Chapter 1

Introduction, aim and outline of this thesis

Introduction

The WHO estimated in 2004 [1] that 16 billion injections are given annually, of which 800 million vaccinations and the remaining 95% for therapeutic purposes. Most of the vaccines are given by subcutaneous or intramuscular injection. The proportion of needle free vaccines consists mainly of oral polio vaccine and must be seen in the light of the polio eradication program by the WHO.
The needle and syringe (N&S) was developed in 1853 by Pravaz and Wood. With

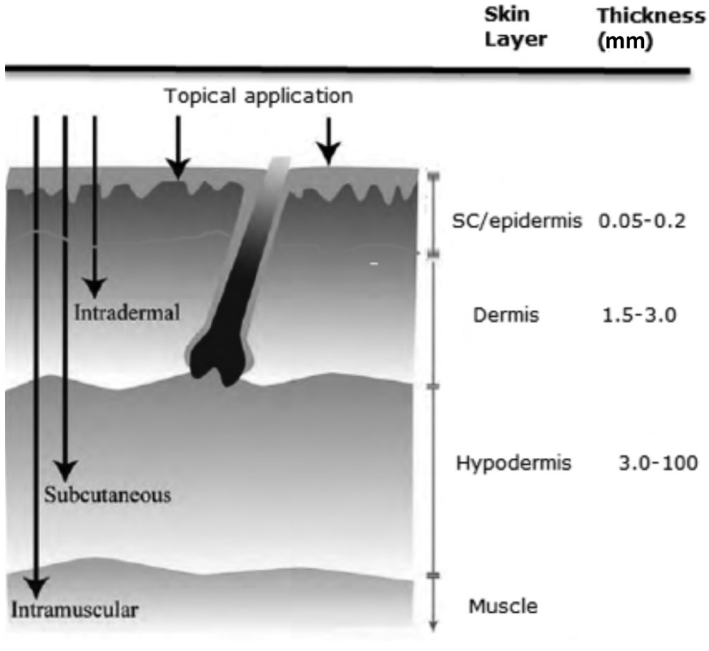

Figure 1. Cutaneous delivery (topical + intradermal), subcutaneous delivery and intramuscular delivery adapted from [2].

the N&S, vaccines can be delivered (see figure 1) in the epidermis or dermis (intradermal, i.d), in the subcutaneous layer (subcutaneous, s.c) or in the muscle (intramuscular, i.m). To date, all vaccines given via the skin are delivered s.c or i.m except for four vaccines, which are intradermally injected: smallpox, influenza, BCG (bacillus Calmette Guerrin) and rabies (only i.d in some countries).
The skin is composed of three layers. The upper layer is the Stratum Corneum (SC), which consists of mainly dead corneocytes. The SC forms a barrier, protecting

the body against pathogens. Under the SC lies the viable epidermis, composed of keratinocytes and antigen presenting cells. The SC and viable epidermis form together the epidermis. The dermis is located underneath the epidermis and is composed of dense fibro-elastic connective tissue with lymph vessels, nerves, sebaceous glands, sweat glands and hair follicles. The subcutis is the deepest layer of the skin and is composed of mainly fat tissue.
The skin is an interesting site for vaccination. In literature, many synonyms are used to indicate the delivery towards the different layers of the skin. In this chapter, the terms “topical application”, “intradermal delivery” and “cutaneous delivery” will be used. With “topical application”, the vaccine is applied onto intact skin or pretreated skin (see figure 1). The vaccine formulation travels through (part of) the SC or hair follicles, into the epidermis and the dermis. With “intradermal delivery”, the SC is physically overcome and the vaccine is delivered directly in the epidermis or dermis by for example needle injections, liquid jet injections, powderject injections or microneedles. “Cutaneous vaccination” will be used to assign both topical and intradermal application.

Why do we need needle free vaccines?

Injected vaccines (needle injections) are very successful but have a number of drawbacks that warrants the development of alternative delivery systems.

Safety

Reuse of needles and syringes as well as needle stick injuries cause many infections in patients as well as medical personnel. The WHO estimates in 2004, 23,3 million infections per year due to unsafe injections [1]. This concerns not only vaccines but all injections given.
Most infections transmitted by needles are hepatitis B (21 million), followed by hepatitis C (2.3-4.7 million) and HIV (80.000-160.000). The transmission risk from an infected person to a health care worker following a needlestick injury is estimated at 0.3 % for HIV, 3% for hepatitis C and 3-10% for hepatitis B. Of 35 million healthcare workers in the world, about 2 million are infected each year via needlestick injuries [3].

The reuse of needles is mainly a problem in developing countries. For this reason, needle free alternatives should be cheap and/or should have additional advantages like increased thermostability and shelf life. These alternatives are not yet in the market but safer needle-based alternatives are already available. Several different auto-disabling syringes have been developed such as the Soloshot (from BD), the Destroject (from Bader), The K1™ (from Star syringe Ltd), Univec™ (from Univec) and the Uniject (from BD) devices [4]. Although a huge improvement, these solutions do not circumvent needlestick injuries.

