UniVax

Publishable summary at project month 36

A “Universal” Influenza Vaccine through Synthetic, Dendritic Cell-Targeted, Self-Replicating RNA Vaccines
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Publishable summary

While vaccination is the cornerstone of influenza (Flu) prophylaxis, current inactivated vaccines provide only moderate protection, requiring annual updating due to poor long-term immunity and antigenic drift of the virus. Efficacious, protective immunity requires humoral and cell-mediated defenses. The UniVax goal is to develop the first multimeric and synthetic universal Flu vaccine based on self-replicating RNA replicons targeted to dendritic cell receptors by synthetic delivery vehicles, inducing humoral and cell-mediated immunity for broad, long-lasting protection. The project aims to conduct a clinical phase I study assessing safety, tolerability and initial immunogenicity of a universal Flu vaccine. Replicons are derived from defective virus genomes, from which at least one structural protein-encoding gene has been deleted – replicons replicate and translate, but cannot produce virus. Inherent problems with many current replicons are their cytopathic nature (reducing the duration of immune response induction), the need for virus-like particles for delivery, and their derivation from human pathogens. Moreover, they are not targeted to dendritic cells (DCs) – critical cells for immune response induction. UniVax employs replicons (RepRNA) derived from a non-cytopathic porcine pestivirus, which is non-infectious for humans. The RNA is delivered into human DCs by synthetic means, wherein the RepRNA efficiently translates and replicates. Our current RepRNA carries insertion sites that efficiently facilitate the accommodation of Flu genes of interest for broad universal protection against Flu. Co-formulation with novel mucosal adjuvants, such as MALP-2 or c-di-AMP, potentiates robust humoral and cellular immune responses, including cytotoxic and multifunctional T cells – the latter have been related to robust protective T-lymphocyte immunity.

Description of Work and main results until month 36

1. Replicon Constructs

The active component of the multimeric influenza vaccine being developed in this project consists of replicon RNA (RepRNA) based on a pestivirus genome. Replicons are genetically modified viral genomes, designed to act as a self-amplifying (replicating) vaccine, whilst being incapable of generating progeny infectious virus. This is achieved by deleting genes encoding for essential viral structural proteins.

The choice of the pestivirus replicon was made for two major reasons. Firstly, unlike the majority of replicons in use today – primarily alphavirus or flavivirus derived, and cytopathogenic – pestivirus RepRNA is non-cytopathogenic and well supported by dendritic cells for its translation and replication. Importantly, the pestivrus RepRNA will not kill dendritic cells in which it is translating and replicating, rendering it more ideal as a vaccine for targeting dendritic cells. The second important characteristic is that the leader protein encoded by the pestivrus genome interferes with the cell-signalling pathway leading to the interferon induction that can impair replicon function. Moreover, the pestivrus genome and replicon do not carry 5’-triphosphates, which would signal a cell’s innate defences to attack

the replicon. Alphaviruses do carry 5'-triphosphates, and can signal the cell innate defence mechanism.
The characteristics of the pestivirus replicon facilitate its retention in dendritic cells for prolonged periods, which fits well to the manner by which dendritic cells slowly process antigen for presenting to the adaptive immune system over a prolonged period, thus promoting robust immune defences. Initially existing constructs encoding reporter genes and influenza virus haemagglutinin (HA (H5)) were produced as starting material to test with different biodegradable, nanoparticulate delivery technologies. Additional constructs have been generated, encoding for influenza virus HA (H1), neuraminidase (NA (N1)), M protein, PB1 protein and nucleoprotein (NP). By such means a series of antigens can be translated to provide the means for inducing both humoral and cell-mediated arms of immune defence. Thus, a multimeric Flu vaccine can be formulated using the biodegradable delivery technologies, with the aim of protecting against different influenza virus strains at the same time. During the first part of the project, a series of new replicon prototypes encoding some of these Flu antigens were generated. This has been elaborated to include all the aforementioned Flu antigens. Moreover, replicons have been constructed to lack one, several or all the structural proteins of the original pestivirus sequence. The expression of the encoded Flu proteins have been assessed alongside expression of certain pestivrus proteins still encoded by the replicon. By such means the relative efficiency of translating the "gene of interest" – the Flu antigen – can be compared with the endogenous genes, which are translated via different ribosomal entry sites in the 5'-NTR or inserted after the gene of interest. This allows determination of the efficiency with which the gene of interest near the 5' end of the RepRNA can be recognised and translated compared with the genes downstream of this. In addition, it can determine the influence of the biodegradable delivery vehicle on the delivery of the replicon to promote interaction with the cellular ribosomal translational machinery. By such assessment, efficacious formulations of the replicon with the biodegradable delivery vehicle can be identified, and selected as prototypes for testing this vaccination approach *in vitro* and *in vivo*. For this purpose, most of the partners in UniVax have now been trained by the lead partner in replicon generation, on producing the replicon RNA for association with the different biodegradable delivery vehicle formulations, and assessment of the capacity to promote translation of the encoded Flu antigens ultimately to induce both arms of the immune defence *in vivo*.

