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A novel fusion protein containing the receptor binding domains of *C. difficile* toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models

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ABSTRACT

Antibodies targeting the *Clostridium difficile* toxin A and toxin B confer protective immunity to *C. difficile* associated disease in animal models and provided protection against recurrent *C. difficile* disease in human subjects. These antibodies are directed against the receptor binding domains (RBD) located in the carboxy-terminal portion of both toxins and inhibit binding of the toxins to their receptors. We have constructed a recombinant fusion protein containing portions of the RBD from both toxin A and toxin B and expressed it in *Escherichia coli*. The fusion protein induced high levels of serum antibodies to both toxins A and B capable of neutralizing toxin activity both *in vitro* and *in vivo*. In a hamster *C. difficile* infection model, immunization with the fusion protein reduced disease severity and conferred significant protection against a lethal dose of *C. difficile* spores. Our studies demonstrate the potential of the fusion protein as a vaccine that could provide protection from *C. difficile* disease in humans.

1. Introduction

The increased incidence of infectious diarrheal disease associated with *Clostridium difficile* and the marked morbidity and mortality resulting from such disease has made treatment of *C. difficile* associated disease (CDAD) a priority for the health care system [1,2]. Its virulence, spore forming ability and persistence contribute to its identification as a leading cause of diarrhea in hospitals worldwide. As the major nosocomial pathogen responsible for gastrointestinal diseases ranging from mild diarrhea to fulminant pseudomembraneous colitis, CDAD has been primarily associated with antibiotic treatment, however it is also a risk associated with immunosuppression, chemotherapy and gastrointestinal procedures. Within the hospital setting, between 10 and 25% of patients who undergo antibiotic treatment become infected [3,4]. Most illnesses resolve following discontinuation of antibiotic treatment followed by administration of metronidazole or vancomycin, however, the incidence of clinical relapse and secondary infection with multi-resistant organisms can be as high as 25% within this group [2,5].

The pathogenicity of *C. difficile* is mediated by the release of two potent exotoxins, toxin A and toxin B. Both toxins are high molecular weight (~300 kDa) secreted proteins that possess multiple functional domains [7]. The N-terminal domain of both toxins contains ADP-glucosyltransferase activity that modifies Rho-like GTPases. This modification induced by the *C. difficile* toxins causes a loss of actin polymerization and cytoskeletal changes resulting in the disruption of the colonic epithelial tight junctions. This leads to excessive fluid exudation into the colon and a resulting diarrhea. The central domain of the toxins contains a hydrophobic domain and is predicted to be involved in membrane transport of the ADP-glucosyltransferase domain from the endosome into the cytoplasm. The carboxy-terminal domain of both toxins contains a series of repeating units of 21-, 30- or 50-amino acids referred to as "clostridial repetitive oligopeptides" [8,9]. These repetitive units comprise the toxins' receptor binding domain(s) (RBD) responsible for the binding of toxin to cell surface oligosaccharide receptors on the target cells [10–12]. The repeat units are thought to exert their function by amplifying the toxin binding affinity through an avidity effect [13]. Thirty-eight repetitive oligopeptides have been identified in toxin A and 24 in toxin B [13].

Abbreviations: CDAD, *Clostridium difficile* associated disease; RBD, receptor binding domains; C-TAB, carboxy-terminal toxin A and B; C-TAB.G5, C-TAB generation 5; PRAS, pre-reduced anaerobically sterilized; MLD_{100}, minimum 100% lethal dose; ED_{50}, 50% effective dose.

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Animal studies have demonstrated that protection against CDAD and disease relapse correlates with the presence of anti-toxin serum antibody. Immunization of mice and hamsters with inactivated toxin (toxoid) and various toxin fragments induced protective immunity which is associated with high levels of toxin-neutralizing antibody [14–19]. These results have been supported by the use of anti-toxin antibody in passive transfer studies which further showed that that titers of serum antibodies to toxin A and toxin B correlate with levels of protection [14,20,21]. Antibodies to both toxin A and toxin B were required to provide optimal protection against morbidity and mortality, as well as CDAD relapse and *C. difficile* reinfection [22–24].

Human studies indicated a protective role for anti-toxin antibodies in CDAD outcome. In one study, a significant correlation between serum anti toxin A antibody concentrations and protection from CDAD was observed [3]. In patients colonized with *C. difficile* and treated with antibiotics, those with low levels of anti-toxin serum IgG were 48 times more likely to develop CDAD. In a second study, protection from *C. difficile* relapse correlated with the early development of anti-toxin A antibody [25]. Additional studies have shown that the administration of pooled human IgG containing anti-toxin antibodies led to clinical improvement in patients with severe or protracted CDAD [26–28]. In a recent phase 2 clinical trial, the passive transfer of monoclonal antibodies to toxins A and B provided protection against recurrent *C. difficile* diarrhea in human subjects [29].

Observations obtained in a number of studies have indicated that the critical antigenic determinants recognized by anti-toxin antibodies are localized to the repetitive oligopeptides contained within the carboxy-terminal RBD of both toxin A and toxin B. Immunization with toxin A fragments containing only the repetitive oligopeptide sequences induced toxin neutralizing antibody and was protective in hamsters receiving a lethal *C. difficile* spore challenge [19–21,29,30]. Human monoclonal antibodies against toxins A and B shown to confer protection against CDAD in hamsters [23] and providing protection against recurrent *C. difficile* diarrhea in human subjects [31] have also been mapped to this region. These observations indicate that blocking the binding of the toxins to their receptors is critical for the prevention of CDAD.

In this study we have constructed a recombinant fusion protein containing portions of the carboxy-terminal RBD from both toxin A and toxin B. The carboxy-terminal toxin A and B fusion protein (C-TAB) was evaluated in multiple animal models for its immunogenicity, and its ability to induce toxin neutralizing antibody and *in vivo* anti-toxin protective immunity. The data presented in this report demonstrate that the C-TAB fusion protein represents a novel and highly efficacious vaccine candidate for the protection against CDAD.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice 6–7 weeks of age were purchased from Charles River Labs, Wilmington, MA and were maintained under specific-pathogen-free conditions. Female Cynomolgus monkeys 4–6 years of age (body weight 2.0–4.0 kg) were provided by Avanza Laboratories, Gaithersburg, MD. Golden Syrian adult female hamsters weighing ~100 g (6–7 weeks of age) were purchased from Harlan Laboratories, Bristol, TN and were housed individually in micro-isolator cages (Micro-Vent Environmental System; Allentown Caging and Equipment Co., Allentown, NJ). Blood was collected from mice by tail sniping, from hamsters *via* the retro-orbital route and from the femerol vein for monkeys. Serum was separated by centrifugation of whole blood and transferred to a labeled tube and stored frozen at −20 °C until tested. All studies were approved by Animal Care and Use committees and conducted in accordance with institutional guidelines.

