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Research paper

Preparation and evaluation of a freeze-dried oral killed cholera vaccine formulation

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ABSTRACT

Different oral liquid cholera vaccines have proved to be safe and effective, but their formulations present problems for use in low-income countries, since large package volumes have to be transported and cold chain maintenance is required. A solid state formulation would here be more advantageous, and consequently, the possibility to develop a dry cholera vaccine formulation by freeze-drying was investigated. The ability of sucrose, trehalose and mannitol to provide process stabilization during freeze-drying was tested on a formalin-killed whole-cell *Vibrio cholerae* model vaccine. A matrix of sucrose or trehalose prevented bacterial aggregation, preserved cell morphology and maintained practically completely the protective lipopolysaccharide (LPS) antigen on the cell surface and its reactivity with specific antibody *in vitro*. After reconstitution, this formulation also retained the capacity to elicit a strong serum and gut mucosal anti-LPS antibody response in orally immunized mice, as compared to the corresponding liquid vaccine formulation. The full preservation of the *in vivo* immunogenicity was also maintained when the internationally widely licensed oral cholera vaccine Dukoral™, which comprises a cocktail of inactivated *V. cholerae* together with cholera toxin B-subunit (CTB), was freeze-dried using sucrose for stabilization. Thus, we present a process generating a dry oral inactivated whole-cell cholera vaccine formulation with attractive features for public health use in cholera-afflicted settings.

1. Introduction

Liquid formulations for parental administration are still the first choice for most vaccines on the market, but high manufacturing costs, large package volumes, stability problems and the requirement of trained personnel for administration are some disadvantages connected with such formulations. In recent years, more effort has, therefore, been put into finding alternative ways of vaccine administration, especially for vaccines against infections at mucosal surfaces, which often may need mucosal administration in order to be effective. The oral route of vaccination is an advantageous alternative to parenteral administration, especially for vaccines against gastrointestinal infections. Better patient compliance due to the avoidance of painful injections and the possibility of self-administration make non-parenteral vaccines to an interesting research topic. Besides the route of administration, the development of an appropriate formulation that meets the specific requirements for a successful use also plays an important role in the development of new effective vaccines. Especially in low-income countries, there is a need for vaccines that can easily and cost efficiently be administered, distributed and stored. The development of dry vaccine formulations would be an important step towards improved use of vaccination in public health control of infections causing high morbidity and mortality in developing countries.

Cholera is an example of such a disease, and it continues to be one of the main causes of severe dehydrating diarrhoea in low-income countries affecting more than 3 million persons annually and causing more than 100,000 deaths. Cholera is a significant negative factor for the economic development, and the prevalence of cholera shows no tendency to decrease [1,2]. Cholera is an intestinal infection predominantly caused by *Vibrio cholerae* (*V. cholerae*) of the O1 serogroup [2,3]. The critical pathogenic molecule produced by *V. cholerae* is cholera toxin (CT) which consists of a single toxic-active A-subunit (CTA) and a five-membered cell-binding B-subunit ring (CTB). Following the binding of the B-subunits to their receptor, the GM1 ganglioside, on the surface of intestinal epithelium cells, the toxin is endocytosed and causes the severe and often even life-threatening watery diarrhoea characterizing cholera disease [4–6].

Prophylaxis against cholera by orally administered vaccines based on killed whole-cell *V. cholerae* O1 bacteria has shown to

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be successful, especially when combined with CTB as in the oral vaccine Dukoral™ [7–9]. The protective efficacy of Dukoral™ is based on the local intestinal production of both antibacterial and antitoxic IgA antibodies, and it is currently the only widely internationally licensed oral cholera vaccine, with a demonstrated efficacy of up to 90 % [9]. The needle-free administration of Dukoral™ has clear advantages, but the formulation has also certain disadvantages for mass vaccinations in cholera-afflicted settings. Since the vaccine is formulated in a suspension, the package volume is very large and cold chain storage and transport is required. Both factors increase costs for long-distance transport and hamper the use in low-income countries. As mentioned previously, a dry and less voluminous formulation would, therefore, be of advantage.

Freeze-drying or lyophilization is an often attractive and advantageous drying method for labile materials like bacteria since it is a low-temperature process where the liquid is removed by direct sublimation, i.e., from the frozen state to the gas state, leading to a dry and porous, powder-like matrix that can easily be reconstituted or further processed into other formulations [10]. Most freeze-dried products contain an active component and excipients such as polymers or sugars [10], which serve a specific function. When small amounts of an active compound are freeze-dried, excipients can function as bulking agents, e.g., mannitol. Here, the excipient addition is intended to provide a satisfactory product appearance and to give the obtained lyophilized cake a sufficient mechanical strength. However, most often the function of an excipient is to increase the stability of the product. Drying of bacteria leads to the removal of hydrogen-bonded water on their surface. Here, the addition of excipients as protecting agents, so called lyoprotectants, that replace the removed water on the surface of protein structures can be used to decrease the risk for membrane damage and loss of function, i.e., to provide stabilization of the product during the drying process [10–12]. The up-concentration of the material to be dried during the water removal also increases the possibility for interactions between the bacteria and thus the risk for bacterial aggregation. These interactions might be so strong that the bacteria still remain aggregated when they are reconstituted, and in the case of *V. cholerae*, this might lead to damage or a decreased recognition of the cell surface lipopolysaccharide (LPS) antigen that is necessary for the induction of a protective immune response. Furthermore, aggregates might become too large to allow them to be taken up via the intestinal mucosa. Challenges in the development of a freeze-dried whole-cell *V. cholerae* vaccine are, therefore, both the avoidance of bacterial aggregation and the retention of the biological structures important for the mucosal immunogenicity of the vaccine formulation.

Non-reducing disaccharides are well-known lyoprotectants capable to replace the removed water around polar residues in phospholipid membranes of, for example, bacteria, so that their integrity can be maintained [11]. Reducing sugars, such as lactose, normally react with proteins and are, therefore, poor stabilizers [10]. In this study, the ability to provide stabilization and to avoid aggregation of *V. cholerae* bacteria was investigated with the disaccharides trehalose and sucrose. Both are able to form a matrix around protein structures and are mostly in the amorphous state. Trehalose is often seen as the 'golden standard', but for every biological material, one has to find the best lyoprotectant, since the same excipient can stabilize biological structures with varying degrees of effectiveness. In addition, another excipient in freeze-drying, the sugar alcohol mannitol, was also investigated. Mannitol is commonly used as bulking agent in drug formulations, and depending on the properties of the formulation and the freeze-drying process, it appears partly or completely crystalline [10], so that the stabilizing properties are often not sufficient. Since mannitol might give the lyophilized material advantageous properties for a potential further processing, e.g., compaction into tablets, it was included in this study.

In the development of a freeze-dried killed whole-cell bacteria formulation, there are two stages where stabilization is required. The first is process stabilization, so that no aggregation occurs, the important structures and properties of the bacteria are preserved and the immunogenicity is maintained. The second goal of stabilization is to produce a dry product with sufficient shelf-life time, preferably without the requirement of cold chain conditions. This study focuses on the first challenge, process stabilization, and the aim was to investigate potential stabilizers for the freeze-drying of both formalin-killed and heat-inactivated *V. cholerae* bacteria as well as the CTB protein. For this purpose, the study was divided into two parts. Initial experiments were carried out using formalin-killed *V. cholerae* O1 bacteria (strain JS 1569, serotype Inaba) as a model vaccine. JS 1569 is a non-toxic (*ctxA* deleted) derivative of the classical O1 *V. cholerae* strain 569B. From the obtained *in vitro* and *in vivo* data on this model vaccine, one excipient was then chosen and tested as stabilizer for freeze-drying of the commercial vaccine Dukoral™, which contains both formalin-killed and heat-inactivated *V. cholera*e bacteria together with CTB protein. The lyophilized Dukoral™ formulations were then tested in oral immunization studies in mice, and the systemic and mucosal immune responses were analysed.

