Chapter 8

Discussion

Summary and perspectives

Summary

Today most human vaccines are given by intramuscular or sometimes by subcutaneous injection. Of all currently licensed vaccines against infectious diseases, estimated between 25 to 30 vaccines, only a few are available as formulations for alternative routes:

- Vaccines against polio, typhus, rotavirus and cholera can be delivered via the oral route.
- Vaccines against smallpox, influenza, bacillus Calmette-Guérin (BCG), and rabies are available as intradermal vaccines.
- Flumist (Fluenz in Europe) is a nasal vaccine against influenza.

Needle injections are effective in achieving the desired immune response but have several drawbacks such as safety (needle stick injuries and risk of cross-contamination), costs and limited acceptance by the public. To overcome these drawbacks, alternative delivery systems are being developed.
In this thesis, three delivery systems have been investigated:

1) Elastic vesicles composed of phosphatidylcholine and a detergent or only detergent. The vesicle formulations are applied topically. The highly deformable bilayers of these vesicles increase the transport across the stratum corneum (SC).
2) The Bioneedle, a hollow thermoplastic starch implant, which can be filled with vaccine. After freeze drying, the implant can be delivered subcutaneously.
3) Liquid jet injector, a disposable syringe jet injector (DSJI) which delivers the vaccine under high velocity.

One of the main problems in vaccine development is the lack of correlation between results obtained in experimental animals and in humans. In the specific case of dermal vaccination, apart from immunological differences also

anatomical differences (e.g. skin thickness, anatomy and composition) exist. This hampers the development of dermal vaccines substantially. In **chapter 2**, the differences between human and animal species, which should be considered when developing dermal vaccines, are discussed. The main challenge of dermal vaccination is overcoming the SC. Human skin and animal skin has different SC thickness. Even within an animal species, the choice of application site can have implication for absorption into the skin because epidermis thickness might differ. Differences in skin immunology between the species are described, which may contribute to differences in immunological response between human and mouse studies. The physiological and structural differences of the skin and immune system between humans and laboratory animals should be taken into consideration to facilitate translation of the results of animal models to clinical studies.

In **chapter 3**, a study in mice with topically applied hepatitis B surface antigen (HBsAg) associated to vesicles, is described. Elastic vesicles were prepared composed of either sucrose-laurate ester and octaoxyethylene-laurate ester (L595 vesicles) or of soybean–phosphatidylcholine and Span-80 (sPC vesicles). HBsAg was associated to these vesicles and the formulations were applied on intact skin and on microneedle-pretreated skin of the abdomen of mice. The vesicles induced in mice via TCI an antibody response only when the skin was pretreated with microneedles. Cholera toxin, a commonly used skin adjuvant, increased the immune response of the vesicle formulations with higher number of responding animals. The sPC-HBsAg vesicle formulations showed to be the most immunogenic for transcutaneous immunization (TCI), which was related to the higher degree of HBsAg association. The response was however significantly lower than the conventional i.m injection of aluminum hydroxide ($Al(OH)_3$)-adjuvated HBsAg formulations. Further research is needed to fully optimize the TCI procedure.

Bioneedle formulations were developed with tetanus toxoid (TTx) or HBsAg. The thermo-stability was determined at different temperatures and the immunogenicity in mouse models was determined. *In vitro* thermo-stability tests demonstrated that Bioneedle formulations with TTx (**chapter 4**) and HBsAg (**chapter 5**) were, up to 60 ^{o}C, more stable than liquid formulations. Bioneedle