2.2. Bacterial strains

The *C. difficile* strain 630 was purchased from the ATCC (ATCC BAA-1382). *C. difficile* spore stocks were prepared by inoculating 500 ml of pre-reduced, anerobically sterilized (PRAS) brain heart infusion (BHI) media with a 5 ml, 24 h, BHI culture of *C. difficile* 630. After 7 days incubation at 37 °C the culture fluids were centrifuged (9000 × *g* for 20 min at 4 °C) and the pelleted spores resuspended in chilled PBS (250 ml). This material was heated in a water bath to 80 °C for 20 min before a second centrifugation. The new pellet was resuspended in 25 ml chilled PBS before dispensing 1 ml aliquots into sterile glass tubes that were stored at −70 °C. The stored material was characterized by thawing two vials on ice, making 10-fold dilutions in PRAS diluents and culturing the diluted material on PRAS blood agar for 7 days at 37 °C all inside an anaerobic chamber. The mean spore concentration after thawing was $10^{6.7}$/ml.

2.3. Antibodies and toxins

The *C. difficile* anti-toxin A monoclonal antibody (clone PCG4.1, IgG2a) was obtained from Meridian Life sciences and the anti-toxin B monoclonal antibody (B426M, IgG1) from GenWay Biotech, Inc. A guinea pig polyclonal antibody against toxin A was prepared in guinea pigs by hyper-immunization with purified toxin A. *C. difficile* toxin A and toxin B were purchased from TechLab, Inc., Blacksburg, VA.

2.4. Construction of recombinant fusion protein expression system

Nucleotide sequences encoding a fragment of the RBD of the *C. difficile* toxin A and toxin B were obtained by PCR amplification of genomic DNA isolated from *C. difficile* strain 630. The PCR amplified toxin A RBD gene fragment (nt 6817–8130) was ligated to toxin B RDB gene fragment (nt 5613–7164) with an intervening 12 nt linker (CGTAGCATGCAT). This recombinant nucleotide sequence was designated C-TAB.G5. The composition of the *C. difficile* toxin fusion protein is shown in Fig. 1.

The DNA encoding sequence of C-TAB.G5 was optimized for expression in *E. coli* (Geneart AG) to avoid internal Pribnow boxes, chi-sites, ribosomal entry sites, AT- or GC-rich sequence stretches, RNA instability motifs or RNA secondary structures. The sequence used for codon optimization corresponded to the *C. difficile* strain VPI10463 which differed from the strain 630 sequence at two nucleotides within the toxin A CDB sequence. These differences resulted in two amino acid changes within the toxin A portion of fusion protein; aspartic acid to histidine at residue 155 and alanine to asparagine at residue 156. The codon optimized nucleotide sequence was designated C-TAB.G5.1. Both C-TAB sequences were inserted into the pET28a(+) *E. coli* expression vector (Stratagene, La Jolla, CA) at the *Nco* I/*Xho* I restriction site.

2.5. Expression of recombinant fusion protein

The C-TAB/pET28b(+) vectors were expressed in the *E. coli* BL21(DE3) cell line (Novagen, Madison, WI). Seed cultures were expanded by overnight growth in LB broth supplemented with kanamycin (50 μg/ml). Overnight cultures were used to inoculate the fermenter (8–15 l). Fermentation was done in a fed-batch process, consisting of a batch phase (11 h; unlimited growth until total consumption of glucose), an exponential feed phase (6 h; controlled glucose feed at $\mu = 0.25\ h^{-1}$, biomass production), induction

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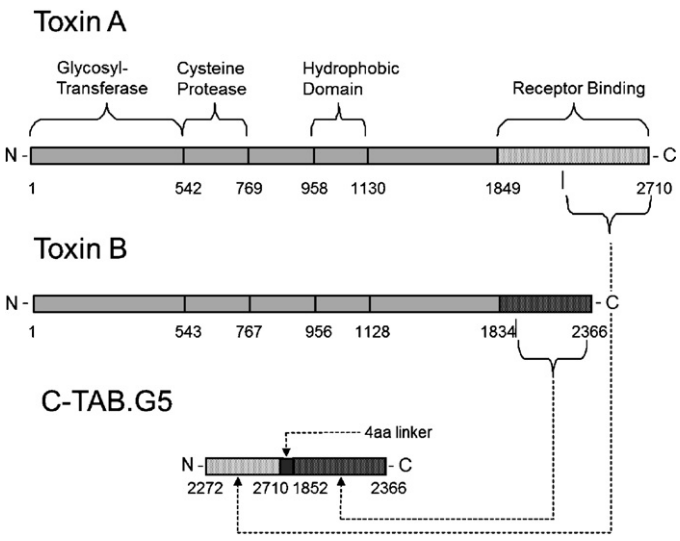

Fig. 1. Composition of the *C. difficile* toxin fusion protein C-TAB.G5. The carboxy-terminal portion of toxin A containing 19 of the 39 repetitive oligopeptides of the receptor binding domain (RBD) was fused to the carboxy-terminal portion of the toxin B RBD containing 23 of the 24 repetitive oligopeptides. The two regions are separated by a 4 amino acid linker sequence (arg–ser–met–his).

with isopropyl-B-D-thiogalactopyranoside (1 mM final concentration) (Sigma, St. Louis, MO) and a constant feed phase (5 h; protein production). To minimize formation of inclusion body, culture temperature was reduced from 37 °C ± 1 °C to 30 °C ± 1 °C one hour prior to induction until the end of fermentation.

Bacteria were isolated by batch centrifugation at 5500 g (average rcf) for 20 min at 2–8 °C. The bacterial cell paste was thawed and resuspended in a 4-fold volume of lysis buffer (20 mM HEPES, pH 7.5). A high pressure homogenizer was then used for cell disruption (3 cycles at 640 ± 25 bar) and soluble supernatants collected. Supernatants were depth filtrated through a depth filter capsule (Pall Supracap or equivalent). After depth filtration, the homogenate was conditioned with 1 M Tris stock solution pH 7.5 to 25 mM Tris, 20 mM HEPES, pH 7.5 to be ready for the first chromatography column step.

The C-TAB.G5 fusion protein was isolated following sequential chromatography: DEAE Sepharose FF, SP Sepharose FF and High Performance Phenyl Sepharose. Following each step, fractions containing C-TAB were identified by SDS-PAGE and Western blot. Positive fractions were pooled for the next step. Final fractions from the Phenyl Sepharose column were pooled, and concentrated using UF/DF membranes (MW cut off 30 kDa, modified PES membrane, Millipore or Pall) to approximately 2.5 mg/ml. The material was then diafiltrated against 10–12-fold volume of the final formulation buffer (20 mM histidine; 75 mM NaCl; 5% sucrose; pH 6.5), passed through a 0.2 μm filter and stored at −70 °C.

