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Research Paper

Thermostability of the coating, antigen and immunostimulator in an adjuvanted oral capsule vaccine formulation

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Sorbitol (PubChem CID: 5780)
Kolliphor HS 15 (Solutol® HS 15) (PubChem CID: 71311956)
Eudragit L30D 55 and Eudragit L100 55 (Alcogum) (PubChem CID: 107665)

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ABSTRACT

Oral vaccines present an attractive alternative to injectable vaccines for enteric diseases due to ease of delivery and the induction of intestinal immunity at the site of infection. However, susceptibility to gastrointestinal proteolysis, limited transepithelial uptake and a lack of clinically acceptable adjuvants present significant challenges. A further challenge to mass vaccination in developing countries is the very expensive requirement to maintain the cold chain. We recently described the effectiveness of a Single Multiple Pill® (SmPill®) adjuvanted capsule approach to enhance the effectiveness of a candidate enterotoxigenic *Escherichia coli* (ETEC) oral vaccine. Here it was demonstrated that this delivery system maintains the antigenicity of ETEC colonisation factor antigen I (CFA/I) and the immunostimulatory activity of the orally active α-Galactosylceramide (α-GalCer) adjuvant after storage of SmPill® minispheres under room temperature and extreme storage conditions for several months. In addition, the internal structure of the cores of SmPill® minispheres and antigen release features at intestinal pH were found to be preserved under all these conditions. However, changes in the surface morphology of SmPill® minispheres leading to the antigen release at gastric pH were observed after a few weeks of storage under extreme conditions. Those modifications were prevented by the introduction of an Opadry® White film coating layer between the core of SmPill® minispheres and the enteric coating. Under these conditions, protection against antigen release at gastric pH was maintained even under high temperature and humidity conditions. These results support the potential of the SmPill® minisphere approach to maintain the stability of an adjuvanted whole cell killed oral vaccine formulation.

1. Introduction

Enteric infections causing diarrhoeal episodes are a major worldwide health problem leading to high levels of mortality and morbidity, especially in developing countries (Cheng et al., 2005). Even though oral rehydration therapy has helped to reduce the number of deaths, enteric infections still kill approximately 600,000 children under the age of 5 years and result in millions of hospitalizations each year in developing countries (Czerkinsky and Holmgren, 2009; Walker and Clifford, 2015). Moreover these diseases may impact on intestinal absorption, nutrition and child development (Petri et al., 2008). Severe acute diarrhoea can be caused by a number of pathogens including rotaviruses, *Vibrio cholerae*, *Shigella* spp., *Salmonella* spp. and pathogenic strains of *Escherichia coli* (*E. coli*) (Girard et al., 2006). One of the most common diarrhoeal diseases detected among children in developing countries and in people travelling to these regions is due to enterotoxigenic *Escherichia coli* (ETEC) (Qadri et al., 2005). ETEC strains are ingested with contaminated water or food and induce diarrhoea, abdominal cramps, vomiting as well as fever (Qadri et al., 2005). ETEC surface proteins named colonisation factors antigens (CFAs) (pili/

Abbreviations: α-GalCer, α-Galactosylceramide; BSA, bovine serum albumin; CFA, colonisation factor antigen; Con A, concanavalin A; *E. coli*, *Escherichia coli*; DMSO, dimethyl sulfoxide; ETEC, enterotoxigenic *Escherichia coli*; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IFN, interferon; iNKT, invariant natural killer T cells; MAb, monoclonal antibody; PBS, phosphate buffered saline; RH, relative humidity; SEM, scanning electron microscopy; SmPill®, single multiple pill

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fimbrial or nonfimbrial) such as CFA/I and powerful secreted enterotoxins are key virulence factors (Tobias et al., 2008; Tobias and Svennerholm, 2012; Lundgren et al., 2014). The CFAs bind to glycoprotein or glycolipid receptors on host epithelial cells of the small intestinal mucosa, which leads to bacterial colonisation and multiplication (Jansson et al., 2006). Colonising bacteria induce fluid and electrolyte secretion into the lumen by the production of heat-labile or heat-stable enterotoxin, or both (Qadri et al., 2005). Local secretory antibody responses against CFAs were shown to play a key role in protective immunity both in animal studies and humans (Svennerholm et al., 1988; Ahren and Svennerholm, 1982; Qadri et al., 2007) and consequently CFAs have been the focus for incorporation into ETEC vaccine candidates (Tobias et al., 2008; Tobias and Svennerholm, 2012; Lundgren et al., 2014; Walker et al., 2007).

Oral vaccine delivery is regarded as the optimal means to fight enteric infections as it induces intestinal immunity through the gut-associated mucosal tissues and is also attractive in avoiding the use of needles and enhancing patient compliance. However, oral vaccines have to overcome significant challenges including the low pH of the stomach, proteolytic enzymes and bile salts, limited uptake across the epithelium and a generally poor immunogenicity of orally delivered antigens (Marasini et al., 2014; Rhee et al., 2012; Davitt and Lavelle, 2015).

