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Challenges and opportunities in nasal subunit vaccine delivery : mechanistic studies using ovalbumin as a model antigen

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General introduction and outline of this thesis

Vaccination

Since its application by Dr Edward Jenner in the 18th century, vaccination has revolutionized medicine. Large scale vaccination campaigns have resulted in the eradication of smallpox and the World Health Organization has set targets to eradicate polio, rubella and measles using a world wide vaccination strategy [1]. The goal of vaccination is to prime an individuals' immune system against a specific pathogen, so on second encounter the immune system is capable of quickly removing the threat. The classical approach is to use a non-pathogenic strain that closely resembles the pathogen (live-attenuated vaccines), or an inactivated pathogen. These vaccines generally provide good protection as they resemble the original pathogen the most. This approach, however, also brings safety risks. Vaccines based on live-attenuated or whole inactivated bacteria or viruses can contain a variety of biologically active compounds (e.g. toxins, bacterial cell membrane products) that can cause symptoms like fever and nausea. In the case of live-attenuated vaccines, reassortment with a wildtype virus could lead to regaining their pathogenicity. Moreover, in immuno-compromised patients these types of vaccines can cause disease symptoms, as these individuals are not capable of clearing the vaccines. Finally, most of these vaccines are injected intramuscularly or subcutaneously, as generally the large size and instability of the antigen does not allow application via mucosal routes. Injectable vaccines often cause pain/discomfort, local swelling (inflammation) and stiffness.

Vaccine coverage in the Western world is not optimal, as the turn up of the various state vaccination programs hardly ever passes 70% [2-5]. Although part of the vaccination refusals is of religious nature, an increasing population does not want to be immunized fearing the side effects of the vaccine and the discomfort upon injection [6]. This has prompted governments and health organizations to enlarge their funds for research and development of (safer) subunit and non-invasive vaccines (e.g. dermal, nasal, oral, pulmonary) [7-9] as better patient compliance may be expected.

Subunit vaccines

Adverse effects can be reduced by stripping the virulence factors from a pathogen, leaving only the part to which the immune system has to make antibodies or a T-cell response. Subunit vaccines only contain this antigenic part of the pathogen (often only a single protein) and are therefore safer and pharmaceutically better defined [10]. Although some subunit vaccines have been applied very successfully (e.g. diphtheria toxoid, pertussis toxoid and tetanus toxoid), most of them do not completely protect the vaccinated population [11]. Paradoxically, because of the lack of co-stimulatory factors, the immunogenicity of these types of vaccines is reduced. This is a direct consequence of the nature of the immune system; it will only develop a response if the encountered material is considered dangerous [12]. Antigen presenting cells (APCs; e.g. macrophages, dendritic cells) play a crucial role in the decision making by the immune system whether or not to respond and are therefore the key target in vaccination (Figure 1). On encounter with a pathogen APCs engulf it and break it down into small fragments (epitopes). Meanwhile various constituents of the pathogen contribute to activation of the APC (Figure 1a), making the cell capable of initiating an adaptive (T- and B-cell mediated) immune response [13]. These constituents are evolutionary conserved motives that are shared by many pathogens, so called pathogen associated molecular patterns (PAMPs), making it possible for the APC to distinguish between dangerous and innocuous antigens, via pathogen recognition receptors (PRR) [14, 15]. Plain subunit antigen will also be sampled by APCs, but because of the lack of PAMPs will be considered harmless and will not induce maturation of the APC (Figure 1b). It is therefore imperative to formulate the antigen in such a way that APCs do get activated e.g. by addition of PAMPs or the use of vaccine delivery systems [16].

Non-invasive vaccination

Currently, most vaccines are injected subcutaneously or intramuscularly as it is a simple procedure and allows accurate dosing. This does not mean however that muscle and subcutaneous tissue are sites that provide the best environment for inducing an immune response. Before the introduction of the hollow needle in the 19th century, vaccines were usually applied nasally or scratched into the skin [17]. As the exterior of the body is under constant attack by invading pathogens, the skin and the mucosal linings are densely equipped with APCs [18], whereas subcutaneous tissue and muscle contain very low numbers of APCs.

Not surprisingly, studies comparing the antibody response after intramuscular and intradermal vaccination clearly show superior antibody titers after intradermal injection [19-21]. Nonetheless intradermal vaccination did not establish itself as the standard administration method, because of poor protection in the elderly population but most of all because the intradermal injection technique is more difficult to master than the intramuscular one [22].