2.6. SDS-PAGE and Western blot

SDS-PAGE and Western blot analysis were used to confirm the identity of the purified fusion proteins. Whole bacterial cell lysates and purified C-TAB.G5 or C-TAB.G5.1 fusion proteins were resuspended in Nu-Page sample buffer containing beta-mercaptoethanol and boiled for 10 min. Samples (25 μl) were loaded onto 3–8% Tris-acetate gels (Invitrogen, Carlsbad, CA). Following electrophoresis (150 V for 1 h), proteins were visualized by staining the gels with simply blue stain or used for Western blot analysis.

C-TAB.G5 or C-TAB.G5.1 specific expression was determined by Western blot analysis using toxin-specific antibodies. Proteins were transferred at 23 V for 30 min onto a PVDF membrane using 1× transfer buffer in 10% methanol. Membranes were blocked for 1 h at room temperature with 0.5% casein in phosphate buffered saline. Transfer membranes were incubated for 2 h at room temperature with either a monoclonal antibody against toxin B or an in-house derived guinea pig polyclonal antibody against toxin A. Washed membranes were incubated with horseradish peroxidase conjugated anti-guinea pig IgG or anti-mouse IgG (Kirkagaard & Perry Laboratories, Gaithersburg, MD) for 1 h. The blots were washed and AEC substrates were added. The blots were incubated with gentle mixing for 5–10 min. The blots were rinsed with water to stop color development.

2.7. Murine immunogenicity study

Female C57BL/6 mice (7–8 weeks old) were immunized with escalating doses of C-TAB.G5 (3, 10 and 30 μg ± aluminum hydroxide) by intramuscular (i.m.) injection (50 μl) into the right thigh muscle on day 0 followed by i.m. injection into the left thigh muscle on day 14. When adjuvanted with aluminum hydroxide (Rehydragel, Reheis Inc.), all doses of C-TAB.G5 were admixed with 50 μg aluminum hydroxide. Serum samples for serological analysis were collected on day 42. Mice were allowed to rest for one week following the serum bleed prior to lethal challenge with *C. difficile* toxin A or toxin B. Toxin challenge was performed by inoculating mice intraperitoneally with the toxin minimal 100% lethal dose (100 μl/injection). Mice were observed for 7 days and survival was determined for each vaccination group. The minimal lethal dose (MLD_{100}) of each toxin was determined by titration on age match control mice prior to challenging experimental mice. The MLD_{100} for toxins A and B were determined to be 25 ng and 50 ng, respectively.

2.8. Hamster immunogenicity study

Hamsters received 3 vaccinations by i.m. injection in the thigh, on day 0, 14 and 28, with 100 μg C-TAB.G5.1 adjuvanted with 125 μg aluminum hydroxide. Controls were vaccinated with a placebo of histidine buffer. Serum was collected on days 0, 14, 28 and 42. On day 43 each hamster received 10 mg/kg clindamycin p.o. and on day 44 each received an oral dose of spores (see above). Two groups of animals ($n = 8$), one placebo and one C-TAB.G5.1 group received an intra-gastric challenge of either 10^2 or 10^4 spores. The health of hamsters was identified and recorded using a ranked scale shown in Table 1. Once symptomatic, hamsters were observed at 4-h intervals and based on signs of diarrhea and weight loss, culled if necessary. Hamsters identified as having severe disease twice in succession and animals identified as moribund were euthanized. All manipulations were done under a vertical laminar flow hood (Animal Transfer Station; Allentown, PA) to protect humans from *C. difficile* and the hamsters from uncontrolled colonization. The study ran for 55 days. Cecal digesta or feces were cultured for *C. difficile* on cycloserine-cefoxitin-fructose agar. Isolates from each hamster were ribotyped for comparison with the challenge strain, ribotype 012 as described [32]. As anticipated only toxin producing ribotype 012 isolates were recovered (data not shown). Intestinal material and cultured isolates were also assayed for GDH and toxins A and B using the C. diff Complete assay (TechLab, Inc., Blacksburg, VA).

2.9. Monkey immunogenicity and local reactogenicity

Female Cynomolgus monkeys (3/group) were immunized by i.m. injection (0.5 ml/injection) in the deltoid muscle with 200 μg C-TAB.G5.1 ± 250 μg aluminum hydroxide. Animals received three injections; day 0 in the left deltoid, day 14 in the right deltoid

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Table 1
CDAD disease stages.

Stage	Disease symptoms
Healthy	No symptoms
Mild	Healthy weight Active and alert Can be observed eating and drinking Light staining of hair around anus Perianal region slightly moist with minimal matting (<10 mm)
Moderate	May begin to lose weight Active, but may seem docile May have slightly hunched posture Moderate staining of hair around anus Perianal region moist with matting (~10 mm)
Severe	Not active Coat appears starey May have hunched posture May be cool to touch Moderate to heavy staining of hair around anus Perianal region wet with matting (>10 mm)
Moribund	Hunched posture No activity May be comatose

Once symptomatic, hamsters were observed at 4-hour intervals and disease stage identified.

and day 28 in the left deltoid. Following each injection, the injection site was monitored over 7 days for any local reactogenicity (edema and erythema). Serum samples were obtained on day 0 (pre-vaccination) day 14, 28 and 42 for serological analysis.

2.10. Serum IgG ELISA

Serum antibodies elicited to C-TAB.G5 or C-TAB.G5.1, toxin A and toxin B (TechLab, Blacksburg, VA) were evaluated in an enzyme linked immunosorbent assay (ELISA). Briefly, stock solutions of 1.0 μg/of toxin A, toxin B or the recombinant C-TAB.G5 fusion protein were prepared in PBS and 100 μl were added to each well of a 96-well plate. After overnight incubation at 4 °C, plates were washed and blocked with 0.5% casein blocking buffer. Plates were washed again and serial, 4-fold dilutions of test sera added to the plates. After a second overnight incubation at 4 °C, plates were washed and incubated with peroxidase-conjugated anti-mouse, anti-hamster or anti-monkey IgG (H + L) (Kirkagaard & Perry Laboratories, Gaithersburg, MD). After 2 h incubation at room temperature, the plates were again washed, peroxidase substrate (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) added and color allowed to develop for 30 min at room temperature. The reaction was stopped by adding 50 μl of 2% SDS to the wells. Plates were read with an ELISA plate reader at an absorbance of 405 nm. Serum antibody titers were reported as the geometric mean of ELISA Units (EU), with EU defined as the reciprocal of the serum dilution that results in an absorbance at 405 nm reading of 0.5. As a negative control a pooled, sample of pre-immune serum obtained from animals pre-bled before the first immunization was used to evaluate an antibody response.