Number of injections

If given the choice between a vaccination by needle or a needle free route, a vast majority chooses the latter. In a clinical study comparing an intranasal virosomal influenza vaccine with a classical syringe and needle formulation, participants could choose between the two formulations. 97% of the participants chose for the nasal vaccine [5]. When they were asked for their motivation, 14% answered they were afraid of injections. This is in accordance with other studies; about 10% of the people has needle-phobia [6]. It is however certain that the number of vaccines, for instance in national pediatric vaccination programs, will expand in the coming decades. The Dutch Health Council has published in 2007 a report on the future of the Dutch national vaccination program [7]. Two of the conclusions were that all vaccines currently in use should stay in the program and that another 15 of 23 vaccines (existing or not yet existing) have a high enough disease burden to justify inclusion. Currently, most Dutch children receive 14 injections against 12 diseases, most of them in their first 14 months of life. The number of injections per session is maximally two. Participation in immunization programs is voluntary and this careful policy and the fact that vaccines are given free of charge, results in a vaccine coverage of more than 95%. In a Dutch study conducted by Mollema *et al.* [8], 95% of the parents reported they intended to participate in the remaining vaccinations (booster vaccinations and other future vaccinations) of the National Immunization Program. Concerns that played a role in whether or not to accept the remaining vaccinations included safety of vaccinations, maximum number of injections, vaccine efficiency and whether vaccinating healthy children is necessary.
With alternative delivery systems, some of these concerns can be taken away.

Mass vaccinations

Classical vaccines are not very suitable for mass vaccinations during emergencies. These circumstances occur when there is an outbreak of a disease that is usually contained by vaccination (e.g. polio), in case of emerging diseases (pandemic influenza, SARS) and during attacks with infectious agents. In these cases, important parameters are speed (number of vaccinations per unit of time), ease of application (no trained personnel needed) and stability (less logistical problems). Vaccines given by needle and syringe do not meet these criteria.

The drawbacks of combination vaccines

The current solution to reduce the number of vaccine injections is to combine vaccines. The applicability of combination vaccines has its limits. Combination vaccines, although very successful, have drawbacks.

High development costs

Combination vaccines are expensive to develop since combining two existing components into a combination is almost as expensive as developing the individual components. The combination formulation has to be re-developed and release tests have to be re-validated. Stability- and toxicity for the individual components and part of the clinical studies have to be repeated.

Pharmaceutical interference

The stability profiles of antigens may be different, for instance as a function of pH. This may result in reduced shelf life of the combination vaccine or the need for additional formulation work to select stabilizing excipients. Bulk concentrations may be limiting. Eventually, all components must be formulated in preferably 0.5 ml but at most 1 ml. The more components in the combination, the more concentrated the bulk materials must be. Sometimes concentration limits are reached because the production process cannot be optimized further or because the antigen in the bulk aggregates to undesired levels or too quickly at high concentrations. This may require optimization of the formulation of the ‘monovalent’ bulk materials.

Impurities

The impurity profile (proteins, nucleic acids, endotoxins) in the combination vaccine may reach unwanted levels. Specifications, apart from clear cut regulations, are often set based on the impurities in the separate components or existing vaccine. Exceeding impurity limits will increase the risk of failure during clinical trials. Therefore, attempts should be made to match the impurity profile of the old, non-combined vaccines. This may result in substantially adapted production processes and increased costs (more unit operations, lower yields), if possible at all. Sometimes the better defined antigens turn out to be less immunogenic because the removed impurities have some adjuvant effect.

Immunological interference

The optimal immunization schedules may differ between antigens in a combination vaccine. Some antigens, polysaccharides for instance, are not very immunogenic in very young children, whereas others, like vaccines against whooping cough, must be given as early as possible since most victims fall in this category.

Another problem that can occur is inhibition of the response after mixing with another antigen. Although the reason for these kinds of phenomena are often not known, it has been observed regularly [9, 10]. Absence of immune interference in preclinical studies is not reliable and therefore expensive clinical studies are needed. An example of immunological interference with serious consequences is the Hexavac vaccine, consisting of diphtheria, acellular pertussis, tetanus, inactivated polio, H. influenzae (Hib) and hepatitis B (HepB). The existing pentavalent vaccine was extended by adding the hepatitis B component. Nine clinical studies were done and the product was approved in Europe in 2000. In 2005, registration was suspended because there were concerns due to lower and varying immunogenicity of the HepB component. HepB and Hib responses after immunization with the hexavalent vaccine were lower as compared to the pentavalent vaccine plus HepB stand alone[10].

Economic risks

Production of complex combination vaccines poses economic risks. If one component in the final product fails, the whole combination fails and has to be discarded.

Alternatives

To counter the drawbacks of N&S injections, as mentioned in the previous part, alternative delivery systems and vaccines are being developed mainly for vaccination via the mucosae (oral, nasal, pulmonary, vaginal) and via the skin (cutaneous, subcutaneous and intramuscular). Here, we will only focus on alternative delivery methods via the skin.

