Journal of Controlled Release 183 (2014) 43–50

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Polymeric penetration enhancers promote humoral immune responses to mucosal vaccines

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ARTICLE INFO

Article history:
Received 22 January 2014
Accepted 10 March 2014
Available online 20 March 2014

Keywords:
Mucosal
Vaccination
Penetration
Delivery
Insulin
Immunity

ABSTRACT

Protective mucosal immune responses are thought best induced by trans-mucosal vaccination, providing greater potential to generate potent local immune responses than conventional parenteral vaccination. However, poor trans-mucosal permeability of large macromolecular antigens limits bioavailability to local inductive immune cells. This study explores the utility of polymeric penetration enhancers to promote trans-mucosal bioavailability of insulin, as a biomarker of mucosal absorption, and two vaccine candidates: recombinant HIV-1 envelope glycoprotein (CN54gp140) and tetanus toxoid (TT). Responses to vaccinating antigens were assessed by measurement of serum and the vaginal humoral responses. Polyethyleneimine (PEI), Dimethyl-β-cyclodextrin (DM-β-CD) and Chitosan enhanced the bioavailability of insulin following intranasal (IN), sublingual (SL), intravaginal (I.Vag) and intrarectal (IR) administration. The same penetration enhancers also increased antigen-specific IgG and IgA antibody responses to the model vaccine antigens in serum and vaginal secretions following IN and SL application. Co-delivery of both antigens with PEI or Chitosan showed the highest increase in systemic IgG and IgA responses following IN or SL administration. However the highest IgA titres in vaginal secretions were achieved after IN immunisations with PEI and Chitosan. None of the penetration enhancers were able to increase antibody responses to gp140 after I.Vag immunisations, while in contrast PEI and Chitosan were able to induce TT-specific systemic IgG levels following I.Vag administration. In summary, we present supporting data that suggest appropriate co-formulation of vaccine antigens with excipients known to influence mucosal barrier functions can increase the bioavailability of mucosally applied antigens promoting the induction of mucosal and systemic antibody responses.

1. Introduction

Trans-epithelial penetration of pharmaceutically relevant drugs has been widely studied. Here pharmaceutical excipients able to influence epithelial penetration have long been utilised to increase trans-dermal drug absorption [1]. However such approaches have not been fully exploited for the delivery of biopharmaceuticals across mucosal surfaces, providing a needle-free delivery strategy that could be easily administered with minimal training. In this respect, trans-mucosal delivery provides an attractive approach for regular repeat dosing of biologics. This may have particular relevance for mucosal vaccination, where gastrointestinal, respiratory and genital mucosal surfaces are the major sites of entry for most pathogens. For this reason elicitation of both systemic and mucosal immune responses is a highly desirable property for preventative vaccines and may increase their capability for controlling infection at the mucosal portals of entry. This is especially true since it is believed that mucosal immune responses are more efficiently activated after direct mucosal application, compared to parenteral routes of vaccination [2]. This has driven development and commercialisation of a number of vaccines against mucosally associated pathogens [3]. Mucosally delivered vaccines are thought to function by localised imprinting of antigen-activated T and B cells facilitating their re-homing to tissues that are the source of original vaccine/antigen insult. However, a major obstacle to effective trans-mucosal vaccination is thought to be the effectiveness of mucosal epithelial barriers, limiting local bioavailability of recombinant vaccine antigens for sampling by professional antigen presenting cells (APCs) [2]. To test this hypothesis and provide deeper insight into the importance of penetration properties in relation to the efficiency of mucosal vaccination, we evaluate side-by-side the activity of three commonly used penetration enhancers with varying immune activating properties, namely Polyethyleneimine (PEI), Dimethyl-β-cyclodextrin (DM-β-CD) and chitosan glutamate. First we assess their differential ability to alter tight junction integrity in model mucosal epithelial transwell systems. Next to determine *in vivo* relevance, we evaluate their impact on the bioavailability of insulin, as an *in vivo* marker of mucosal permeability. Finally we determine their differential impact on antigen-specific systemic and mucosal

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http://dx.doi.org/10.1016/j.jconrel.2014.03.018
0168-3659/

humoral immune responses to two model vaccine candidates: recombinant HIV-1 envelope glycoprotein (CN54gp140) and tetanus toxoid (TT). Our data provide important insight as to how these penetration enhancers differentially influence the effectiveness of mucosal vaccination according to route of administration.

2. Materials and methods

2.1. Polycationic compounds and vaccine antigens

Polyethyleneimine (MW 25 kDa, branched), Dimethyl-β-cyclodextrin (MW 1.33 kDa) and nonoxynol-9 (Tergitol®NP-9) were obtained from Sigma-Aldrich (UK). Chitosan glutamate (PROTASAN™ UP G 213, MW 200–600 kDa, degree of deacetylation 85%) was obtained from Novamatrix, FMC Biopolymer (Norway). PEI and N-9 were dissolved in sterile water at a concentration of 2% (v/v) while Chitosan and DM-β-CD were diluted in sterile water to 2% (w/v) and 10% (w/v) respectively. TT was obtained from the Statens Serum Institute (Denmark). Trimeric recombinant CN54gp140 and demannosylated CN54gp140 were a kind gift from Dietmar Katinger (Polymun Scientific, Austria).

2.2. Assessment of compound mediated cytotoxicity using colorimetric MTT (tetrazolium) assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphethyl tetrazolium bromide) assay was performed as described before [4] with some modifications. A detailed description of the protocol can be found in the Supplementary materials and methods.

