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RESEARCH ARTICLE

Immunization of hamsters against *Clostridium difficile* infection using the Cwp84 protease as an antigen

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Abstract

Clostridium difficile is a pathogen responsible for diarrhoea and colitis, particularly after antibiotic treatment. We evaluated the *C. difficile* protease Cwp84, found to be associated with the S-layer proteins, as a vaccine antigen to limit the *C. difficile* intestinal colonization and therefore the development of the infection in a clindamycin-treated hamster model. First, we evaluated the immune response and the animal protection against death induced by several immunization routes: rectal, intragastric and subcutaneous. Antibody production was variable according to the immunization routes. In addition, serum Cwp84 antibody titres did not always correlate with animal protection after challenge with a toxigenic *C. difficile* strain. The best survival rate was observed with the rectal route of immunization. Then, in a second assay, we selected this immunization route to perform a larger immunization assay including a Cwp84 immunized group and a control group. *Clostridium difficile* intestinal colonization and survival rate, as well as the immune response were examined. *Clostridium difficile* hamster challenge resulted in a 26% weaker and slower *C. difficile* intestinal colonization in the immunized group. Furthermore, hamster survival in the Cwp84 immunized group was 33% greater than that of the control group, with a significant statistical difference.

Introduction

Following the disruption of the normal bowel microbiota by antibiotic therapy, *Clostridium difficile* colonizes the gut, resulting in a spectrum of diseases ranging from asymptomatic carriage to pseudomembranous colitis (PMC) (Kelly & LaMont, 1998; Wilcox, 2003).

The disease symptoms are mediated by two secreted enterotoxins: TcdA and TcdB. *Clostridium difficile* is shed in the faeces as spores that persist in the environment and facilitate the colonization of new individuals. *Clostridium difficile* is thus a particular problem in health care facilities, where transmission easily occurs between patients and from carriers to patients (McFarland *et al.*, 1989).

Measures to prevent *C. difficile* infection (CDI) through patient isolation are costly and have had variable success.

Although previously considered rare, the incidences of community-acquired CDI and colitis are on the increase. After the acquisition of *C. difficile* and subsequent colonization by the bacterium, facilitated by the disruption of the intestinal microbiota, the host immune response is considered of prime importance to prevent disease (Kelly & Kyne, 2011; Mulligan *et al.*, 1993; Giannasca & Warny, 2004). Vaccines containing formaldehyde-inactivated TcdA and TcdB have been developed. In healthy volunteers, this vaccine induced high levels of specific neutralizing immunoglobulin G (IgG) and some promising initial experience has been gained in a few patients with recurrent CDI (Sougioultzis *et al.*, 2005).

Although the role of antitoxin immunity in protection from CDI is clear, vaccines based on toxins are unlikely to prevent colonization, and carriage and transmission of *C. difficile* will therefore remain a persistent threat. Hence, a more complete approach against CDI should consider not only the inhibition of toxicity but also the prevention of bacterial colonization (O'Brien *et al.*, 2005).

Cwp84 is a cysteine protease of *C. difficile*, found to be associated with the S-layer proteins (SLPs). This protease is highly immunogenic in patients with *C. difficile*-associated disease (CDAD) (Pechine *et al.*, 2005), suggesting that Cwp84 could play an important role in the physiopathology of *C. difficile*. In particular, Cwp84 could contribute to the cleavage of the extracellular matrix host proteins to facilitate

the degradation of host tissue integrity and thus dissemination of the infection (Janoir *et al.*, 2007). In addition, it has been shown recently that Cwp84 plays a role in the maturation of SlpA. The inactivation of the *cwp84* gene in *C. difficile* 630ΔErm resulted in a bacterial phenotype in which only immature, single-chain SlpA comprises the S-layer (Kirby *et al.*, 2009). The role of Cwp84 in the cleavage of the SlpA precursor in the two structural SLPs (HMW and LMW) has been further confirmed (Dang *et al.*, 2010).

