Stabilized influenza vaccines

Stabilization of different vaccine-subtypes

In this thesis it is shown that influenza vaccines (subunit and virosomes) incorporated in sugar glass can be stored at room temperature which reduces the dependency on the cold chain. It was found that the stability of the dried vaccine is dependent on the formulation (composition), the sugar in which the vaccine is incorporated and of course storage conditions. The extent of stabilization seems further to be related to the type of vaccine. Especially the differences in complexity between WIV, virosomes and subunit or split vaccines deserve attention. The sugar-glass stabilization of subunit preparations was highly successful even at elevated temperatures (40°C). In contrast, virosomes (and also WIV [1]) incorporated in a glassy sugar matrix showed to be less stable at comparable storage conditions. This difference may be explained by the "intact" lipid vesicular structure of virosomes (and WIV). Lipid vesicles are known to be more difficult to stabilize than proteins. An improved stability of vesicular lipid bilayer systems (like liposomes, red blood cells, or other mammalian cells) was found when a stabilizer (like trehalose) was present at both sides of the lipid bilayer instead of the outside only [2-6]. Lack of a stabilizer at the inside of the vesicle can result in (partial) reorganization of the lipids and subsequent phase transitions. In the studies performed in this thesis on stabilization of virosomes, the vaccine dispersion was mixed with a sugar solution before lyophilization. Consequently, the inside of these vaccine particles lacks the presence of sugar. As a result virosomes (and WIV) incorporated in sugar glasses are less successfully stabilized than subunit vaccines. Stabilization of lyophilized virosomes may be improved by incorporating sugar inside the virosome before formulation and lyophilization. Moreover, also stabilization of WIV vaccines may be improved by loading WIV-particles with sugar from an "extracellular" medium through a combination of osmotic imbalance and phospholipid phase transitions as has been shown successful for stabilization of red blood cells [6]. In addition this sugar uptake by the WIV-particles may be facilitated by increasing the membrane fluidity with compound such as benzyl alcohol or other weak surfactants [6]. In conclusion, stabilization of each type of vaccine should be optimized individually.

In this thesis, subunit vaccine was brought in the dry state by both freeze drying and spray-freeze drying, while virosomes were dried by freeze drying only. Also other drying methods could have been applied for the incorporation of vaccine compounds in sugar glass matrices. However, additional research is needed regarding the use of these methods and in particular the comparison between the different drying methods for the production of stabilized influenza vaccines. In addition, each vaccine type may possess its own intrinsic sensitivity to different process stresses and have its own limitations. E.g. due to their particulate nature, virosomes and WIV may possess a higher sensitivity to shear stresses during atomization applied by spray (freeze) drying than subunit and split vaccines being proteinous vaccines. As a result the incorporation of a vaccine com-
pound in sugar glasses should be optimized by both formulation and drying process considerations.

**Methods to examine vaccine stability by determining the antigen structure**

One of the methods that has been used in this thesis to reveal integrity of HA in vaccine formulations after the formulation process and/or storage is the single radial immunodiffusion (SRID) assay. For several decades, this standardized method has been used to determine the antigen content (HA potency) of all human inactivated influenza vaccines, as recommended by the European Pharmacopoeia and the WHO [7, 8]. SRID is based on the diffusion of viral antigen in an agarose gel containing specific antibodies to the antigen measured.

However, for the determination of antigen integrity a SRID assay may not be sufficient. The SRID assay is based on haemagglutinin binding and does not address the antigen's stability. In case no structural alterations in HA are detected by SRID this does not guarantee a complete absence of conformational changes, since the method may not reveal every structural change.

Combining SRID analyses with additional analytical techniques, like fluorescence spectroscopy, circular dichroism spectroscopy, surface plasmon resonance transfer (SPR), asymmetric flow field flow fractionation (AFFF), reversed-phase high performance liquid chromatography (RP-HPLC), electron microscopy and the use of proteolytic assays may give more complete information on structural state of HA in the product. However, even with the combination of all techniques it is hard (impossible) to detect every possible structural change in large and complex proteins like HA.