2.3. Binding interactions of compounds to gp140 by interferometry

Recombinant CN54gp140 at a concentration of 50 μg/ml was immobilised on ForteBio Second-Generation amine reactive biosensor probes (ARG2) using N-hydroxysuccinimide/1-ethyl-3-(3dimethylaminopropyl)carbodiimide (NHS/EDC) linkage. Probes were blocked using 5 mg/ml BSA in PBS (pH 7.6) containing 0.01% Tween-20. PEI, Chitosan and monoclonal antibody 5F3 IgG were used at 25 μg/ml, DM-β-CD was used at 100 μg/ml and anti-gp140 polyclonal rabbit serum was diluted to a final concentration of 5 mg/ml of total protein. Samples were loaded into a ForteBio BLITz machine (Fortebio, Inc., Menlo Park, CA) to evaluate binding interactions. Binding is measured as a wavelength shift in nanometers [5]. Analysis was performed using Graphpad Prism (version 4 for Mac). The average rates of change were calculated using the 25% and 75% signal intensities.

2.4. Polycationic compound mediated changes in tight junction permeability

The influence of PEI, DM-β-CD and Chitosan on tight junction integrity was tested using HEC-1A or Caco-2 cells as model epithelial cell layers in a transwell assay. For full details see Supplementary materials and methods.

2.5. In vivo assessment of mucosal permeation using insulin as a model delivery agent

Female BALB/c mice at 6 to 8 weeks of age were obtained from Harlan Laboratories (UK) Ltd. All mouse procedures were performed under the appropriate project licence (PPL 70/6613) in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and were ethically approved by the animal ethics committee of St. George's University of London. Mice were maintained in conditions conforming to UK Home Office guidelines to ameliorate suffering and were euthanised by cervical dislocation.

The average weight of the mice was 20 g ± 2 g. Prior to application all animals were fasted overnight. Water was provided ad libitum. Mice receiving I.Vag application were treated with 2 mg of DepoProvera (Pharmacia Limited, UK) prior to administration. All animals in the test groups were administered glucose at a dose of 1 g/kg bodyweight intraperitoneally (IP) prior to application. Recombinant human insulin (hINs) (MW 5.8 kDa) (Sigma-Aldrich, UK) was administered at 1 IU/kg bodyweight alone or in combination with penetration enhancers. Penetration enhancers were used at a concentration of 100 μg except from PEI, which was used at 40 μg for nasal and sublingual administration due to toxic side effects at higher concentrations at these routes. One group of animals received only IP PBS to obtain baseline glucose levels. For IN, I.Vag and IR application mice were anaesthetised using isoflurane. For IN application 20 μl of formulation was put onto both nostrils. For I.Vag and IR application 20 μl of formulation was applied into the entrance of the vagina or the rectal cavity respectively. For SL application the mice were heavily anaesthetised using isoflurane. A volume of 15 μl of formulation was delivered with a pipette underneath the tongue. The mice were maintained anaesthetised with their head positioned in ante-flexion for a further 10 min to avoid swallowing. Blood samples were taken to assess glucose levels at 0, 30, 60, 90 and 120 min after formulation administration using a OneTouch®UltraEasy® blood glucose monitor and OneTouch®UltraEasy® Test Strips (LifeScan, UK). At the end of each experiment mice were humanly killed by cervical dislocation.

2.6. Immunisations and sampling

Female BALB/c mice were immunised three times at two-week intervals by IN, I.Vag and SL routes as described above. Serum and vaginal wash samples were taken prior to the first immunisation and two weeks after each immunisation. Serum was collected from blood from the tail vein, which was allowed to clot for one hour at room temperature before centrifugation at 400 ×*g* for 10 min and then frozen until analysis. Vaginal lavage samples were collected by gently flushing 3 times 25 μl of sterile PBS in the vagina using a positive displacement pipette with round-ended tips. Washes were placed on ice and supplemented with 4 μl of protease inhibitor cocktail (Roche Diagnostics, UK) for 30 min before centrifugation at 400 ×*g* for 20 min and freezing. At the end of each experiment mice were humanely killed by cervical dislocation.

2.7. Antigen-specific IgG, IgG1, IgG2a and IgA ELISA

Serum and vaginal samples were analysed for antigen specific IgG, IgG1, IgG2a and IgA using an in-house ELISA protocol. A full description of the methods used is shown in the Supplementary methods.

2.8. Statistical analysis

Statistical analysis was performed using a Mann–Whitney test with a confidence interval of 95% using Graphpad Prism (version 4 for Mac) software.

3. Results

3.1. Impact of polymeric penetration enhancers on mucosal epithelial tight junction integrity

We first sought to determine the effects of the various polymers on epithelial cell viability using a colorimetric MTT assay. After a 4 hour exposure using the model colorectal epithelial cell line Caco-2, 100 μg PEI mediated high levels of cytotoxicity, reducing cell viability by 79% (±4.8%) (Fig. 1A). This contrasted significantly to DM-β-CD, which resulted in a 24.3% (±23.7%) reduction in cell viability, and to Chitosan which reduced cell viability by 10% (±11.5%). Nonoxynol-9 (N-9), a compound known to display high levels of cytotoxicity, was used as a control and resulted in 83.3% (±2.5%) reduced cell viability (Fig. 1A).

Next bilayer interferometry was used to evaluate potential binding interactions with gp140 as a model vaccine antigen. Binding to the