Systemic antibody responses induced by a two-component *Clostridium difficile* toxoid vaccine protect against *C. difficile*-associated disease in hamsters

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*Clostridium difficile* infection (CDI) has been identified as the leading cause of nosocomial diarrhoea and pseudomembranous colitis associated with antibiotic therapy. Recent epidemiological changes as well as increases in the number of outbreaks of strains associated with increased virulence and higher mortality rates underscore the importance of identifying alternatives to antibiotics to manage this important disease. Animal studies have clearly demonstrated the roles that toxins A and B play in gut inflammation as well as diarrhoea; therefore it is not surprising that serum anti-toxin A and B IgG are associated with protection against recurrent CDI. In humans, strong humoral toxin-specific immune responses elicited by natural *C. difficile* infection is associated with recovery and lack of disease recurrence, whereas insufficient humoral responses are associated with recurrent CDI. The first generation of *C. difficile* vaccine that contained inactivated toxin A and B was found to be completely protective against death and diarrhoea in the hamster *C. difficile* challenge model. When tested in young healthy volunteers in Phase I clinical trials, this investigational vaccine was shown to be safe and immunogenic. Moreover, in a separate study this vaccine was able to prevent further relapses in three out of three patients who had previously suffered from chronic relapsing *C. difficile*-associated diarrhoea. Herein we examined the immunogenicity and protective activity of a next-generation Sanofi Pasteur two-component highly purified toxoid vaccine in a *C. difficile* hamster model. This model is widely recognized as a stringent and relevant choice for the evaluation of novel treatment strategies against *C. difficile* and was used in preclinical testing of the first-generation vaccine candidate. Intramuscular (i.m.) immunizations with increasing doses of this adjuvanted toxoid vaccine protected hamsters from mortality and disease symptoms in a dose-dependent manner. ELISA measurements of pre-challenge sera showed that the median anti-toxin A and anti-toxin B IgG titres in the group of surviving animals were significantly higher than the median values in the group of animals that did not survive challenge. Assessment of the neutralizing activity of these sera revealed a statistically significant difference between the levels of both toxin A and toxin B neutralizing titres in protected versus unprotected animals as the median anti-toxin A and anti-toxin B neutralizing titres from surviving animals were higher than the median values from animals that succumbed to challenge. Statistically significant correlations between the toxin-specific binding titres and toxin neutralizing titres were seen for both toxin A and toxin B responses. The role of circulating anti-toxin antibodies in immunity against disease was evaluated by passive transfer of immune sera against *C. difficile* toxoids to naïve hamsters. Passively immunized animals were protected against morbidity and mortality associated with *C. difficile* challenge. Taken together, these results indicate the ability of i.m. immunization with inactivated toxins A and B to induce robust dose-dependent anti-toxin A and anti-toxin B IgG responses, the principal role of circulating anti-toxin antibody in immunity against disease and that antibody toxin binding and neutralization titres can serve as correlates of protection in the hamster challenge model of *C. difficile*.

**Abbreviations:** CDAD, *C. difficile*-associated diarrhoea; CDI, *C. difficile* infection; i.g., intragastrically; i.m., intramuscular; i.p., intraperitoneal; TNA, toxin neutralization assay.
INTRODUCTION

*Clostridium difficile*, a Gram-positive, spore-forming anaerobic bacillus, is a leading cause of nosocomial diarrhoea and pseudomembranous colitis worldwide (Rupnik et al., 2009). *C. difficile* infection (CDI) is estimated to cause over $7 billion in healthcare costs annually in the US and EU combined (O’Brien et al., 2007). Since 2003, the emergence and spread of the now endemic hypervirulent strain BI/NAP1/027 in North America and later in Europe (Freeman et al., 2010) have been associated with increased morbidity and mortality (Miller et al., 2010; Kuijper, et al., 2006). Mortality rates from CDI have more than quadrupled in the US from 1999 to 2004 and in the UK from 2004 to 2007 (Freeman et al., 2010; Redelings et al., 2007). Current treatment options largely rely on antibiotics (Cohen et al., 2010). Metronidazole is considered the front line defence for mild to moderate cases followed by vancomycin for severe and recurrent CDI. A narrow-spectrum macrolide antibiotic, fidaxomicin, was recently approved and licensed for treatment of *C. difficile*-associated diarrhoea (CDAD) in adults over 18 years of age, but it remains a very expensive treatment option (Johnson & Wilcox, 2012; Louie et al., 2011). These antibiotic-based treatment choices have serious limitations such as high recurrence rates (Cohen et al., 2010; McFarland et al., 2002), increasing treatment failure (Kuijper & Wilcox, 2008) and selection of vancomycin-resistant enterococci (Pepin, 2008). There are currently no preventative products approved for use (Gerding, 2012).