2.11. Toxin neutralizing antibody assay (TNA)

Toxin neutralizing properties of antisera were determined using Vero cells (monkey kidney cells, ATCC CCL-81) and purified toxin A and B. Vero cells were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum. For *in vitro* analysis, 125 μl of either toxin A (5 ng/ml) or toxin B (1 ng/ml) was incubated with 125 μl of serial dilutions of antisera obtained from immunized animals. After 1 h of incubation at 37 °C, the toxin:serum mixtures were added to microtiter wells containing $1x10^5$ Vero cells, and the microtiter plates incubated at 37 °C in 5% CO_2 for 18 h. Incubation of either toxin A or B with Vero cells resulted in a change in cell morphology and a loss of cell adherence which was measured by neutral red staining of toxin treated cells after removal of non-adherent cells. The neutral red dye was solubilized by the addition of solubilization buffer (50% ethanol in 1% acetic acid) and the plates read on a plate reader at a wavelength of 542 nm. The toxin neutralization titer of a serum is reported as the serum dilution which gives a 50% reduction in toxin activity (ED_{50}).

3. Results

3.1. Construction and purification of the C-TAB fusion protein

The gene encoding the C-TAB.G5 fusion protein was constructed as described in Section 2. The fusion protein (Fig. 1) contains the carboxy-terminal portion of the toxin A RBD fused to the carboxy-terminal portion of the toxin B RBD separated by a 4 amino acid linker sequence (arg–ser–met–his). The fusion protein was constructed to contain 19 of the 39 repetitive oligopeptides found in the full length toxin A RBD and 23 of the 24 repetitive oligopeptides found in the full length toxin B RBD.

Recombinant C-TAG.G5 was expressed in *E. coli* BL21(DE3) and purification from *E. coli* cell paste was performed using a three-column strategy (see Section 2). The purification process described consistently yielded a product with >99% purity based on size exclusion chromatography. Purified C-TAB contained <10 EU Endotoxin/mg, <2 ng host cell protein/mg and <0.2 ng DNA/mg. The purified C-TAB.G5 fusion protein was isolated as a single 109 kDa protein (Fig. 2), in which 46% of the sequences are derived from toxin A (438 aa) and 54% from toxin B (516 aa).

3.2. Immunogenicity of C-TAB.G5 in mice

To evaluate the immunogenicity of C-TAB.G5, mice were vaccinated i.m. with two doses of C-TAB.G5 in the absence or presence of aluminum hydroxide adjuvant. When serum samples obtained two weeks following the second immunization were evaluated by ELISA (Fig. 3), the data indicated that immunization with the C-TAB.G5 fusion protein resulted in the generation of antibodies to C-TAB.G5 as well as native toxin A and toxin B in a dose dependent manner. The co-administration of aluminum hydroxide with the fusion protein resulted in significant antibody titer increases (10–50-fold) for anti-C-TAB.G5 as well as anti-toxin A and B. It is important to note that antibody titers to toxin A were consistently higher than those to toxin B independent of vaccine dose, both in the absence (20–25-fold) and presence of aluminum hydroxide (5–8-fold).

To determine the functional capacity of anti-C-TAB.G5 induced antibodies to neutralize native toxins, toxin neutralization assays were performed (Table 2). Pooled sera from the different experimental groups were all found to have toxin A neutralizing activity. The neutralizing activity was found to be dose dependent and the use of aluminum hydroxide adjuvant resulted in 5–20-fold increases in neutralizing antibody activity. In contrast to toxin A neutralization, only pooled sera from the C-TAB.G5.1 + aluminum hydroxide adjuvant group demonstrated toxin B neutralizing activity.

A mouse toxin challenge model was also used to evaluate the anti-toxin antibody functional activity. Immunized mice were injected i.p. with a minimal lethal dose of either toxin A (25 ng) or toxin B (50 ng) three weeks following the second C-TAB.G5 immunization (Table 2). Following toxin A challenge, 7 of 8 mice in the control group died. In all experimental groups, regardless of dose or the use of aluminum hydroxide adjuvant, 100% of the mice

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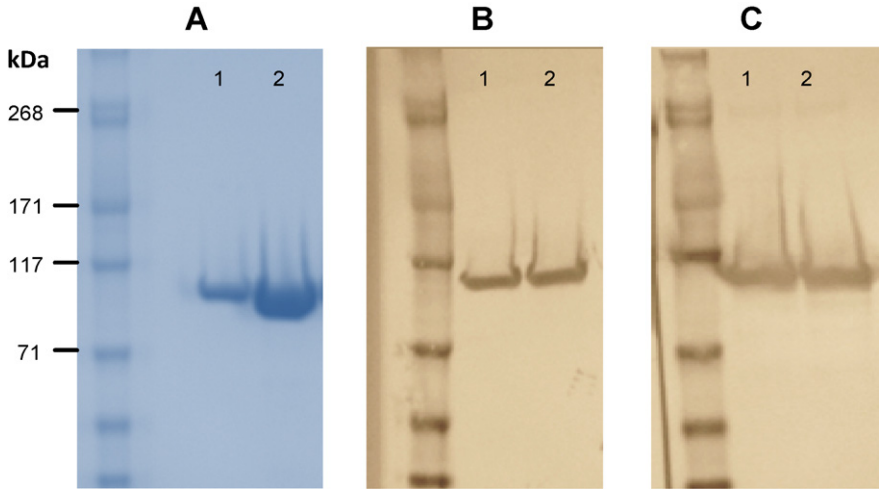

Fig. 2. Analysis of the purified 109 kDa fusion protein by SDS-PAGE (A) and Western blotting with anti-toxin A antibody (B) and anti-toxin B antibody (C). Gel lane 1: 0.5 μg, lane 2: 5.0 μg.

