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Research paper

Encapsulation of Cwp84 into pectin beads for oral vaccination against *Clostridium difficile*

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ABSTRACT

We have designed an oral vaccine against *Clostridium difficile* infection. The virulent factor Cwp84, that is a cystein protease highly immunogenic in patients with *C. difficile*-associated disease, was entrapped within pectin beads. Beads encapsulating Cwp84 were shown to be stable in the simulated intestinal medium and to release the cystein protease once in the simulated colonic medium. Three groups of hamsters were immunized, the first receiving pectin beads encapsulating Cwp84, the second unloaded beads and the third one free Cwp84. After three immunizations by the intragastric route, all groups received clindamycine. Post-challenge survival with a strain of *C. difficile* showed that 2 days after infection, all hamsters treated with unloaded beads and all hamsters treated with free Cwp84 have deceased after 7 days, whereas about 40% of hamsters administered with Cwp84-loaded beads survived 10 days after challenge, proving that oral vaccination provides partial protection. These first data obtained with an oral vaccine against *C. difficile* appear promising for preventing this infection.

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1. Introduction

Clostridium difficile is a Gram-positive anaerobic bacterium responsible for many nosocomial infections. The spectrum of diseases caused by *C. difficile* infection varies from antibiotic-associated diarrhoea to life-threatening clinical manifestations such as pseudomembranous colitis [1]. Antimicrobial therapy is the main risk factor responsible for the infection. Indeed, in susceptible individuals, disruption of the normal colonic microbiota by antibiotic treatment leads to colonization by *C. difficile* [2]. Pathogenicity of *C. difficile* is mediated by the release of two exotoxins, toxins A and B, which are potent cytotoxic enzymes inducing severe damages to the human colonic mucosa [3]. Other factors such as proteases were recently shown to be involved in the intestinal damages by degradation of the basement membrane which contributes to the intestinal epithelium necrosis. Among these factors, Cwp84 is *a C. difficile* surface protein that has recently been shown to possess cysteine protease activity and which may have such behaviour [4,5].

The current treatment regimens as well as the important rate of relapse make the eradication of the organism from infected patients difficult to achieve. In addition, the increasing prevalence of patients with *C. difficile* in elderly care wards or nursing homes suggests that a more rational strategy would be to design a vaccine against the infection. This is the reason why several studies have focused on the use of toxin preparations to stimulate active immunization in animal models infected by *C. difficile* inducing the secretion of serum antibodies against toxins [6–8]. In healthy volunteers, this vaccine induced high levels of specific neutralizing IgG. Initial studies have been conducted with promising results in a few patients with recurrent CDI [9]. However, if a toxin-based vaccine prevents toxin binding and neutralizes inflammatory effects, it is unable to affect colonization. This type of strategy therefore does not allow preventing patient-to-patient transmission. More recent studies have shown that surface proteins from *C. difficile*, such as flagellar proteins: FliC, FliD and the Cwp84 protease were able to induce an immune response that could play a role in the host defense mechanism [10,11]. The possibility of mucosal immunization against *C. difficile* by intra-nasal, rectal and oral route through the administration of FliD, and Cwp84 was tested [12]. Only the rectal route was able to induce an increase in IgA antibody specific to *C. difficile*. Although the rectal route is interesting, an orally administered vaccine would be vastly superior because of the increased patient compliance, patient

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comfort and low cost. Unfortunately, delivery of vaccine antigens by the oral route is plagued with challenges such as gastrointestinal destruction of labile molecules, poor immunogenicity of orally delivered soluble antigens and inefficient uptake of the antigens [13–16]. Much of the research directed at overcoming these barriers has focused on the development of microparticles containing antigens, as oral vaccine delivery vehicles [17]. Biocompatible and biodegradable polymers, either natural or synthetic, have become popular as materials for encapsulation. Moreover, since the lower GI-tract may provide an advantageous absorption site for such molecules and may induce an effective immune response in animals [18,19], it was interesting to deliver the above-mentioned virulence factor of *C. difficile* Cwp84 to the colon. Since the development of the *C. difficile* infection is localized at the colonic level, a colonic immune response could be appropriate to limit the development of the infection. Several colon-specific drug delivery systems are based on an enzyme-triggered degradation of the polymer they are made of [20–22]. Among these, pectin, a polysaccharide naturally present in plant cell walls have clearly shown interesting potentialities for protein encapsulation and delivery to the colon [23–26]. Indeed, pectin is non-toxic, not digested by gastric or intestinal enzymes and almost totally degraded by pectinolytic enzymes produced by the colonic microbiota. Low methoxylated pectin, amidated or not, can form a gel in the presence of divalent cations, such as calcium or zinc [25–31]. Beads can therefore be obtained by ionotropic gelation in very mild conditions favourable to the encapsulation of proteins [26,32,33], such as the *C. difficile* protease Cwp84 and afterwards to deliver it to the colon, promoting a vaccination against *C. difficile*. The aim of this study was to confirm the suitability of the chosen matrix to deliver the protein to the colon and to evaluate Cwp84, encapsulated into pectin beads as an oral vaccine candidate against *C. difficile* in a hamster model.