The hamster mimics the human course of infection and is believed to be a good model for prediction of protective immunity in humans (Best et al., 2012). In previous hamster studies it was reported that while parenteral vaccination with culture filtrate of non-toxigenic strains of *C. difficile* did not confer protection against lethal ileocecalitis induced by clindamycin and *C. difficile* challenge, vaccination with formalin-inactivated culture filtrates containing both toxins A and B or toxin A alone, but not toxin B alone, was protective (Kim et al., 1987). Moreover, this protection could be transferred via the immune sera from the immunized animals to naïve infant hamsters. It was also demonstrated that immunization with partially purified adjuvanted preparations of toxoid A and B delivered via mucosal and parenteral routes of administration led to the development of high neutralizing activity to both toxins and conferred varying degrees of protection from diarrhoea and death associated with *C. difficile* challenge (Torres et al., 1995). While hamsters immunized by the intranasal, intraperitoneal and subcutaneous routes were fully protected from death, but not disease, the animals immunized by a combination of intranasal and intraperitoneal routes were fully protected against both death and diarrheal disease. To achieve this protection, cholera toxin and Ribi adjuvants were used for mucosal and parenteral administration, respectively. Analysis of saliva and faeces in these immunized and challenged animals did not reveal the presence of toxin-specific antibodies suggesting that serum antibodies were major effector molecules in protection against this mucosal disease.

Similar to findings in the animal models, evidence from clinical studies on naturally occurring anti-toxin antibodies suggested that inability to mount an immune response to toxin A was associated with increased risk for recurrent CDI, whereas development of IgG to toxin A was associated with asymptomatic carriage of *C. difficile* (Kyne et al., 2000, 2001). Another study found that high ELISA IgG titres to toxin B and/or presence of neutralizing activity in the serum correlated with clinical recovery from CDAD without relapse (Aronsson et al., 1985). Therapeutic activity of intravenous immunoglobulin obtained from the sera of healthy humans was demonstrated to be effective in the treatment of individuals with severe and recurrent CDAD and colitis (Beales, 2002; Salcedo et al., 1997; Warny et al., 1995); this finding almost certainly relates to the fact that the majority of the healthy adult population (>60%) have measurable levels of serum *C. difficile* toxin-specific IgG (Viscidi et al., 1983). More recent reports from various animal models (Kink & Williams, 1998; Giannasca & Warny, 2004; Sun et al., 2011; Babcock et al., 2006; Wang et al., 2012; Marozsan et al., 2012) and a recent clinical trial (Lowy et al., 2010) confirmed the importance of holotoxin or toxin domain-specific neutralizing antibodies in protection against *C. difficile* disease. All these studies clearly indicate that presence and magnitude of systemic toxin-specific immunity play a major role in protection from enterotoxic and inflammatory actions of *C. difficile* toxins and that neither toxin A nor toxin B can be downgraded in terms of importance for novel treatments and therapeutics.

A previous generation of investigational *C. difficile* vaccine, comprising a partially purified preparation containing inactivated toxins A and B, provided protection in a preclinical hamster challenge model, when administered by a combination of rectal and intramuscular routes (Giannasca et al., 1999). *Escherichia coli* heat-labile toxin was used as mucosal adjuvant for rectal and alum as an adjuvant for intramuscular immunizations.

This vaccine was subsequently tested in human clinical trials and demonstrated to be safe and immunogenic in young healthy volunteers (Kotloff et al., 2001). To address the ability of the vaccine to mount toxin-specific responses in individuals that are unable to develop substantial immunity despite multiple exposures to *C. difficile* through infection, a pilot study with three patients with chronic relapsing CDAD was conducted. Vaccination of these individuals led to all three subjects being free of CDAD for the two-month follow up period despite no antibiotic therapy (Sougioultzis et al., 2005). These data indicated that parenteral vaccination with the toxoid A and B vaccine may be effective not only in prevention, but also in treatment of CDI.

The roles of toxins A and B in the virulence of *C. difficile* disease have long been shown in both animal models and
humans, and the primary role of systemic toxin-specific humoral immunity in protection against symptomatic *C. difficile* infection has also been demonstrated. Therefore, the specific aims of this study were to build on those previous findings with a preclinical assessment of the efficacy of the next-generation Sanofi Pasteur two-component parenteral toxoid vaccine candidate in the hamster model. As this vaccine is currently undergoing Phase II clinical trials (Greenberg et al., 2012; Foglia et al., 2012), assessment of the vaccine in the hamster model was used to evaluate immune correlates of protection. Passive protection studies were also performed to validate the role of the humoral immune response to the vaccine in mediating protection.