Furthermore, it should be realized that until today only limited information is available on the effects of structural changes of the vaccine compounds on the final immune response in humans. The effects of low pH, detergents or process stresses on the immunogenicity of the vaccine may not be simply represented by changes in HA potency determined in vitro. For this purpose well designed studies using appropriate animal models or even human volunteers should be conducted. In addition the critical endpoints that are to be defined should be based on the desired or expected type of immune response in relation to the presentational form of HA, e.g. subunit, virosomal and WIV. In a study of Babiuk et al it was shown that changes in the production process of influenza split vaccine can have remarkable effects on the immunogenicity of the vaccine [9], while no changes in HA potency may be revealed by SRID. Due to a new viral splitting procedure the amount of un-split virions and aggregates, in the split vaccine were increased. This led to a change in the immune response to a greater Th2 cytokine pattern with potential implications for vaccine safety and efficacy. This shows that solely determining the HA content will not suffice as determinant for the immunity of the vaccine.

In addition, criteria should be formulated to address the relevant physical and functional properties for each vaccine type. For example WIV and virosomal vaccines might have immunological advantages over subunit vaccine related to their particulate form and/or ability to deliver material to the cytoplasm of APCs. Therefore appropriate criteria should be formulated for these functional characteristics like fusion activity. Strikingly,
there is no criterion for vesicular size mentioned in the European Pharmacopoeia for WIV vaccines, while on the other hand the size of virosomes should be between 100 and 500 nm [7].

**Development of mucosal dosage forms**

In this thesis, it has been shown that administration strategies of influenza vaccines via the oral or pulmonary route induce/promote mucosal and systemic humoral as well as cell-mediated immune responses. In contrast to parenteral vaccination, these mucosal vaccinations induce secretory antibody responses in the respiratory tract. As a result these mucosal vaccinations might give protection against influenza infection at the port of entry not only against homologous strains, but also against drifted, heterologous strains.

**Towards an oral influenza vaccine**

*Delivery to the gastrointestinal tract*

In the development of an oral influenza vaccine various pre-clinical studies addressed the issue of the use of adjuvants. However, in this thesis we paid attention to the delivery site of the vaccine in the gastro-intestinal tract. In the murine study presented in Chapter V it was found that the delivery site within the gastro-intestinal tract had several effects on the immune response for a subunit vaccine. Moreover, co-administration of the adjuvant LT enhanced the immune responses in different ways depending on the site of delivery. It was concluded that the right combination of a (strong) mucosal adjuvant and antigen delivery site within the gastro-intestinal tract might result in effective vaccination via the oral route.

Although no clinical trials have been performed to reveal the optimal delivery site for vaccine in the gastro-intestinal tract, a number of clinical studies with oral influenza vaccines for delivery in the intestinal tract have been performed. In most of the clinical studies relative high doses of antigen were applied to overcome the limited absorption by M cells, all these immunizations resulted in IgG responses below detection level [10-12]. Some authors explained the lack of IgG responses with oral tolerance caused by repeated ingestion of significant doses of antigen. It was concluded that the dose of antigen needs to be optimized to ensure a maximal and safe immune response [11]. A critical note regarding this study is that the antigen integrity in the formulations used (inactivated vaccines air-dried with D-xylose) was not determined. It should, however, be emphasized that a vaccine powder with known antigenicity is required to perform adequate dose response study. For this purpose a well designed influenza vaccine powder may guarantee the antigenicity of the vaccine.

On the other hand, most clinical studies demonstrated a significant increase in IgA antibodies in both saliva and nasal lavage fluids. It is unknown whether these IgA antibodies alone could provide sufficient protection against influenza infection in humans. Sufficient protection by mucosal IgA induced by oral immunization might be achieved.
However, to our knowledge, no clinical study has been designed so far to reveal the level of protection provided by the orally-induced local IgA immune response in humans.