2. Materials and methods

2.1. Preparation of formalin inactivated bacterial strains

V. cholerae serogroup O1 serotype Inaba (strain JS1569) were stored at −70 °C in Luria–Bertani (LB) medium (1% Bacto™ Bactotryptone and 0.5% Bacto™ Yeast extract, BD, USA; 0.17 M sodium chloride, Merck, Germany) containing 17% (v/v) glycerol (Apoteket AB, Sweden). Bacteria were cultured on LB agar streak plates containing rifampicin (50 μg/ml, Sigma Aldrich, Germany), and the plates were incubated overnight at 37 °C. Single colonies were then used to inoculate 25 ml LB medium supplemented with rifampicin (50 μg/ml, Sigma Aldrich, USA). After incubation overnight at 37 °C with shaking (Orbital Shaker, Thermo Forma, USA), 5 ml of this starter culture were taken to inoculate 500 ml fresh LB medium supplemented with rifampicin. The resulting culture was grown at 37 °C with shaking to an OD_{600} of 1.2. Bacteria were harvested by centrifugation, washed once in 500 ml phosphate buffered saline (PBS, 0.01 M, pH 7.2–7.4, Sigma Aldrich, Germany) and resuspended in PBS to a concentration that gives an OD_{600} of 1.18 when diluted 1:10. To inactivate the *V. cholerae* O1 Inaba (strain JS 1569), 4% formaldehyde (Sigma Aldrich, Germany) was added to a final concentration of 0.2 M. After incubation at room temperature overnight with shaking at 100 rpm, the bacteria were washed three times in PBS and finally resuspended in PBS to a concentration of $\sim 10^{10}$ bacteria/ml, giving an OD_{600} of 1.0 when diluted 1:10. In order to confirm complete killing before storage at 4 °C, aliquots of the resulting suspension were spread onto horse blood agar plates and incubated at 37 °C for 48 h.

2.2. Isolation of lipopolysaccharide antigen (LPS)

LPS was extracted from *V. cholerae* O1 Inaba strain 569B (Inaba) using the phenol–water method of Westphal and Jann [13] and further purified as described elsewhere [14]. Protein contamination was <1%, as judged by Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA) and by optical density at 280 nm relative to total weight.

2.3. Freeze-drying of bacterial preparations

Formulations of formalin-killed (fk) JS 1569 *V. cholerae* bacteria and Dukoral™ bacterial suspension (Crucell, Netherlands; previously SBL Vaccines, Sweden) were freeze-dried in the presence and absence of different excipients. The investigated excipients were Mannitol 35® (Roquette, France) and D-(+)-Trehalose dihydrate (Sigma Aldrich, Germany) for JS 1569 and sucrose (Sigma Aldrich, Germany) for both JS 1569 and Dukoral™. Solutions of three different concentrations (0.25, 2.5 and 25 mg/ml) were prepared in sterile water. 500 μl aliquots of bacterial suspension containing 5×10^9 cells/ml were diluted with equal volumes of either water or excipient solution in 10 ml freeze-drying glass vials (BergmanLabora, Sweden). All samples were frozen overnight at −70 °C in a freezer and then subjected to freeze-drying (Christ Alpha 2-4, Germany) during 48 h. The shelf temperature was set to −5 °C and the pressure control to 0.78 mbar. All vials were manually closed, sealed with aluminium locks and stored at 4 °C until usage.

2.4. Characterization of lyophilized V. cholerae JS 1569

2.4.1. Microscopy

In order to visualize if and to what extent the freeze-drying process leads to bacterial aggregation depending on the type and concentration of excipient, both confocal microscopy (CM) and scanning electron microscopy (SEM) were used. For the SEM investigations, all freeze-dried samples were coated with a thin layer of gold and examined using a Leo Ultra 55 FEG scanning electron microscope (Zeiss, Germany) with an accelerating voltage of 3 kV. For CM, freeze-dried samples were reconstituted, diluted 1:4 in PBS and mixed with a staining solution of safranin (Sigma Aldrich, Germany) in purified water (0.5 mg/ml). Images were acquired using a Carl Zeiss LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Germany). The excitation wavelength was 488 nm, and the emission was detected in the wavelength range 500–700 nm. A Plan-Apochromat 63x/1.40 Oil DIC objective was used and the pinhole setting was 1 Airy unit, giving an optical section of 0.7 μm. Z-stucks were taken by scanning the samples with a spacing of 0.34 μm.

2.4.2. Residual moisture content

The residual moisture content of all lyophilized *V. cholerae* JS 1569 formulations was determined by Karl-Fischer titration using a KF 684 Coulometer (Metrohm, Switzerland). The precision of the instrument was determined to −0.6% using Hydranal water standard 10.0 (Sigma Aldrich, Germany). All formulations were tested in duplicates.

2.4.3. Differential scanning calorimetry

DSC analysis of the lyophilized *V. cholerae* JS 1569 formulations containing the highest tested concentration of excipient was performed with a Pyris 1 DSC (Perkin Elmer, Sweden), equipped with an Intracooler. Samples were weighed into 50 μl sealed DSC aluminium pans (Perkin Elmer, Sweden), heated from 5 °C to 220 °C, cooled to 5 °C and reheated to 220 °C at 10 °C/min. The obtained DSC patterns were used to characterize the grade of crystallinity of the samples, and the glass transition temperatures (Tg) were determined using the associated software.

2.4.4. X-ray diffraction

In order to confirm the presence of crystalline material in the freeze-dried formulation containing mannitol powder, X-ray diffraction (XRD) analysis was performed with a Siemens diffractometer D5000 using a Cu Kα radiation with a wavelength of 1.54 Å. Scanning was performed over the angle range $2\theta = 20–60°$.

2.5. Analysis of LPS and CTB preservation after freeze-drying

The generation of an immune response by a vaccine is dependent on the processing of antigenic material by the immune system. In the present study, both the preservation of surface LPS and CTB epitope structures during freeze-drying was examined.

2.5.1. LPS inhibition ELISA

High binding ELISA trays (Greiner, Germany) were coated overnight at 4 °C with O1 LPS (5 μg/ml in PBS). Non-coated plates were blocked with 1% (w/v) bovine serum albumin (BSA, Sigma Aldrich, Germany) in PBS for 30 min at 37 °C. Samples, a blank (1% BSA/PBS) and a standard of known activity (LPS 569B, 120 μg/ml) included in each tray, were titrated in threefold falling dilutions, and each dilution was then mixed with an equal amount of a monoclonal anti-O1 LPS antibody (MAb 8:4) diluted 1:160 with 1% BSA in PBS. The plates were incubated for 1 h at room temperature, and the samples were then transferred to LPS coated plates that have been washed three times with PBS, blocked with 1% BSA in PBS for 30 min at 37 °C and washed twice with 0.05% (v/v) Tween 20 (Merck, Germany) in PBS (Tween/PBS). After 90 min incubation at room temperature, the plates were washed twice with Tween/PBS and once with PBS. Then, horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG/M (Jackson ImmunoResearch Europe Ltd., UK) was added to all wells, and after incubation overnight at −4 °C, the plates were developed with a solution containing 1 mg/ml ortho-phenylenediamine (OPD, Sigma Aldrich, USA) and 0.012% hydrogenperoxide (Merck, Germany) in 0.1 M citrate buffer of pH 4.5 (prepared from trinatriumcitrate-trihydrate, Merck, Germany). The reaction was stopped after 15 min with 0.5 M sulphuric acid (prepared from sulphuric acid, 95–97%, reagent grade, Scharlau Chemie, Spain). The absorptions of all samples were measured in a plate reader (BioTek Power Wave XS, BioTek Instrumentals, USA) at 490 nm and analysed using the associated software Gen5 version 1.09 (BioTek Instruments, USA). The 50% inhibition titres were defined as the dilution of the bacterial samples that gave an absorbance of 50% of the maximum value obtained in the absence of bacteria. These titres were used to calculate the mean LPS recovery in percent ± SE (standard error of the mean) for each formulation in relation to the untreated suspension. One-way ANOVA with Bonferroni's post-test was used for statistical comparisons with the untreated control.