The SLPs of *C. difficile* are potential colonization agents thought to be involved in bacteria–host interaction (Drudy *et al.*, 2001; Calabi *et al.*, 2002; Cerquetti *et al.*, 2002). In a recent study, O'Brien and colleagues tested whether anti-SLP antibodies, assessed independent of the toxins, could have a protective effect against CDI *in vivo*. In fact, a passive immunization using anti-SLP antibodies significantly delays the progress of CDI in a lethal hamster challenge model (O'Brien *et al.*, 2005). The same laboratory tested SLPs as a vaccine component in a series of immunization and challenge experiments with hamsters. None of the regimens tested conferred complete protection of animals and antibody stimulation was variable and generally modest or poor (Ni Eidhin *et al.*, 2008).

In a previous study, we showed that the protease Cwp84 of *C. difficile* used as an immunogen was able to delay the colonization by *C. difficile* in a human microbiota-associated mouse model (Pechine *et al.*, 2007). The aim of this study was thus to evaluate the *C. difficile* protease Cwp84 as a vaccine candidate in a hamster model. We observed the kinetics of colonization and animal death after immunization and challenge with *C. difficile*, to evaluate the putative protective effect of a vaccination with Cwp84.

Materials and methods

Bacterial strain and culture

The *C. difficile* strain 79-685 is a toxigenic strain (toxin A and toxin B positive) from serogroup S3, according to Delmée. This strain was isolated from a patient with PMC, and was a gift from the Department of Microbiology of the University of Strasbourg, France. This strain was grown under anaerobic conditions in a tryptone glucose yeast infusion broth (Difco Laboratories) at 37 °C for 24 h, unless indicated otherwise, and onto Columbia agar plates supplemented with 4% horse blood (Biomerieux). The *Escherichia coli*/pET-28a(+)Ω*cwp84* strain was grown on Luria–Bertani agar or in broth (Difco Laboratories) supplemented with 50 μg mL^{-1} kanamycin to maintain the pET plasmid.

Purification of recombinant protein Cwp84

Recombinant Cwp84 was purified as described previously (Pechine *et al.*, 2005). Briefly, Cwp84 was obtained from the *E. coli*/pET-28a(+)Ω*cwp84* clone by induction of protein expression with 1 mM isopropyl-β-D-thiogalactopyranoside and subsequent purification by single-step affinity chromatography using BD Talon cobalt affinity resin (BD Biosciences) as described in the protocols supplied by the manufacturer. The eluted fraction containing the recombinant protease was dialysed overnight against phosphate-buffered saline (PBS) and then frozen at − 80 °C for storage.

Preparation of spores for hamster challenge

Spores were prepared as described previously (Sambol *et al.*, 2001). Briefly, cultures of the 79-685 toxigenic strain of *C. difficile* were grown anaerobically at 36 °C for 5–7 days, on blood agar plates. The cultures were harvested into 10 mL of PBS, washed in PBS and then heat shocked at 56 °C for 10 min. The spores were centrifuged, resuspended in Dulbecco's modified Eagle medium and frozen at − 80 °C. The frozen spores were quantified by 10-fold serial dilutions plated onto Columbia agar plates supplemented with 4% horse blood and sodium taurocholate (0.1%).

Animals

Adult *Mesocricetus auratus* female hamsters (weight, 80–100 g) were obtained from Charles River Laboratories and were housed in polypropylene isolator cages fitted with filter covers holding disposable polyester air filters. All food, water, bedding, cages, wire lids and filter covers were autoclaved before being used. Procedures were commenced after 1 week of receipt. Animals were caged in groups of five during the immunization period and then caged individually during the *C. difficile* challenge. All animal procedures were conducted according to protocols approved by the Animal Central Department of University Paris-Sud.

Before treatment and inoculation, a sample of the hamsters' faecal pellets was cultured using selective media added with taurocholate to exclude prior *C. difficile* colonization.

Immunization regimens

First experiment: selection of the best route of immunization

Three different active regimens of immunization were tested: one parenteral (subcutaneously) and two mucosal (intragastrically and rectally) (Table 1). Groups of six animals were used for all immunization regimens. For subcutaneous and rectal immunization, hamsters were anaesthetized by an intraperitoneal route with a cocktail composed of Rompun® 2% (0.25 mL kg^{-1}) and ketamine chlorhydrate (1 mL kg^{-1}).

