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Recombinant antigens based on toxins A and B of *Clostridium difficile* that evoke a potent toxin-neutralising immune response

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ABSTRACT

Infection with the bacterium *Clostridium difficile* causes symptoms ranging from mild to severe diarrhoea with life-threatening complications and remains a significant burden to healthcare systems throughout the developed world. Two potent cytotoxins, TcdA and TcdB are the prime mediators of the syndrome and rapid neutralisation of these would afford significant benefits in disease management. In the present study, a broad range of non-toxic, recombinant fragments derived from TcdA and TcdB were designed for soluble expression in *E. coli* and assessed for their capacity to generate a potent toxin-neutralising immune response as assessed by cell-based assays. Significant differences between the efficacies of isolated TcdA and TcdB regions with respect to inducing a neutralising immune response were observed. While the C-terminal repeat regions played the principal role in generating neutralising antibodies to TcdA, in the case of TcdB, the central region domains dominated the neutralising immune response. For both TcdA and TcdB, fragments which comprised domains from both the central and C-terminal repeat region of the toxins were found to induce the most potent neutralising immune responses. Generated antibodies neutralised toxins produced by a range of *C. difficile* isolates including ribotype 027 and 078 strains. Passive immunisation of hamsters with a combination of antibodies to TcdA and TcdB fragments afforded complete protection from severe CDI induced by a challenge of bacterial spores. The results of the study are discussed with respect to the development of a cost effective immunotherapeutic approach for the management of *C. difficile* infection.

1. Introduction

The Gram positive, anaerobic, spore-forming bacterium *Clostridium difficile* continues to be a significant problem within healthcare facilities [1–3] with an estimated global financial burden of over $12 billion. CDI is caused by ingested spores and is usually preceded by the use of antibiotics which perturb the normal gut flora. The bacterium colonises the digestive tract and produces potent cytotoxins which damage the gut epithelium and cause its characteristic symptoms [4,5]. These range from mild, self-limiting diarrhoea to sometimes life-threatening pseudomembranous colitis and toxic megacolon [6].

A 19.6 kb region (PaLoc) of the chromosome of *C. difficile* encodes its two principal virulence factors, toxins A (TcdA) and B (TcdB) [7]. Structurally, TcdA and TcdB are organised as complex, multi-domain proteins (see Fig. 1) which define its multi-step action [8]. Sequence variations in the 19.6 kb region (PaLoc) of the chromosome, which encodes TcdA and TcdB have been identified and these variants, termed toxinotypes, result in sequence differences between the toxins [9,10].

Current antibiotics, while successful in treating the majority of CDI cases, are less effective at managing recurrent or severe CDI [11]. As a consequence, several alternative therapies are under development [12]. With respect to therapeutic strategies directed at TcdA and TcdB, a considerable evidence base suggests that antibody-mediated neutralisation of these toxins affords protection against CDI [13,14]. These include passive immunisation studies [15–20] with antibodies to TcdA and TcdB and also vaccines designed to evoke a toxin-neutralising immune response to these toxins [21]. Recombinant vaccine candidates based on polypeptide fragments representing the C-terminal repeat regions of TcdA and TcdB have been the focus of a number of studies [22–28].

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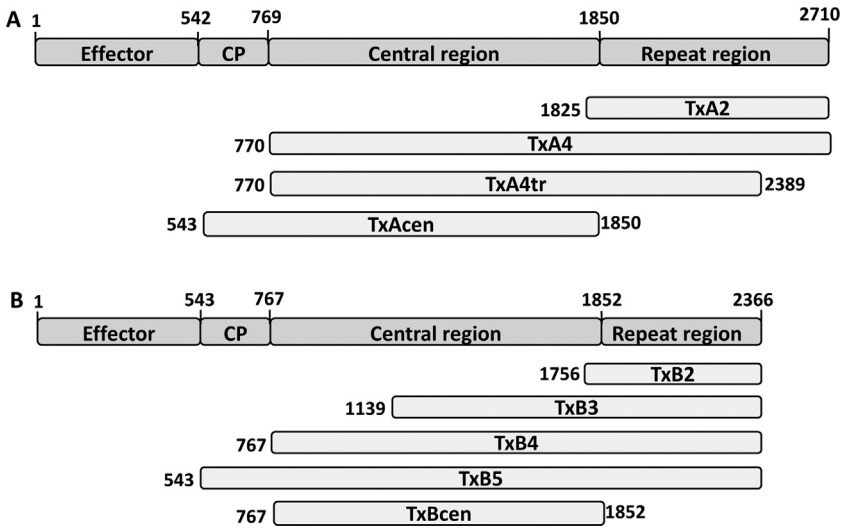