2.5.2. GM1 ELISA

The detection of CTB was performed using a GM1 ELISA according to a method previously described for the detection of *Escherichia* coli heat-labile toxin (LT) [15]. Briefly, low-binding polystyrene plates (Nunc, Denmark) were coated with GM1 ganglioside (0.3 nm/ml in PBS, kindly provided by late Professor Lars Svennerholm) at 4 °C overnight. After three washes with PBS, all plates were blocked with 0.1% BSA in PBS. Samples and a standard of known activity included in each tray were titrated in a threefold falling dilution, and the plates were incubated for 60 min at room temperature. After three washes with Tween/PBS, monoclonal anti-CTB antibody was added to all wells followed by incubation for 1–2 h at room temperature. The plates were washed again three times, and then HRP-conjugated goat-anti-mouse IgG/M was added. After incubation overnight at 4 °C, plates were finally developed with OPD and analysed by UV as described previously. The CTB concentration in the samples was determined by comparison with the CTB standard curve.

2.6. Immunizations

BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and used at 8–10 weeks of age. All vaccine

formulations tested in the present study were administered via the peroral route (PO) as described before [14,16], although using a lower non-saturating dose. In experiments with JS 1569 formulations, the doses were given in three rounds at days 0 and 2, 13 and 15, 41 and 43, i.e., six inoculations per mouse in total. In the experiments with Dukoral™ formulations, doses were given at days 0 and 1, 14 and 15, 27 and 28. Freshly lyophilized samples were prepared prior to the vaccinations and stored at 4 °C until use. Immediately prior to the immunizations, the lyophilized formulations were reconstituted in PBS to a concentration of 5×10^9 cells/ml, i.e., new samples were used for each immunization day. Non-treated suspension was used in the reference group. Subsequently, all suspensions were mixed with an equal volume of a 6% (w/v) sodium bicarbonate (Sigma Aldrich, Germany) solution in PBS in order to neutralize gastric acidity. Mice to be immunized were anaesthetized with isoflurane (Isoba vet. Schering-Plough Animal Health, Sweden). All peroral immunizations were performed with a dose of 200 μl bacterial suspension containing 5×10^8 cells administered by intragastrical inoculation using a disposable feeding needle with silicon tip (Fuchigama Ltd., Kyoto, Japan). All animals in this study were housed under specific-pathogen free conditions, and the experiments were approved by the Ethical Committee for Laboratory Animals in Gothenburg.

2.7. Sample collection

From mice that had received JS 1569 formulations, samples were taken 4 weeks after final immunization and from those that received Dukoral™ formulations 1 week after final immunization. For the estimation of intestinal IgA responses, supernatants were prepared from homogenates of fresh faecal pellets (FP) according to a method described elsewhere [16]. Seven FP were collected from each mouse, emulsified in 600 μl of ice-cold PBS containing 0.1 mg/ml of soybean trypsin inhibitor (STI), 1% (w/v) bovine serum albumin (BSA, Sigma Aldrich, USA), 25 mM ethylenediaminetetraacetic acid (EDTA), 0.035 mg/ml Pefabloc (Coatech AB, Sweden) i PBS and 50% (v/v) glycerol and kept at 4 °C for 4 h. Debris was removed by centrifugation (15.5k × *g*, 10 min, 4 °C), and the supernatants were collected and stored at −20 °C.

For blood sampling, mice were euthanized, blood was taken from the subclavian vein and sera were separated by centrifugation prior to storage at −20 °C. Immediately after the bleeding, the mice were sacrificed. In addition to FP extracts, intestinal tissue extracts were prepared from animals in the Dukoral™ immunization study using a modified version of the perfusion–extraction technique (PERFEXT) [17]. Animals were perfused with at least 20 ml heparin (0.1% solution of 5000 IU/ml) (Lövens Kemiske Fabrik, Danmark) in PBS per mouse before removal of the small intestines. All tissue samples were stored overnight at −20 °C in 450 μl PBS containing 2 mM PMSF, 0.1 mg/ml STI and 0.05 mM EDTA and then thawed and permeabilized by the addition of saponin (Riedel-de Haën, Germany) to a concentration of 2% (w/v). After incubation at 4 °C overnight, samples were centrifuged (15.5k × *g*, 10 min, RT) and supernatants were collected and frozen at −20 °C.

2.8. Analysis of antibody responses

2.8.1. Enzyme-linked immunosorbent assay (ELISA)

Serum and mucosal anti-LPS and anti-CTB antibody responses were both determined by ELISA according to previously described methods [14,17]. For the anti-LPS ELISA, high binding ELISA trays (Greiner, Germany) were coated overnight at 4 °C with O1 LPS (5 μg/ml in PBS), and for the anti-CTB ELISA, low-binding polystyrene plates (Nunc, Denmark) were coated with GM1 ganglioside (0.3 nm/ml in PBS) overnight at 4 °C and then incubated with recombinant CTB (0.5 μg/ml). After three washes with PBS, all plates were blocked with 1% BSA in PBS. Samples and a standard of known activity included in each tray were titrated in a threefold falling dilution. Plates for IgG + M analysis were incubated for 90 min at room temperature and those for IgA determination for 4 h at 37 °C. All plates were washed twice with 0.05% (v/v) Tween 20 in PBS and once with PBS. HRP-conjugated goat-anti-mouse IgG/M was added to the plates with serum samples and goat-anti-mouse IgA-HRP (Southern Biotech, USA) to the plates with FP or small intestine extracts. The plates were incubated at 4 °C overnight and finally developed with OPD and analysed by UV as described previously. All IgA antibody responses were standardized as ELISA units (EU) per mg of total IgA according to a previously described method [18], but with the following modifications: High binding ELISA trays were coated with 1 μg/ml goat-anti mouse IgA (Southern Biotech, USA) in PBS; samples and purified mouse IgA (Southern Biotech) as standard were titrated in threefold falling dilutions; goat-anti-mouse IgA-HRP conjugate was used as above.

2.8.2. Bactericidal assay

Serum samples from the immunizations with Dukoral™ formulations assayed were tested for the presence of (complement-dependent) bactericidal antibodies using *V. cholerae* O1 serotype Inaba (strain T19479) and Ogawa (X25049) with a modified microtitre plate assay according to a previously described method [14]. Briefly, *V. cholerae* were cultured overnight at 37 °C on horse blood agar plates. Three colonies of bacteria were then inoculated in 20 ml LB. The cultures were grown at 37 °C with shaking at 180 rpm up to an OD_{600} of 0.6–1.0. If necessary, the concentration was adjusted to an OD_{600} of 0.6 with sterile physiological saline solution (PSS) and further diluted 1:50 in PSS resulting in a suspension containing $\sim 4 \times 10^6$ bacteria/ml. This suspension was then added to a microtitre tray (50 μl/well) containing equal volumes of guinea pig serum (BioJet service, Sweden) in PSS as the source of complement, followed by heat-inactivated serum samples (56 °C, 30 min) serially titrated in twofold dilution series with PSS. In each tray, standard rabbit serum of known activity was included. Wells containing only PSS without serum and complement served as positive control for bacterial growth and additional wells where no bacteria were added served as negative control. After incubation for 60 min at 37 °C and shaking at 150 rpm (Innova 2000 platform shaker, New Brunswick Scientific, USA), pre-warmed 4× LB medium was added to all wells and incubation was continued for approximately 2–4 h at 37 °C until the bacteria growth in the control wells had increased to OD_{600} ca. 0.35. The vibriocidal titres of all samples were determined as the highest dilution of serum that inhibited bacterial growth.

2.9. Statistical analyses

For all statistical analyses, the Prism software system GraphPad 4.03 (GraphPad Software Inc., San Diego) was used. Before calculations, all values were $\log_{10}$ transformed and geometric means were calculated. Multigroup comparisons were performed using one-way ANOVA with Bonferroni's post-test. The relationship between intestinal antibody estimates was evaluated using a Pearson correlation test. Two-sided *P*-values <0.05 were considered as significant.