**METHODS**

**Animals.** Female Golden Syrian hamsters (*Mesocricetus auratus*) from Charles River (Wilmington, MA) or Harlan (Indianapolis, IA), 70–90 g, were used for immunization and challenge studies. For challenge studies the animals were housed individually. All procedures involving animals were conducted under protocols approved by the Institutional Animal Care and Use Committee.

**C. difficile toxoid vaccine.** Toxins A and B of *C. difficile* reference strain VPI10463 (ATCC 43255) were individually purified and fully inactivated per a Sanofi Pasteur manufacturing process, yielding highly (>90%) pure toxoids. Purified inactivated toxins A and B were formulated with either aluminium hydroxide or a proprietary Sanofi Pasteur adjuvant to create a two-component vaccine.

**Active protection model in hamsters.** Hamsters were vaccinated twice via the intramuscular (i.m.) route, 2 weeks apart, with three dose levels (5, 0.5 and 0.05 μg) of Sanofi Pasteur’s *C. difficile* toxoid adjuvanted vaccine. Ten to fifteen animals per group were used. On day 34, 10 mg kg⁻¹ clindamycin solution was administered via the intraperitoneal (i.p.) route. Twenty-four hours later, animals were challenged intragastrically (i.g.) with the VPI10463 live *C. difficile* culture; 10⁸ c.f.u. per dose using a feeding needle. In the case of spore challenge, 100 c.f.u. (LD₅₀) dose of strain VPI10463 was used. Post-challenge animals were observed at least twice a day for morbidity and mortality. Diarrhoeal disease was reported as a group median score of 0, no disease; 1, loose faeces; 2, wet tail and perianal region; 3, wet perianal region, belly and hind paws. To evaluate systemic antibody responses, blood samples were taken on day 28 after immunization. Blood (200–400 μl) was withdrawn from the retro-orbital sinus of Isoflurane-anaesthetized animals and left to clot for 1–3 h at room temperature or overnight at 2–8 °C. Serum from each blood sample was separated and placed in fresh tubes following centrifugation. Sera were frozen at ≤−65 °C prior to analysis by ELISA or IMR-90 cell-based toxin neutralization assay (TNA).

**Passive protection model in hamsters.** Hamsters were passively immunized with the immune antisera raised in hamsters against aluminium hydroxide adsorbed *C. difficile* toxoids. Five animals per group were used. Anti-toxoid A and anti-toxoid B antisera were mixed at 1:1 ratio and 1 ml of this mixture was administered i.p. to the hamsters on four consecutive days. On the last day of antisera administration, a spore dose of 300 c.f.u. (LD₅₀) of *C. difficile* strain 630 was delivered i.g. via feeding needle. Twenty-four hours prior to challenge all animals received i.p. pre-treatment with clindamycin. Post-challenge animals were observed for morbidity and mortality as described in the active protection model.

**C. difficile vegetative culture and spore preparation.** For vegetative culture preparation, TYMG medium (Fang et al., 2009) was prepared in 100 ml glass bottles; 100 ml fresh, autoclaved and equilibrated to 37 °C media was inoculated with 1 ml of a thawed *C. difficile* cell bank. The bottle caps were tightly sealed and the bottles were incubated at 37 °C for 24 h without agitation. Optical density at 600 nm (OD₆₀₀) was determined using a 1 ml aliquot of this culture and TYMG medium as a blank. The culture density was calculated using the conversion scheme: 1.0 OD₆₀₀ = 1 × 10⁷ c.f.u. ml⁻¹. The bulk of the culture was transferred to a 250 ml sterile centrifuge bottle and spun at 2000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended with sterile TYMG medium. The bacteria were washed two additional times with TYMG medium before being resuspended in PBS at 1 × 10⁶ ml⁻¹.

For spore preparation, *C. difficile* was grown for 24 h in thioleuculate medium (BBL). This culture was inoculated on anaerobic blood agar plates and incubated at 37 °C until confluent (3–4 days). After reaching confluence, plates were incubated for an additional 3 days to induce spore formation (approximately 7 days’ duration). Spores were harvested into PBS without Ca²⁺ or Mg²⁺, washed once then heat shocked at 56 °C for 10 min to kill the vegetative cells. Spore suspension was centrifuged at 500 g for 30 min and resuspended in 20 % glycerol in PBS. Spore preparations were frozen at ≤−65 °C for long-term storage. Viable spore counts (c.f.u. ml⁻¹) were assessed by thawing the spore stock at 37 °C and performing serial tenfold dilutions in water. Dilutions were plated in triplicate onto pre-reduced CDSA (*C. difficile* selective agar, Becton Dickinson) plates. Plates were incubated under anaerobic conditions at 37 °C for no less than 48 h. The colonies were counted and c.f.u. ml⁻¹ was calculated.