2. Formulation for delivery to dendritic cells

Different biodegradable delivery systems have now been tested and compared for formulation with the RepRNA. Assessment looks at their capacity to package RepRNA and protect from RNase, together with delivery of the cargo to dendritic cells for promoting translation of the encoded Flu antigens or reporter genes. Selected cell lines were also employed for comparative purposes and also to facilitate rapid identification of candidates for testing in dendritic cells. The main criteria for the delivery vehicles were biodegradable, readily tolerated by cells and host, capable of interacting efficiently with dendritic cells to promote cargo uptake, efficient at complexing RepRNA, and efficient also at promoting the RepRNA release within cells to facilitate translation of the encoded Flu vaccine antigens. For this purpose, the delivery vehicle components were classified as (i) cationic lipids (lipoplexes), (ii) PEI-based polymers (polyplexes), (iii) cationic lipids combined with polymers (lipopolyplexes), and (iv) chitosan-based complexes and nanoparticles. The initial work performed for the 1st reporting period looking at a large number of these compounds and formulations continued during the 2nd reporting period. Again, this employed variations of

each compound formulated in a number of different manners with RepRNA encoding different genes of interest, including reporter genes and the genes encoding Flu antigens.
All resulting nanoparticles were physico-chemically characterized with regards to their shape, structure, charge, zeta-potential and stability. Secondly these particles were tested for their capacity to deliver functional nucleic acid to a relevant cell culture model. The majority of delivery formulations were shown to be highly efficient at associating the RepRNA; most provided protection against RNase and were shown to release RepRNA under acidic conditions. Initial assessment of delivery efficiency employing both dendritic cells and cell lines (see section 5 below) was also defined in terms of promoting translation of the RepRNA. This allowed identification of the most promising prototype formulation for assessment *in vivo* (see section 6 below).

3. Targeting

The nanoparticulate delivery vehicles were also modified to target ligands on dendritic cells. On the one side, this employed ligands such as hyaluronic acid, well know for targeting the CD44 found on dendritic cells and indeed many cell types. The other approach was to employ specific glycan structures that should bind to members of different families of dendritic cells, such as C-type lectins, SigLecs and galectins. The approach with hyaluronic acid proved particularly successful. There was no toxicity from these formulations, and delivery to cells proved most efficient. Translation of the delivered RepRNA was also observed, and this also proved successful *in vivo*, whereby these delivery vehicles were among the most successful for inducing immune responses (see section 2 above and sections 5 and 6 below).
For the glycans, it was necessary to determine their relative capacity for binding to dendritic cells compared with other mononuclear cells. Over 300 glycoconjugates were assessed on mononuclear cells obtained from porcine and human donors, and clustered into different groups dependent on their relative binding to the different cell populations present. This clustering facilitated a definition of glycan binding capacity of the different mononuclear cells, with particular emphasis on the dendritic cells. The analyses created cluster groups and heat maps, which also permitted the identification of species-common and species-specific glycan-binding receptors. A selection of the probes showing the highest binding was further analysed by microscopy to define binding capacity and internalisation efficiency. This was related to the structure of the probe and the likely receptors with which they would be reacting. Certain of these probes have been modified to interact with the delivery vehicles, to assess their influence on RepRNA cargo delivery and translation efficiency.