Fig. 1. Diagrammatic representation of the TcdA and TcdB regions and expressed recombinant constructs. Numbers correspond to the amino acid sequence.

Previously, we described the administration of ovine antibodies, which potently neutralise TcdA and TcdB, as a potential therapeutic option for the treatment of severe CDI [18]. In the current study, we describe recombinant fragments derived from the *C. difficile* toxins which can underpin the large-scale production of such therapeutic antibodies. Toxin regions critical to the generation of neutralising antibodies were also identified.

2. Materials and methods

2.1. C. difficile strains and purification of toxins

C. difficile VPI 10463, CCUG 20309 were from the ATCC. *C. difficile* ribotype 027 (NCTC 13366) was a gift from the Anaerobe Reference Laboratory, Cardiff and *C. difficile* ribotype 078 (clinical isolate) was obtained via the *C. difficile* Ribotyping Network (Southampton). These were toxinotyped and maintained as previously described [9,18]. TcdA and TcdB were purified from *C. difficile* strains by a modification [18] of a previously described protocol [29].

2.2. Expression and purification of recombinant fragments

TcdA and TcdB gene constructs optimised for *E. coli* expression were synthesised (Entelechon GmbH) (supplemental Fig. S1) and incorporated into the pET28a vector system. *E. coli* BL21(DE3) and BL21 Star (DE3) (Invitrogen) were used as expression hosts for recombinant toxin fragments. Protein expression was performed in Phytone Peptone Terrific Broth (PPTB) supplemented with kanamycin (50–100 μg/ml). *E. coli* BL21(DE3) containing expression constructs were grown in PPTB supplemented with kanamycin in a 3.0 l fermenter (Applikon Biotechnology) and expression induced by autoinduction at 25 °C or 1 mM isopropyl-beta-D-thiogalactopyranoside for 16 h at 16 °C.

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Cell paste (40 g) was resuspended in 400 ml of 50 mM Tris-HCl pH 8.0 buffer containing 500 mM NaCl, 4 mM EDTA, sonicated on ice (5 × 1 min) and the lysate centrifuged (25,000 × *g*, 20 min) before being dialysed against 50 mM Tris–HCl pH 8.0 buffer containing 500 mM NaCl at 4 °C. The dialysed material was made up to 20 mM imidazole using high imidazole buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 500 mM imidazole) and applied to a GE Chelating Sepharose (nickel) column (100 ml, ∅ 50 mm). After washing with 50 mM Tris–HCl pH 8.0 buffer containing 500 mM NaCl, 20 mM imidazole, bound material was eluted with a 10-column volume gradient to 100% of the high imidazole buffer. Thrombin cleavage was carried out in 20 mM Tris–HCl pH 8.4 containing 150 mM NaCl, 2.5 mM $CaCl_2$ overnight at 20 °C using restriction grade thrombin (Novagen) added at 1 U/mg protein; HRV 3C (Novagen) cleavage was performed in 50 mM Tris–HCl pH 7.5 buffer with 500 mM NaCl, 2.5 mM dithiothreitol for 20 h at 4 °C using a protease:protein ratio of 1:200 (wt/wt). Cleaved fragments were dialysed against 50 mM Tris–HCl pH 7.5 buffer containing 500 mM NaCl and 20 mM imidazole at 4 °C and applied (5 ml/min) to a GE Chelating Sepharose Ni column (100 ml, ∅ 50 mm) and the toxin fragment eluted in the flow through. Proteomic analyses (GeLC–MS/MS) using in-gel tryptic digestion of constructs were conducted at the Centre for Proteomic Research, Southampton University [30].