2. Materials and methods

2.1. Materials

Infusion broth was obtained from Difco Laboratories (France). Colombia agar plates supplemented with 4% horse blood were obtained from Biomérieux (France). Unipectine™ OG 175C (degree of esterification from 22% to 28% and degree of amidation from 19% to 23%) was a gift from Cargill (France). All the following compounds were purchased from Sigma–Aldrich (USA): Zinc acetate, Calcium chloride, Azocasein, EDTA, Sodium Citrate, NaCl, HEPES, Pepsine, Pancreatin, the mixture of pectinases from *Aspergillus aculeatus*, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, 5-(6)-carboxy-X-rhodamine *N*-succinimidyl ester, bovine serum albumin (BSA), rabbit anti-hamster IgG, IgM, IgA (H&L) conjugated to biotin (Biovalley), 3,3′-5,5′ tetramethylbenzidine (TMB), ammonia buffer solution and 1-(2-pyridylazo)-2-naphthol (PAN). TRIS was provided by from Prolabo (France), and hydroxy methylcellulose phthalate (HPMCP), type HP55S, was a gift from SEPPIC (France). Acetonitrile and ethanol were purchased from Carlo Erba Reagents (Italy), the Bradford reagent from BioRad (France), streptavidin-horseradish peroxidase conjugate from Amersham and Maxisorp™ 96-well microtiter plates from Nunc (France). All other chemicals used were of reagent grade.

2.2. Bacterial strain and culture

The *C. difficile* toxigenic strain 79–685 was isolated from a patient affected by pseudomembranous colitis and was a gift from the Department of Microbiology of the University of Strasbourg, France. This strain was grown under anaerobic conditions in Tryptone Glucose Yeast (TGY) infusion broth (Difco Laboratories, France) at 37 °C for 24 h unless indicated otherwise, and onto Columbia agar plates supplemented with 4% horse blood (Biomérieux, France). The *Escherichia coli*/pET-28a(+)_*cwp84* strain was grown on LB (lysogeny broth) agar or in broth (Difco Laboratories, France) supplemented with 50 μg/mL kanamycin to maintain the pET plasmid.

2.3. Purification of recombinant protein Cwp84

Recombinant Cwp84 was purified as previously described [10]. Briefly, Cwp84 was obtained from the *E. coli*/pET-28a(+)_*cwp84* clone by the induction of protein expression with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich, France) and subsequent purification by single-step affinity chromatography employing BD TALON cobalt affinity resin (BD Biosciences, France) as described in the protocol supplied by the manufacturer. The eluted fraction containing the recombinant protease was dialysed overnight against phosphate-buffered saline and then frozen at −20 °C for storage. The molecular weight of Cwp84 is 84 kDa [4].

2.4. Cwp84 fluorescent labelling

Five milligrams of 5-(6)-carboxy-X-rhodamine *N*-succinimidyl ester, dissolved in 300 μL of acetonitrile, were added to the purified protein solution (phosphate buffer 0.1 M pH 8.5) and kept under magnetic stirring at 4 °C for 2 h [34]. The excess of reagent was eliminated by dialysis against phosphate buffer, and subsequently, a last dialysis was carried out against TRIS buffer (25 mM pH 7.5) to exchange buffer. The labelled protease was frozen at −30 °C for storage.

2.5. Preparation of pectin beads

Unipectine™, with or without Cwp84 (720 μg in a final volume of 2.7 mL), was dissolved in TRIS buffer (25 mM, pH 7.5) at the concentration of 6% (w/V). After agitation for 2 h at room temperature, the solution was left undisturbed overnight and then dropped from a syringe into a cross-linking solution of Zn acetate (12% w/V), using a syringe pump (Havard Apparatus, 11 PLUS, France) at a rate of 1 mL/min. The nozzle diameter was 0.8 mm and the dropping distance 8 cm. Beads (about 50) formed instantaneously by contact with zinc ions were left in the cross-linking solution for 30 min at room temperature under magnetic stirring. Free zinc ions in excess were removed by filtration and three washings with distilled water for 1 min. Finally, beads were dried at 37 °C for 0, 1, 2 or 3 h.

2.6. Optimization of the formulation

Preliminary studies were carried out to optimize Cwp84 encapsulation within pectin beads. The drying time was investigated mainly because beads stability and protease enzymatic activity could be influenced by the water content and the thermal process. The drying temperature was fixed at 37 °C, and four drying times (0, 1, 2 and 3 h) were tested. Beads were then characterized by thermo-gravimetric analysis, stability in various media, and Cwp84 enzymatic activity was also assayed.

2.6.1. Determination of weight loss upon drying

Beads weight loss during drying was determined by thermogravimetric analysis. It was expressed as percentage according to the following equation:

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