**Toxin ELISA.** Serum IgG responses against native highly purified *C. difficile* toxin A and toxin B were measured by indirect ELISA. In this assay, ELISA plates were coated with 1 μg ml⁻¹ of either toxin A or toxin B in carbonate–bicarbonate coating buffer, pH 9.6, overnight at 2–8 °C. Plates were washed between each step with PBS–0.05 % Tween 20 using a BioTek plate-washer and 200 μl blocking buffer containing 1 % BSA was added to each well of the plate for 1 h at 37 °C. Two-fold serial dilutions of serum samples were prepared in diluent 0.2 % BSA in PBS–0.05 % Tween 20 and added to the wells. After 1 h of incubation, bound hamster antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-hamster IgG (H + L) (Southern Biotech). Plates were developed with the addition of the TMB (KPL) substrate for 20 min at 37 °C and then read at 650 nm using a Molecular Devices plate reader and SoftMax Pro software. ELISA titres in EU ml⁻¹ were assigned using an in-house generated serum standard from vaccinated and challenged animals. A four-parameter dose–response curve was outlined for the standard serum by plotting the absorbance as a function of the antibody concentration in EU ml⁻¹.

**TNA.** Serum from individual animals was tested for neutralizing activity against native *C. difficile* toxin A and toxin B in the IMR-90 cell-based neutralization assay. Briefly, IMR-90 cells were seeded at 2.5 × 10⁴ per well in complete Minimum Essential Eagle Media (Invitrogen) in a 96-well plate and incubated overnight at 37 °C with 5% CO₂. Individual native purified toxin A or B, at concentrations 8 × M₉₀ (median concentration causing rounding of 50% of cells) was pre-incubated in equal volume with serial dilutions of immune sera for 1 h at 37 °C with 5 % CO₂ prior to their addition to the IMR-90 cells. Cell rounding was scored microscopically 18–20 h after the addition of the toxin/immune sera mixture using the following scoring system: 0, 0 % rounded cells; 1, 20–30 % rounded cells; 2, 40–50 % rounded cells; 3, 70–80 %
rounded cells; 4, 95–100 % rounded cells. The highest serum dilution resulting in cell rounding with the score 2 was defined as NT50.

**Statistical analyses.** Median group ELISA or neutralizing titres were compared using Mann–Whitney non-parametric t-test with a 95 % confidence interval. The correlation between ELISA and neutralizing titres was analysed using a non-parametric Spearman test.

## RESULTS

**Active vaccination with a *C. difficile* toxoid vaccine given by the parenteral route protects hamsters against *C. difficile*-associated disease and death**

We tested the protective activity of the Sanofi Pasteur adjuvanted toxoid vaccine in the hamster model of *C. difficile* infection. Hamsters were vaccinated twice with increasing concentrations of the toxoid A and B combinations via the i.m. route and then challenged i.g. with a live vegetative *C. difficile* culture of a high toxin producing reference strain VPI10463 (Fig. 1a, b). In agreement with our previous studies (Sanofi Pasteur’s unpublished observations), all diluent/adjuvant alone immunized animals, as well as unimmunized controls, succumbed to this stringent challenge and rapidly died even prior to developing a severe diarrheal disease. In contrast, vaccine-immunized animals were protected against death and symptoms of disease in a dose-dependent manner. Vaccine doses of 5, 0.5 and 0.05 µg elicited protection from death in 89, 45 and 3 % of animals, respectively (Fig. 1a). Disease symptoms measured as a diarrhoeal score were also lower in animals receiving higher vaccine doses (Fig. 1b). Active immunization with the vaccine protected hamsters equally well against a lethal challenge with *C. difficile* spores of strain VPI10463 as a 5 µg dose of the adjuvanted vaccine conferred protection to 91 % of the challenged animals (Fig. 1c).