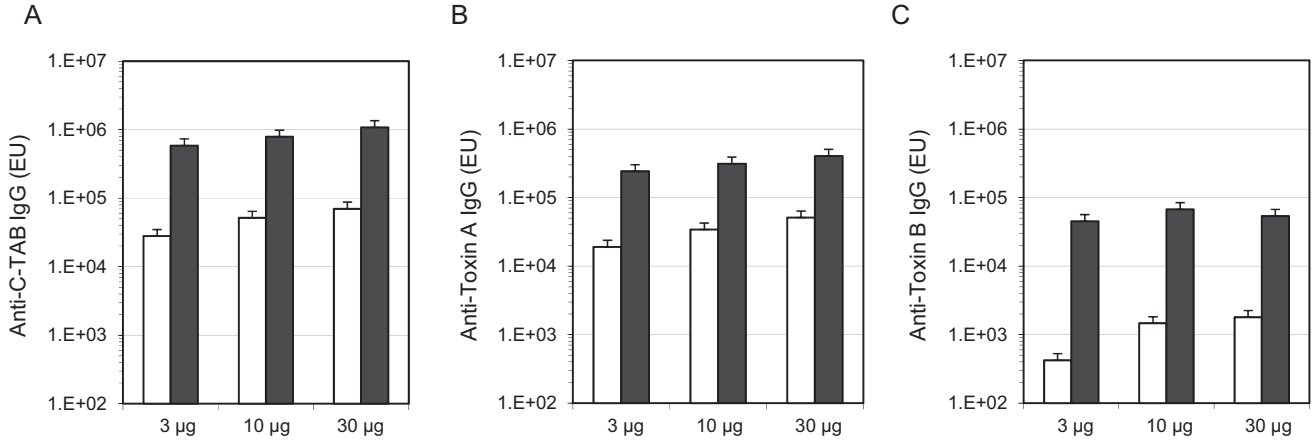

Fig. 3. Immunization with C-TAB.G5 fusion protein elicits antibody specific responses for C-TAB.G5 as well as toxin A and toxin B. Female C57BL/6 mice (8/group) were immunized twice with increasing doses of C.TAB.G5 in the absence (□) or presence (■) of 50 μg aluminum hydroxide adjuvant. Serum IgG was determined in an ELISA with plates coated with either for C-TAB.G5 (panel A), toxin A (panel B) or toxin B (panel C). Results are shown as geometric mean titers ± SE.

Table 2
In vitro and *in vivo* evaluation of toxin neutralizing antibody following immunization of mice with C-TAB.G5.

Vaccine[a]		Toxin neutralizing titer ED_{50}[b]		Toxin challenge survival[c]	
C-TAB.G5 dose (μg)	Aluminum hydroxide dose (μg)	Anti-toxin A	Anti-toxin B	Anti-toxin A	Anti-toxin B
0	0	0	0	17%	0%
3	0	103	0	100%	0%
3	50	683	369	100%	100%
10	0	171	0	100%	12.5%
10	50	7789	300	100%	100%
30	0	150	0	100%	50%
30	50	1010	669	100%	87.5%

[a] Mice received two immunizations (study day 0 and 14).
[b] Pooled sera were used for the analysis of toxin A TNA (±aluminum hydroxide) and for toxin B TNA without aluminum hydroxide due to the limited amount of serum available. Sera were obtained two weeks following the second immunization.
[c] C57BL/6 mice (8 mice/group) were challenged i.p with either 25 ng toxin A or 50 ng toxin B three weeks following the second immunization.

survived the lethal challenge. In the toxin B control group, all mice died following toxin B challenge. In the experimental groups immunized with C-TAB.G5 in the absence of aluminum hydroxide, a dose dependent protective effect was observed. Survival rates of 0%, 12.5% and 50% were observed in mice immunized with 3, 10 and 30 μg C-TAB.G5 respectively. When aluminum hydroxide adjuvant was co-administered with C-TAB.G5, survival rates increased to 100%, 100%, and 87.5%.

3.3. Protective efficacy of C-TAB.G5 in the hamster model

Hamsters provide an infectious disease model in which CDAD can be induced following a challenge with *C. difficile* spores. To evaluate the protective efficacy of the fusion protein, hamsters ($n = 24$) received three immunizations of either a placebo vaccine (histidine buffer) or 100 μg C-TAB.G5.1 (*E. coli* codon optimized C-TAB.G5) co-administered with 125 μg of aluminum hydroxide adjuvant.

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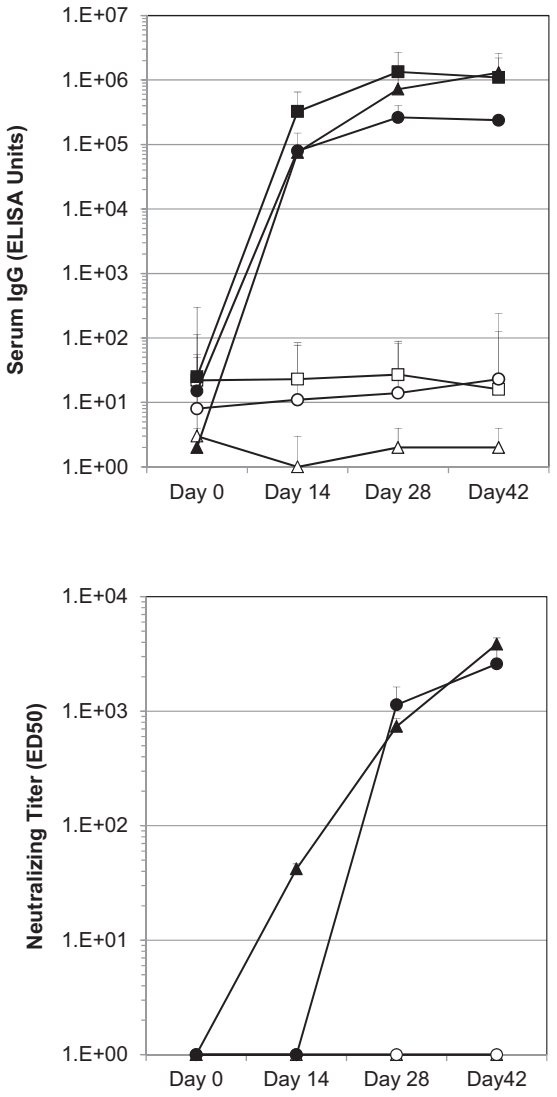

Fig. 4. Immunization of hamsters with C-TAB.G5.1 induces high titer anti-toxin serum antibody with toxin neutralizing activity. Hamsters (24/group) were immunized three times with either histidine buffer (placebo vaccine, open symbols) or 100 μg CTAB.G5.1 + 125 μg aluminum hydroxide adjuvant (closed symbols). Serum IgG was determined in an ELISA (A) with plates coated with either for C-TAB.G5.1 (■), toxin A (▲) or toxin B (●). Results are shown as geometric mean titers ± SE. Toxin neutralization (B) for toxin A and B were assessed by co-incubation of sera with native toxins followed by addition of the serum:toxin mixture to the Vero indicator cell line. Results are shown as the geometric mean ED_{50} titers ± SE animals in which activity could be measured at that time point (see Section 3.3).

Pre-immunization sera and serum samples obtained two weeks following each immunization were evaluated by ELISA to assess the development of the antibody response (Fig. 4 A). Significant levels of anti-C-TAB.G5 antibody as well as anti-toxin A and B antibody were observed after a single immunization of C-TAB.G5.1with aluminum hydroxide adjuvant. Only baseline titers were observed in placebo immunized controls. A second immunization provided an added boost in antibody titer to all proteins. Antibody titers to C-TAB.G5.1 and toxin B appeared to plateau after the second immunization while a further increase in anti-toxin A antibody was observed following the third immunization. As observed in the mouse immunogenicity study, hamster anti-toxin A titers (day 42 GMT = 1,300,882 EU) were consistently higher than those observed for anti-toxin B (day 42 GMT = 237,579 EU). In addition to anti-toxin serum antibody, hamster fecal samples obtained following the third immunization were shown to contain anti-toxin IgG specific for both toxin A and toxin B (data not shown).