formulations with TTx were prepared with and without the adjuvant aluminum phosphate ($AlPO_4$). Mice, immunized with $AlPO_4$-adsorbed TTx formulated in Bioneedles, induced comparable antibody responses as mice immunized with the equivalent liquid formulations. A dose titration study with the adjuvanted TTx Bioneedle formulations and the regular liquid vaccine, showed that with Bioneedle formulations, four times less antigen is needed.
HBsAg-Bioneedle formulations were prepared with either no adjuvant or with the adjuvant aluminium hydroxide ($Al(OH)_3$) or LpxL1 (LPS derivative). Bioneedle formulations containing the lipopolysaccharide based adjuvant LpxL1, induced after prime immunization higher antibody responses as compared to the prime immunization with conventional liquid HBsAg-$Al(OH)_3$ formulation or the HBsAg-Bioneedles without adjuvant or the $Al(OH)_3$ - adjuvated HBsAg-Bioneedle formulations. After booster vaccination with LpxL1-HBsAg Bioneedles, comparable antibody responses were induced as with i.m injection of the conventional liquid $Al(OH)_3$-HBsAg vaccine. Immunization studies with heat-treated formulations showed no correlation between immunogenicity and antigenicity: although antigenicity was reduced up to 54% (liquid formulations) and 67% (Bioneedle formulation) after heat treatment, obtained IgG titers were comparable to the non-heat treated (100% antigenicity) formulations. Bioneedle formulations with TTx and HBsAg-LpxL1 are more thermo-stable than the liquid formulations and showed comparable immune responses as the conventional liquid vaccines. Therefore, the Bioneedle formulation is a good alternative for needle injections.

In a phase I clinical trial (**chapter 6**), massive, non-hollow Bioneedles were delivered in a safe way. Clinical and histological assessment demonstrated good local tissue tolerance for the thermoplastic starch. The thermoplastic starch was mostly degraded within several hours and remnants were cleared by macrophages. The delivery procedure was pain free and volunteers graded global tolerance of the Bioneedles as 'very good' or 'good'.

For the PharmaJet liquid jet injector, preclinical studies in rats and ferrets failed because the animal models were too small (data not published) for this needle-free delivery device. In **chapter 7**, the suitability of the minipig model was evaluated for intradermal vaccine delivery with the PharmaJet injector. Minipigs were

immunized with HBsAg by using the PharmaJet injector (intradermal), and the conventional needle and syringe (intradermal and intramuscular). Intradermal delivery of the vaccine by either DSJI or by needle and syringe resulted in a bleb under the surface of the skin. Pigs that were immunized by DSJI showed less signs of pain or stress than pigs that were immunized intradermally by needle injections. Prime immunization by using the DSJI resulted in a higher antibody titer than did conventional intradermal immunization and a comparable titer to that after intramuscular needle vaccination with HBsAg and $Al(OH)_3$ adjuvant.

Perspectives

The "ideal" vaccine

Many technologies are under development to administer vaccine through and in the skin in an effective and painless way. The ideal vaccine is effective, causes no adverse effects, is cheap, stable at elevated temperatures, easy, safe, fast (i.e. number of vaccines per unit of time) reliably to apply and painless . As this is realistically speaking not possible, the requirements for needle free devices should be selected on their intended use (figure 1). The main differentiating factors are low costs, absence of pain and logistical factors like ease of application and stability of the formulation. In the studies described in this thesis, three alternative delivery systems have been assessed and these are discussed in the next section.