**Immunization elicited toxin-specific IgG responses where binding and neutralizing antibody titres correlated with one another for both toxins A and B**

To assess the development of toxin-specific IgG, sera were collected from immunized animals prior to challenge on day 28. These serum samples were tested in both ELISA and IMR-90 cell-based TNA assays. Both toxin binding and neutralizing antibody responses exhibited dose dependence. While immunization with all three dose levels elicited substantial anti-toxin IgG responses, immunization with the lower doses led to the development of low toxin-neutralizing activity. Neutralizing antibody responses for each individual animal and a group median log<sub>10</sub> NT<sub>50</sub> for each dose-level group are shown (Fig. 2). Median titres were compared using the non-parametric Mann–Whitney t-test. The median toxin A neutralizing titre in the 0.05 µg dose group was significantly lower than the titre in the 0.5 µg dose group (P<0.0001) (Fig. 2a). Similarly, the titre in the 0.5 µg dose group was significantly lower than that in the 5 µg dose group (P=0.0009). Similar comparison between the groups was performed for the neutralizing toxin B antibody responses. The median toxin B titre in the 0.5 µg dose group was significantly lower than that in the 5 µg dose group (P<0.0001) (Fig. 2b). P values for the compared groups are shown in Fig. 2.

Toxin binding antibody for each individual animal and a group median titre in log<sub>10</sub> EU ml<sup>-1</sup> are shown for each dose-level group (Fig. 2). The titres in the group immunized with the 0.05 µg dose were the lowest and the titres in the 0.5 µg dose group were lower compared to

![Fig. 1](http://jmm.sgmjournals.org)

**Fig. 1.** Protective effects of *C. difficile* toxoid vaccine in lethally challenged hamsters. Twenty days after completion of immunization regimen, hamsters were pre-treated with clindamycin and challenged 24 h later with the LD<sub>100</sub> dose of vegetative cells or spores of the VPI10463 *C. difficile* strain. (a) Percentage of surviving animals post-challenge with vegetative cells for each dose level is shown. (b) Illness is shown as a group median of the disease score for the animals challenged with vegetative cells. 0, no diarrhoea; 1, wet faeces; 2, wet tail and perianal region; 3, wet tail, paws and lower abdomen. (c) Percentage of surviving animals post-challenge with spores is shown.
the 5 µg dose group with \( P < 0.0001 \) for all comparisons. Therefore the median toxin A and toxin B ELISA titres in each dose-level group were shown to be statistically significantly lower than the groups that received higher doses.

Correlation between the toxin-specific titres measured by ELISA and toxin neutralizing titres measured by the IMR-90 cell-based TNA was analysed for 90 individual day 28 serum samples. As previously described, ELISA values were reported in \( \log_{10} \text{EU ml}^{-1} \) (right \( y \)-axes). Median \( \log_{10} \text{EU ml}^{-1} \) for each dose-level group is shown. TNA values are reported in \( \log_{10} \text{NT}_{50} \) (left \( y \)-axes). Median \( \log_{10} \text{NT}_{50} \) for each dose-level group is shown. Medians of the respective groups were compared using non-parametric Mann–Whitney test. \( P \) values for the compared groups are shown.

**Fig. 2.** Toxin A and B neutralizing and binding serum responses in vaccinated animals. Pre-challenge antiserum from immunized individual animals was tested in IMR-90 cell-based TNA and ELISA for toxin A (a) and toxin B (b) specific activity. ELISA values are shown in \( \log_{10} \text{EU ml}^{-1} \) (right \( y \)-axes). Median \( \log_{10} \text{EU ml}^{-1} \) for each dose-level group is shown. TNA values are reported in \( \log_{10} \text{NT}_{50} \) (left \( y \)-axes). Median \( \log_{10} \text{NT}_{50} \) for each dose-level group is shown. Medians of the respective groups were compared using non-parametric Mann–Whitney test. \( P \) values for the compared groups are shown.

**Responses to both toxins were the best predictors of survival in the hamster efficacy model**

To better understand the value of the *in vitro* ELISA and IMR-90 TNA in predicting protection following immunization with a two-component toxoid vaccine and challenge with live *C. difficile*, survival data obtained in these studies were analysed in the context of binding and neutralizing toxin-specific titres.

**Fig. 3.** Concordance between ELISA and TNA in measurement of toxin A and toxin B specific antibody activities in sera of vaccinated hamsters. Graph shows plots of anti-toxin A (a) and anti-toxin B (b) day 28 sera titres from immunized animals measured in both indirect toxin ELISA and TNA for each individual animal. Number of individual animals analysed for the toxin responses was 90. ELISA values are reported in \( \log_{10} \text{EU ml}^{-1} \) and TNA values are reported in reciprocal \( \text{NT}_{50} \). Correlation analysis between the titres measured in both assays by Spearman test showed statistically significant correlation for both anti-toxin A \( (P < 0.0001) \) and anti-toxin B \( (P < 0.0001) \) titres. Spearman \( r \) values are shown.
Ninety per cent of unprotected animals had anti-toxin A and anti-toxin B IgG ELISA titres equal or below $5.4 \log_{10}$ EU ml$^{-1}$ and $5.3 \log_{10}$ EU ml$^{-1}$, respectively; therefore these titres were defined as 'non-protective' (Fig. 4a). The anti-toxin A and anti-toxin B IgG ELISA titres above $5.4 \log_{10}$ EU ml$^{-1}$ and $5.3 \log_{10}$ EU ml$^{-1}$, respectively, were defined as 'protective'. Similarly, 90% of unprotected animals had anti-toxin A and anti-toxin B neutralizing titres equal to or below 1:3000 and 1:240, respectively, which were considered 'non-protective' (Fig. 4b). The titres above 1:3000 and 1:240 were defined as 'protective'.