All groups received a total of three doses of the vaccine on days 1, 15 and 30. Each hamster was sampled under anaesthesia directly by heart puncture before the first immunization

Table 1. Schemes of immunization of hamsters with the protease Cwp84 of *Clostridium difficile* on days 1, 15 and 30

Route of immunization (group)	Dose of protein per immunization (μg)	Adjuvant and dose per immunization (immunization 1 or 2 or 3)	Volume administered (μL)
Subcutaneous (s.c.)	100	Freund complete adjuvant 50 μL (1) Freund incomplete adjuvant 50 μL (2, 3)	100
Rectal (r.)	100	Cholera toxin 10 μg (1, 2, 3)	150
Intragastric (i.g.)	100	None	500

and 15 days after the last one, in order to evaluate the immune response induced.

Fifteen days after the last immunization, hamsters were administered by gavage clindamycin (Dalacine®) at a single dose of 50 mg kg^{-1} to disrupt the barrier microbiota in order to predispose them to CDI. Five days later, hamsters were challenged orogastrically with 2×10^3 CFU of spores of the 79-685 toxigenic strain of *C. difficile*. From the day after infection, hamsters were observed three times a day.

Second experiment: comparison of animal challenge survival of a group immunized with Cwp84 by the rectal route vs. a control group

The conclusions of the first experiment led us to perform a second one, with a higher number of animals, with the route of immunization inducing the best animal survival results. Hence, the second experiment was performed with the use of the rectal route, as per the same immunization regimen as described above. A group of 18 animals was immunized by 100 μg of the protease Cwp84 and 10 μg of cholera toxin and a control group of 16 animals was immunized by PBS and cholera toxin 10 μg.

Clostridium difficile detection in hamster faecal samples

To confirm the excretion of *C. difficile* after challenge with spores (12 animals immunized with Cwp84 and 10 animals of the control group randomly selected), faeces were sampled each day and *C. difficile* was numerated by culture. Hamster faecal pellets were cultured before clindamycin administration and daily for 1 week after *C. difficile* challenge, to assess the colonization rate and its onset. Faecal sample were processed as described previously (Pechine *et al.*, 2007). The limit of detection was estimated to be 10^4 CFU g^{-1} of faeces.

Measurement of antibody response in immunized animals by an ELISA

To evaluate the antibody response in sera, blood samples (200–400 μL) were withdrawn before the first immunization and 15 days after the last immunization, before *C. difficile* challenge. The blood was left to clot for 1 h at room temperature and 3 h at 4 °C. Serum was obtained by centrifugation and frozen at − 20 °C until use.

Indirect ELISA was used to detect antibodies in the sera as described before (Pechine *et al.*, 2007). Wells of a 96-well microtitre plate (MaxiSorp, Nunc) were coated with 100 μL of a 5 μg mL^{-1} solution of recombinant purified Cwp84. Sample dilutions tested were 1 : 100; 1 : 200; 1 : 400; 1 : 800; 1 : 1600; 1 : 3200; 1 : 6400; and 1 : 12 800. After washings, positive reactions were detected by successive incubations with a rabbit anti-hamster immunoglobulins conjugated to biotin (1 : 8000 dilution; Biovalley) for 30 min at 37 °C and with a streptavidin–horseradish peroxidase conjugate (1 : 1000 dilution; Sigma) for 30 min at 37 °C. The specificity of the ELISA was confirmed by immune absorption. A preincubation for 30 min at 37 °C of control and immunized hamster serum samples with the protease Cwp84 at 50 μg mL^{-1} was carried out. All samples in this study were tested in duplicate and treated simultaneously to avoid interassay variation. Assays with antigen in the absence of sera served as negative controls. Immunoglobulin titres are expressed as OD units, with a value obtained for 1 : 100 diluted serum samples.