In vitro toxin neutralization was assessed for all time points of the hamster study (Fig. 4 B). Toxin A neutralizing antibody was observed after a single immunization in 14 of 24 animals and was in 100% of animals following two immunizations. In contrast, toxin B neutralizing antibody was not observed until after the second immunization (5 of 24 animals) and was only observed in 14 of 24 animals after the third immunization.

To evaluate *in vivo* protective efficacy, three weeks following the third immunization, hamsters were treated with clindamycine and challenged intra-gastrically the following day with either 10^2 or 10^4 *C. difficile* spores. The health of spore challenged hamsters were monitored using the health criteria described in Table 1 and the data is presented in Fig. 5. Following the 10^2 spore challenge, CDAD was observed in 50% of the placebo immunized hamsters within 24 h. By 48 h all hamsters in this group exhibited moderate to severe disease. Disease was observed in 2 of 6 surviving animals through day 7. Hamsters immunized with C-TAB.G5.1 with aluminum hydroxide adjuvant did not show signs of CDAD until 48 h. The disease noted in this group was less severe and all hamsters returned to normal health by day 5.

Following the 10^4 spore challenge, CDAD was observed in 100% of the surviving placebo group (5 of 8) within 24 h. By 48 h all hamsters in this group had died. Hamsters immunized with C-TAB.G5.1 with aluminum hydroxide adjuvant did show signs of CDAD at 24 h (3 of 8). Disease symptoms peaked with hamsters showing mild to moderate disease followed by recovery to normal health in the majority of the hamsters. The disease noted in this group was less severe and all hamsters returned to normal health by day 5. No mortality in this group was observed.

In addition to reducing the severity of CDAD, immunization with C-TAB.G5.1 with aluminum hydroxide adjuvant significantly enhanced the survival from lethal disease (Fig. 6). Placebo immunized hamsters that received 10^2 spores exhibited a 62.5% survival (5 of 8 animals) on day 3 and 50% survival on day 8. The 100% survival for C-TAB.G5.1 with aluminum hydroxide adjuvant immunized hamsters on day 12 post challenge (end of study) was significant ($p = 0.025$) compared to the placebo group. Placebo immunized hamsters that received 10^4 spores exhibited a 37.5% survival (5 of 8 animals) on day 1 and 100% mortality was observed on day 3. Again, 100% survival for C-TAB.G5.1 + aluminum hydroxide adjuvant immunized hamsters observed on day 12 post challenge (end of study) was significant ($p = 0.0001$) compared to the placebo group.

3.4. Immunogenicity of C-TAB.G5 in Cynomolgus monkeys

The immunogenicity of C-TAB.G5.1 was also analyzed in a non-human primate model. Monkeys ($n = 3$) received three immunizations of 200 μg C-TAB.G5.1 ± 250 μg aluminum hydroxide adjuvant by i.m. injection. Evaluation of the injection site indicated a lack of protein reactogenicity as evidenced by the absence of local erythema and/or edema. As shown in Fig. 7, high titers of serum IgG specific for C-TAB.G5.1, toxin A and toxin B were induced. The co-delivery of aluminum hydroxide adjuvant with the C-TAB fusion protein resulted in an enhanced antibody response against all three proteins. The effects of aluminum hydroxide adjuvantation on the antibody response were more pronounced for the first two immunizations for C-TAB.G5.1 (390-fold) and toxin A (104-fold) and to a lesser extent for toxin B (21-fold). Following the third immunization, serum IgG titers in the aluminum hydroxide groups remained enhanced when compared to the non-aluminum hydroxide groups (C-TAB.G5.1, 6-fold; toxin A, 17-fold; and toxin B, 3-fold). A comparison of the serum IgG titers in monkeys directed against the toxin A component with the toxin B component of C-TAB, confirmed the

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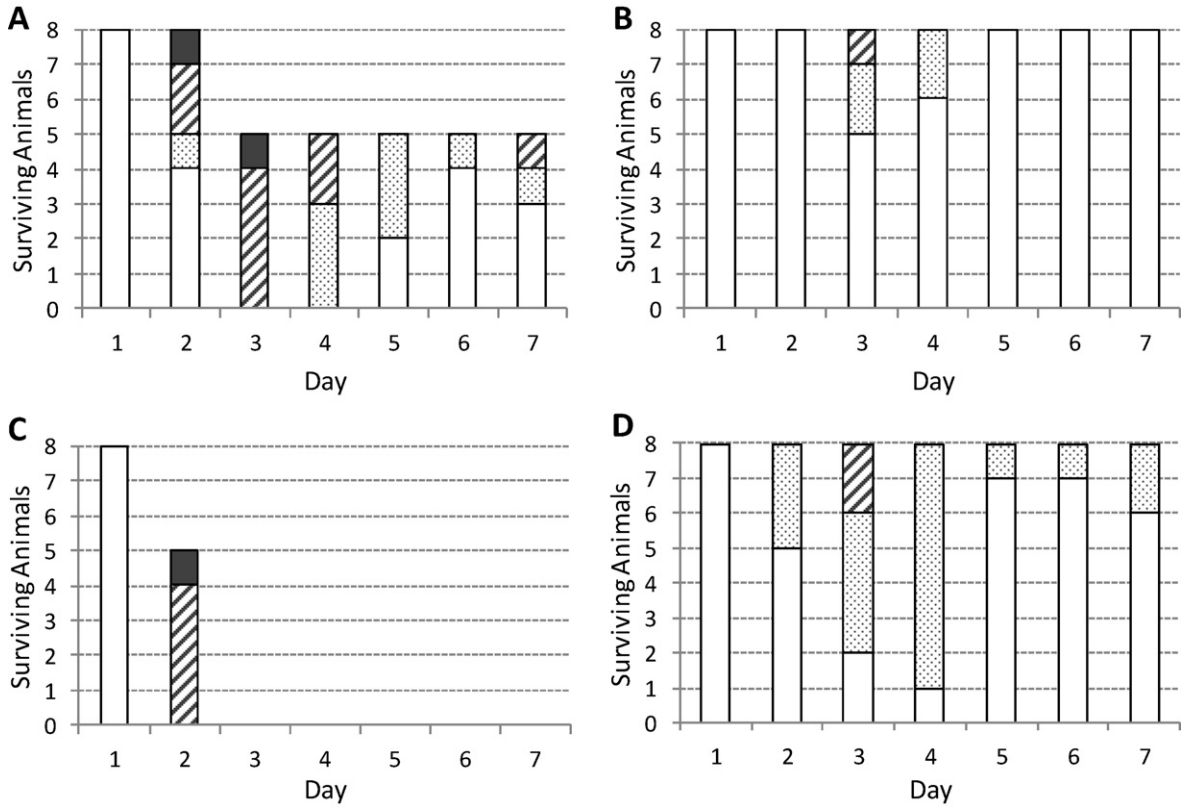