Based on the protective and non-protective titres from the *in vitro* assays, true and false positive and negative rates were calculated for each individual vaccine component as well as for both components. The definitions are summarized in Table 1, where in brief, A=animals that survived and had a protective titre, B=animals that died with an apparent protective titre, C=animals that survived with a non-protective titre and D=animals that had a non-protective titre and died. The columns in the table indicate the actual outcome, i.e. survival or death. The rows indicate the assay results, protective or non-protective titre. The true negative rate was defined as the probability that a test indicates lack of protection among those that indeed did not survive (D/(D+B) × 100%). The false positive rate is when the test indicates protection, but animals do not survive, and was calculated for all assays using the formula: 100–true negative rate (%). The true positive rate was defined as the probability that the test indicates protection among those that indeed survive (A/(A+C) × 100%), and the false negative rate is when the test indicates lack of protection, but animals survive and was calculated using the formula: 100–true positive rate (%).

This analysis indicated that both TNA and ELISA were good at predicting lack of protection for each individual component of the vaccine on the basis of 'non-protective' titres. Toxin A TNA had the highest true negative rate of 98%, followed by the toxin A ELISA with a value of 94%. In comparison, both toxin B TNA and ELISA had slightly lower true negative rates of 89% and 92%, respectively. The assays had lower true positive rates on the basis of 'protective' antibody titres with a toxin A ELISA being the most predictive (79%). Individual toxin A and B neutralization assays had a lower sensitivity in predicting protection among true survivors (Table 2).

When used in combination, the results from the toxin A and toxin B ELISA were highly predictive of lack of protection (true negative); as 87% of the animals that did not survive challenge had titres below the cut-off for both toxins. There were very few false positive cases when the ELISA titres were assessed in combination; only 1% of the animals that succumbed to challenge had titres that exceeded the cut-off values for both toxins A and B.

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**Table 1.** Definition of true positive, false positive, false negative and true negative values: true survival condition versus test result

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<thead>
<tr>
<th>Test result</th>
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<tr>
<td></td>
<td>Survival</td>
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<tr>
<td>Protective titre (positive)</td>
<td>True positive (A)</td>
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<tr>
<td>Non-protective titre (negative)</td>
<td>False negative (C)</td>
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False positive rates based on a single toxin titre exceeding the cut-off value were slightly higher, 5 % and 7 % for toxins A and B, respectively (Fig. 5b and Table 2).

For animals that survived challenge, the combined ELISA titres were moderately predictive of protection when both toxin A and toxin B titres were above the cut-off. There was a true positive rate of 46 %. There was also a high false negative rate as 18 % of the animals that survived challenge had titres below the cut-off for both toxins. The antibody titres in those false negative animals were just below the cut-off and potentially reflected limitations of the assay sensitivity. Predicting protection based on either toxin A or toxin B titre alone was even lower, 33 % and 3 %, respectively (Fig. 5a). True positive rate on the basis of toxin A only was 79 % and toxin B only was 49 % (Table 2). These analyses indicate that serum responses to both A and B toxins were highly specific in predicting lack of protection.

**Passive transfer of immune sera protects hamsters against C. difficile challenge**

As low serum responses to both A and B toxins could be correlated with lack of protection in the hamster challenge model, we wanted to confirm the role of anti-toxin antibodies in mediating protection. To eliminate a possible contribution of cellular immune response and to better define the characteristics of a protective humoral response, a passive immunization approach was used. Hamsters were passively immunized with the immune sera raised in hamsters against the adjuvanted *C. difficile* toxoids. The toxin-specific antibody titres in the passively transferred immune sera were $6.15 \log_{10}$ EU ml$^{-1}$ for anti-toxin A and $6.39 \log_{10}$ EU ml$^{-1}$ for anti-toxin B. Because of limitations in achieving blood titre levels similar to those reported in previous active immunization studies, a lower toxin-producing *C. difficile* toxotype 0 strain, clinical isolate 630 (Merrigan et al., 2010), was used in these passive transfer challenge studies in contrast to the active immunization studies, where the high toxin-producing reference strain VPI10463 was used.