Most of the vaccines are delivered as liquid solutions in the subcutaneous tissue or into the muscle. Alternative delivery systems, replacing the N&S, for liquid formulations have the advantage that no- or considerable less- reformulation work is needed. Examples are liquid jet injectors and hollow microneedles (see table 1). Alternative delivery systems making use of solid formulations (powder jet injectors/ballistic formulations/dissolving microneedles, coated microneedles) have the advantage of higher product stability, but need considerable reformulation.

Some delivery systems deliver the vaccine in the skin. The skin is an attractive location for immunization. The epidermis is densely populated with antigen presenting cells. These cells process antigen or micro-organisms that managed to pass the SC, the upper 15 µm of the epidermis. The SC consists of corneocytes containing mainly keratin and water surrounded by a cornified envelope. The corneocytes are embedded in a lipidic matrix consisting of mainly ceramides, fatty acids and cholesterol. When intact, the SC is rather impermeable for micro-organisms, macromolecules and to a lesser extent also for many small molecules. Therefore, the main challenge in dermal vaccination is to pass the antigen through the SC. Immunization via intact skin is only possible with a strong adjuvant or with the help of penetration enhancing methods such as chemical enhancers, electroporation, ultrasound, and abrasion (see chapter 2 & 3 of this thesis).

I.d injection of a vaccine leads to comparable or higher immune responses than s.c or i.m immunization [2]. Work of Mikszta and coworkers suggests that the kinetics of the response after i.d delivery is different [68]. I.d delivery of anthrax protective antigen in rabbits resulted in more potent early antibody responses compared to i.m injection, especially when low antigen doses were given. This may be beneficial in situations of emergency vaccinations. The differences became less pronounced at longer time period intervals after vaccination. A

Table 1. Advantages and disadvantages of different alternative delivery systems. i.m= intramuscular, s.c= subcutaneous, i.d = intradermal, SC = stratum corneum, MUNJI = multi-use nozzle jet injector.

Delivery systems	Delivery site	Advantage	Disadvantages
Liquid Jet injector	i.m / s.c / i.d	-No or less formulation work -Appropriate for mass vaccination	-adverse effect slightly higher than S&N but well-tolerated -Cross-contamination with MUNJIs (obsolete) -High costs of device
Powder jet injector	cutaneous	-Small package volume -Increased stability/no cold chain -Natural targeting to antigen presenting cells in the skin	-Extensive reformulation -High costs (device/formulation) -Limited clinical history -Occasional bleeding
Monolythic formulations	s.c	-Small package volume -Increased stability/no cold chain	-Extensive reformulation
Topical application	s.c	-Easy application -High patient compliance -Natural targeting to antigen presenting cells in the skin	-Strong adjuvant or permeation enhancers needed; some permeabilization methods require expensive devices -Standardized dose delivery is difficult -Limited clinical history
Solid microneedles & Patch	cutaneous	-Technically relatively simple	-Two-step administration process -No precise and inefficient dosing
Coated Microneedles	i.d	-No patch or pump is required -Precise dosing	-Requires an efficient coating procedure -Reformulation of the drug needed -Reduction of microneedle sharpness/ penetration ability
Dissolving microneedles	i.d	-No patch or pump is required -No sharp waste (dissolving microneedles) -Precise dosing	-Impaired microneedle strength -Often less sharp microneedles -Reformulation of the drug needed
Hollow microneedles	i.d	-No- or limited- reformulation work -Delivery of higher volumes -Rate of delivery can be regulated by a pump	-Risk of clogging or blocking -Impaired microneedle strength -Increased risk of leakage and backflush for arrays -More complex device

clinical trial with polio in human showed slightly lower antibody response after 28 days when using i.d reduced dose polio, as compared to a full dose i.m injection (N&S). After 1 year, the differences were no longer apparent [11]. I.d vaccination by classical injection cannot be applied routinely because intradermal injection is difficult to perform and more painful than subcutaneous or intramuscular injection, although this may be related to the skills and experience of the vaccinating personnel.
Several alternative delivery systems will be discussed in further details.

Liquid jet injection

Liquid jet injectors make use of a high-speed (more than 100 m/sec) jet to puncture the skin. The power is furnished by compressed gas or a mechanical spring. Gas-powered jet injectors can, due to their greater driving force, deliver a liquid volume up to 1 ml. For spring-powered injectors, the volume is limited to 0.5 ml. The liquid is delivered either into the skin, in the subcutaneous tissue or into the muscle. The nozzle diameter and jet velocity determines the depth of delivery [12].
Liquid jet injectors have a long history. Already in 1866, a jet injector (aqua puncture) was described in France. In the first half of the 20th century, the procedure was reinvented and used for mass vaccination purposes for 20 to 30 years. These multi-use nozzle jet injectors (MUNJIs) were developed for US army recruits. These devices allow injection of several doses using the same nozzle and vaccine reservoir. Up to 1000 vaccinations per hour could be given. The use of MUNJIs was abolished when it became clear that cross contamination from subject-to-subject could occur. In 1995, a joint meeting of the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) concluded that MUNJIs presented an unacceptable risk to the vacinee. To minimize the risk of contamination, protective devices have been developed for the MUNJIs. Since 1998, PATH (Program for Appropriate Technology in Health) has collaborated in the development and testing of protector cap needle-free injectors, which uses a disposable plastic cap as a shield between the injector nozzle and the skin. However, clinical studies revealed the caps were unable to prevent contamination [13]. Today, a new generation safe disposable cartridge jet injectors (DCJIs) are available.