In order to overcome these challenges, a number of oral vaccine delivery technologies are currently under pre-clinical and clinical development (Gupta et al., 2007; Wang et al., 2015; Aguirre et al., 2016). Self-emulsifying lipid based systems, composed of oils, surfactants, co-surfactants as well as co-solvents, are widely used (Aguirre et al., 2015). Oil-in-water emulsions formed in aqueous gastrointestinal fluids enhance bioavailability by the formation of small oil droplets which may be lysed by pancreatic lipases, leading to drug release (Kohli et al., 2010). Oil-in-water emulsions may be delivered in solid oral dosage forms such as soft gelatine capsules, pellets, microspheres and minispheres (Aguirre et al., 2015). A novel oral delivery system, Single Multiple Pill® (SmPill®), containing emulsions formulated as minispheres, is able to enhance local, topical drug (e.g. cyclosporine (Keohane et al., 2016), celecoxib (McDonald et al., 2015), calcitonin (Aguirre et al., 2015) or dimethyloxalylglycine (Tambuwala et al., 2015)) efficacy through site-specific release in intestinal regions. This can be achieved through the application of a specific exterior coating to the core of the minispheres. It has been hypothesized that this novel oral delivery system in combination with an efficacious oral adjuvant could be used to deliver oral vaccines and increase antigen immunogenicity by protection from gastric acidity and enhancing site-specific delivery in the gastrointestinal tract, particularly the jejenum and ileum as these intestinal regions contain gut-associated lymphoid tissues such as Peyer's patches and mucosal immune cells to elicit gut immune responses (Mowat and Agace, 2014). It has been recently demonstrated that the integrated SmPill® system incorporating a recombinant formalin-killed whole cell *E. coli* overexpressing CFA/I and the orally active invariant natural killer T (iNKT) cell activator α-Galactosylceramide (α-GalCer) was able to potentiate immune response to this ETEC candidate vaccine (Davitt et al., 2016).

Similarly to pharmaceuticals and biologics, stability tests are essential and mandatory steps in the development of vaccines. However, additional considerations apply to vaccines comprising complex mixtures of active components and in this case, the integration of antigen and adjuvant in the SmPill® delivery system. Environmental factors such as temperature, humidity or light might affect SmPill® minispheres containing the ETEC vaccine formulation and impact on the efficacy of the integrated system. The goal of this study was to evaluate the effects of temperature and humidity on CFA/I antigenicity, α-GalCer immunostimulatory properties, SmPill® minisphere structure and bacterial release. The effects of long-term storage at room temperature: 25 °C/60% Relative Humidity (RH) (normal condition), were compared to the effects of more extreme storage conditions such as 30 °C/65% RH (intermediate condition) and 40 °C/75% RH (accelerated condition). It has been observed that the core of SmPill® minispheres fully retained its initial structure and its antigen release properties at intestinal pH under normal and extreme storage conditions up to 12 months. Importantly, the CFA/I antigenicity and adjuvant α-GalCer activity were very well maintained inside the core even when SmPill® minispheres were stored under extreme conditions for several months. It was also found that the protection of antigen facilitated by a specific Eudragit® coating avoiding its release at gastric pH was effective at 25 °C/60% RH. However, the resistance of Eudragit L30D 55 enteric coating to acidic pH was shown to decrease due to surface modifications at 30 °C/65% RH and especially at 40 °C/75% RH. However, the use of a double-layer coating made of an inner Opadry® White film and an outer Eudragit® L100 55 film demonstrated an improved resistance of SmPill® minispheres at acidic pH when stored under elevated temperature and humidity.

This study supports the potential of SmPill® minispheres to maintain the stability of the antigen (CFA/I *E. coli*) and adjuvant (α-GalCer) vaccine components over long-term storage under unrefrigerated conditions.

2. Materials and methods

2.1. Composition and manufacture of SmPill® minispheres

Three SmPill® minispheres contained 3×10^8 whole-cell killed *E. coli* overexpressing CFA/I with or without 10 μg α-Galcer (Davitt et al., 2016). Placebo SmPill® minispheres containing neither bacteria nor α-Galcer were also prepared.

SmPill® minispheres were manufactured as previously described (Davitt et al., 2016). Briefly, when included in a formulation, α-Galcer (Avanti Polar Lipids Inc., USA) was dissolved in Kolliphor HS 15 (BASF GmbH, Germany) under magnetic stirring. The loading of α-Galcer was approximatively 0.1%. Whole-cell formalin killed recombinant *E. coli* C600 strain overexpressing CFA/I (JT-49; generously provided by Prof. Ann-Marie Svennerholm) in a PBS suspension was then added and the resulting dispersed phase mixed until homogeneous. Sorbitol (Roquette Freres, France) was dissolved in water at room temperature, then type A porcine gelatine (Nitta Gelatin NA Inc., USA) was added, the temperature was increased to 60–70 °C and the mixture stirred until complete dissolution of the components. The aqueous phase and dispersed phase were mixed to achieve homogeneity. The homogeneous dispersion was ejected through a single orifice to form droplets that fell into a cooling oil medium (Miglyol 810N, Cremer Oleo GmbH;Co. KG, Germany) at 8–10 °C. After approximately 30 min, the minispheres were recovered from the cooling oil medium, centrifuged to eliminate excess oil and dried at room temperature. When required, SmPill® minispheres were coated using a Vector MFL.01 Fluid Bed System in the bottom spray configuration with an enteric polymer, Eudragit L30D 55 or Eudragit L100 55 (Evonik Industries AG, Germany) that are soluble in intestinal fluid from pH 5.5. In some cases, Opadry® White film 20A28380 (Colorcon) was also applied between the core and the enteric polymer to provide a double-layer coating. Minisphere size was measured by light microscopy (BX51 Olympus) and was between 1 and 2 mm.

2.2. SmPill® storage conditions

Coated, uncoated and placebo SmPill® minispheres were stored in open glass vials at 25 °C/60% RH, 30 °C/65% RH and 40 °C/75% RH. The vials were placed in smaller sealed chambers where the RH was controlled using a saturated salt solution and the chambers were placed in incubators.

The storage conditions were chosen according to the ICH guidelines 'Stability Testing of new Drug Substances and Products' (ICH Topic Q 1A (R2), European Medicines Agency, 2003) (2003).