An anti-toxoid A and anti-toxoid B antisera mixture was administered to the hamsters i.p. on four consecutive days. On the last day of antiserum administration, an LD$_{100}$ of *C. difficile* spores was delivered i.g. to clindamycin pre-treated animals. The spore challenge was chosen to mimic a natural course of infection in the human population. The control group treated with normal hamster serum succumbed to the CDI and died within 5 days post-challenge, while the immune sera-treated animals were protected against morbidity and mortality. Furthermore, hamsters passively immunized with increasing dilutions of immune antiserum (1:5, 1:25 and 1:125) were protected.
from mortality in a dose-dependent manner. Antiserum diluted at 1:125 did not confer protection, as all animals succumbed to disease and died by day 3; in contrast the 1:25 and 1:5 dilutions protected 83 and 100% of animals, respectively.

Hamsters in the group treated with normal serum had the highest median disease score and all animals died by day 5 (Fig. 6a). Animals treated with a 1:25 dilution of serum had a median disease score of 2 at a peak of illness, whereas animals in the 1:5 dilution group remained healthy throughout the study (Fig. 6b). Passive transfer of immune sera protected hamsters in a dose-dependent manner, demonstrating the principal role of circulating toxin-specific antibodies in immunity from C. difficile toxin-mediated mucosal disease.

**DISCUSSION**

Sanofi Pasteur’s C. difficile candidate vaccine is currently being developed for the prevention of primary symptomatic disease caused by C. difficile. In contrast to the original investigational vaccine, this vaccine candidate contains highly purified, formalin-inactivated alum adsorbed preparations of toxins A and B. The target population for this vaccine is adults at risk of CDI; this group includes those with planned hospitalization, long-term care/nursing home residents, and those with co-morbidities that require frequent and/or prolonged antibiotic use. The vaccine is currently undergoing Phase II clinical trials and has been proven to be immunogenic and well tolerated in adults and the elderly (Greenberg et al., 2012; Foglia et al., 2012). In this study, the vaccine candidate’s efficacy was evaluated in a preclinical hamster model.

Reports from multiple animal models and human studies clearly indicate importance of anti-toxin A and anti-toxin B antibodies in protection against CDI. There has been an ongoing discussion in the field focused on the relative contribution of toxin A and toxin B and their specific domains in mediating a protective response. Neutralizing antibodies to the receptor binding domain (RBD) of both toxins have long been implicated in efficacy. Out of all recombinant peptides spanning the entire toxin sequences, only antibodies raised against RBDs were completely protective against purified toxins in hamsters (Kink and Williams, 1998). Supporting the essential role of the RBD is a recent study in which hamsters immunized with a fusion protein containing RBD of toxin A and toxin B developed neutralizing antibodies and were protected against C. difficile spore challenge (Tian et al., 2012). In agreement with these studies, a pair of anti-toxin A and anti-toxin B RBD-specific mAbs developed by Medarex/Merck was found to be efficacious in the hamster model (Babcock et al., 2006) and in humans against recurrent CDI, when tested in a Phase II clinical trial (Lowy et al., 2010). A recent study using the infant pig model showed that when animals were treated with the Medarex/Merck anti-toxin B antibody alone or in combination with the anti-toxin A antibody, those animals displayed reduced gastrointestinal
(GI) inflammation and were fully protected against systemic CDI following *C. difficile* challenge with hyper-virulent epidemic strain NAP1/027/BI. However, when anti-toxin A antibody alone was used, animals experienced exacerbated GI inflammation and there was a greater rate of fatality when compared to the placebo-treated control (Steele *et al.*, 2013). This anti-toxin A-mediated exacerbation, which has not been previously reported in rodent models, serves as a reminder to investigators not to over-interpret the results of the animal models and their potential correlation with humans. Apart from this phenomenon, this paper highlights the importance of anti-toxin B antibody in treatment of CDI. These data are not in full agreement with the previously reported data from other animal models, which demonstrated a leading role of anti-toxin A antibody in protection (Lyerly *et al.*, 1986; Babcock *et al.*, 2006; Permpoonpattana *et al.*, 2011).