One of the concerns with liquid jet injections is whether the shear forces, generated when the vaccine is forced though the small orifice, could damage the antigen. Benedek *et al.* showed with several model proteins no damaging effects concerning aggregation and degradation. This should however be tested for each individual vaccine-injector device combination [14].
Many clinical studies have been performed with liquid jet injectors (see table 2). Clinical studies show consistently that the number of responders and the mean antibody response are comparable to N&S injections, regardless the injection depth [15-18]. Some studies even show better immune responses as compared to N&S injection [19, 20]. This may be caused by a better tissue distribution of the vaccine. Instead of a bolus, the fluid is dispersed more homogeneous. Several intradermal studies with jet injectors showed conflicting results. The skin is populated with many antigen presenting cells (APCs). Targeting vaccine to the skin promotes its contact with these APCs and this might reduce the antigen dose. Clinical trials with polio (IPV) have been conducted in Cuba [21] and in Oman [15]. Fractional dose of IPV, delivered i.d with a jet injector, was compared to full dose i.m injection with N&S. In the Cuban study, infants were vaccinated with 3 doses given four weeks apart, beginning at 6 weeks of age. The i.d fractional dose, delivered with the jet injector resulted in lower seroconversion rates and antibody titers as compared to the full dose i.m injection (N&S). The researchers concluded that IPV vaccination at 6 weeks of age is too early and that existing maternal antibodies partly inhibited the immune response. Parallel to the Cuban study, a clinical trial under similar conditions was conducted in Oman. The vaccination schedule was however different: infants were vaccinated at 2, 4 and 6 months. In this study, the two vaccination strategies (i.d and i.m) resulted in comparable seroconversion rates but the i.d route showed lower antibody titers as compared to i.m injection. Both clinical studies did not include an arm with i.m fractional dose and could therefore not distinguish whether the lower antibody titers in both studies should be attributed to the lower antigen dosage or the i.d delivery with the jet injector. Recently, Soonawala *et al*. [11] conducted a clinical trial with IPV, including this third fractional i.d arm. Adults were vaccinated either intramuscularly with a full dose IPV (N&S or jet injector) or intradermally with a fractional dose of IPV (N&S or jet injector). They showed that i.m vaccination with a fractional dose (N&S) was statistically inferior to full dose i.m (N&S). In contrast, the i.d fractional dose delivered with a jet injector

Table 2. Clinical studies with DCJIs and MUNJIs.

Device	System	Vaccine	Result summary	Reference
PharmaJet	DCJI Spring-loaded	Polio	-Less painful, more local effects than N&S -Fractional i.d immunization with jet injector similar titers as full dose i.m immunization with N&S and higher titers than fractional i.m with N&S	[11]
Biojector®	MUNJI Gas-powered	Hepatitis A	-Higher level of seroconversion compared to N&S -Higher local reactivity	[19]
PharmaJet	DCJI Spring-loaded	Polio (IPV)	-Fractional dose i.d with jet injector was less effective than full dose i.m vaccination (N&S)	[25]
Mini-Imojet®	DCJI Spring-loaded	Influenza, Typhoid, Tetanus toxoid, Hepatitis A	-Similar or improved immunogenicity compared to N&S	[20]
Biojector™ 2000	MUNJI Gas-powered	Polio	-Similar level of seroconversion, but significant lower titers, after fractional dose i.d vaccination compared to i.m full dose with N&S	[15]
Biojector™ 2000	MUNJI Gas-powered	Polio	-Lower seroconversion with fractional dose i.d vaccination with jet injector compared to full dose i.m with N&S	[21]
HSI-500® Protector Cap Needle free injector	MUNJI Spring-loaded	(Saline)	-Study was ended early because HBV contamination was found in the sterile saline immediately following injection	[13]
VitaJet™ Biojector 2000®	DCJI Gas-powered	Influenza	-Higher pain ratings on immunization than N&S -More local site reactions than N&S -Comparable immune response as N&S	[16]
LectraJet®	DCJI Spring-loaded	Influenza	-Increased reactogenicity -Comparable immune responses	[17]
Injex Jet	DCJI Spring-loaded	MMR	-No significant difference in pain as compared to N&S -Comparable immune response	[18]
DermoJet	MUNJI Spring-loaded	Several vaccines	-Jet injector can be safely used, without contamination risk	[26]