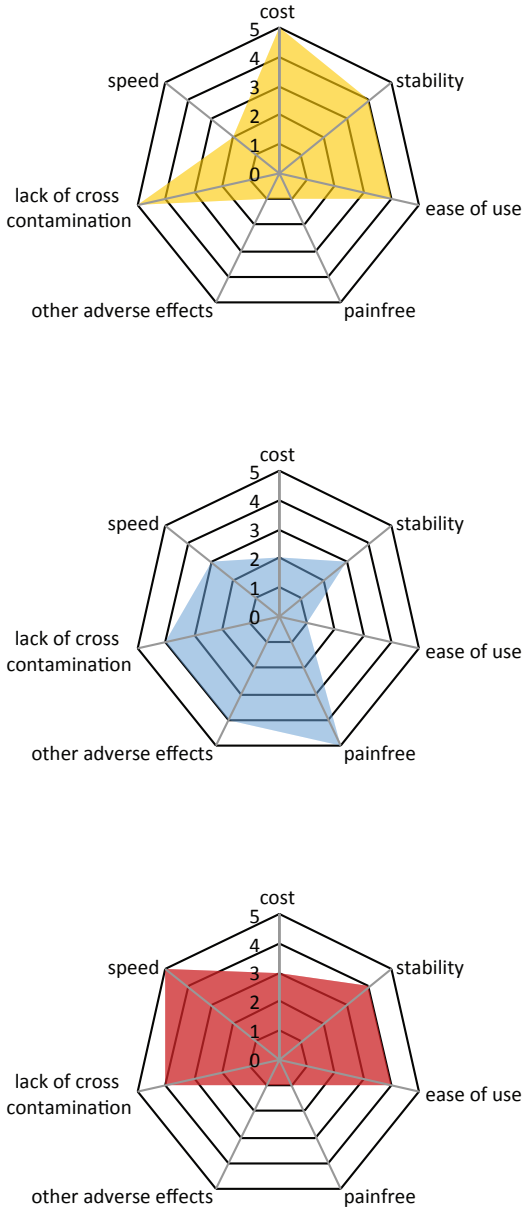

Figure 1. Product characteristics of needle free vaccine delivery systems for developing countries (top), industrialized countries (middle) and emergency vaccinations (bottom). Scale: 0 less important 5: important [1].

Alternative delivery systems

-Elastic vesicles and microneedles

The topical application of vaccine formulations is a non-invasive delivery method with the advantages of being needle-free, easy to apply and painless. The antigens are targeted into the skin where many antigen presenting cells (APCs) are present. This delivery method would be the ultimate alternative delivery system. However, the SC barrier is a major hurdle that has to be overcome.

Elastic vesicles have been reported to enhance the delivery of vaccine components through intact skin [2, 3]. Antibody responses are induced comparable to i.m injections. In chapter 3, HBsAg-elastic vesicles were prepared and applied topically on intact skin. The transcutaneous enhancement obtained by the vesicles was not sufficient to induce an immune response. The HBsAg-elastic vesicles were only able to induce an immune response when the SC was physically disrupted by microneedle application. The microneedles create small channels in the SC and underlying viable epidermis, thereby enhancing the penetration of topically applied formulations. The presence of cholera toxin (CT), a common skin adjuvant, increased the efficiency by reducing the number of non-responder mice.

Preparing vaccine-elastic vesicles is not straightforward. Many formulation parameters influence the properties of elastic vesicles such as size, elasticity and vesicle-vaccine association. All these properties might influence penetration efficiency, targeting to dendritic cells, the uptake in dendritic cells, and as a result, the immunogenicity. More knowledge about the mechanism and properties of elastic vesicles as well as the immune system of the skin will lead to more rational development of elastic vesicles for topical application.

The microneedle treatment prior to the topical application of elastic vesicles was effective in enhancing the immune response. Much research has been performed using microneedles for vaccine delivery into the skin [4]. The efficiency of the solid microneedle approach in combination with topical application of vaccines has not yet been shown in clinical trials. Laurent *et al.* [5] performed a clinical trial with rabies vaccine that was topically applied on microneedle-pretreated skin. They showed that abrasion with a patch of 50 microneedles of 200 µm in length was not enough to induce neutralizing antibodies. These were only induced with longer needles, delivering the vaccine into the dermis. More research is needed to elucidate whether this is dependent on the type of vaccine, on the delivery

approach, or both.
The two-step approach of microneedle pretreatment and topical application is probably difficult to implement in the regular immunization practice. Moreover, the inefficient delivery, because only a fraction of the dose will penetrate into the epidermis, is economically not attractive.
The microneedle research field has moved more towards other approaches such as hollow, dissolvable, and coated microneedles. Although these approaches also have their disadvantages, clinical trials have already shown promising results [6, 7]. These approaches could be combined with nanoparticulate delivery systems, such as liposomes, to improve the penetration through the SC and the uptake of antigens in antigen presenting cells.

-Bioneedles
Bioneedles formulated with tetanus toxoid adjuvated with $AlPO_4$ and HBsAg adjuvated with LpxL1 showed comparable antibody titers as the liquid alum-adjuvated liquid formulations delivered by i.m injections.
Aluminium salts are the most common adjuvants in liquid vaccine formulations but showed to be less convenient in Bioneedle formulations. The freeze drying process in the preparation method of Bioneedle formulations, causes coagulation of the aluminium salts, entrapping the antigens and leading to lower immunogenicity of the vaccine antigens. Maa *et al* [8, 9] showed that immunogenicity is maximized when alum particle coagulation is minimized. Coagulation of $AlPO_4$ was reduced by using trehalose (chapter 2). Beside the stabilization of aluminum particles during freeze drying, trehalose is also known to be a good stabilizer for proteins. Trehalose showed to be an effective stabilizer for Bioneedle formulations of tetanus toxoid-$AlPO_4$ but not for HBsAg-$Al(OH)_3$ formulations. Bioneedle formulations with trehalose and HBsAg-$Al(OH)_3$ resulted in several non-responders after immunization. For HBsAg, the adjuvant LpxL1 was therefore assessed as an alternative. LpxL1 is an LPS derivative and resistant to freeze drying. The LpxL1-HBsAg Bioneedle formulations showed comparable immune responses as the liquid formulations and better thermo stability than the liquid formulations and the HBsAg-Bioneedle formulations without adjuvant. It was demonstrated that LpxL1 plays a role in conserving the antigenicity upon freeze-drying, but the mechanism of stabilization remains unclear. The studies with HBsAg- and tetanus toxoid Bioneedle formulations