2.3. Production of ovine antisera and antibodies

Antigens were used to immunise groups of 3 sheep using Freund's adjuvant as described [18]. For formaldehyde treatment, antigens in HEPES buffer (50 mM, pH 7.4) containing 500 mM NaCl at 0.5–1 mg/ml, were made 0.2% (v/v) with formaldehyde and incubated at 37 °C for 24 h and then stored at 4 °C. Immunisations were carried out every 28 days and blood samples taken 14 days after each immunisation. Once adequate antibody levels were achieved, larger volumes of blood were taken and the IgG purified as previously described [18]. ELISA on serum and purified IgG (detection limit, 5–10 ng toxin-specific IgG/ml) was conducted by the method described previously [18].

2.4. Toxin neutralisation assay

A cell-based neutralisation assay using Vero cells was performed as described previously [18,29]. Cells were assessed by microscopy for rounding and the highest serum/IgG dilution providing complete protection from the cytotoxic activity of TcdA/B was recorded as the neutralisation titre. Antibody toxin neutralisation titres were also estimated by colorimetric assays based on cell staining with crystal violet [31]. These assays were performed as described above using final concentrations of TcdA and TcdB in antibody mixtures of 50 ng/ml and 2 ng/ml respectively. After overnight incubation, cells were washed gently with 200 μl of Dulbecco's PBS (Sigma) and fixed with 70 μl ice-cold ethanol for 2 min. The ethanol was then removed and 70 μl crystal violet (1%, w/v, in ethanol; Pro-Lab)

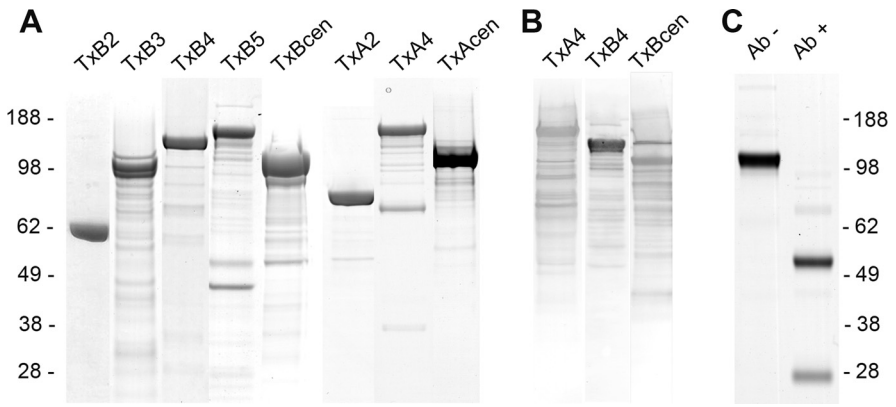

Fig. 2. Characterisation of recombinant antigens. (A) SDS-PAGE of purified TcdA and TcdB constructs as depicted in Fig. 1. (B) Western blots for key fragments using either ovine antibody raised against TcdA for TxA4 or to TcdB for TxB4 and TxBcen. Each antibody was used at a dilution of 1/30,000. (C) SDS-PAGE of a typical IgG preparation in the presence (Ab+) and absence (Ab−) of dithiothreitol (20 mM). IgG was purified by a caprylic acid precipitation method as described [18].

added to the fixed cells for 30 min at 22 °C. Plates were washed carefully in water to remove excess dye, dried at 37 °C and then 200 μl of 50% (v/v) ethanol added. Plates were incubated in a shaker incubator (37 °C; 300 rpm) for 2 h then read at 492 nm. ED_{50} values were derived from the resulting toxin neutralisation curves using 4 or 5-pl nonlinear regression models (SigmaPlot 12.0).