showed comparable responses as the full dose i.m injection. In line with previous studies, more transient vaccination site erythema and swelling was observed with i.d jet injection.
To date, several liquid jet injectors are available on the market. In the US, seasonal influenza vaccine has been delivered using a jet injector.
The new generation DCJIs have countered the cross contamination problems but acceptance of jet-injectors has been low because the system is not always painless and because of occasional bleeding at the site of injection. These side effects occur because penetration depth and jet velocity is not well controlled. Therefore, several experimental injectors have been developed, focusing on minimizing pain and bruising. These include pulsed microjet injectors [22], variable velocity injectors [23] and feed-back controlled injectors [24]. With pulsed microjets, a piezoelectric pulse generator drives a piston, delivering 2 – 15 nl fluid per stroke through a micronozzle. At a frequency of 1Hz about 1 microliter/min can be delivered into the skin. Due to the small volume per pump cycle the injection depth is only 200 – 400 micrometers, i.e. true dermal delivery is easier to achieve. This may reduce or prevent pain, bleeding and other local adverse effects sometimes seen after 'conventional' jet injection. Delivery of larger volumes may be achieved by the use of nozzle arrays and increased piston frequency. These improved designs may also be suitable for standard vaccination.

Ballistic delivery

Balistic delivery make use of solid particles. To this purpose, vaccine formulations have to be developed with freeze drying and spray drying techniques. The removal of a protein's hydration shell can result in aggregation via protein unfolding. To overcome this problem, the addition of lyo-and cryoprotectant excipients is needed to preserve the native protein structure. This implicate extensive formulation work but result in formulations which are generally more thermo-stabile than liquid formulations.

Powder Jet injections

Powder jet injections make use of helium powered injectors to deliver drugs or vaccines as a dry powder into the epidermis. This immunization method is called epidermal powder immunization (EPI) [27]. Uniform dosing is difficult

since relatively small differences in particle size results in large differences in kinetic energy and, as a result, in penetration depth. An interconnected optimization process for injector device parameters [28] and vaccine powder particle characteristics [29] is needed to deliver the vaccine in a consistent way to the narrow target epidermal region. Particles less than 100 μm in diameter have been reported as pain-free, while particles smaller than 20 μm were unable to penetrate into the epidermis [30].
The EPI approach shows promise with respect to DNA vaccination. Several preclinical studies have shown comparable or superior efficacy of EPI to i.m and s.c injections [31-34].
Human clinical trials with influenza vaccines have reported painless delivery of DNA vaccines using powder injections [35] and antibody responses that were comparable to i.m injections [36]. However, seroprotection was higher with i.m injection as compared to EPI.

Monolithic formulations
Solids can also be injected as monolithic formulations, circumventing the problem of particle size differences. The biodegradable implant contains the antigen and is injected by air pressure or a released spring. The implant dissolves and the vaccine is released.

- Glide pharma developed a solid drug delivery system for the injection of drugs and vaccines in solid doses (see figure 2 (1)). The implant is a pointed rod of about 4 mm in length and 0.8 mm in diameter. The implant is delivered by an actuator powered by a mechanical spring. Non-clinicial data with influenza suggests enhanced immune response for antigens delivered with the Glide solid dose injector [37].

- Myschik and coworkers developed lipid implants, which promote the sustained release of antigen. They prepared liposomal dispersions of Quil-A, cholesterol and phosphatidylcholine and compressed the lyophilized powder into implants. Crystalline cholesterol was included to achieve sustained release. The compressed implants had a cylindrical shape with a diameter of 2 mm and a weight of approximately 9 mg. In mouse studies, ovalbumine containing lipid implants stimulate an immune response equivalent to that induced by a prime and boost with a comparable injectable vaccine [38, 39].

- Bioneedles (see figure 2 (2)) are fabricated from starch. Studies with hepatitis B antigen and tetanus toxoid are described in this thesis.

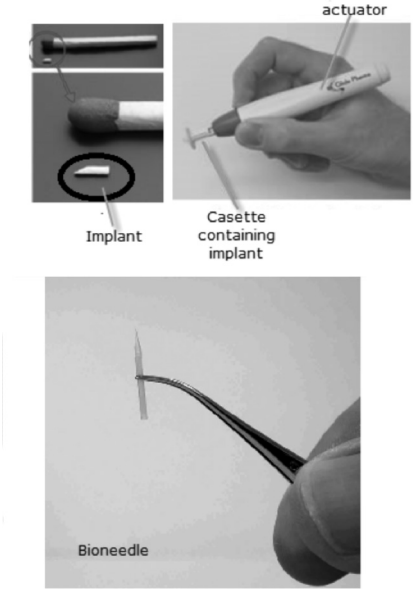

Figure 2. 1) Glide Pharma injector: the vaccine in solid form is formulated as an implant. A special designed actuator delivers the implant through the skin; 2) Bioneedle: the vaccine is freeze dried in the Bioneedle. The Bioneedle is delivered subcutaneously by air pressure. Subsequent dissolution releases the vaccine.