Since no clinical trials have been performed to reveal the optimal delivery site for vaccine in the gastro-intestinal tract, it would be highly interesting to perform clinical studies and/or studies with primates in which specific areas of the gastro-intestinal tract are targeted. For these studies, state of the art technologies, like formulation technologies (tablets with dried vaccine) and special coatings, should be used to enable targeting of specific GI-sites [13-16].

Influenza vaccination via the GI-tract may be improved by specific colon delivery of vaccine that is normally degraded and/or poorly immunogenic in the upper part of the GI-tract. This might be envisaged by a pH-controlled pulsatile delivery system (system comprising a pH-sensitive coating material wherein a swellable agent is embedded) as developed by Schellekens et al. that enables pulsatile colon-specific release of the antigen [15, 16].

**Delivery to the oral cavity**

Although the oral cavity was not evaluated in this thesis for vaccination against influenza, the oral cavity could be a promising site for induction of immunity. In contrast to GI-administration, formulations for delivery to the oral cavity do not need protection of the antigen (e.g. enteric coating) against the harsh environment of the stomach. Moreover, in the oral-cavity many enzymes found in the GI-tract that may degrade the antigen are absent. The approach to use the oral cavity as an access to the immune system is best explored by research done on allergen specific immunotherapy, especially by sublingual application of allergens. Although the mechanism by which sublingual immunotherapy exerts its effects remains unclear, this therapy recently resulted in the launch of a tablet-based vaccine against grass pollen allergy (GRAZAX®, ALK-Abelló, Danmark). GRAZAX® is a sublingual tablet of a lyophilisate containing standardized allergen extract of grass pollen, gelatin (fish source), mannitol and sodium hydroxide.

Recently, oral spray immunization with WIV was evaluated in a phase I/II trial [17]. Although no significant increase in salivary IgA antibodies was found, the oral spray immunization induced serum HI antibodies in 75% of the volunteers already after 2 doses. A critical note with respect to this study is that the authors did not ensure that no aerosol particles were inhaled. Therefore, it cannot be excluded that a part of the found immune response is caused by pulmonary deposited WIV. However, delivery of an influenza vaccine in the oral cavity, e.g. by sublingual or buccal tablets or patches, might be an interesting alternative route for vaccine delivery. For this approach a lyophilized influenza vaccine powder as presented in Chapters III and IV may be adequate starting points. In addition, delivery in the oral cavity might offer the opportunity to use mucosal adjuvants that are not suitable for nasal and/or pulmonary use.
Towards a pulmonary influenza vaccine

From the pre-clinical study on pulmonary vaccination with an influenza subunit vaccine powder in mice as described in this thesis (Chapter VI) it was concluded that pulmonary delivery of influenza vaccines is a promising strategy for vaccination against influenza.

Already, in the 1960s and 1970s pulmonary delivery has been investigated in a number of clinical studies. However, in many of these studies efficient targeting to the lungs is unlikely. First of all atomizers generating aerosols with droplet sizes between 1 and 100 μm were used, which is too large for deep lung penetration (since this requires 1 to 5 μm droplets). Secondly a number of studies were performed with pressurized metered dose inhalers (pMDIs). In these studies it is unclear whether the vaccine was compatible with the propellant. Moreover, the old pMDIs gave lung deposition below 15% [18, 19]. However, some general trends can be seen:

- Aerosol immunization with inactivated influenza vaccine prevented influenza related illness in humans [20-22].
- Aerosol immunization in humans stimulated higher levels of respiratory secreted antibodies than subcutaneous immunization [23, 24].
- The respiratory antibodies were more cross-reactive with heterologous viruses [25].
- Inhaled inactivated vaccine can provide protection against a heterologous Variant [26].
- These last two results confirm that cross-reactivity of the respiratory antibodies elicited by inhaled influenza can be achieved.