2.5. Animal model for C. difficile infection

The Syrian hamster model was performed as described previously using groups of 10 animals [30]. All hamsters were weighed and administered clindamycin (2 mg in 0.2 ml sterile H_2O) by the orogastric route on Day 0. On Day 2, test animals were challenged (orogastrically) with between 10^2 and 10^3 colony forming units of *C. difficile* spores in 0.2 ml DMEM. Animals were weighed daily and monitored 6 times/day for 15 days for disease symptoms (diarrhoea, weight loss, lethargy and tender abdomen) [19,32]. Survival curves were analysed by log rank tests (non-parametric distribution analysis, right censoring). For passive immunisation, ovine IgG was purified from antisera generated using TxA4 and TxB4 fragments. Doses (0.5–2 ml) were administered at various times by the intraperitoneal route (see Fig. 4).

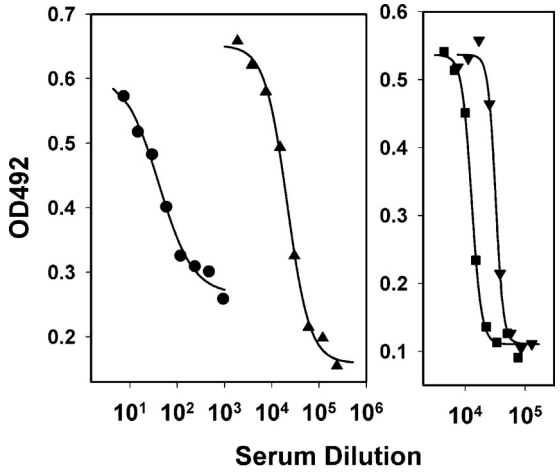

Fig. 3. Antibody-mediated neutralisation of TcdA and TcdB as measured by the Vero cell ED_{50} assay. The capacity of various dilutions of antiserum to TxB2 (●) and TxB4 (▲) to prevent the cytotoxic effects of purified TcdB (2 ng/ml) was assessed using crystal violet staining to measure cell viability [31]. Neutralisation by TxA2 (■) and TxA4 (▼) antiserum was assessed using TcdA at 50 ng/ml. Each antiserum was a pool obtained from the immunisation of 3 sheep.

3. Results

3.1. Expression, purification and assessment of recombinant TcdB constructs

The panel of TcdB-derived fragments is summarised in Fig. 1. Construct TxB5 contained the mutation Cys700 → Ser to reduce substantially the activity of the cysteine protease (CP) domain [33]. With the exception of antigen TxB2, levels of total protein expression and levels of soluble expression were low without the addition of an N-terminal fusion protein. Several fusion protein candidates were screened and thioredoxin and NusA were found to promote the highest levels of soluble expression. Details of the design of antigen constructs are provided as supplemental data (Fig. S1). Purified fragments were analysed by SDS-PAGE (Fig. 2) and immunoblotting. For each construct, the principal protein band reacted strongly with antibodies raised to TcdB [18] (data not shown). Proteomic analysis of TxB4 by GeLC–MS/MS using in-gel tryptic digestion confirmed its identity and presence of >98% of the predicted construct sequence.