Topical application

Small molecules (<500 Da) might pass through intact skin [40] but for vaccine antigens, which are often larger than 30 kDa , the SC is a true barrier. Vaccination through topical application is only possible with the help of a strong adjuvant (ADP ribosylating exotoxins) or penetration enhancer such as chemical molecules, electroporation, ultrasound, abrasion. Cholera toxin (CT) and heat-labile enterotoxin (LT) are molecules of ~85 KDa, which have strong adjuvant effect for co-applied antigens when administered topically.

ADP-ribosylating exotoxins

ADP-ribosylating exotoxins like LT and cholera toxin (B subunit), are able to induce potent immune responses and are strong mucosal adjuvants. CT and

LT have ADP-ribosyl transferase activity, and are believed to bind to the GM1-ganglioside receptor [41] on the cell membranes of the skin dendritic cells. Glenn and coworkers demonstrated that application of a patch containing LT to the skin leads to potent immune responses in man [42]. Enterotoxigenic *Escherichia coli* (ETEC) can secrete heat-labile (LT) and heat-stable enterotoxins, which cause diarrhoea. Vaccines that induce immunity to heat-labile toxin offer protection against diarrhoea from ETEC. Phase 2 clinical studies with LT patches against ETEC travelers' diarrhea showed promising results [43]. A vaccine patch containing LT was safe and feasible, with benefits to the rate and severity of travellers' diarrhoea. A recent phase 3 clinical trial showed however only limited protection against LT positive ETEC diarrhea [44]. Although LT in a dermal patch vaccine was unsuccessful, LT may be used as a dermal adjuvant. LT is currently available as purified recombinant *E. coli* [45] or plant expressed material [46]. The dose required for an injected vaccine can be lowered by combining a vaccine injection with a skin patch containing the adjuvant. This dose sparing approach has the advantage that no reformulation of the existing vaccine is needed. Adjuvant patch and the antigen injection need to target the same draining lymph node [47] and should therefore be delivered in close anatomical proximity. The hypothesis that antigen and adjuvant should be delivered simultaneously and in close physical contact has been countered with this study. Delivery of other antigens than LT and CT in a sufficient high dose via a patch is only possible after physical disruption of the SC [48].

(Chemical) penetration molecules

Chemical penetration enhancers are mostly amphiphilic molecules like surfactants and fatty acids. The mechanism of action of some types of elastic vesicles (see below), consisting of surfactants, may be the adsorption enhancing capabilities. Other adsorption enhancers are less suitable because of their poor solubility and effect on protein stability. Hammond *et al*. showed in mice a delivery improvement of CT when the skin was pretreated with a combination of glycerol and isopropanol [49]. These types of compounds are compatible with many protein antigens and would be suitable in vaccine formulations. Their penetration enhancement effect has not yet been tested on human skin.

Certain peptides containing so called Protein Transduction Domains that facilitate transport across cell membranes also facilitate transdermal transport.

This requires association between transporter peptide and cargo. Possibly a recently discovered peptide may provide new opportunities [50]. In rats, a cyclic 11-mer peptide facilitates transport of insulin to the circulation without the need of association. The mechanism is yet unclear. Transport via hair follicles seems to play a role, making the applicability in humans maybe less interesting.

Elastic vesicles

Elastic vesicles are liposome-like structures consisting of surfactants alone or in combination with phospholipids with a low transition temperature. Due to the high bilayer fluidity and/or presence of 'destabilising' micelle forming surfactants, they are ultradeformable. When applied to the skin, they are able to penetrate the SC, possibly via channel-like imperfections in the SC [51]. The first generation elastic vesicles (Transfersomes®) was introduced by Cevc *et al.* Transfersomes consist of phospatidylcholine and edge activator, such as sodium cholate [52, 53]. Van den Bergh *et al.* introduced elastic vesicles consisting of only surfactants [54]. Antigens formulated in Transfersomes have been reported to induce comparable IgG responses in mice as the same formulation delivered by subcutaneous injection [55]. The IgA levels in serum were however higher with the Transfersome formulations. The mechanism of action, apart from the above mentioned adsorption enhancement is proposed to be movement from the skin surface into the epidermis via a transepidermal osmotic gradient [52, 56]. According this mechanism of action, deformable liquid state vesicles will diffuse into the skin, especially when the vesicles are applied in a non-occlusive manner. Occlusive application on the other hand, does not lead to penetration of intact vesicles but lipid plaques are formed in the SC [51]. This may however be enough for immunization purposes. It has been reported that physical association of antigen and vesicle makes the process more efficient although mixing antigen and vesicle (which may result in unnoticed association) also can result in potent immune responses [57]. Association of antigen to the delivery vehicle may affect the elastic properties of the vesicles, reducing transport ability, although Mishra *et al.* achieved extraordinary results with hepatitis B surface antigen associated with elastic vesicles. Immune responses in mice were comparable to parenterally given, equal doses of alum adsorbed antigen. This indicates very efficient transport into the dermis, which is in accordance with in vitro transport efficiency of more than 60% [57]. Other types of elastic vesicles

are ethosomes, which consist of high percentage ethanol in the formulation. Topical applied ethosomes formulated with hepatitis B surface antigen (HBsAg) induced comparable immune responses as i.m injections of alum-adjuvanted HBsAg [58].