Fig. 5. C-TAB.G5.1 with aluminum hydroxide adjuvant confers protective efficacy against CDAD in the hamster spore challenge model. Hamsters (8/group) were immunized three times with histidine buffer as a placebo treatment (panel A and C) or with 100 μg CTAB.G5.1 + 125 μg aluminum hydroxide adjuvant (panel B and D). Two weeks following the third immunization, hamsters were treated with clindamycin p.o. (10 mg/kg) and the following day received an intra-gastric challenge of either 10^2 (panels A and B) or 10^4 *C. difficile* spores (panels C and D). Hamsters were monitored at 4-h intervals for disease symptoms as defined in Table 1 and illustrated as healthy (open bars), mild disease (dot filled bars), moderate disease (stripped bars), severe disease (black bars).

findings in the mouse and hamster models that toxin A induced higher antibody titers than toxin B.

Toxin neutralizing activity was also observed in monkey immune sera. As seen in Table 3, sera from both groups of animals (C-TAB.G5.1 ± aluminum hydroxide adjuvant) were able to neutralize both toxin A and toxin B activity. While high titers of toxin A neutralizing antibody were detected in sera from the aluminum hydroxide adjuvant group after a single immunization, toxin A neutralizing antibody in the non-adjuvanted group and toxin B neutralizing activity in both the adjuvanted and non-adjuvanted group took longer to develop.

4. Discussion

The pathogenesis of CDAD is mediated by two *C. difficile* secreted toxins, toxin A, both a cytotoxin and endotoxin, and toxin B, a more potent cytotoxin. The action of these toxins on the intestinal epithelium results in the stimulation of intestinal fluid secretion and the release of proinflammatory mediators which results in mucosal injury, inflammation of the colon and diarrhea. The role of serum anti-toxin antibodies in mediating systemic and mucosal protection against CDAD in both animal models and patients with toxigenic *C. difficile* infection is well established. The presence of anti-toxin antibody, induced by either active immunization or passive transfer, has been shown in animal models to be associated with protection from CDAD [14–24] and is also associated with protection from CDAD re-occurrence in human patients [3,25–28].

The role of toxin A *versus* toxin B in CDAD has been addressed in numerous studies. Early studies in which purified toxin A or toxin B were administered intragastrically, disease was only observed after the administration of toxin A suggesting toxin A was the primary pathogenic factor [33]. However, when toxin B was co administered with toxin A, disease symptoms were more severe. When pathogenic *C. difficile* variants that produced toxin B in the absence of toxin A were identified, a role for toxin B in *C difficile* pathogenicity was established [34]. Indeed, immunization with toxoid B was protected from lethal challenge with an A−/B+ strain of *C. difficile* indicating the protective properties of anti-toxin B antibody [35]. Later studies questioned the role of Toxin A as the primary pathogenic factor after genetically derived isogenic strains of *C. difficile* deficient in the production of either toxin A or toxin B were derived [36]. These studies demonstrated that toxin B was essential for virulence as isogenic toxin B- strains showed a significantly reduced lethality in the hamster model. A more recent study using a similar approach found that A+/B− mutants caused significant disease in hamsters indicating that both toxin A and toxin B both play significant roles in CDAD [37]. Since both toxin A and toxin B contribute to the virulence of *C. difficile* and the induction of CDAD, any immunotherapeutic approach needs to target both toxins.

In this report we describe the immunotherapeutic potential of a novel recombinant protein, C-TAB.G5, produced using an *E. coli* expression system. C-TAB.G5 is a fusion protein consisting of the carboxy-terminal portion of the toxin A RBD (15 of the 31 repetitive oligopeptide sequences) and the toxin B RBD (23 of the 24 repetitive oligopeptide sequences) joined by a 4 amino acid linker sequence. This fusion protein is expressed as a homogenous 109 kDa protein, 46% derived from toxin A and 54% derived from toxin B. The focus on the RBD sequences in this vaccine candidate is supported by studies demonstrating that: (i) hamsters immunized with carboxy-terminal portions of toxin A containing RDB are protective in CDAD

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Table 3
C-TAB.G5.1 induction of toxin neutralizing antibody in Cynomolgus monkeys[a].

Target	Vaccine	Toxin neutralizing titer (ED_{50})			
		Pre-bleed	Day 15	Day 29	Day 43
Toxin A	C-TAB.G5.1	0	4	18	579
Toxin A	C-TAB.G5.1 + aluminum hydroxide	0	954	3191	11,877
Toxin B	C-TAB.G5.1	0	1	5	722
Toxin B	C-TAB.G5.1 + aluminum hydroxide	0	5	81	4415

[a] Monkeys (3/group) received three immunizations (study day 0, 14 and 28) with 200 μg C-TAB.G5.1 ± 250 μg aluminum hydroxide adjuvant.

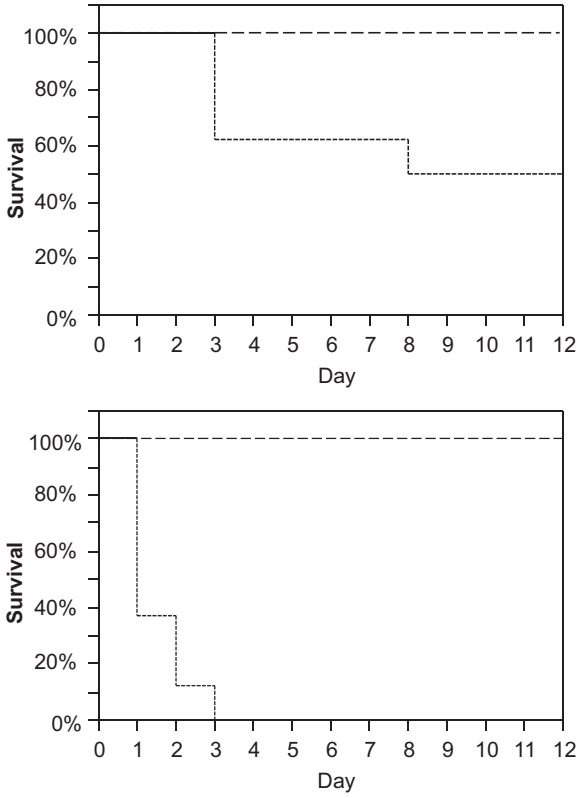

Fig. 6. C-TAB.G5.1 with aluminum hydroxide adjuvant reduces mortality in the hamster spore challenge model. Hamsters (8/group) were immunized three times with 100 μg CTAB.G5.1 + 125 μg aluminum hydroxide adjuvant (dashed lines) or histidine buffer as a placebo treatment (dotted lined). Two weeks following the third immunization, hamsters were treated with clindamycin p.o. (10 mg/kg) and the following day received an intra-gastric challenge of either 10^2 (top panel) or 10^4 (bottom panel) *C. difficile* spores. Hamsters were monitored at 4-hourly intervals for disease symptoms and mortality. At study termination, all surviving animals were disease free for ≥5 days. Statistical analysis of Kaplan–Meier plots were done by Log-Rank analysis.