End points in Vero-cell assays used to assess the levels of toxin-neutralising antibodies to fragments were determined by both microscopy (complete cell protection as the endpoint) and as an ED_{50} in which cell integrity was assessed using crystal violet staining (Fig. 3 and Table 1). While there was a generally good correlation between the two methods used to determine toxin-neutralising titres in the cell assay, there was little correlation between these and titres obtained by ELISA. For the latter, <25-fold variation in titres was observed for the various fragment sera compared to a >1000 fold difference in the serum neutralising titres. This suggests that neutralising antibodies represent a variable sub-set of the total toxin specific antibodies. With the exception of TxB5, toxin-neutralising titres obtained from animal sera immunised with native fragments were low. Mild treatment with formaldehyde significantly enhanced toxin neutralising titres of all fragments with improvements of >100-fold for TxB3 and TxB4 constructs. For the formaldehyde-treated fragments, inclusion of the central toxin domains markedly increased neutralising titres compared to TxB2 which consisted of TcdB repeat regions only. Highest toxin-neutralising titres were obtained with fragment TxB4 which elicited titres >100-fold that obtained with TxB2. Of the central domain-containing fragments, TxB4 was also expressed in highest yields (approximately 30 mg purified antigen per litre) making it the preferred antigen for generating antibodies to TcdB.

Table 1
Ovine antibody response to TcdB-derived recombinant antigens.

Antigen	ELISA titre	Neutralising titre (visual)	Neutralising titre ED_{50}		
			1	2	Mean
Native					
TxB2	2×10^4	<10	nd[a]	nd[a]	nd
TxB3	1×10^5	40	29 ± 3	20 ± 9	25
TxB4	7×10^4	80	32 ± 3	32 ± 3	32
TxB5	5×10^4	640	1196 ± 118	996 ± 158	1096
HCHO-treated (0.2%, v/v)					
TxB2	2×10^5	80	41 ± 11	59 ± 13	45
TxB3	5×10^5	5120	7495 ± 1418	9984 ± 2209	8740
TxB4	2×10^5	10,240	23,364 ± 3363	21,309 ± 1620	22,337
TxB5	5×10^4	5120	12,673 ± 1878	14,682 ± 1587	13,678
TxB(cen)	1×10^5	5120	7946 ± 1027	8225 ± 418	8086

For each antigen, 5 doses of 100 μg were given monthly to each of 3 sheep and the serum analysed at 18 weeks. ELISA titres, derived from 14-week samples, represent serum dilutions (pool from 3 animals) which gave a signal of 0.5 A_{450} above background and are the mean of duplicate determinations. Visual neutralising titres (units/ml) were determined by light microscopy and represented the dilution of antiserum that was able to completely protect Vero cells from a fixed concentration of TcdB at 0.5 ng/ml. Data represent the mean of duplicate determinations. For the crystal violet ED_{50} assay, TcdB was used at a fixed concentration of TcdB at 2 ng/ml.

[a] Neutralising titres were too low to achieve 50% neutralisation in the assay.

3.2. Expression, purification and assessment of recombinant TcdA constructs

A panel of recombinant TcdA fragments was expressed and purified in a similar manner to that described for the TcdB fragments above (Figs. 1 and S1). In toxin neutralising assays for several of the constructs, and notably TxA2, the microscopy-based assay end point (100% cell protection) was poorly defined with a low level of cell death occurring over several dilutions within the assay. This resulted in a poorer correlation between the neutralising titres derived by the two methods, with the ED_{50} values arguably providing a better relative measure of toxin-neutralising activity (Table 2 and Fig. 3). Limited treatment of antigens with formaldehyde significantly enhanced the neutralising titre elicited by TxA4, but the effects were less marked than those observed for the TcdB-derived constructs. The highest toxin neutralising titres were obtained with formaldehyde-treated TxA4. Yields of this fragment were lower than that for corresponding TcdB fragment with yields of 18–20 mg/l purified fragment obtained. Proteomic analysis of TxA4 by GeLC–MS/MS revealed that an impurity band of approximately 70 kDa was a breakdown product of TxA4 representing the N-terminus of the fragment.