Neutralizing properties of hamster sera against Cwp84 proteolytic activity

The proteolytic activity of Cwp84 was quantified with azocasein (Sigma); 50 μg of protease was added to 500 μL of a 5 mg mL^{-1} azocasein solution in 25 mM Tris (pH 7.5). After 16 h of incubation, intact azocasein was removed by 3% trichloroacetic acid precipitation, and the amount of released dye was measured spectrophotometrically at 336 nm.

The neutralizing activity of the specific anti-Cwp84 hamster sera was tested by monitoring Cwp84-mediated degradation of azocasein. Various amounts of sera were added to the protease, resulting in 1 : 50 dilutions, and after 30 min of incubation at 37 °C, an azocasein mixture was added and assays were performed as described above. To assess the specificity of the neutralizing activity of immunized hamster sera, and to exclude the possibility of a steric hindrance effect, negative control experiments were performed with preimmune hamster sera, using the same dilutions.

Statistical analysis

Statistical analyses were performed to compare the antibody level ($OD_{405\,nm}$ values) directed to Cwp84 in the hamster sera sample of the control group to the Cwp84 immunized group. It shows that antibody levels were not normally distributed. Therefore, we used the Mann–Whitney *U*-test for nonparametric data to test the null hypothesis that there was no difference between the immunized group and the control group. Analyses were performed using the STATA 8.0 (Statacorp, College Station, TX). Statistical significance was set at $P = 0.05$. All *P*-values were two-sided.

The survival of animals following infection was analysed using Kaplan–Meier estimates. Survival rates across groups were compared using log-rank tests. *P*-values of < 0.05 were considered to be statistically significant. Statistical analyses were performed using STATA 8.0 (Statacorp).

Results

Antibody response and survival of hamsters immunized by the rectal, intragastric or subcutaneous route

Three groups of hamsters were immunized by 100 μg of the protease Cwp84 by several routes of immunization: rectally, intragastrically and subcutaneously. Then clindamycin was administered to animals and, 5 days later, hamsters were challenged by *C. difficile* spores.

Each hamster was sampled under anaesthesia directly by heart puncture. Cwp84-specific IgG, IgA and IgM were quantified by ELISA and the capacity of serum antibodies to neutralize Cwp84 activity *in vitro* was measured.

Serum antibodies against Cwp84 were measured before immunization and 15 days after the second boost. The response was variable within groups (Fig. 1). The poorest response was seen with the intragastric route; the mean $OD_{405\,nm}$ was 0.5 and there was no significant difference before and after immunization ($P = 0.13$). Hamsters receiving the protease by the subcutaneous route exhibited a relatively strong response, with a mean of $OD_{405\,nm}$ of 1.8, and the difference between pre- and postimmune sera was significant ($P = 0.002$). By the rectal route, specific antibodies measured after immunization increased, but less than by the subcutaneous route, and not significantly ($P = 1.06$); the mean $OD_{405\,nm}$ is 0.9.

Whatever the route of immunization (rectal, intragastric and subcutaneous), the antibody titres were highly variable between animals in the same group. The SDs were very high.

After challenge, the median survival times were highly variable within groups. The challenge outcome in all groups is presented in Fig. 2. The three immunization routes were significantly different from each other ($P = 0.05$). There was no correlation between serum anti-Cwp84 titres and post-challenge survival. Animals immunized by the subcutaneous route had the highest antibody level, but only 17% of them (1/6) survived to the *C. difficile* challenge on day 11. Fifty percent of hamsters (3/6) immunized by the rectal route survived to *C. difficile* challenge. The group immunized by the intragastric route did not seem to be protected against the challenge; no hamsters from this group survived on day 11.

Survival of hamsters immunized with the Cwp84 protease by the rectal route compared with a control group, antibody response and colonization onset by *C. difficile*

As the animal challenge results observed for the rectal route were promising, we decided to perform a second assay, under exactly the same conditions, but increasing the number of animals and including the analysis of the faecal pellet samples in order to monitor the colonization and to analyse the results observed in the protection assay.