showed, comparable to the conventional liquid formulations, that an adjuvant is required to optimize the immune response. The optimization and stability of these formulations requires an understanding of the sensitivity for freezing and drying of not only the adjuvant and the vaccine but also of the combination of adjuvant and vaccine.
In vaccination programs, cost reduction is an important factor for the future success of the Bioneedles. Several factors play a role in this:
The improved thermo-stability at higher temperatures as compared to liquid formulations is a big advantage. Especially in developing countries, where temperatures are high and the infrastructure is poor, the cold chain represents a major economic and logistical burden as well as the risk of use of sub-potent (i.e. heat exposed) vaccines. Vaccine formulations such as Bioneedles, which show significantly better thermo-stability than liquid formulations, could offer a solution to the cold chain burden. The cold chain is estimated to cost vaccine programs worldwide between 200-300 million dollars per year [10] and represents the major logistic costs. Logistic costs can significantly be reduced with Bioneedle formulations, when no cold chain is needed.
Bioneedle formulations are compact in size and require less packaging and less storage space. All this, contributes to a reduction of the total costs of vaccination programs. In contrast, the freeze drying procedure is an expensive process. When comparing costs, costs from medical care necessary due to blood-born pathogen transmission caused by needle stick injuries and unsafe vaccination practice have also to be considered. Bioneedles are single implants and are delivered with a special designed device. The device is not in contact with the patient's body. Therefore, the chance of cross-contamination from one person to another is negligible.

The development and production of Bioneedles are still in the early stage of development. Beside tetanus toxoid and HBsAg, also influenza [11], BCG [12] and inactivated polio vaccines (Kraan *et al*., in preparation) were successfully formulated in Bioneedles and showed comparable immune responses (comparable titers and type of response) as the equivalent injected vaccine. A clinical trial with 'empty' (no vaccine) Bioneedles was conducted and showed that Bioneedles are safe and well tolerated (chapter 6). Clinical trials with vaccine containing Bioneedles will demonstrate whether Bioneedles are effective in

inducing immune responses in man. To this purpose, collaboration with vaccine developers and manufacturers is needed to further develop the delivery system in full compliance with good manufacturing procedures (GMP). The current lab scale manufacturing process of Bioneedles consists of preparing massive (non-hollow) implants, which are subsequently hollowed out. The cavity is thereafter filled with the vaccine formulation. The development of a feasible and robust large scale manufacturing process, under GMP conditions, will be a challenge.
The clinical trial with empty Bioneedles (chapter 6) demonstrated that Bioneedle material could not be detected anymore, 1 day after application, most probably due to its dissolving behaviour. This was however a trial with a low number of participants. Additional research is needed to study in detail the dissolving kinetics of the Bioneedle material and the release kinetics of the vaccine component upon delivery.
A consistent Bioneedle filling procedure is crucial to assure a consistent dose. The limited fill volume of 4-5 µl hampers a filling procedure without too much vaccine spillage. The limited fill volume requires high concentrations of both vaccine antigen and adjuvant. For this reason, and the fact that they are difficult to lyophilize, aluminium salt suspensions are less suitable as adjuvant in Bioneedle formulations. Whether this is a disadvantage remains to be seen. The effect of aluminium salts on human immune responses is not always evident. Antigens formulated in Bioneedles without aluminium salt may be non-inferior as compared to alum salt adsorbed fluid vaccine.