3.3. Contribution of the regions of TcdA and TcdB to toxin neutralisation

Comparison of the data within Tables 1 and 2 with respect to the ED_{50} values derived for formaldehyde-treated fragments reveals significant differences with respect to the principal toxin domains contributing to the toxin-neutralising immune response. With respect to neutralisation of TcdB, serum raised against a central domain fragment (residues 767–1852; TxBcen) had >150-fold toxin-neutralising activity compared to the C-terminal fragment, TxB2. That these fragments displayed similar antibody ELISA titres (approx. 10^5) against TcdB suggests that this difference is not due to a poor immune response against the latter fragment. In contrast, comparison of the toxin-neutralising immune response elicited by similar fragments derived from TcdA, showed that the C-terminal fragment, TxA2 had approximately 6-fold increase in toxin-neutralising activity compared to a central domain fragment (residues 543–1850; TxAcen). These data indicate significant differences in the key domains that contribute to a toxin-neutralising immune response between TcdA and TcdB: the C-terminal region playing the dominant role in the case of TcdA as opposed to the central region domains in the case of TcdB.

3.4. Cross-neutralisation of TcdA and TcdB toxinotypes

Neutralising efficacy was assessed against TcdA and TcdB produced by key epidemic ribotype 027 and 078 *C. difficile* strains, which produce toxinotype 3 and 5 toxins, respectively [10] and TcdB (toxinotype 10) produced by a TcdA-negative, ribotype 036 strain [34] (Table 3). Antibodies raised against TxA4 were broadly neutralising with little or no loss of efficacy against toxinotype 3 and 5 toxins. A greater variation in cross-neutralising efficacy was observed with antibodies raised to TxB4. While a reduction of <3-fold was observed against TcdB toxinotypes 3 and 5, a more marked reduction in neutralising potency was observed against a toxinotype 10 TcdB.

3.5. Protection from CDI by passive immunisation

For passive immunisation studies, the high-toxin producing *C. difficile* strain, VPI 10463 was used. After perturbation of the normal gut flora using clindamycin, passively immunised and control group animals were challenged with *C. difficile* spores [18]. In

Table 2
Ovine antibody response to TcdA-derived recombinant antigens.

Antigen	ELISA titre	Neutralising titre (visual)	Neutralising titre ED_{50}		
			1	2	Mean
Native					
TxA2	2×10^5	640	21,619 ± 418	16,793 ± 25	19,206
TxA4	5×10^5	1280	13,232 ± 982	14,010 ± 421	13,621
HCHO-treated (0.2%, v/v)					
TxA2	2×10^5	1280	13,233 ± 384	12,292 ± 614	12,763
TxA4	7×10^5	15,360	28,826 ± 988	32,148 ± 981	30,487
TxA4(tr)	3×10^5	5120	8879 ± 395	8271 ± 246	8575
TxAcen	2×10^5	640	2472 ± 199	2096 ± 222	2284

Determinations were as described in Table 1 with the exception that TcdA was maintained at a fixed concentration of 50 ng/ml in both cell-based assays.

Table 3
Cross-neutralisation of toxinotypes of TcdA and TcdB.

Immunising antigen	Assay toxinotype	Neutralising potency ED_{50} (μg/ml IgG)		
		1	2	Mean
TxA4	TcdA (0)	1.6 ± 0.06	1.5 ± 0.07	1.5
TxA4	TcdA (3)	1.7 ± 0.23	1.4 ± 0.10	1.5
TxA4	TcdA (5)	1.7 ± 0.04	1.7 ± 0.14	1.7
TxB4	TcdB (0)	1.8 ± 0.13	1.5 ± 0.34	1.6
TxB4	TcdB (3)	1.9 ± 0.36	2.0 ± 0.29	2.0
TxB4	TcdB (5)	4.1 ± 0.43	4.0 ± 1.01	4.0
TxB4	TcdB (10)	9.4 ± 2.13	13.7 ± 0.55	11.6
TxBcen	TcdB (0)	2.5 ± 0.22	2.1 ± 0.20	2.3
TxBcen	TcdB (3)	7.9 ± 0.64	7.5 ± 1.20	7.7
TxBcen	TcdB (5)	7.7 ± 0.73	8.1 ± 0.71	7.9
TxBcen	TcdB (10)	7.2 ± 0.67	9.0 ± 0.75	8.1