3. Results

3.1. Effect of different excipients on freeze-drying of a model whole-cell *V. cholerae* vaccine

Studies with fk *V. cholerae* O1 strain JS 1569 as a model vaccine were performed to assess the possible stabilization provided by

mannitol, sucrose and trehalose with regard to prevention of bacterial aggregation and preservation of the surface LPS antigen as well as the *in vivo* immunogenicity of the vaccine. Since not only the type of excipient but also the concentration is of importance for the stabilizing effect during freeze-drying, all excipients were tested in three concentrations (0.25, 2.5 and 25 mg/ml).

3.1.1. In vitro studies

The lyophilized cakes all looked porous were easy to handle and could quickly be reconstituted in PBS. From the visual observations, it was also noticed that the lyophilized cakes containing high concentrations of sucrose and trehalose had collapsed during the freeze-drying since the obtained volumes were much decreased compared to the liquid starting material and the cakes looked also shrunken together, whereas the sample containing mannitol almost maintained the same volume and the cake looked more powdery. The freeze-dried samples were analysed by scanning electron microscopy (SEM) in order to visualize the bacteria's appearance in the obtained cakes. Small amounts from different parts in the cakes were randomly sampled and analysed. The obtained SEM images are presented in Fig. 1 and show a clear impact of both the type and concentration of excipient. Less magnified pictures giving an overview of the cakes were first taken and it was found that the sucrose and trehalose samples with the highest sugar concentration contained a lot of small denser cakes whereas the mannitol sample looked much more porous (pictures not shown).

In all samples that contained 0.25 or 2.5 mg/ml excipient during the freeze-drying process, large clusters of intact looking bacteria were found (as indicated by the arrow in Fig. 1a). At the highest excipient concentration (25 mg/ml), the presence of sucrose or trehalose (Fig. 1f and i, respectively) resulted in several smooth lyophilized small cakes with single bacteria visible in the matrix, thus suggesting that the bacteria were well embedded in a sugar matrix. In contrast, the presence of mannitol in the highest concentration did not lead to similar structures. Instead, mannitol crystals were found in the high concentrated sample (as indicated by the arrow in Fig. 1c), and it looked as if the bacteria were deformed and distributed between these crystals. Similar deformed bacterial structures were found in the absence of excipient (Fig. 1j).

To confirm the presence of crystalline mannitol, both DSC and XRD analysis were performed on the freeze-dried formulations with the highest excipient concentration. The obtained DSC patterns of the sucrose and trehalose formulations did not show any indications for the presence of crystalline material since no melting peaks were found. In contrast, the pattern of the mannitol formulation had a melting peak at 135 °C, thus indicating the presence of crystalline material in the sample. In accordance, also, the XRD pattern of the mannitol sample showed two peaks indicating the presence of partly crystalline mannitol in the sample.

Following reconstitution of the freeze-dried samples with PBS, an LPS inhibition ELISA was performed to test the preservation of the bacterial O1 surface LPS antigen, the main protective antigen

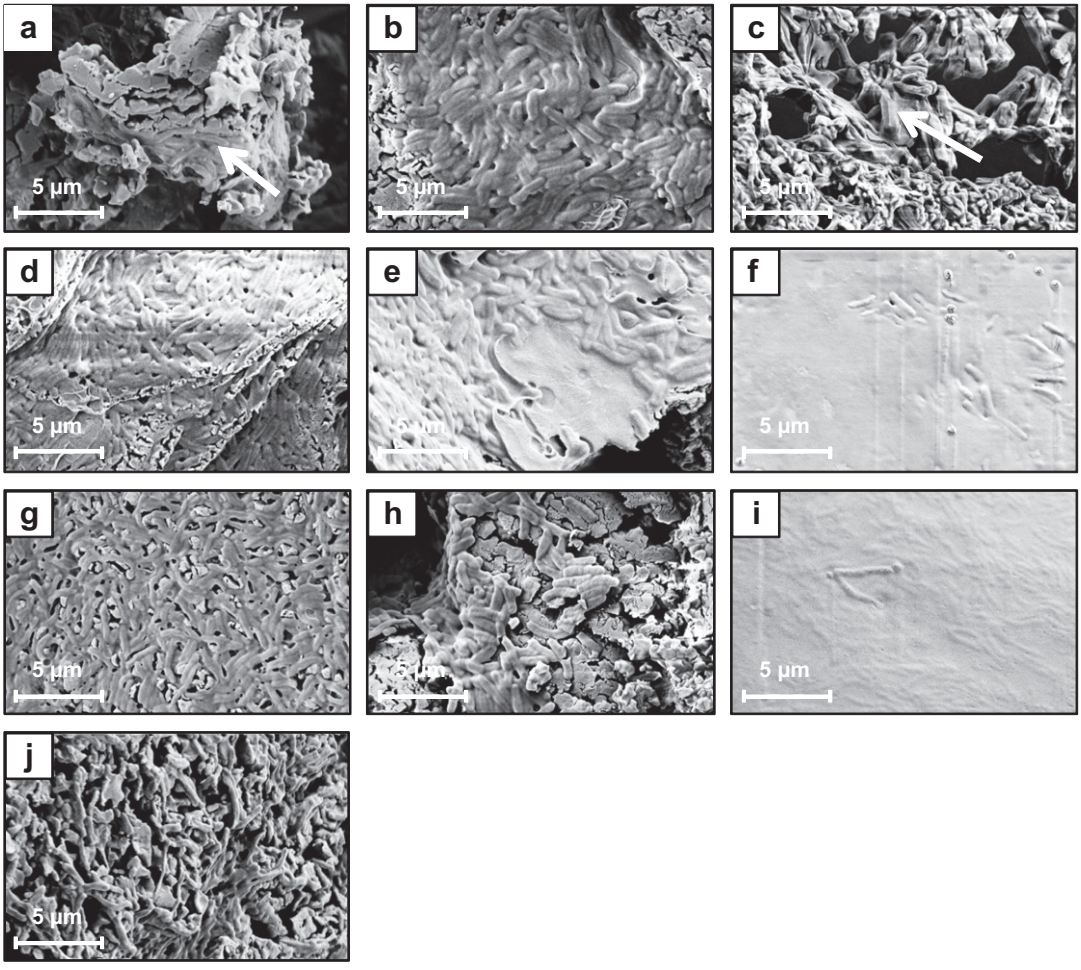


Fig. 1. SEM images of JS 1569 *Vibrio cholerae* freeze-dried in presence and absence of excipients in different concentrations. (a) 0.25 mg/ml mannitol, (b) 2.5 mg/ml mannitol, (c) 25 mg/ml mannitol, (d) 0.25 mg/ml sucrose, (e) 2.5 mg/ml sucrose, (f) 25 mg/ml sucrose, (g) 0.25 mg/ml trehalose, (h) 2.5 mg/ml trehalose, (i) 25 mg/ml trehalose and (j) without excipient. The arrows indicate in (a) large clusters of intact looking bacteria and in (c) mannitol crystals.

of *V. cholerae*, during the freeze-drying process. The mean percentage LPS antigen recovery values were calculated from the 50% inhibition titres for each formulation as shown in Fig. 2 and the obtained values differed clearly depending on the added excipient. In accordance with the results from the SEM investigations, the excipient concentration also had an impact. A concentration dependent LPS recovery was observed for both sucrose and trehalose containing samples where an increasing concentration of sugar resulted in increasing recovery values. For mannitol, a concentration depending effect was observed between the two lowest concentrations (0.25 and 2.5 mg/ml), but a further increase in the concentration could not increase the LPS recovery. The best and almost complete LPS recovery was found for *V. cholerae* freeze-dried with 25 mg/ml sucrose, with a mean value of 89%. The LPS of bacteria freeze-dried with 25 mg/ml trehalose and mannitol, respectively, was not preserved to the same extent as indicated by recovery values of 70% and 60%, respectively. As expected, the LPS recovery of *V. cholerae* freeze-dried without any excipient was even less (36%). In order to control reproducibility, all samples were freeze-dried in triplicates in three independent runs and no major differences were observed (Table included in Fig. 2).