-Liquid Jet injector
The liquid jet injector can deliver vaccines at the same depth as needle injections: subcutaneous, intramuscular and intradermal. The main difference is that the liquid jet injector is a needle free device, and the vaccine is delivered under high speed. The high delivery speed is a fixed parameter for liquid jet injectors whereas with conventional needles and syringe, the speed is much lower and can be adjusted. This makes the intradermal delivery with needle and syringe in small animals such as mice possible, although trained personnel are needed. Intradermal injection with the PharmaJet injector was not possible in mice and ferrets. The fixed injection speed was too high for these small animals, resulting in skin– to–skin penetration in the fold of the skin used to assure dermal vaccination. Therefore, the mini-pig as animal model was assessed

(chapter 7) with a hepatitis B vaccine and showed to be suitable for intradermal delivery. Mini-pigs are larger than mice and need more space and special care. Consequently, experiments with mini-pigs will be much more expensive and the number of animals per group is therefore limited. More extensive experiments are needed to judge the efficacy and stability of hepatitis B vaccine delivered by the liquid jet injector. Alternatively moving to clinical trials sooner is also an option. In the past, many clinical jet injector studies have been conducted, including dermal jet injection.

Liquid jet injectors have a long history and have already been used for mass vaccinations (polio (i.m), smallpox (i.d)). Compared to other needle-free delivery systems, such as patches and Bioneedle formulations, liquid jet injectors have the advantage that they make use of existing formulations. This brings significant cost savings through reduced times of development and clinical trials. Ekwueme *et al.* [13] estimated material costs for delivery of vaccines by needles and syringes around US$ 0.06 per injection. When taking into account the medical care costs due to pathogen transmission caused by needle stick injuries and improper needle handling, the overall costs was estimated to be US$ 26.77 per injection. However, jet injectors are relatively expensive devices but when overall costs (including costs related to iatrogenic transmission of blood- borne pathogens) were considered, prices of US$1 and US$ 0.37 were estimated for respectively the multi-use nozzle jet injectors and disposable cartridge jet injectors.

Liquid jet injectors have been used in the US to deliver seasonal influenza. In 2011 the FDA recommended not to use these devices because the combination jet injector-vaccine was not yet approved. More knowledge about efficacy and safety of the combination jet injector device - antigen is required to obtain a registered product. Safety and efficacy studies will be needed for each individual vaccine in combination with the device.

Translation from laboratory animals to clinical studies

When doing research in laboratory animals, the physiological skin differences between animals and humans have to be considered. The SC and viable epidermis in humans is much thicker and less hairy than in mice and rats. These differences have implications on the effect of cutaneous delivered vaccines. Moreover, mice and rats are small animals and can be anesthetized for a limited time

(approximately one to two hours). This time slot is an important limitation for topical applications. Naito *et al.* [14] showed that prolonged topical application leads to higher antibody responses even in the absence of an adjuvant.
The pig as laboratory animal is bigger in size, less hairy and has a SC and epidermis thickness that resembles more that of a human. Pigs might therefore be a better choice as laboratory animal than mice and rats. Pig studies are however much more expensive (more space, more food, special training of caretakers) than mice/rat studies and therefore less convenient in an early stage of research.

Much effort is put in developing better and safer vaccination methods. Each delivery method studied in this thesis has advantages and limitations (see figure 2). Not one single delivery method will be generally applicable but depending on vaccine antigen, target population and market. For each antigen and target population the most optimal delivery system and administration route need to be selected, based on scientific findings. With the new technologies and increasing knowledge to this field, more alternatives for the needle and syringe will be available in the future.

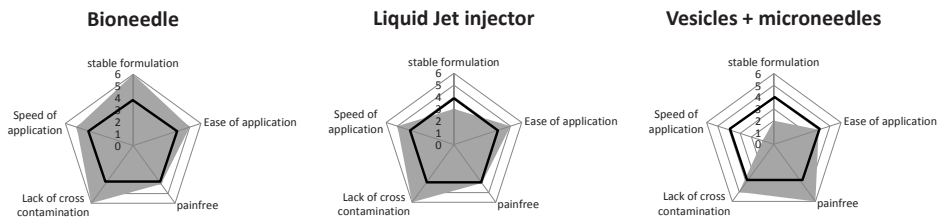

Figure 2. Pros and cons of the three delivery strategies investigated in this thesis, compared to needle injections. 0-2 = in favor of needle injection; 3 = needle injection; 4-6 = in favor of alternative.

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