Antibodies to TxA4 and TxB4 antigens (toxinotype 0 sequences) were assessed for their capacity to neutralise other TcdA and TcdB toxinotypes. Neutralising potencies are expressed in μg/ml IgG required for 50% neutralisation of either TcdA or TcdB at the concentrations defined below. Thus, lower values represent higher neutralising efficacy. Purified TcdA toxinotypes (0, 3 and 5) were each titrated in the cell assay and used at fixed concentration of 32× the minimum toxin concentration, which causes cell death in a 24 h incubation period. Purified TcdB toxinotypes (0, 3, 5 and 10) were used at 16× the minimum lethal concentration.

animals immunised with a mixture of antibodies raised against antigens TxA4 and TxB4, statistically significant protection from CDI ($p < 0.001$) was obtained with survival of 80% of the animals in the lower antibody doses. At the highest antibody dose, 100% of the animals were protected from severe CDI at 15 days post challenge; 30% of the animals in this group showed transient diarrhoea for 1–2 days. Animals which received either no antibody or non-specific ovine IgG, all succumbed to severe CDI within 3 days post challenge (Fig. 4). Protective efficacy was similar to that observed previously using antibodies produced using the full-length toxoids of TcdA and TcdB [18].

4. Discussion

Infection with *C. difficile* remains a problem within healthcare systems of the developed world [35] and additional therapeutic options are needed [36]. Previously, we described development of an immunotherapeutic for CDI based on the administration of polyclonal antibodies to TcdA and TcdB [18]. In the present study, we define antigens which can underpin the large-scale production of antibodies which potently neutralise TcdA and TcdB. We also show significant differences between TcdA and TcdB with respect to the protein regions which induce a toxin-neutralising immune response.

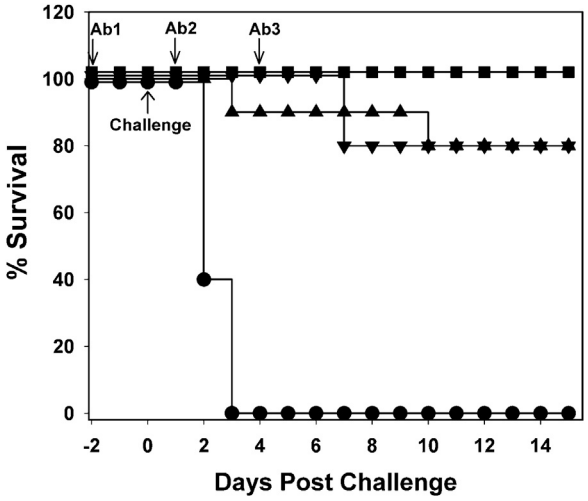

Fig. 4. Protection from CDI by passive immunisation with ovine anti-toxin A/B. Graph shows survival post challenge with *C. difficile* VPI 10463 spores in the hamster model. Animals (10 per group) were administered (2 ml, i.p.) with 3 doses of IgG produced using TxA4 and TxB4 and which consisted (■) 50 mg IgG containing 1×10^4 units anti A, 2×10^4 units anti B; (▼) 25 mg IgG containing 5×10^3 units anti A, 1×10^4 units anti B; (▲) 8.3 mg IgG containing 1.7×10^3 units anti A, 3.3×10^3 units anti B. Toxin neutralising units were determined by the cell-based assay (microscopy-based endpoint) as described in Table 1. A control group (●) received no antibody. Comparison of survival curves by log rank analysis showed ■, ▼ and ▲, $p < 0.001$ compared to the control group (●).

In a previous study [18] and consistent with others [17], we showed that a TcdB fragment representing the toxin's effector (glucosyltransferase) domain (residues 1–543) induced only a weak toxin-neutralising response as measured by cell-based assays. The present study focussed on various TcdB-derived recombinant fragments derived from C-terminal and central regions of TcdB. With all TcdB fragments assessed, their capacity to generate toxin-neutralising antibodies was markedly enhanced by limited treatment with formaldehyde. In the case of TcdB fragments, short-term formaldehyde treatment led to enhancement in toxin-neutralising potency of >100-fold for the majority of constructs. The mechanism of these enhancing effects is unclear, but stabilisation of protein structure through intra-molecular cross-linking (via methylene bridges) [37] is a possibility and such a mechanism has been proposed from similar observations with botulinum toxin fragments [38].