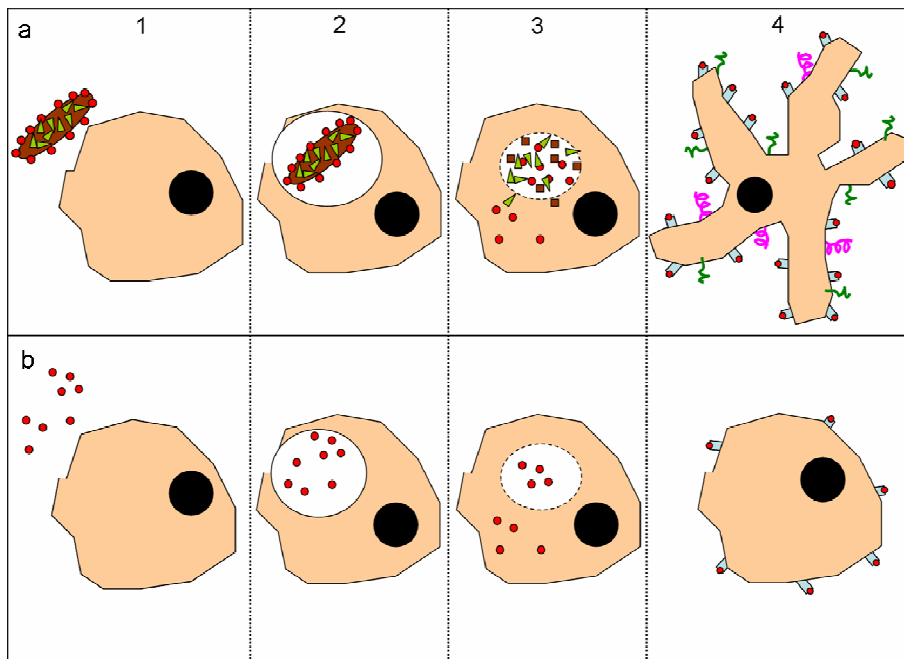


Figure 1: a) A bacterium is encountered by the APC (1) and subsequently engulfed (2). In the endosome the pathogen is degraded into epitopes (red) (3). Co-stimulatory factors (brown, green) on the bacterium activate the APC and make it express various co-stimulatory factors (4) enabling it to activate T- and B-cells. b) Plain subunit antigens encountered by a DC (1) are also taken up (2) and degraded into epitopes (3), but lack of virulence factors prohibits the activation of the APC (4).

Similarly, very encouraging results were obtained by pulmonary vaccination against measles in Mexico [23], but the need for a delivery device (nebulizer) whereas intramuscular vaccination was just as effective, prohibited the widespread use of the pulmonary vaccine [24]. In this respect nasal and oral vaccination provide a more promising alternative. Oral application (e.g., as a tablet or capsule) may seem easy and convenient for the vaccinee, but the harsh gastrointestinal conditions compromises the vaccines' stability. Currently, 1 inactivated and 4 live-

attenuated oral vaccines have been licensed (the polio vaccine already being administered over 1 billion times!), but no oral subunit vaccine has been marketed yet, illustrating the difficulty of making effective oral vaccines.

Table I. Advantages and disadvantages of different routes of immunization.

Immunization route	Advantages	Disadvantages
Intramuscular/ Subcutaneous	Powerful systemic immune response Accurate dosing	Invasive Limited mucosal immune response
Nasal	Non invasive Mucosal & systemic immune response Easily accessible Little degradation (compared to oral)	Mucociliary clearance Inefficient uptake of soluble antigens Application device needed
Oral	Non invasive Mucosal & systemic immune response Large surface area	Vaccine digestion in stomach and gut Inefficient uptake of soluble antigens Mucosal tolerance
Pulmonary	Non invasive Mucosal & systemic immune response Little degradation (compared to oral)	Delivery of antigen highly variable from person to person Dry powder inhaler or nebulizer needed Clearance from lungs
Dermal	Non or minimally invasive Large, easily accessible application area High density of immune cells in skin Mucosal immune response possible	May require (minimally) invasive technology (e.g. tattooing, microneedles) Patch or application device needed Less established technology

Nasal vaccination

The nasal cavity is easily accessible (e.g., nasal spray or nose drops) and the low enzymatic activity compared to the gastro-intestinal tract provides better antigen stability, making nasal administration very promising. Nonetheless, only 1 live attenuated flu vaccine (Flumist®) is on the market, showing that nasal vaccination is possible, but also challenging

Although the nasal flu vaccine has been well perceived by the public [25], because of the live attenuated nature of the vaccine, it is only recommended for use in a population between the age of 2-50. Obviously this is not ideal as influenza is most threatening in young children

and the elderly. A subunit vaccine would be preferable, but until now only 1 nasal subunit vaccine had been licensed which had to be withdrawn from market because of presumed side effects of the adjuvant [26].