Summarizing, both the SEM investigations and the LPS recovery values showed that only the highest investigated excipient concentration provided sufficient potential to stabilize *V. cholerae* during freeze-drying, where sucrose and trehalose seemed to have the best properties, followed by the less efficient mannitol. Freeze-drying without any excipient did not seem to be promising, especially according to the LPS recovery values.

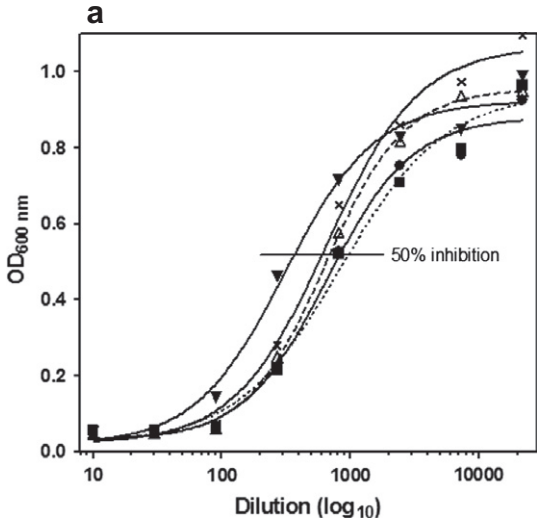
In order to gain more knowledge about the impact of the different excipients, the highest excipient concentration (25 mg/ml) was chosen for further investigations. Reconstituted samples were analysed by confocal microscopy to obtain information about the bacterial cell morphology and the presence of bacterial aggregates in suspension. The observed images showed again clear differences between the investigated formulations. In the presence of both sucrose (Fig. 3b) and trehalose (Fig. 3c), the bacterial suspension looked like the untreated suspension (Fig. 3a) showing a well preserved cell morphology and no formation of bacterial aggregates. In contrast, the presence of mannitol could not prevent bacterial aggregation; in these samples, several aggregates were visible (Fig. 3d). In the samples without any excipient (Fig. 3d), even more aggregates were observed and the appearance of these aggregates differed from the mannitol samples. The aggregates were larger and looked more densely packed.

In addition to the microscopy analyses, all freeze-dried JS 1569 formulations were analysed for their residual moisture content and Tg. All formulations had residual moisture contents between 2% and 4%, except the ones containing 25 mg/ml sucrose or trehalose. The sucrose formulation had an average moisture content of 7% and the trehalose formulation of 8%. The Tg's of the freeze-dried formulations with the highest excipient concentration were also analysed. For the trehalose and sucrose formulation, a Tg of 67 °C and 59 °C, respectively, was found. For the mannitol formulation, it was not possible to detect any clear Tg.

3.1.2. In vivo studies

To determine the immunogenicity of the different formulations characterized previously, groups of four mice were immunized PO as described in Section 2 with reconstituted samples of each formulation freeze-dried with 25 mg/ml excipient. A reference group of four mice given untreated JS 1569 suspension was also included. Several previous experiments in our laboratory [14,16] have shown that neither naïve mice nor mice that were sham-immunized with PBS alone elicit any significant anti-LPS or anti-CTB antibody response. The antibody titres from seventeen such negative controls were, therefore, included in these studies.

All groups of immunized mice developed serum and mucosal anti-LPS antibody titres higher than the unimmunized control group (Fig. 4). From the *in vitro* assessments, it could be anticipated that JS 1569 freeze-dried with sucrose would elicit similar antibody titres as untreated JS 1569, whereas JS 1569 freeze-dried in the presence of mannitol might elicit lower antibody titres. As shown in Fig. 4a, these expectations were confirmed with regard to the obtained systemic responses. The serum anti-LPS antibody response of the mannitol group was significantly lower than that of the reference group ($P < 0.05$), whereas the serum titres of the sucrose group were of the same magnitude as the reference group (untreated suspension). The trehalose group had slightly but not significantly lower serum titres compared to the reference and the sucrose groups. This is in consistence with the *in vitro* results



b

Formulation	mg/ml	LPS recovery (% ± SE)	ANOVA
Untreated	n.a.	100 ±12	n.a.
FD	n.a.	37 ± 3	$P < 0.001$
FD + mannitol	0.25	52 ± 12	$P < 0.001$
	2.5	61 ± 3	$P < 0.05$
	25	60 ± 4	$P < 0.01$
FD + sucrose	0.25	36 ± 3	$P < 0.001$
	2.5	62 ± 1	$P < 0.01$
	25	89 ± 10	**$P > 0.05$**
FD + trehalose	0.25	28 ± 1	$P < 0.001$
	2.5	63 ± 5	$P < 0.01$
	25	70 ± 7	$P < 0.05$

Fig. 2. Preservation of bacterial surface LPS antigen determined as LPS antigen recovery by LPS inhibition ELISA in reconstituted samples of *V. cholerae* JS 1569 freeze-dried with different excipients of varying concentrations (0.25, 2.5 and 25 mg/ml) compared to untreated bacteria. Results from three freeze-drying experiments were used to plot inhibition curves for each formulation to calculate the mean LPS recovery in percent ± SE for each formulation in relation to the untreated suspension as reference. (■) untreated suspension as reference, (▼) freeze-dried without excipient, (×) freeze-dried with 25 mg/ml mannitol, (●) freeze-dried with 25 mg/ml sucrose and (△) freeze-dried with 25 mg/ml trehalose. One-way ANOVA with Bonferroni's post-test was used for statistical comparisons with the untreated control. (n.a. = not applicable).

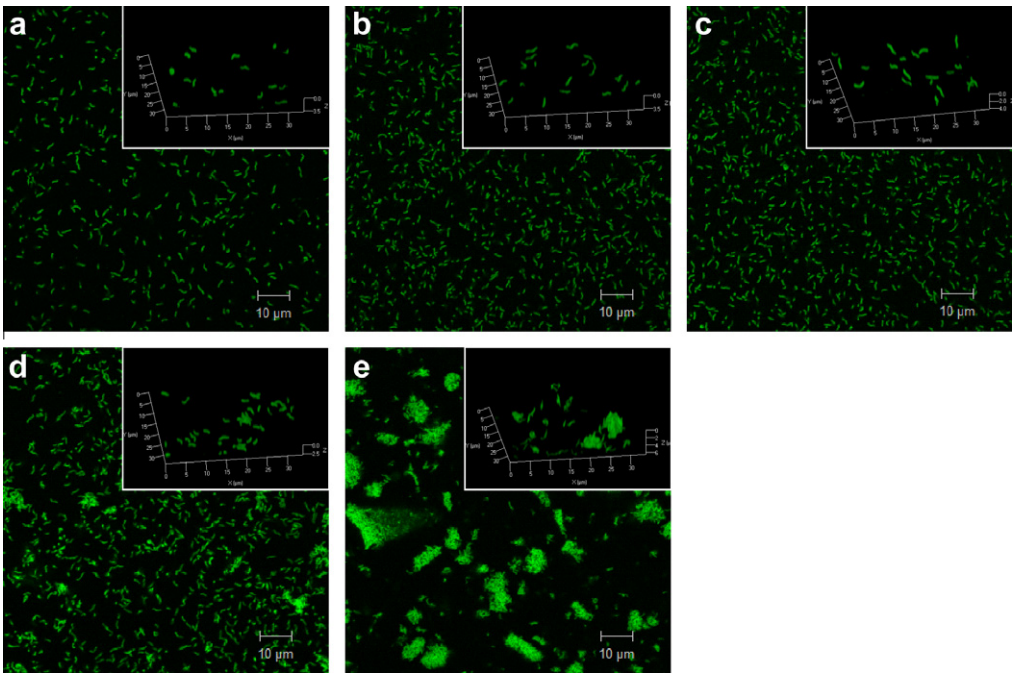


Fig. 3. Confocal microscopy images of formalin-killed JS 1569 *Vibrio cholerae* suspended in PBS: (a) untreated, (b) freeze-dried with sucrose, (c) freeze-dried with trehalose, (d) freeze-dried with mannitol, (e) freeze-dried without excipient. The arrow bars in the 2D images indicate 10 µm. The *x*- and *y*-axes of the inserted *z*-stack images are scaled in 5 µm units, and the dimensions are 33.81 µm × 33.81 µm in *x*- and *y*-direction and vary between 2.75 and 6.52 µm in *z*-direction depending on the properties of the sample.