Electroporation

With electroporation, high-voltage pulses result in structural perturbation of the lipid bilayer in the SC, thereby enhancing the penetration in the skin. New available non-invasive probes, make this technique less invasive than needle injections. A study in mice [59] with ovalbumin showed OVA-specific CTL responses to the vaccine delivered by electroporation, that were comparable to i.d injected vaccine. In a more recent study, electroporation mediated DNA vaccination conferred protection comparable to that observed following vaccination with FDA-approved anthrax vaccine [60]. Disadvantages of the electroporation method is the bulky equipment and the pain caused when the pulses are no longer confined to the SC and stimulate the lower lying nerves and neurons [61].

Ultrasound

Ultrasound at frequencies in the range of 20 kHz-16 MHz has been used to enhance skin permeability. Delivering vaccine by ultrasound can be performed by including the vaccine into the coupling medium or by pre-treatment of the skin with ultrasound and subsequently applying the vaccine on the skin [62]. Including the vaccine in the coupling medium might lead to damage of the antigen, which is exposed to the ultrasound waves. Tezel *et al.* applied tetanus toxoid on ultrasound pretreated skin of mice. IgG responses with 1.3 µg toxoid, delivered with ultrasound, were as high as 10 µg delivered by s.c injection [63]. The application of ultrasound resulted in activation of Langerhans cells in the epidermis and migration to the lymph nodes. Ultrasound is still a poorly understood technique which is influenced by many experimental parameters and which require high doses of vaccine (only 1% of the applied dose penetrates in the skin [64]).

Abrasion

Piercing or abrasion of the SC can facilitate entrance of antigens to the epidermis by several orders of magnitude. If the damage is restricted to the SC no pain will be perceived. Several abrasion methods exists such as rough surfaces, tape-stripping and microdermabrasion devices.

The Skin Prep System (SPS) [65] provides a controlled method to disrupt the SC. The SPS has been tested in human volunteers and was well tolerated and showed to be appropriate for self-application. Apart from SC disruption as pretreatment, followed by application of antigen [48], microstructures have been described that were coated with DNA [66]. The device is wiped over the skin, resulting in genetic immunization.

Microneedles

Rather than avoiding needles, needles have been downscaled such that they are long enough to penetrate effectively into the skin, to target the antigen presenting cells, but small enough to improve acceptability and safety. Four general microneedle approaches have been developed using solid microneedles (poke and patch approach, coat and poke approach, poke and release approach) and hollow microneedles.

Solid Microneedles: Poke and patch approach

Solid microneedles pierce the SC after which the patch with vaccine is applied. The vaccine enters the skin by passive diffusion. Solid miconeedles to pre-treat the skin can be fabricated from titanium, silicon, ceramics stainless steel or glass [64, 67]. With this approach, part of the vaccine formulation stays in the patch and is not delivered. Long application times are needed in order to minimize vaccine loss. Several preclinical studies showed that intradermal injection is much more effective than topical application of vaccine on microneedle pretreated skin [66, 68-70].

Solid microneedles: Coat and poke approach

With the coat and poke approach, solid microneedles coated with vaccine are used. Multiple coating methods and coating devices have been developed [71]. Dip coating procedures onto stainless steel microneedles have been described [72]. A variety of materials from proteins to microparticles, could be coated in

a reproducible manner and released into cadaver skin in a quantitative manner. Several influenza preclinical studies have been performed in mice and showed improved immunity as compared to s.c or i.m injection [73-77]. Studies with BCG in guinea pigs and with hepatitis B surface antigen in pigs showed improved immunity as compared to i.d injections [78, 79].

Solid microneedles: Poke and release approach

A way of delivering vaccines with the poke and release approach is by using dissolving microneedles [80-85]. These microneedles are fabricated from safe, inert, water-soluble materials such as polymers and sugars. The microneedles dissolve in the skin after insertion. Upon dissolving, the vaccine is released in the skin. Since the microneedle may not insert fully in the skin, some dissolving microneedles are formulated with only vaccine encapsulated in the tips of the needles. Guo *et al.* [80] developed a dissolving polyvinylpyrrolidone microneedle array where the tips were loaded with cationic liposomes containing ovalbumine as model antigen and CpG as adjuvant. Mice were vaccinated with these microneedles and induced significant higher IgG antibodies as compared to i.m injection with OVA solution.