More recently, pre-clinical studies have shown that respiratory delivered influenza vaccine generated systemic, mucosal (local) humoral and cellular virus-specific immune responses that increased with increasing the depth of vaccine deposition within the respiratory tract [27, 28]. The higher efficacy of deep-lung vaccination has been explained by the prolonged residence time of the influenza vaccine within the lungs [28]. Especially, deposition in the alveolar region which lacks mucociliary clearance, is considered to result in an increased residence time of the vaccine [28]. In addition, immunization via the deep-lung instead of i.m. injection resulted in Th1 skewing of the cellular immune response [28].

These pre-clinical studies suggest that deep-lung deposition may be a critical parameter for immunogenic efficacy of influenza vaccines in humans. Since current inhalation technologies provide a higher deep-lung deposition, it may be expected that pulmonary vaccination will be more effective than those from 1960s and 1970s. Therefore it would be highly interesting to perform clinical trials using modern inhalation devices and formulations. Especially the use of so-called "soft mist inhalers" would be interesting since they apply the concept of low-velocity aerosol in combination with the generation of mono-disperse aerosols [19, 29]. In contrast to pressurized metered dose inhalers, soft mist inhalers can directly aerosolize the aqueous vaccine formulation without the use of propellants that may affect the integrity of the influenza vaccine [29]. However,
next to the clinical trials in humans that are needed to prove efficacy, it will first have to be investigated whether the vaccine is resistant against the aerosolization.

Dry-powder inhalers (DPIs) are potentially the most attractive inhalation systems for the delivery of vaccines to the lungs. Advantages such as the stability of the vaccine in the solid state, the high lung depositions that can be generated with simple and cheap devices, and their robustness, make these systems in principle superior to the liquid inhalation systems [29-31]. Moreover, they can be disposable systems [32].

The first development studies on formulations suitable for dry-powder inhalation of influenza vaccine are described and discussed in Chapter II. In the pre-clinical study described in Chapter VI, pulmonary vaccination with a new influenza subunit vaccine powder was evaluated in mice. Vaccine powder was produced by spray-freeze drying (SFD) using inulin as stabilizer. Pulmonary vaccination of mice with the vaccine powder induced mucosal, systemic humoral as well as cell-mediated immune responses. These responses were superior to those elicited by conventional i.m. vaccination or pulmonary vaccination with a liquid aerosolized subunit vaccine. The superiority of the SFD vaccine powder compared to the aerosolized liquid subunit vaccine was ascribed to:

- the deposition in the lower respiratory tract of the powder, resulting in increased residence time in the lungs,
- the increased viscosity at the site of deposition caused by inulin, which increases the residence time.

To confirm these hypothesis, further studies should be designed that address deposition, tracking and immunity, e.g. with labeled particles, with labeled HA and labeled carbohydrates. In this way the relation between deposition, residence time and immunological outcome might become clear(er).

After the lessons learned from studies on inhalable measles vaccines, like the use of less hygroscopic formulations and more purified vaccine compounds in the formulation [33] and addressing the questions related to toxicity, clinical trials should be performed using recently designed inhalers and dry-powder formulations. These inhalers and formulations have to guarantee high and reproducible deposition of the vaccine in the lung area of interest. Finally, these studies might prove the efficacy and safety of pulmonary dry-powder vaccination against influenza in humans.

**Nasal influenza vaccine considerations**

Although not investigated in this study, vaccination via the intranasal route (i.n.) is the only mucosal route that has been successfully applied in the form of the live-cold adapted trivalent influenza intranasal vaccine (Flumist™). So far a number of (hypothetical) safety concerns exist regarding the live-cold adapted vaccine, e.g. the safety in immunocompromised patients which has not been established, the attenuated vaccine strain may reassort with other influenza viruses and the possible risk of vaccine induced central nervous system complications [34].