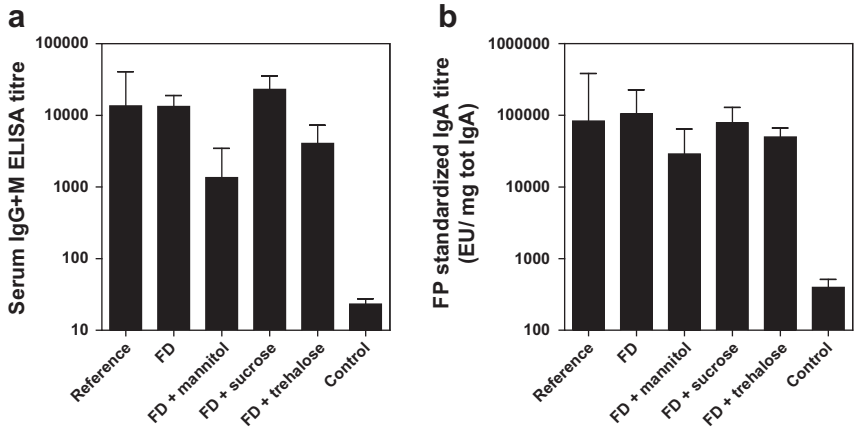


Fig. 4. Serum and intestinal anti-O1 LPS antibody responses in mice immunized PO with formalin-killed *V. cholerae* strain JS 1569. All groups of mice (four animals per group) were immunized with doses containing 5×10^8 bacteria on days 0 and 2, 13 and 15, 41 and 43. Four groups received bacteria that had been freeze-dried (FD) in the absence or in the presence of an excipient (mannitol, sucrose or trehalose) and one group received untreated bacteria (reference). (a) Serum IgG + M responses and (b) standardized faecal pellet (FP) IgA responses were measured against O1 LPS by ELISA and are expressed as $\log_{10}$ antibody titres (GM ± SE).

where few aggregated bacteria were found in the samples freeze-dried with trehalose and confirms the difference between the two investigated disaccharides, of which sucrose seems to be somewhat more advantageous. Surprisingly, the serum antibody titres in the group of mice that had been immunized with freeze-dried JS 1569 without any excipient were not significantly lower compared to the reference, which is in contrast to the *in vitro* results from the LPS inhibition ELISA. These results suggest that not only the LPS recognition in the samples after reconstitution is of importance for an *in vivo* effect. The mucosal anti-LPS antibody titres measured as standardized IgA titres in FP extracts showed the same tendency between the different groups as seen in the systemic responses. The titres in the sucrose group were slightly higher than in the trehalose group and the mannitol samples elicited the lowest antibody titres, but the antibody levels were not significantly lower than for the reference. The significantly decreased systemic immune response and the slightly lower mucosal response in the mannitol group indicate a reduced immunogenic potential of this excipient.

In summary, the initial investigations on fk *V. cholerae* O1 Inaba strain JS 1569 showed that mannitol did not seem to be a promising excipient as a stabilizing agent. Although trehalose, often seen as the 'golden standard' in freeze-drying of biological materials, showed good stabilizing properties, some bacterial aggregates and a lower LPS recovery as compared to the sucrose samples were observed in the reconstituted sample. Furthermore,

it is a much more expensive disaccharide than sucrose. Consequently, for these reasons, sucrose was chosen as excipient for further investigations.

3.2. Freeze-drying applied on the commercially available Dukoral™

After the interesting and promising results from the initial freeze-drying studies on the model vaccine *V. cholerae* JS 1569, our attention turned to the freeze-drying of the commercial vaccine Dukoral™. This vaccine consists of a mixture of fk and heat-inactivated *V. cholerae*, both of serotype Inaba and Ogawa. Since Dukoral™ also contains CTB, which has been shown to have a synergistic protective effect when it is combined with *V. cholerae* vaccine [9], the challenge was to maintain both the relevant bacterial LPS structures as well as the CTB structure.

3.2.1. In vitro studies

In order to ensure that the structure of CTB had not been affected by the freeze-drying process, a GM1 ELISA was used to examine the receptor and antibody binding capacity of CTB in Dukoral™ freeze-dried in the presence and absence of sucrose compared to untreated Dukoral™. The data obtained did not reveal any differences between the tested samples (data not shown), thus indicating that the CTB structure was not affected by the freeze-drying process. Samples freeze-dried in three independent runs were also compared in order to control reproducibility, and no differences between the samples from different runs were found.

3.2.2. In vivo studies

For the *in vivo* investigations, groups of seven mice were immunized via the PO route with lyophilized and reconstituted Dukoral™ samples. Animals were immunized PO by three rounds of vaccine administration, each round with the vaccine given on two consecutive days, similar to the schedule used with JS 1569 above. A reference group of 7 mice received untreated Dukoral™ suspension, and the negative control group was the same as described previously for JS 1569.

In addition to the mucosal antibody determinations using FP extracts, IgA ELISA titres were also assessed in small intestinal tissue extracts prepared as described in Section 2.7. Although there is a strong correlation between FP and PERFEXT IgA estimates [14], the latter samples provide direct evidence of the site-specific local production of IgA in the gut and avoid any possible impact of naturally excreted IgA from the liver via bile to the intestinal lumen compared to FP extracts. In both cases, IgA titres were standardized to correct for the variable total amount of this immunoglobulin in the different samples.

3.2.2.1. Antibacterial antibodies. The estimated serum and mucosal antibody titres against O1 LPS are shown in Fig. 5a and b. All groups developed significantly higher serum and mucosal antibody titres than the unimmunized control group, and no significant differences between the groups were found in serum, FP and small intestine extracts. As already seen with the model vaccine JS 1569, also the Dukoral™ formulation freeze-dried without any excipient elicited high serum antibody titres against O1 LPS, but slightly lower than both freeze-dried Dukoral™ with sucrose and the reference group (untreated). In FP extracts, the standard deviations of the titres were unexpectedly high and thus hard to interpret. The analysis of the small intestinal tissue extracts showed equivalent mucosal responses, with titres in the sucrose group similar to the reference group, whereas mice in the group without excipient mounted a slightly lower response. All serum samples from the Dukoral™ immunizations were also analysed for its bactericidal activity. The results are presented in Fig. 5c and show that all tested Dukoral™ formulations elicited serum antibodies with vibriocidal activity, both against *V. cholerae* serotype Inaba and Ogawa. No significant differences were found between the different groups, but the group immunized with the freeze-dried formulation containing sucrose had slightly higher vibriocidal antibody titres compared to the other two groups.

3.2.2.2. Antitoxic antibodies. The estimated serum and mucosal antibody titres against CTB are shown in Fig. 6. All groups developed significantly higher serum and mucosal antibody titres than the unimmunized control group. The assessed antibody levels in serum, FP and small intestinal extracts did not differ significantly between the three groups, which is consistent with the *in vitro* assessments by GM1 ELISA and confirms that the CTB structure is preserved during the freeze-drying process. Interestingly, the mean serum response in the sucrose group was about twofold higher than in the reference group. Importantly, the levels of the anti-CTB IgA responses examined using intestinal tissue extracts were comparable to those detected in the corresponding FPs with significant correlations ($r \geqslant 0.91$ and $P < 0.0001$), thus confirming that the latter is an acceptable valuable method for the