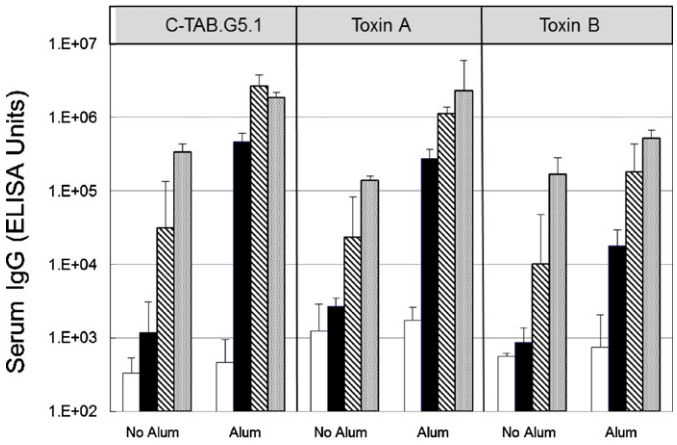

Fig. 7. C-TAB.G5.1 induces high titer anti-toxin antibody in Cynomolgus monkeys. Monkeys were immunized three times (day 0, 14, and 28) with 200 μg CTAB.G5.1 in the absence or presence of 250 μg aluminum hydroxide adjuvant. Sera were obtained on days 0 (open bars), 14 (black bars), 28 (hatched bars) and day 35 (gray bars). Serum IgG was determined in an ELISA (A) with plates coated with either for C-TAB.G5, toxin A or toxin B. Results are shown as geometric mean titers ± SE.

[15,18,19], (ii) antibodies targeting the RDB region of both toxin A and toxin B have toxin neutralizing activity [29,30] and (iii) the passive transfer of antibodies targeting the RDB region of both toxin A and toxin B are protective in hamster CDAD models [23].

Our studies demonstrate that C-TAB.G5 is highly immunogenic. Immunization of mice, hamsters and monkeys induced the generation of both anti-toxin A and anti-toxin B antibodies which were capable of neutralizing toxin in both *in vitro* and *in vivo* assays. The consistent induction of higher anti-toxin A serum antibody titers as assessed by ELISA suggests that the toxin A portion of the fusion protein is more immunogenic than the toxin B portion. This was observed in both the absence and presence of aluminum hydroxide adjuvant. The importance of achieving sufficient anti-toxin B antibody titers is illustrated in the mouse toxin challenge study in that immunization with C-TAB.G5 in the absence of aluminum hydroxide provided 100% protection against a toxin A MLD challenge but only 50% or less protection against a toxin B MLD challenge. This supports the observations that correlated the presence of serum anti-toxin B antibody with protection from recurrent *C. difficile* infection [24]. The reduced immune response to toxin B *versus* toxin A is also observed when evaluating the induction of toxin neutralization antibody. This difference, however, may in part be due to a reduced sensitivity of the toxin B neutralization assay as suggested by the protection against an *in vivo* toxin B challenge in the absence of measurable toxin B neutralizing antibody (Table 2).

C-TAB.G5 demonstrated protective efficacy in the hamster CDAD model, reducing the severity and time to onset of CDAD and significantly protecting animals from mortality induced by challenge with *C. difficile* spores. The significance of this model is characterized by a very rapid progression of CDAD and high mortality. In the placebo group, hamsters challenged with 10^4 spores had all died by day 3, but 100% survival was observed in the vaccinated group at day 12. While animals in the vaccinated group exhibited CDAD characterized as mild to moderate, all recovered and were symptom free by study end.

In addition to the induction of high titers of anti-toxin A and toxin B serum antibody with toxin neutralizing activity and the protection from a lethal challenge of *C. difficile* spores following immunization with C-TAB.G5, anti-toxin IgG and IgA were present in fecal samples obtained from vaccinated hamsters and monkeys (data not shown) indicating the induction of mucosal and systemic immune responses. Given that the enterotoxicity of both toxin

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A and toxin B are associated with a loss of actin polymerization and cytoskeletal changes, resulting in the disruption of the colonic epithelial tight junctions [7], the induction of mucosal anti-toxin antibody by C-TAB.G5 and its neutralization of intestinal toxin may be central to the efficacy of the vaccine. While studies have correlated titers of anti-toxin serum antibodies with protection against *C. difficile* infection [14–24], limited information of the levels of mucosal antibody and how it correlates with serum anti-toxin antibody levels is available.

No preventive vaccine against CDAD is currently available, although Sanofi Pasteur has evaluated a *C. difficile* toxoid vaccine, ACAM-CDIFF™ in phase 1 and 2a studies with humans. This vaccine contains formalin-inactivated toxoids A and B where the toxins have been purified from *C. difficile* bacterial cultures. In a phase 1 study, ACAM-CDIFF™ was shown to be safe and immunogenic in healthy volunteers [38]. High titer serum antibody responses to both toxin A and toxin B were observed in nearly all subjects as evaluated by both ELISA and toxin neutralization. In a phase 2a study, subjects with recurrent CDAD were immunized with ACAM-CDIFF™ and vaccine induced immune responses to toxins A and B were associated with resolution of recurrent diarrhea [39]. Switching the development focus of ACAM-CDIFF™ from a prophylactic to a therapeutic indication, a phase 2 trial that targets adults aged 40 to 75 years of age who are at risk of *C. difficile* infection, has been initiated by Sanofi Pasteur. ACAM-CDIFF™ has been granted fast track designation by the FDA for this urgent unmet medical need.

Our studies have shown that C-TAB.G5 induced high titer serum anti-toxin antibody to toxins A and B in three different pre-clinical animal models. These antibodies have functional activity as evidenced by both *in vitro* and *in vivo* toxin neutralizing activity and confer protection from a lethal *C. difficile* spore challenge in a hamster model. As the pathogenesis of CDAD in the hamster model is more aggressive and severe than that observed in humans, the significant level of C-TAB.G5 induced protective efficacy indicates its promise as a clinical vaccine candidate. In addition, the approach taken, the construction of a single recombinant protein containing the immune-protective domains of both toxins A and B lends itself to commercial manufacturing. Furthermore, lack of toxicity of the non-enzymatic recombinant protein obviates the need for formalin inactivation required for preparing toxoid from active toxin. Based on the studies presented here, Intercell has initiated a phase 1 clinical trial in healthy young and elderly adults.

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