The use of inactivated intranasal vaccines may be an alternative which combines the convenience of intranasal administration and the safety of inactivated intramuscular vac-
cine [34]. However, a disadvantage of intranasally delivered inactivated influenza vaccines is that they are poorly immunogenic without the use of special delivery systems and/or mucosal adjuvants [35]. Therefore, in recent preclinical and clinical studies investigators examined the suitability of a number of candidate vaccines which are adjuvanted or specially formulated (as reviewed in Chapter II).

Powder formulations may have additional advantages for nasal delivery of vaccines. In addition to the fact that powders offer the advantages of improved vaccine stability [36, 37], certain powder formulations also have demonstrated increased residence time in the nasal cavity compared with liquid [38, 39], which may translate into higher “bioavailability” and immune responses. A few studies report the development of dry-powder vaccine formulations for intranasal delivery [1, 40-43]. However, the intranasal delivery of these dry-powder formulations prepared by lyophilization could only elicit a sufficient immune response when they were co-formulated with a mucoadhesive polymer or mucosal adjuvant.

The effectiveness of the SFD vaccine powder upon pulmonary delivery, as described in Chapter VI, was ascribed to site specific deposition; increase in residence time in the lungs due to lower respiratory tract deposition and an inulin-induced local viscosity increase at the site of deposition. These characteristics, if proven, may facilitate the effectiveness of such a vaccine powder also upon nasal delivery.

Due to nasal anatomy and physiology, with a non-ciliated area in the anterior part of the nasal cavity and a ciliated region in the more posterior part of the nose, the site of deposition is of importance for the nasal mucociliary clearance [44]. The site of deposition depends on several parameters which are related to the delivery device and formulation, such as velocity of the delivered particles and particle size of the formulation [45]. As a result the vaccine powder should be reformulated to obtain deposition of the vaccine powder at the desired site in the nose. In conclusion, the required (deep) nose deposition may be obtained with the right combination of formulation and delivery device, providing relative long vaccine retention in the nose and an effective immune response.

**Correlates of protection**

Due to current criteria for vaccine immunogenicity, most new developments on influenza vaccines aim only at the induction of an adequate level of virus-neutralizing antibodies in the serum, indicated by an HI titer higher than 40 in humans [46]. However, other immune responses may be just as or even more valuable (protective) as the induction of HI-titers. For example, secretory antibodies in the respiratory tract induced by mucosal immunization have the potency to provide protection against influenza virus at the port of entry [47, 48]. In addition, from human studies it was concluded that cellular immunity, especially CTL activity, is important for recovery from influenza infection even in the presence of protective antibodies [49, 50].

Ideally, criteria for vaccine efficacy should not be based on the induction of adequate virus-neutralizing antibodies in the serum only, but should be based on the level of protection that is desired. The desired protection may depend upon the route of administration as well as on the formulation of the vaccine. The induction of mucosal virus
neutralizing antibodies (IgA) may for example be a better parameter for protection after nasally administered vaccines, whereas for i.m. injected subunit vaccine clearly the level of neutralizing antibodies in the serum is more relevant. As a result, parameters for protection, e.g. serum antibodies, mucosal antibodies as well as cellular immunity, should be determined (and re-evaluated) for each type of vaccine and route of administration by adequate (post licensing) human studies.

**Inulins as adjuvants for needle-free inactivated influenza vaccines**

Today's vaccines may still need adjuvants to improve the immune response elicited by current inactivated vaccines, to facilitate dose sparing vaccination and/or to improve mucosal immunization strategies. Although, investigation of various adjuvants and adjuvanting systems showed in some cases promising results thus far, the search for new, improved and safe adjuvants continues.

In Chapter II, the hypothesis was drawn that inulin may have been acting as an adjuvant in the pre-clinical study on pulmonary delivery of an inulin-stabilized subunit vaccine in mice. The adjuvant activity of inulin was mentioned in several papers as reviewed in [51]. In these publications inulin gamma-crystals were explicitly assigned to have adjuvant activity. However, in the system described in Chapter VI amorphous inulin particles were used, that are believed to dissolve rapidly after deposition at the mucus. However, part of the inulin may be processed by the immune system before dissolution in the mucosal fluid or after dissolution and possible subsequent crystallization to the gamma-crystal.