Postchallenge survival of hamsters

For this survival study, groups were composed, respectively, of 18 animals for the immunized group and 16 animals for the control group. The challenge outcome in the control

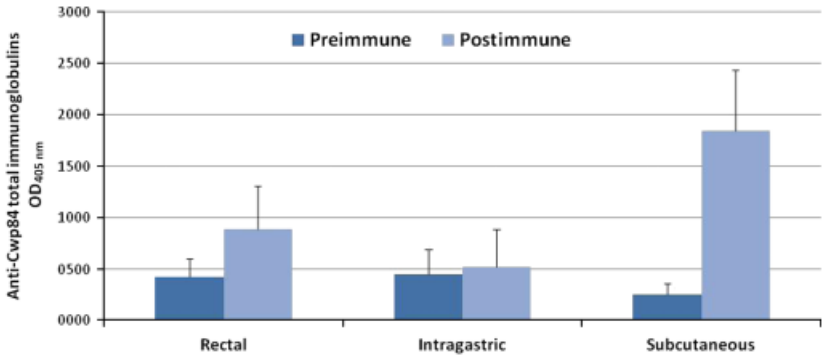

Fig. 1. Serum antibodies response to Cwp84 in hamsters immunized by three different routes of immunization: rectal, intragastric and subcutaneous. Hamster sera were diluted to 1 : 100 and analysed by ELISA.

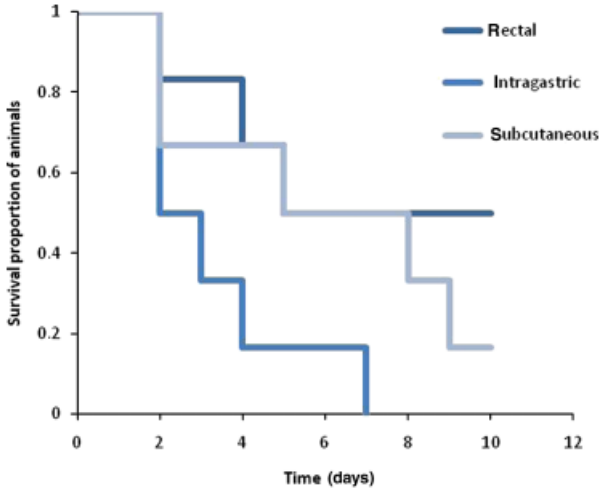

Fig. 2. Kaplan–Meier survival estimates, according to immunization routes (rectal, intragastric and subcutaneous) demonstrating the time between challenge with *Clostridium difficile* and death. Clindamycin (50 mg kg^{-1}) was administered 5 days before spore challenge. Animals were observed for 11 days.

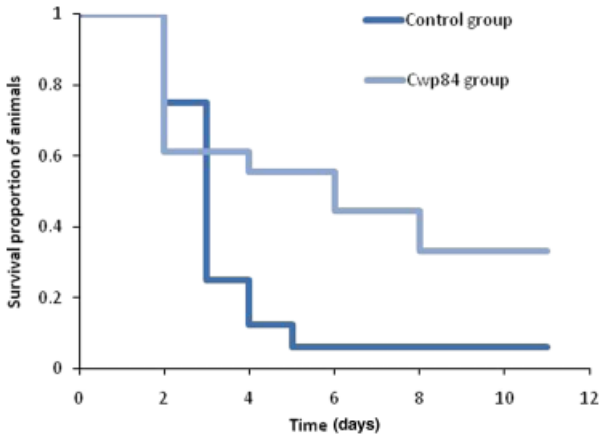

Fig. 3. Kaplan–Meier survival estimates after rectal immunisations demonstrating time between challenge with *Clostridium difficile* and death. Clindamycin (50 mg kg^{-1}) was administered 5 days before spore challenge. Animals were observed for 11 days. Experiments were performed with hamsters immunized rectally with Cwp84 or receiving PBS as a control. The cholera toxin was used as an adjuvant for the two groups.

group and the group immunized by Cwp84 is presented in Fig. 3.

Postchallenge survival was significantly prolonged in animals immunized with Cwp84 as compared with the control group ($P = 0.038$). Within the first 5 days, 90% of hamsters from the control group died (15 out of 16 animals died). Among the animals immunized by Cwp84, 33% survived the challenge (six out of 18 animals survived). Signs of morbidity such as inactivity and wet tail or diarrhoea were not always apparent before dying.