Consistent with other studies [23,27] immunising animals with fragment TxB2 which contained the entire repeat region of TcdB, generated antiserum with low toxin-neutralising titre. Inclusion of TcdB domains from the central (translocation) region of the toxin dramatically increased toxin-neutralising titres; in the case of fragment TxB4, which consisted of the entire central (residues 767–1852) and repeat regions (residues 1852–2366), titres were increased >120-fold. Immunisation of sheep with the central domain fragment (TxBcen; residues 767–1852) elicited a potent toxin-neutralising response confirming the presence of neutralising epitopes within this region. While the neutralising titre afforded by fragment TxB4 serum was approximately 2–3-fold increased compared to the central domain fragment TxBcen serum, the neutralising titres of purified IgG fractions differed by <2-fold (Table 3) which underlines the dominant role played by the TcdB central region in eliciting neutralising immune response. Previous studies on central domain fragments from TcdB reported derived antibodies with poor neutralising titres [17]. However, as none of these fragments represented the entire central domain, it is possible that key toxin-neutralising epitopes were either absent or compromised.

Assessment of toxin-neutralising titres of serum produced using TcdA-derived fragments revealed significant differences in the toxin regions which dominate the neutralising immune response compared to TcdB. While the highest titres were obtained with fragment TxA4 which consisted of both central and repeat regions, fragment TxA2 which comprised solely the repeat region induced a potent neutralising response and this is consistent with several previous studies [17,23]. A fragment representing the TcdA central region (TxAcen) gave neutralising titres markedly lower than TxA2. Thus, in contrast to TcdB, the repeat region rather than the central region appears to dominate the toxin-neutralising immune response within the TcdA fragments assessed. That a C-terminally truncated fragment, TxA4(tr), which contains only 4 of the 7 repeat unit modules compared to the full-length fragment, gave a significantly reduced neutralising immune response (approx. 3-fold) provides further evidence of the importance of this region. Previous vaccine design strategies for combating CDI have largely been based on antigens consisting of inactivated TcdA and TcdB holotoxins or fragments derived from the toxins' repeat regions [17,23,27]. Data presented in this study suggest that for TcdB, the latter approach is far from optimal as it omits key toxin-neutralising epitopes.

A further important consideration in the antigen design is whether the generated antibodies provide protection against a broad range of *C. difficile* isolates. Antibodies produced with TxA4 potently neutralised TcdA toxinotypes, 0, 3 and 5 with similar efficacy. Potent neutralisation by TxB4 antibodies was also observed against various TcdB toxinotypes albeit with some reduction in neutralising efficacy: <3-fold against TcdB toxinotypes 3 and 5 and approximately a 7-fold reduction against a TcdB toxinotype 10. It is notable that the latter unusual TcdB variant [39] showed least sequence homology compared to TcdB toxinotype 0 (85.7% overall and 88.1% within the central region).

In conclusion, the designed constructs TxA4 and TxB4 have several properties which make them attractive as antigen candidates. They can be expressed in a soluble form in scalable, low cost *E. coli*-based expression systems and were shown to induce the production of antibodies which neutralise potently key toxinotypes of TcdA and TcdB. In addition, a mixture of the resulting antibodies was shown to afford protection from severe CDI using the hamster infection model. Data presented in the study reveal significant differences between TcdA and TcdB with respect to the domains which evoke a toxin-neutralising immune response. The described antigens will support large-scale antibody production and so underpin the development of an immunotherapeutic platfom for the treatment of CDI.

Disclaimer

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Conflict of interest statement: The authors declare that they have no conflict of interest.

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