In contrast to the previous belief that antibodies directed to the RBD only are important, recent data in hamsters suggest that a combination of antibodies to the RBD of toxin A and the enzymic glucosyltransferase domain (GTD) of toxin B may be efficacious as well (Marozsan *et al.*, 2012). In a mouse model, a DNA vaccination screening of different fragments of toxin A and toxin B revealed that only a subset of the toxin fragments, including the RBD of toxin A and an enzymic domain of toxin B, were able to elicit protective antibody responses as determined by a cell-based neutralization assay and prevent death of animals in a passive protection study (Jin *et al.*, 2013). Hamster experimental data also suggest that domains other than the RBD may be protective as a combination of humanized mAbs that bind to the RBD of toxin A and enzymic GTD of toxin B protected 19 of 20 hamsters against *C. difficile* challenge (Marozsan *et al.*, 2012).

While fusions (Tian *et al.*, 2012), recombinant domain (Kink & Williams, 1998; Marozsan *et al.*, 2012) and chimeric (Wang *et al.*, 2012) approaches to a *C. difficile* toxin vaccine have been very attractive due to the complexity of manufacturing native toxins, the contradictory findings in the field on the relative importance of toxin A or toxin B in the pathogenesis of disease and protective immunity, as well as the lack of full agreement on the major neutralizing epitopes associated with a particular toxin domain, lends weight to a holotoxins approach targeting both A and B toxins as a vaccine candidate.

It has been previously demonstrated that parenteral vaccination with formalin-inactivated toxins A and B in culture filtrate protected hamsters against lethal ileocecticis induced by clindamycin and *C. difficile* challenge (Libby *et al.*, 1982; Fernie *et al.*, 1983; Kim *et al.*, 1987). Giannasca *et al.* (1999) reported that the vaccine containing partially purified and inactivated toxin A and B (toxoids) delivered by sequential immunization via intramuscular (with alum) and mucosal (with *E. coli* heat-labile toxin adjuvant) routes conferred full protection against live toxinogenic *C. difficile* challenge, implying that a combination of mucosal and systemic immunity was necessary for optimal protection. In this study, a proof-of-product assessment of the native toxin A and toxin B inactivated two-component vaccine candidate delivered via i.m. administration was performed. Antibody responses elicited by this candidate in a *C. difficile* preclinical animal model were evaluated and immune correlates of protection were identified. Antigen purity, choice of adjuvant and route of administration were dictated by the desire to make a model suitable for a clinical application.

Analogous to a previous report (Giannasca *et al.*, 1999), we have shown that i.m. immunization with the alum adjuvanted vaccine candidate, comprising highly purified toxoids A and B, protected hamsters from death associated with *C. difficile* challenge. Toxin-specific serum IgG titres as well as efficacy were also shown to be dependent upon the administered dose of vaccine. Likewise, amelioration of diarrhoeal disease and faster recovery was associated with receiving higher doses of the vaccine.

Two in vitro assays, a toxin ELISA and an IMR-90 cell-based TNA, were identified as immune correlates of protection. We demonstrated concordance between the serum toxin-specific antibody levels measured in the ELISA and the serum neutralizing activity measured in the TNA for both toxins A and B. Furthermore, there was a correlation between these serum measurements and the outcome of *C. difficile* challenge. Insufficient responses to both toxins were predictive of lack of protection against challenge, suggesting the importance of an adequate response to both components of the vaccine. A sufficient response as measured by ELISA was highly predictive of survival as the false positive rate of animals that had titres exceeding the cut-off values for both toxins A and B was only 1%. However, the higher false negative rates are indicative of limitations in the assay sensitivity. The higher false negative rate could also be attributed to protection associated with parameters other than toxin-specific IgG, such as duration of exposure to *C. difficile* infection, normal gut flora, innate cellular responses and responses to non-toxin components of the bacterium. Individual toxin A and B neutralization assays had lower sensitivity in predicting efficacy among true survivors. The discontinuous, step-wise nature of the TNA may contribute to its diminishing power in comparison with the continuous colorimetric ELISA.

The absolute importance of the humoral immune response in mediating vaccine-induced protection was demonstrated in the passive transfer studies. Similar to previous reports which demonstrated the importance of anti-toxin antibodies in protection from CDI (Giannasca *et al.*, 1999; Babcock *et al.*, 2006; Kink & Williams, 1998; Wang *et al.*, 2012; Marozsan *et al.*, 2012; Torres *et al.*, 1995), we have shown that antisera raised against the adjuvanted toxoids A and B conferred passive protection in a hamster model in a dose-dependent manner confirming the role of toxin-specific circulating antibodies in protection. In summary, an alum adjuvanted toxoid A and B vaccine delivered i.m. protected hamsters against a lethal challenge with either vegetative cells or spores of a *C. difficile*
toxinotype 0 strain. Toxin A and B specific IgG levels measured in sera by holotoxin ELISA and TNA correlated with protection against CDI in the hamster model. Future studies will address the ability of this vaccine to protect against other clinically important toxinotypes of C. difficile.

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