4. Adjuvants

While the characteristics of the pestivirus replicon facilitate retention by dendritic cells and therefore increase the chances for developing robust immunity, the aforementioned characteristics render it is less likely to activate the innate immune mechanisms required for maturation of the dendritic cells. Accordingly, the delivery vehicles employed with the pestivrus replicon require a potent adjuvant. In this context, adjuvants for parenteral or mucosal immunisation are being studied. The most promising candidates were MALP-2 and cyclic-di-AMP, both having a distinct mode of action. These offer the further advantage of being manufactured synthetically and applicable via parenteral or mucosal routes.
The adjuvants were formulated with a number of the delivery vehicles mentioned under section 2. For some of the lipoplex and initial polyplex formulations, a limited number of experiments on *in vitro* assessment of efficacy were performed. Although these showed some promise, many of the experiments were inconclusive. Additional *in vivo* immunisations

were performed by sub-cutaneous injection, but also failed to produce conclusive results. Further studies employed particular polyplex and chitosan-based formulations selected after extensive assessment *in vitro*. These displayed clear efficacy after intra-pulmonary injection for delivery leading to translation in dendritic cells and induction of specific immune responses. These *in vivo* experiments provided clear evidence of immunogenicity (see section 6 below). An important discovery was the particularly powerful immunomodulatory capacity of cyclic-di-AMP, both parenterally and mucosally.

5. *In vitro* Evaluation

Both primary dendritic cells (murine, porcine, and human) and cell lines relevant for either replicon translation or as models for dendritic cells were employed. The majority of delivery formulations were shown to be highly efficient at associating the RepRNA and releasing it under acidic conditions; most provided protection against RNase. Delivery to both dendritic cells and cell lines was highly efficient for the majority of the delivery formulations, particularly notable with the cationic lipids (lipoplexes). The efficiency of delivery did not relate to efficiency at promoting translation of the RepRNA. Early versions of the PEI-based polyplexes gave similar results, but modifications of the PEI increased the translation efficiency of the delivery RepRNA. Certain PEI-based polyplexes and in particular the chitosan-based delivery vehicles – showed *in vitro* delivery promoting translation – provided clear immunogenicity of the RepRNA-encoded antigens *in vivo* when assessed by mucosal (intra-pulmonary) vaccination.

It was considered that the RepRNA may have proven to be eventually non-functional in certain complexes, or compacted to a degree that would not be reversed adequately for ribosomal entry and translation of RepRNA-encoded antigens. Thus, the functionality of the RepRNA was assessed using virus replicon particles (VRP). These are constructed in complementing cell lines to create the original virus-like particles, but carrying the replicon in place of the virus genome. VRPs were efficiently delivered to cells, promoting efficient translation of the encoded antigens, including the Flu antigens. These results clearly demonstrated that the RepRNA was indeed translation- and replication-competent. Analyses with the different PEI-based formulations may be adding some clarity to the situation. While certain formulations were efficient at promoting translation of all encoded antigens, other formulations provided for an imbalanced translation, often favouring translation of the endogenous replicon genes with little or no read-out from the gene of interest. This would imply a differential degree of compaction along the RepRNA, either preventing ribosomal entry at the first site but not the second, or inefficiently protecting one site from RNase. Nonetheless, it is now clear that careful selection of the delivery vehicle components facilitates formulation with RepRNA to protect from RNase, facilitate delivery to dendritic cells, and promote translation of the vaccine antigens leading to induction of immune responses. This work is continuing to determine the variability between experiments and further modify the structures to improve the translation efficiency.

In addition to the dendritic cell models above, the TCR Ova model (OTI and II) was also employed with certain formulations showing promise with dendritic cells, as part of the high throughput screening process. Selected lipoplexes were found to induce cellular immune responses with isolated $CD8^+$ $CD4^+$ T cells.