Colonization onset after challenge with *C. difficile*

After the *C. difficile* challenge, the numbers of viable *C. difficile* bacteria (vegetative cells and spores) present in faecal samples were determined every day during 1 week in order to examine *C. difficile* intestinal colonization. There were differences in colonization onset among hamsters.

Challenge of hamsters with the 79-685 *C. difficile* strain resulted in colonization of 90% of the control group; each colonized animal developed infection leading to death, which was observed from day 2 to day 6. In the immunized group, the colonization reached 66% (Fig. 4).

For the two groups, 1 day after challenge, *C. difficile* was not detected in any sample. Onset of colonization was variable, ranging from 1 to 5 days after challenge. In the immunized group, two hamsters were never colonized and two others were colonized, but this colonization disappeared later, leading to animal survival.

Antibody response in sera

Antibodies titres were highly variable between animals in the same group. Therefore, the SD calculated for each group was very high. Surprisingly, the background antibody levels observed in the two groups were high (Fig. 5). Even if the mean level of Cwp84-specific antibody was higher for the Cwp84 immunized group than for the control group, the difference was not statistically significant ($P = 0.13$).

We assessed the relationship of Cwp84-specific antibody levels elicited in serum with the protection conferred to hamsters. We found that antibody levels did not appear to correlate directly with protection, because surviving hamsters did not consistently demonstrate higher titres of specific antibody in sera. The specificity of the ELISA was confirmed by immune absorption. Preincubation of control and immunized hamster serum samples with the protease Cwp84 at 50 μg mL^{-1} resulted in a reduction in reactivity in the antiprotein ELISA (data not shown).

The neutralizing activity of antibodies against Cwp84 was tested on azocasein in an *in vitro* assay (data not shown). No significant difference was observed between inhibition of enzymatic activity of Cwp84 by immunized hamster sera and by control hamster sera. Therefore, as observed in the first study, there was no correlation between systemic immune response directed to Cwp84 and postchallenge survivals.

Discussion

Individuals who acquire *C. difficile* may be colonized or develop disease, and the immune status of the host is an important determinant of the outcome. Patients with more severe underlying illnesses are more likely to develop CDI. Asymptomatic carriers, colonized by *C. difficile*, who can

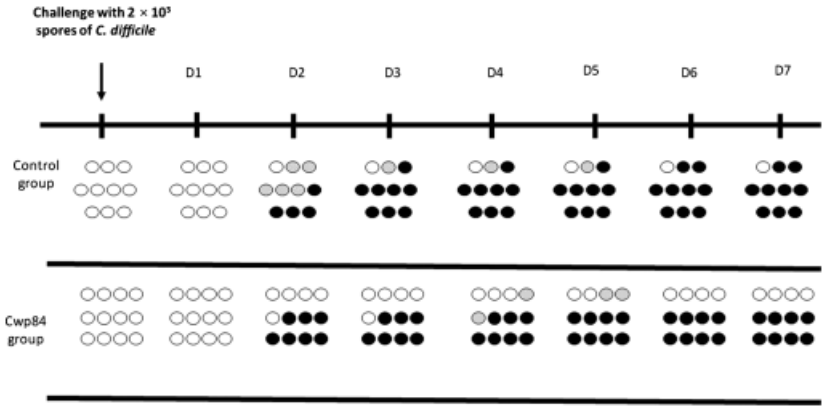

Fig. 4. Course of colonization and death of hamsters pretreated with clindamycin and challenged with a toxigenic *Clostridium difficile* strain after immunization by the rectal route. White ovals, uncolonized hamsters; black ovals, colonized hamsters that died; grey ovals, hamsters colonized with *C. difficile*.