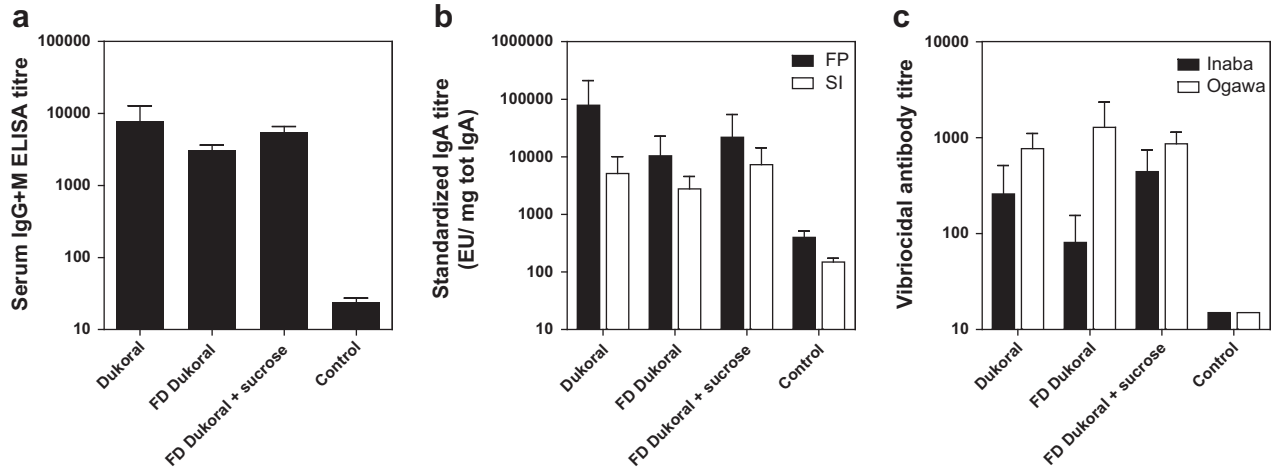


Fig. 5. Antibacterial responses in mice immunized PO with Dukoral™. All groups of mice (seven animals per group) were immunized with doses containing 5×10^8 bacteria in three rounds on days 0 and 1, 14 and 15, 27 and 28. Two groups received Dukoral™ formulations that had been freeze-dried in the absence or in the presence of sucrose and the reference group received untreated bacteria. Immune responses were measured against O1 LPS by ELISA and are expressed as $\log_{10}$ antibody titres (GM ± SE). (a) Serum IgG + M responses, (b) standardized faecal pellet (FP) and small intestine (SI) IgA responses and (c) vibriocidal serum responses against Inaba and Ogawa bacteria.

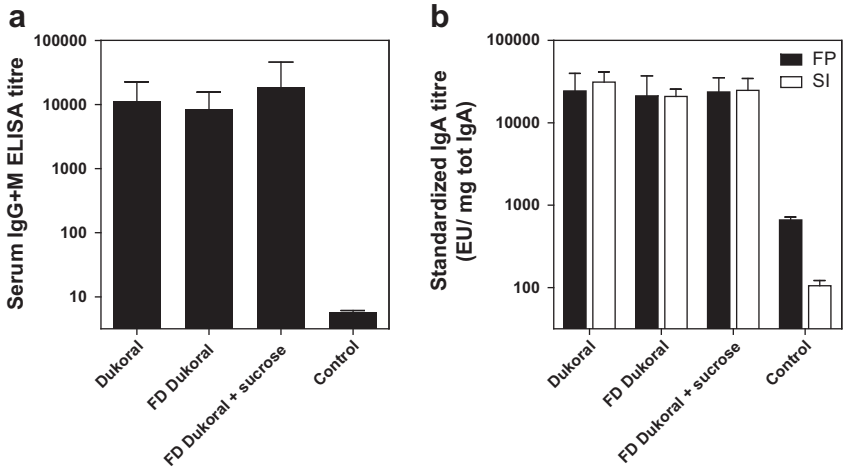


Fig. 6. Antitoxic serum and intestinal antibody responses in mice immunized PO with Dukoral™. All groups of mice were immunized with doses containing 5×10^8 bacteria in three rounds on days 0 and 1, 14 and 15, 27 and 28. Two groups of mice received Dukoral™ formulations that had been freeze-dried in the absence or in the presence of sucrose and the reference group received untreated bacteria. (a) Serum and (b) standardized faecal pellet (FP) and small intestine (SI) IgA responses were measured against rCTB by ELISA and are expressed as $\log_{10}$ antibody titres (GM ± SE).

determination of local IgA antibody production. As previously shown [14], this correlation was also found for the anti-LPS IgA responses ($r \geqslant 0.90$ and $P < 0.0001$).

4. Discussion

Although currently licensed oral cholera vaccines, consisting of a suspension of inactivated *V. cholerae* with or without added CTB protein, are safe and effective, their liquid formulations resulting in large package volumes and potentially in less long-term storage stability make them however not optimal for public health use in many low-income countries. Therefore, it would be a great advantage if a suitable dry vaccine formulation with intact immunological properties could be developed. In the present study, we describe a freeze-drying method for a liquid formalin-killed single-strain oral cholera model vaccine using a sufficient concentration of sucrose for stabilization and the application of this method on the internationally widely licensed oral cholera vaccine Dukoral™, which comprises several inactivated cholera strains together with added CTB protein. The drying process results in a dry vaccine formulation that practically retains both the morphological and immunological properties of the original liquid formulation after reconstitution, including a similar capacity to stimulate antibacterial and antitoxic immune responses both in serum and in the gut mucosa when tested as orally administered vaccine in mice.

A main challenge in preparing dry formulations of biological materials is the prevention of adverse effects due to the removal of water hydrogen bonds. Here, freeze-drying is an advantageous drying method for labile materials like bacteria compared to other common and more cost efficient drying methods in the pharmaceutical industry, e.g., spray drying. In spray drying, the sample is sprayed as droplets into hot gas, which rapidly dries the liquid, but may be harmful to temperature-sensitive biological [19]. In contrast, freeze-drying is a low-temperature process where water or solvent are removed by direct sublimation leading to a dry and porous matrix that is often sufficiently stable for storage and distribution and can easily be reconstituted [10]. A further advantage of freeze-drying is that the vials can be sealed in the freeze-dryer so that moisture control is possible, which can be important for storage stability of dried bacteria. Regarding early stage research and development of suitable processes and formulations, freeze-drying has two further main advantages. Firstly, only small amounts are required for the processing. Secondly, the critical heat and mass transfer characteristics at laboratory scale can often easily be scaled up to full industrial production. In spite of relatively high costs and often long process times in freeze-drying, the economical aspects have to be balanced with relatively small consumption of material during the process and formulation development as well as with the quality of the final product [10]. Therefore, freeze-drying is still the first choice for drying of biological materials in many cases.

Although freeze-drying is a low-temperature process where the liquid is removed via direct sublimation and the bacteria are almost immobile in the ice during the freeze-drying process, there is still a risk for aggregation since some water is already eliminated during the freezing step, when the bacteria still can move in the unfrozen parts and thus an up-concentration of the bacteria can occur [10]. Furthermore, protein structures can be destroyed during the drying process due to the removal of hydrogen-bonded water. Therefore, stabilizing excipients that hinder an aggregation and replace hydrogen-bonded water are usually required in the formulation to be freeze-dried. In this study, the ability to stabilize inactivated whole-cell *V. cholerae* bacteria during freeze-drying was investigated with three different excipients, mannitol, sucrose and trehalose.