Hollow microneedles

Hollow microneedles are fabricated from glass, silicon, polymer or metal. The vaccine is delivered through the needle hole, commonly injected with a syringe. Other hollow microneedle systems are integrated with an actuator and vaccine reservoir. The flow of liquid through the holes is controlled manually by a plunger or by CO_2 gas pressure, a spring, or a pump [86]. There are two kinds of hollow microneedles: 1) a single hollow microneedle, 2) array of hollow microneedles. Although the hollow microneedle array delivers the formulation in a wider area all at once in a fast way, leakage in one of the needles can lead to unequally distributed pressure over the array, resulting in an inconsistent delivery of the formulation. Inconsistent delivery can also occur when some needles in the array are blocked. A clinical trial with influenza vaccine in healthy adults [87] was carried out using an array of 4 silicon microneedles of 450 µm in length. I.d vaccination with 3 µg and 6 µg influenza vaccine induced a similar immune response as an i.m injection with 15 µg. Recently, van der Maaden *et al.* published a new microneedle applicator to ensure controlled and reproducible injection.

With this applicator, penetration depth, angle of insertion, and injection speed and time can be adjusted. They demonstrated *in vitro* reproducible injections. Studies with polio (IPV) in rats showed similar immune responses as with i.m injections [71, 88].
Becton Dickinson developed a single hollow microneedle consisting of a 1.5 mm needle mounted on a pre-filled syringe. Although this needle length is actually too long to call it a microneedle, this device is, in literature, considered as a microneedle. An i.d influenza vaccine of Sanofi (Intanza) using this single hollow needle (Soluvia®) is available on the market [89].

Summary

The number of registered vaccines that is applied via other routes than with classical injections is still very limited. Until about a decade ago vaccine manufacturers solved the problems associated with needle and syringe application via relatively conservative approaches like the development of more and more complex combination vaccines and the design of single use or autodestruct syringes. With the availability of an ever-increasing number of vaccines and the need for easy, painless, fast and safe administration techniques, many alternatives are under development and impressive progress is made in many areas of needle free vaccine delivery. The results of clinical studies indicate that alternatives for the N&S can be safe and result in strong immune responses.

Aim and outline of the thesis

In the Netherlands, children receive within the National Vaccination Program, 14 injections against 12 diseases. This number will only increase in the future. Since needles and syringes have several drawbacks, such as needle stick injuries and needle fear, alternatives have been assessed to deliver vaccines. The aim of the thesis was to identify and evaluate methods suitable for minimally invasive delivery of vaccines in order to become more flexible in complex pediatric immunization programs. Access to these delivery methods would facilitate

incorporation of new vaccines in the program. Three alternatives are assessed in this thesis:
-Bioneedles for s.c delivery
-Elastic vesicles for topical application
-Liquid jet injector for i.d delivery
Chapter 1 gives an overview of the current status of vaccine delivery via the skin, classical needle and syringe delivery excluded.
Chapter 2 is a review on the differences between animals and humans when designing animal studies for specifically cutaneous delivery. Mice and rats are much smaller than humans, limiting the applicability of some devices. They also differ substantially in skin physiology and anatomy. With a liquid jet injector, it was not possible to perform intradermal vaccinations in rats and ferrets. The fixed injection speed was too high for these small animals, resulting skin-to-skin penetration in the fold of the skin used to assure dermal vaccination. Therefore, mini-pigs have been assessed as animal model for cutaneous immunization (chapter 7).
In **chapter 3**, a study with hepatitis B surface antigen formulated in elastic vesicles for topical application is described. The vesicles were prepared and characterized with regard to size, antigen association and elasticity. In vivo experiments were conducted on intact skin and on microneedle pretreated skin, using an applicator for controlled skin piercing.
In the study described in **chapters 4 and 5,** Bioneedles have been used as vaccine delivery platform. To demonstrate the platform capabilities, preclinical studies were done with a bacterial (chapter 4) and viral antigen (chapter 5). In chapter 4, Bioneedles have been formulated with tetanus toxoid as a proof of principle. The formulations were prepared, characterized and tested in vivo. Thereafter, Bioneedles have been formulated with hepatitis B surface antigen, characterized and tested in mouse studies. At the start of the thesis, hepatitis B vaccine was, in the immunization program, only given to children with one of the parents born in an endemic country or from hepatitis B positive mothers. In 2006, the immunization program was extended with the pneumococcal vaccine for all children and an extra hepatitis B vaccine at birth for children of hepatitis B antigen-positive mothers. Moreover, the WHO advised a hepatitis B vaccination for all children, including those of Western Europe, which was a possible further expansion of the immunization program in the Netherlands. These expansions

of the vaccination program with hepatitis B vaccine, and the limited availability of combination vaccines containing hepatitis B antigen, was the basis to choose this vaccine for the development of an alternative delivery system.

Chapter 6 describes a clinical trial with non-hollow Bioneedles, e.g. not containing antigen. The safety and local tolerance was assessed in 18 volunteers.

In the study described in **chapter 7,** mini-pigs have been assessed as animal model for dermal delivery. Minipigs were vaccinated with hepatitis B vaccine using the PharmaJet injector and the regular N&S.

Chapter 8 contains a brief summary and a general discussion and perspectives.

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