772	61 peak height units (phu) 4+ visual score
Clin. iso. KP	Pos.	Pos.	Pos.	Pos.	2.106	N/A
Clin. iso. #1	Pos.	Pos.	Pos.	Pos.	0.908	63 phu , 4+
Clin. iso. #2	Pos.	Pos.	Pos.	Pos.	0.324	34 phu , 4
Clin. iso. #3	Pos.	Pos.	Pos.	Pos.	0.659	47 phu , 4+
Clin. iso. #4	Pos.	Pos.	Pos.	Pos.	0.731	54 phu , 4+
Clin. iso. #5	Pos.	Pos.	Pos.	Pos.	1.177	72 phu , 4+
Clin. iso. #6	Pos.	Pos.	Pos.	Pos.	0.55	57 phu , 4+
Clin. iso. #7	Pos.	Pos.	Pos.	Pos.	0.772	87 phu , 4+
Clin. iso. #8	Pos.	Pos.	Pos.	Pos.	1.685	82 phu , 4+
Clin. iso. #9	Pos.	Pos.	Pos.	Pos.	1.243	74 phu , 4+
Clin. iso. #10	Pos.	Pos.	Pos.	Pos.	1.41	84 phu , 4+
ATCC 43255	Neg,	Neg.	Pos.	Pos.	0.05	0 phu , 0
ATCC 700057	Neg,	Neg.	Neg,	Neg.	0.059	0 phu , 0
Blank	N/A	N/A	N/A	N/A	0.052	N/A

Figure 1. CDTa and CDTb enrichment and isolation process and analysis.

A. SDS PAGE stained with Coomassie blue. Lane 1, molecular weight marker. Lane 2, ammonia sulfate fraction of *C. difficile* CCUG 20309 culture supernatant; lane 3, the lane 2 material immunoabsorbed by a few affinity columns; lane 4, SEC enriched lane 3 material; Lane 5 and 6, CDTa and CDTb separated after ion-exchange chromatography or electroelution.

B. IEF gel electrophoresis of CDTb (lane 1) and CDTa (lane 2) stained with Coomassie blue. A band corresponding to CDTa had a pI around 8.0 (Lane 2) and was close to the theoretical pI of 8.17; a doublet bands corresponding to CDTb at pIs around 4.6-4.7 (lane 1) matched the theoretical pI of 4.78.

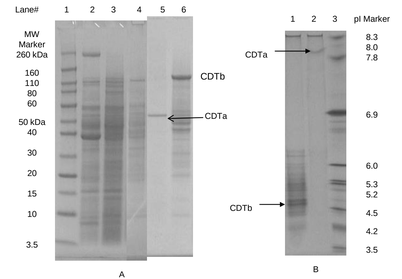
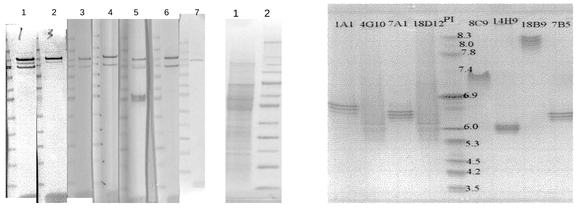


Figure 2. A. Western blotting analysis of CDTb mAbs. Lanes 1 through 7: clones 8C9, 14H9, 7A1, 8D12,1A1, 4G10, and 7B5. B. Immunoabsorbed CCUG 20309 culture supernatant (lane 1) separated on SDS PAGE, transferred to NC membrane and stained with Ponceau S before immunostained by various mAbs in panel A. C. IEF gel analysis of the purified monoclonal antibodies.



Conclusions and Discussion

•Thermo Fisher Scientific Microbiology has produced monoclonal antibodies from native *Clostridium difficile* binary toxin immunogen that specifically react to CDTb.

•These mAbs should be useful tools in studying the effects of the binary toxin expressing *C. difficile* infection on patients *in vivo* and *in vitro*. The antibodies should be also helpful in understanding the mechanism of CDT intoxicating of host cells. Immunoassays (EIA and LF) can be developed with these mAbs.

• It is not clear why no single mAb clone was isolated that reacts to CDTa subunit even though the isolated CDTa immunogen was highly purified. It might be that the polypeptide was not very immunogenic in mice.

• It seems that CDTa or CDTb alone is not lethal for mice based on our immunization experiences. It is logical that the two polypeptides need to work together to intoxicate the host cells based on their reported functions.

• More tests need to be done to understand whether these mAbs cross-react with C2 toxin of *C. botulinum*, iota toxin of *C. perfringens*, *C. spiroforme* toxin (CST), and other related binary toxins.

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