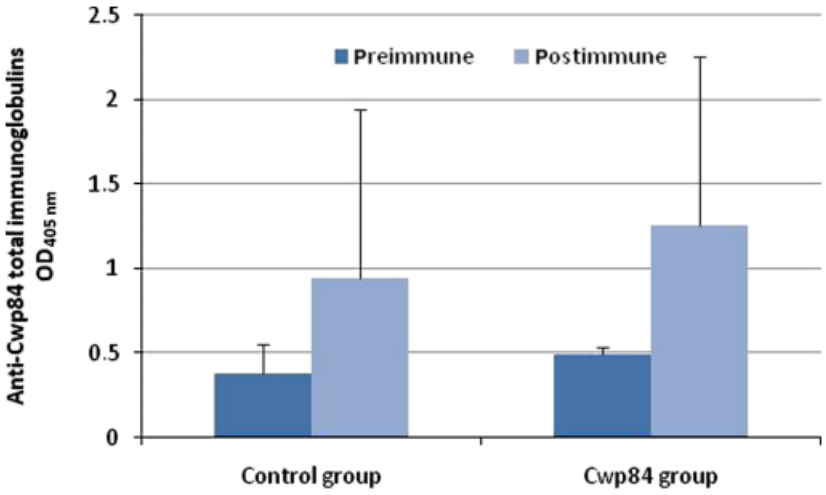

Fig. 5. Mean of Cwp84-specific total immunoglobulins in the serum of hamster (diluted 1 : 100). Sera of hamster immunized by the rectal route with Cwp84 or by PBS for the control group were analysed by ELISA.

constitute up to 20% of patients receiving antibiotics, have elevated levels of serum immunoglobulins to somatic antigens (Mulligan *et al.*, 1993). These results suggest that acquired immunity to toxins (Kyne *et al.*, 2001) or somatic antigens (Kelly, 1996; Kyne *et al.*, 2001) could protect against infection.

The apparent role of immunity in controlling CDI has prompted research into the development of a vaccine. *Clostridium difficile* exerts its pathological effects at the intestinal surface. Thus, a vaccine that stimulates mucosal immunity in the gut should be an appropriate line of defence against this pathogen. However, most of the vaccine trials have been carried out using toxin A, toxin B and subfragments of the C-terminal repeat region as antigens. These experiments have shown that toxins A and B (1) induce mostly systemic, toxin-neutralizing immune responses, but induce poorly local immune responses in the intestine (Ward *et al.*, 1999); (2) have frequently proven effective in protecting animals against toxin-induced damages, but are frequently inept at preventing diarrhoea (Torres *et al.*, 1995; Ryan *et al.*, 1997; Giannasca *et al.*, 1999); and (3) in humans, were reported to be safe and immunogenic in healthy volunteers and were associated with the resolution of recurrent diarrhoea when administered by an intramuscular route to patients with recurrent CDAD (Kotloff *et al.*, 2001; Sougioultzis *et al.*, 2005). However, *C. difficile* strains have now been identified that possess variant toxins that exhibit marked variations in their C-terminal repeat region (Rupnik, 2001).

However, because the toxins are released extracellularly, it is not clear whether an effective antibody response to them will eliminate carriage of *C. difficile*, which would be an ideal outcome of vaccination. It would be interesting to inhibit the first step of the pathogenesis, the colonization process and consequently *C. difficile* dissemination.

In this study, even if the protection observed is less than the one observed previously with the toxoid as an antigen, the difference between the control group and the protease Cwp84 immunized group is statistically significant.

Even if Cwp84 does not play a crucial role in CDI pathogenesis in the animal model (Kirby *et al.*, 2009), it

seems that an immune response induced after active immunization is able to partially protect an animal from death. As the immune response is not yet well characterized, it is difficult to explain the protection mechanism observed here.

Challenge of hamsters with the toxigenic strain 79-695 resulted in the colonization of the majority of hamsters within the first 2 days. Interestingly, the number of hamsters colonized by *C. difficile* was lower in the immunized group than that in the control group and the colonization level was also lower. This difference in *C. difficile* intestinal colonization between the two groups confirms the Cwp84's involvement in the intestinal colonization process.

The association of a toxoid-based vaccine with a vaccine based on colonization factors like Cwp84 could prevent the host-to-host dissemination of the bacteria and could lead to herd immunity.