6. *In vivo* Evaluation

Following the *in vitro* identification of the most potentially efficacious delivery formulations delivering RepRNA for translation in dendritic cells, the prototypes were assessed by immunogenicity studies in mice. As mentioned under section 5 (above), assays with dendritic

cells were supplemented with the TCR Ova model, which offers a means of crossing *in vitro* with *in vivo* assessment. $CD8^+$ and $CD4^+$ ovalbumin-specific T cells were isolated from OTI and OTII mice, respectively. Naïve TCR transgenic CD8+ and CD4+ T cells were enriched, labelled with CFSE, and injected intravenously into C57BL6 mice. After 24h, the animals were vaccinated with the prototype vaccine candidates, carrying RepRNA encoding for ovalbumin through the gene of interest insertion, selected as described under section 5. The proliferative capacity of the injected ovalbumin-specific T cells was analysed after a further 5 days.

Certain lipoplex, polyplex and chitosan-based nanoparticle formulations were identified as showing promise for efficacious immunogenicity. Selected lipoplex formulations were then assessed by more conventional immunogenicity studies by vaccinating normal, naïve mice directly with the formulations of interest, this time employing RepRNA encoding Flu antigens – HA (H1) and NP. The outcome of this very extensive and time-consuming experiment involving several partners was disappointing. Despite the in-depth analysis of most aspects on immune responsiveness, including antibody induction, T-lymphocyte subset induction, cytokine profiling and assessment of individual lymphocyte activities, the lipoplexes formulations were relatively poor at promoting a specific immune response. A smaller *in vivo* experiment with polyplexes also proved disappointing.

Accordingly, the delivery formulations and the route of injection were modified, bringing in different PEI molecular weights and formulation modifications, as well aso studying the chitosan-based nanoparticles. Initial selection employed extensive *in vitro* assessment (see section 5 above). These approaches identified new polyplexes and chitosan-based vehicles with high promise due to the increased translation of the delivered RepRNA. A new *in vivo* assessment, using a different route of injection (intra pulmonary), is confirming the capacity of these new formulations to induce immune responses against the Flu antigens encoded by the RepRNA. Particularly interesting is the efficiency of the chitosan-hyaluronic acid nanoparticles for inducing both humoral and cell-mediated immune responses. Moreover, the power of the cyclic-di-AMP, in particular as a mucosal adjuvant, has been further evidenced. Overall, this demonstrates that particular formulations will facilitate delivery of the RepRNA for translation in the manner observed with RepRNA delivery in a single shot by VRPs, although this may prove more efficacious dependent on the route of administration. These experiments will be repeated and the use of such delivery vehicles further assessed in terms of modifications and increased efficacy at inducing immune defences.

Expected final results and potential impact and use

Influenza is a serious public health problem affecting more than 100,000,000 people per year. Most recover from the symptoms within a week without requiring attention, but there is a high risk for severe illness (3-5 million cases) or death (250,000 to 500,000) (WHO); most deaths occur among the elderly. The most effective way to prevent the disease is vaccination, although the vaccine among the elderly reduces severe illness and complication by only up to 60%. Annual vaccination is currently recommended for pregnant women, children 6 to 59 months of age, healthcare workers with patient contact, the elderly and people with underlying chronic health conditions such as respiratory, cardiac, metabolic, neurological and immunosuppressive diseases. The outcome of the UniVax project will be the first synthetic replicating RNA vaccine against Flu. UniVax will generate essential data on integrating innovative technologies for RepRNA, synthetic delivery, glycoconjugate-based targeting of DCs and mucosal adjuvanting. The data integrates for the first time polysaccharide and lipid technologies, providing a most powerful integrated technology for

delivering functional RNA into DCs. The first ever prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants will be evaluated pre-clinically and clinically, providing data on enhancing efficacy of vaccine delivery for breadth and duration of protection. UniVax has as a direct outcome a novel clinical technology with great benefits for population's health; this also provides tools in basic research (RNA delivery system to DC, new adjuvant, replicative RNA technologies). It will enable the widespread use of nucleic acid delivery to dendritic cells and other "immune cells" such as monocytes, macrophages, natural killer cells or even lymphocytes with the purpose of investigating cellular functions and overexpressing or silencing genes in therapeutic strategies.

Contact and further information: www.UniVax-FP7.eu

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