Inulin derived adjuvant, based on gamma-inulin, efficiently promotes both Th1 and Th2 immune responses against a variety of antigens (e.g. diphtheria toxoid, whole cell meningococci, hepatitis B surface antigen, malarial merozoite surface antigen and protein of human papilloma virus) [51]. Pure inulin gamma-crystals given with virus immunogen (A/JAP, H2N2) have been shown to induce heterotypic protection against lethal challenge with live influenza virus (A/WSN, H1N1).[52] BALB/c mice were injected with (live/gamma-irradiated) influenza virus alone or combined with gamma-inulin. Whereas, only 3.8 % of the mice primed with virus survived, 50% of the mice primed with gamma-inulin/virus mixture survived. This improved protection was assigned to cytotoxic T-cell mediated immunity [52]. Consequently, it would be interesting to investigate whether amorphous inulin has adjuvant activity or the adjuvant activity should solely be assigned to the gamma-crystal-form of inulin. Finally, dried vaccine formulations based on inulin might be investigated for use in epidermal powder, nasal, oral and oro-pharyngeal immunization.
Concluding perspective

In this thesis formulation and delivery strategies are presented, that finally may result in a stabilized influenza vaccine that is administered via a non-parenteral route.

Incorporation of vaccines in amorphous glassy sugar matrices has been shown to have the potential to solve the problems associated with the cold chain requirement of liquid vaccines. However, many aspects of the stabilization of influenza vaccines have to be further investigated. Not only vaccine type dependent formulation and process design, but also methods to establish the critical (formulation, process and immunogenic) parameters for vaccine stability should be addressed.

Solid vaccine powders are interesting starting materials for the development of non-parenteral dosage forms for influenza vaccines. Various strategies, such as oral, nasal or pulmonary delivery may evolve in successful non-parenteral dosage forms for influenza vaccines. Critical re-evaluation of old clinical studies, the use of new (up-to-date) delivery technologies, site specific vaccine delivery, introduction of new criteria for vaccine immunogenicity and the design of more effective vaccine compounds together with new adjuvants may facilitate the development of such needle-free influenza vaccines.

In this thesis the most promising strategy presented is the development of a pulmonary influenza vaccine. The pulmonary delivery of a subunit vaccine powder (inulin based) in mice was shown to be highly immunogenic. In addition, compared to the conventional i.m. immunization pulmonary immunization induced not only higher serum antibodies, but was also capable to induce secretory antibodies in the respiratory tract. The induction of these secretory antibodies, that exhibit cross-reactivity against antigenically distinct viruses, might offer broader protection against drifted, heterologous strains.

Finally, cellular-immunity was shown to be increased by this strategy of immunization. Therefore, future developments should aim at pulmonary immunization with influenza vaccines. Clinical studies should address the immunogenicity of pulmonary delivered vaccine aerosols with modern inhalation devices such as "soft mist inhalers". Or even more interesting, clinical studies should be performed to address the immunogenicity of pulmonary delivered vaccine powders with modern dry-powder inhalers. Such a vaccination strategy, by dry-powder inhalation, may lead not only to influenza vaccinations that provide broader protection against new emerging influenza viruses, but also would facilitate vaccination of people in "hard to reach" areas with a temperature-resistant self-administerable and needle-free influenza vaccine.
References


[7] Influenza vaccine (surface antigen, inactivated)


[22] Haigh W, Howell RW, Meichen FW. A comparative trial of influenza immunization by inhalation and


[31] Laube BL. The expanding role of aerosols in systemic drug delivery, gene therapy, and vaccination. Respir Care 2005;50(9):1161-76.


[46] (CPMP) TEAftEoMPECfPMP. Note for Guidance on Harmonisation of Requirements for Influenza Vaccines, London; 1997 March 12.