Here, we used the hamster model of CDI, which is highly sensitive to this infection. In fact, the Golden Syrian hamster is widely regarded as the most relevant animal model of *C. difficile* disease after oral infection of animals pretreated with antibiotics. It reproduces many symptoms observed in humans. Thus, we tested the animal protection against death after several immunization routes, and more precisely, the rectal route with the *C. difficile* protease Cwp84.

Mucosal surfaces are the primary sites for the transmission of most infectious diseases. The compartmentalization of mucosal immune responses imposes constraints on the selection of vaccine administration route. Significant advances have been made in the study of mucosal immunity and in the use of adjuvants and alternative routes of immunization to achieve a protective local immune response (Staats *et al.*, 1994). Traditional routes of mucosal immunization include oral and nasal routes. Other routes for inducing intestinal immunity could include the rectal route. Rectal vaccination has been tested previously against certain other enteric pathogens such as *Salmonella* (Forrest *et al.*, 1990; Kantele *et al.*, 1998).

Here, we tested how different routes of immunization can be used to generate immune responses inducing a protection against CDI, with Cwp84 as an antigen.

Immunizations by the intragastric route did not induce an increase of seric Cwp84-specific antibody levels and this result was correlated with the very low animal protection from CDI observed. Antigen degradation by gastric and intestinal secretions, dilution in the intestinal fluids, poor sampling via Peyer's patches, may all be factors that contribute to the limited efficiency of the oral route. It seems evident that this route requires that antigens must be protected from degradation by digestive enzymes. The subcutaneous route was the best to induce a high systemic immune response with antibody titres more than twofold higher than that for the intrarectal route. However, in this study, serum Cwp84 antibody titres did not correlate with protection. The best animal protection was observed with the rectal route of immunization.

Further studies are needed to specify the immune effectors induced by rectal immunization. Unfortunately, secondary antibodies directed to hamster IgA are not commercially available. This is why we were not able to determine more precisely the specific immune response at the intestinal level.

We failed to find evidence of significant neutralization activity against the Cwp84 protease activity in the serum of hamster vaccinated with a protective intrarectal formula vaccine. These results indicate that, in this model, protection is probably not only related to neutralizing antibodies and other factors may play an important role in the host immune response against CDI. Because survival correlated poorly with antibodies titres, it is possible that our immunization strategy generated a wider cell-based immunity that induces partial protection. Recent data on *Streptococcus pneumoniae* have demonstrated that multiple immune cell types are required for the induction of a protective immunity in a murine model that lacks mature B cells and fails to produce antibody (Mizrachi-Nebenzahl *et al.*, 2003; McCool & Weiser, 2004).

Recently, surface proteins such as the SLPs, because they cover the cell almost completely, have been tested in a series of immunizations combined with different systemic and mucosal adjuvants and challenge experiments in Golden Syrian hamsters (Ni Eidhin *et al.*, 2008). None of the immunization regimens conferred complete protection in the hamster model, and antibody stimulation was variable within regimens, and generally modest.

Others have demonstrated the benefits of using a protease as components of vaccines against *S. pneumoniae* for example. Mucosal immunization with caseinolytic protease (ClpP) antigen induced both systemic and mucosal antibodies, and in this way, reduced lung colonization and also protected mice against death. ClpP has been found to be highly immunogenic and conserved among different strains of *S. pneumoniae* and to have a surface localization (Cao *et al.*, 2008).

The next step of this work will be to study the immune response induces by the vaccination with Cwp84. This could be performed by the analysis of immunologic mechanisms, by the evaluation of the induction of both Th1- and Th2-type cytokines from both whole spleen and lymphocytes stimulated by the Cwp84.

To conclude, the protection from CDI observed for 33% of hamsters after rectal immunization with Cwp84 demonstrates that this protease is an interesting antigen for mucosal immunization. The hamster immunization studies also demonstrate that Cwp84 is an attractive component for inclusion in a vaccine to reduce *C. difficile* intestinal colonization in humans, which in turn may diminish the risk of CDI. A combination of other associated surface

proteins may improve the protection. Finally, given the potency of *C. difficile* toxins, it may be interesting to incorporate TcdA and TcdB with surface proteins for immunization to confer total protection against CDI.

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