Bacterial aggregation and decreased LPS recognition were regarded to be the main problems in the first part of the study using fk *V. cholerae* O1 strain JS 1569 as a model vaccine. All excipients were tested in three different concentrations, and bacteria without excipient were also freeze-dried. Already the SEM images of all samples made it obvious that both the concentration and the type of excipient had an impact on the properties of the resulting lyophilisates. In almost all samples, large clusters of tightly packed bacteria were observed. It was only in samples containing the highest tested concentration of sucrose or trehalose (25 mg/ml) that single bacteria embedded in a disaccharide matrix were found. As expected, mannitol thus seemed to be a less efficient stabilizer. These obtained differences between the types and concentrations of the stabilizer were supported by an LPS inhibition ELISA, where a similar concentration-dependence was found. Here, the best results were obtained for the samples freeze-dried in the presence of 25 mg/ml sucrose. Only in these samples, the LPS recovery values were not significantly different from the values of the un-

treated reference ($p > 0.05$). All other samples had significantly lower LPS recovery values as compared to the reference. Comparisons of reconstituted samples with untreated suspension of JS 1569 by confocal microscopy could further confirm the different effects of the tested excipients. In the samples containing sucrose or trehalose, no aggregation was found, whereas the prevention of bacterial aggregates by mannitol was clearly not successful since large aggregates were found in these samples. One possible reason for that could be that mannitol is not able to sufficiently form hydrogen bonds with the cell membranes so that interactions during the removal of water are only partly prevented. A more likely explanation is that mannitol occurs mainly in the crystalline state in the freeze-dried cake, i.e., crystalline mannitol particles are formed during the freeze-drying step [10], which could be confirmed by SEM, DSC and XRD analyses in this study. Water and bacteria will be expelled from this crystalline mannitol phase, which leads to a dry cake with areas of locally increased bacteria concentrations surrounded by crystalline mannitol areas which were partly visible in the SEM images taken on the samples containing freeze-dried JS 1569 and 25 mg/ml mannitol. During the reconstitution of the dry cake, the bacteria clusters were not destroyed and closely adjacent bacteria in these samples can easily come in direct contact with each other, which could lead to a further formation of bacterial aggregates. In contrast, if the bacteria are more evenly distributed in the excipient matrix, as found in the trehalose and sucrose samples, the risk for bacterial aggregation during reconstitution decreases. However, although mannitol did not inhibit bacterial aggregation, it seems to have some effect on the dried bacteria since the confocal microscopy images differed from the images of bacteria that had been freeze-dried without any added excipient. In these samples, much larger and more densely packed aggregates were observed. The distinct differences between the formulations found in the LPS ELISA were thus confirmed with the microscopy analysis, and it seems as if an increased aggregation results in a decreased LPS detection.

The elicited immune responses in the following PO immunizations in mice also showed that both the sucrose and the trehalose containing freeze-dried formulations of JS 1569 had similar immunogenicity as the reference formulation (untreated JS 1569 suspension), whereas the mannitol preparation was less immunogenic. These results can be nicely explained by the findings from the *in vitro* investigations. An aggregation of bacteria in the mannitol formulation leads to a decreased accessibility of surface LPS, which was also indicated by a hampered binding of LPS to antibodies in the inhibition ELISA. In addition, tissue penetration specific to the Peyer's patches has been shown to be restricted to microspheres below 10 μm in diameter. It has been shown that only particles smaller than 10 μm are taken up by the M-cells and transported into the Peyer's patches, which are the main induction sites for mucosal immune responses in the small intestine [20]. Thus, it can be suggested that large aggregates of bacteria are unlikely to be taken up by M-cells upon PO immunization, so that a less efficient LPS presentation to antigen presenting cells occurs.

Both with trehalose and especially with sucrose promising *in vitro* and *in vivo* data were obtained for freeze-dried JS 1569 formulations. Since sucrose is a more cost efficient excipient, it was chosen to test freeze-drying in the presence of sucrose of the commercial vaccine Dukoral™, which consists of a mixture of fk and heat-inactivated *V. cholerae*, both of serotype Inaba and Ogawa, in combination with CTB protein. After the surprising *in vivo* data without any excipient, Dukoral™ alone was also freeze-dried. Since CTB has been shown to have a synergistic protective effect when combined with *V. cholerae* whole-cell vaccine [9], it was important to maintain not only the relevant bacterial LPS structures but also the CTB structure and its binding capacity to the GM1 receptor. It could be shown by GM1 ELISA that the binding capacity of CTB was not affected by the freeze-drying process. The successful preservation of CTB was supported by the *in vivo* data from the PO immunizations since all mice immunized with freeze-dried Dukoral™ formulations mounted responses that were equivalent or higher than those of the reference group. Both the serum antibody responses including the bactericidal activity which has been found to correlate with protective efficacy of cholera vaccines and the mucosal IgA antibody responses measured in faecal pellets and small intestine tissue extracts were of the same magnitude in all three groups.

Even more interesting was whether or not the bactericidal immunogenicity of Dukoral™ can be maintained during freeze-drying. In the two groups of mice that had received Dukoral™ freeze-dried with or without sucrose, the elicited serum antibody levels were similar to the reference group with a slightly lower mean titre in the group without excipient. For a vaccine against cholera disease, it is even more important to consider the mucosal responses since protective immunity is mainly mediated by locally produced intestinal IgA antibodies to LPS and CTB [21]. Therefore, it was very promising that the mucosal antibody levels in the sucrose group were of the same magnitude or even slightly higher than those in the reference group, both in small intestine extracts and faecal pellets. Mice that were immunized with freeze-dried Dukoral™ without any excipient mounted slightly lower mucosal antibody levels than the reference. In the development of new oral vaccines, the potential to elicit antibodies with bactericidal activity has been shown to be an important indicator of the protective efficacy [22]. The bactericidal antibody levels were slightly lower in mice that were immunized with freeze-dried Dukoral™ without any excipient than the levels in the reference group, whereas mice immunized with the sucrose containing freeze-dried formulation mounted slightly higher vibriocidal antibody levels compared to the reference group. These findings are consistent with the serum anti-LPS antibody responses. Thus, they give further support to sucrose being a promising candidate as a stabilizing agent for freeze-drying of *V. cholerae* for the production of a dry cholera vaccine.

The most interesting results were obtained for bacteria which were freeze-dried without the addition of excipient. Here, all *in vitro* characterizations on JS 1569 indicated a less efficient freeze-drying. In contrast, systemic and mucosal immune responses similar to the reference group were obtained in mice that were immunized with JS 1569 as well as Dukoral™ freeze-dried without excipient. A possible explanation could have been that too high doses were given in the immunizations so that no remarkable differences can be seen *in vivo*. However, recent studies with co-administered oral adjuvants have shown that significantly increased immune responses can be obtained which indicates that the dose used in the present study is well below the saturation dose. It is more likely that the sensitivity of the used *in vivo* model is not efficient enough to detect the differences between the formulations found *in vitro*. The mouse model used only measures the functionality of the cholera vaccine formulation investigated and only little is known about how well the data obtained can predict the protective efficacy of the vaccine in humans. Considering the poor preservation of the bacteria's morphology and the low antigen recovery in samples freeze-dried with mannitol or without excipient, these formulations do not seem to be potential vaccine candidates. Even though aggregates might also be immunogenic and can possibly be destroyed in the gastrointestinal tract, it will be difficult to control the immunogenicity of such a formulation since different rates of aggregation and disaggregation might occur. Research is also ongoing to develop new vaccine strains that accumulate antigens in the intracellular periplasma, such as the pH sensitive CTB in a *V.* cholerae strain [23]. For such strain con-

structs, it will most likely be highly important to maintain the cell morphology and avoid aggregation during a drying process.

The results from our studies on JS 1569 showed that trehalose but especially sucrose is able to provide good process stabilization so that the *in vitro* antigen detection is not affected and both bacterial morphology and *in vivo* immunogenicity are maintained. Both formulations had Tg's above room temperature and also ambient temperatures in tropical countries and might, therefore, be suitable for storage at non-cooling conditions. The relatively high residual moisture contents of these formulations might on the other hand impair storage stability; however, a low moisture content is not always a guarantee for better storage stability properties [24]. Thus, further detailed studies will be needed in order to evaluate and optimize storage stability as a second step towards a dry vaccine formulation against enteric diseases suitable for use in developing countries.

5. Conclusion

A dry oral whole-cell *V. cholerae* vaccine formulation with preserved bacterial morphology, no bacterial aggregates, maintained protective lipopolysaccharide (LPS) antigen on the cell surface and reactivity with specific antibody *in vitro* could be produced by freeze-drying when 25 mg/ml sucrose was added as stabilizer in the formulation. Strong serum and gut mucosal anti-LPS antibody responses were elicited in immunized mice, comparable to the responses achieved with the equivalent liquid formulation. A dry formulation leads to substantial reductions in package volumes and weights of the vaccine, which would mean significant advantages for shipment and public health use in low-income countries and in mass vaccination campaigns for the prevention or control of large cholera outbreaks. The presented study is, therefore, an important step towards new dry mucosal vaccines against enteric diseases with attractive features for public health use. Process optimization as well as shelf-life stability studies will be required when the presented process is applied on new vaccines.

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