The poor efficacy of nasally administered vaccines is caused by the physiology of the nasal cavity. Compared to muscle or subcutaneous tissue, the nasal cavity has a different immunologic build up, as it is a mucosal site. Moreover, the antigen has to find its way through several barriers (mucus, epithelium), in a limited time frame (nose cleared every 20 minutes removing all constituents trapped in its mucus), before it is absorbed into the body. So, if nasal vaccination is to be successful, the vaccine's formulation should be adapted to the challenges the physiology of the nasal cavity provides. Indeed, in the literature a wide variety of vaccine formulations are described (mainly tested in mice) that increase the efficacy of nasally administered antigen [27-32]. Although these studies are very encouraging and provide valuable information on the use of absorption enhancers and adjuvants, an integral approach combining the positive characteristics of these various formulations is hardly described. Increasing knowledge on the pathways and bottlenecks involved in nasal vaccination will make it possible to optimize the formulations and rationally design nasal vaccines.

Aim and outline of this thesis

Nasal vaccination has the potential to provide protection combined with more patient comfort and a higher safety profile than classical injectable vaccines. However, the nasal physiology and immunological aspects of the nasal epithelium hamper the efficacy of nasally administered vaccines.

The aim of this thesis is therefore three-fold:

- to identify the principal hurdles to successful nasal vaccine delivery;
- to develop preclinical model systems to investigate these hurdles;
- to apply these principles to rationally design nasal subunit vaccine formulations in a preclinical setting.

In **Chapter 2** the main physiological hurdles that have to be overcome to render nasal vaccination successful are reviewed. The progress made in the field of nasal delivery of subunit vaccines is described and emerging opportunities for improving nasal vaccines are discussed.

Throughout this thesis ovalbumin (OVA), a 45-kDa protein purified from chicken eggs and widely used in immunology, was used as a model subunit antigen. As nasal administration of plain OVA does not result in effective seroconversion, the effectiveness of a nasal vaccine formulation can be easily assessed using this antigen. A model system to investigate one of the main physiological hurdles, penetration of the mucosal epithelium, is discussed in **Chapter 3**. A cell culture system based on an intestinal epithelial cell line (Caco-2) including M-cells is introduced as a tool to assess the transport of vaccine delivery systems through the mucosal epithelium. Moreover, monocyte derived human dendritic cells (DC) are explored to investigate the role of DC uptake and maturation. The predictive value of these *in vitro* assays is studied by intraduodenal vaccination with OVA encapsulated in two potential mucosal vaccine delivery systems, chitosan and N-trimethyl chitosan (TMC) nanoparticles.

To address the mechanistics behind nasal vaccination, the characteristics of 3 nasal delivery systems, based on nanoparticles composed of PLGA, TMC, or both, are correlated to their capacity to induce antibody production (**Chapter 4**) and CD4⁺ T-cell activation (**Chapter 5**) or tolerance (**Chapter 5**) after nasal administration in mice. Moreover, a new method to assess the residence time of an antigen in the nasal cavity is introduced (**Chapter 4**).

The effectiveness of cationic liposomes, a promising delivery system for injectable vaccines, as carriers for nasal vaccines is investigated in mice (**Chapter 6**) and compared to other application routes, i.e. epidermal, intradermal and intranodal administration. Furthermore, this chapter describes investigations on the usefulness of (1) encapsulating antigen in vesicles and (2) co-encapsulation or co-administration of an adjuvant.

In **Chapters 7-10** the knowledge gained from the first chapters on the mechanistics behind nasal vaccination is applied to improve the most promising delivery system tested, being nanoparticles based on TMC. In an attempt to make smaller TMC/antigen entities, TMC-antigen conjugates are developed and characterized physicochemically and immunologically in **Chapter 7**. Nasal application of these conjugates and their interaction with various components of the murine immune system are described in **Chapter 8**.

Chapter 9 concerns the replacement of tripolyphosphate, a physical crosslinker used to prepare TMC nanoparticles, with the adjuvant CpG, acting as a crosslinking agent as well as an immune modulator, and the effect on particle characteristic and immunogenicity. In **Chapter 10** a variety of other adjuvants described in the literature are encapsulated in TMC nanoparticles and their effectiveness as immune potentiators as are assessed in mice.

Chapter 11 summarizes the results and conclusions in this thesis. Moreover, future directions concerning the developed and rational design of nasal subunit vaccines are discussed.

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