



Introduction

Influenza virus infection is a major cause of morbidity and mortality World-wide, and a new pandemic strain (capable of increased incidence and severity of influenza) is expected at any time. Despite recent improvements in the treatment of influenza with novel anti-viral agents, prophylactic vaccination remains the primary method for preventing influenza and its severe complications. Although influenza vaccines will continue for the foreseeable future to be the mainstay of influenza control, currently licensed influenza vaccines are of limited efficacy, particularly in the elderly, and there is a great unmet medical need for improved influenza vaccines.

Whilst numerous novel approaches to influenza vaccination have been explored in recent years, all currently licensed influenza vaccines rely upon the generation of antibodies against the hemagglutinin (HA) protein, and anti-HA antibodies (measured by hemagglutination inhibition) are required for the licensure of novel vaccines.

Here we present a novel liposomal influenza vaccine designed to produce antibodies to the HA protein. It is based on a new principle, i.e. the “co-delivery” of influenza protein and DNA immunogens in the same liposomal vaccine vehicle. Unlike co-administration of DNA and protein in the same injectate, ‘co-delivery’ delivers both payloads to the same individual cells, and results in dramatic synergy between the DNA and protein forms of the immunogen.

Co-delivery concept

Proteins that are synthesized within a cell (e.g. from plasmid DNA having a mammalian-active promoter) are continuously sampled, as peptides, by the proteasome/class-I MHC antigen presenting pathway. Conversely, proteins that are acquired exogenously, by antigen-presenting cells, are sampled in an analogous way by the endosomal/MHC-class-II pathway. We postulated that the delivery of both protein and plasmid-DNA-encoded forms of a protein antigen to the same individual antigen-presenting cell would result in the simultaneous presentation of the antigen via both class-I and class-II pathways, thereby providing an opportunity for synergy in the resulting immune response to the antigen. We designed several appropriate liposomal formulations to test the ‘co-delivery’ hypothesis, exploiting the unique attributes of our proprietary ImuXen™ liposomal vaccine technology that efficiently entraps both DNA and protein immunogens. The formulations are described in Figure 1, and comprise various test and control permutations of plasmid DNA and protein, either free or entrapped (together or separately) in the liposomal vehicle. The formulation representing the hypothesis under test (co-entrapment of protein and the corresponding plasmid DNA encoding the protein, in the same liposomal vehicle) is represented in cell-1 of Figure-1.

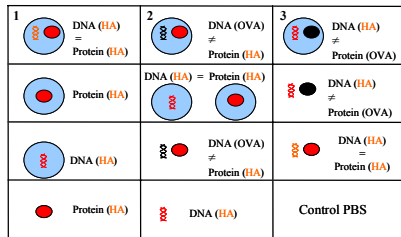


Figure 1. Permutations of homologous (“=”) & non-homologous (“≠”) DNA & protein, used to test the “co-delivery” concept

Immunisation with DNA encoding the influenza haemagglutinin protein has been explored previously with naked [1] or liposomally formulated DNA [2] or rotein antigens [3]. Although immune responses elicited by DNA alone were adequate to achieve protective efficacy against influenza virus challenge in preclinical studies, only weak anti-HA antibody responses were elicited [2]. Our new ‘co-delivery’ concept was designed to rectify this deficiency of DNA-based influenza vaccines.

Experimental Methods

Preparation of Liposomal Formulations

Briefly, small unilamellar vesicles (SUV) were prepared from egg phosphatidylcholine (PC) and dioleoyl phosphatidylcholine (DOPE) and 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) (4:2:1 molar ratio) by sonication were mixed with DNA and protein alone or DNA and protein together (Table 1).

Following rehydration under controlled conditions, the resulting dehydrated-rehydrated vesicles (DRV liposomes) were washed by centrifugation to remove non-incorporated materials.

The washed pellets were resuspended in PBS to the required dose volume. DNA and/or protein incorporation was estimated on the basis of ³⁵S (for DNA) and ¹²⁵I (for protein) radioactivity recovered in the suspended pellets. Liposomes were subjected to photon correlation spectroscopy (PCS) at 25°C in a Malvern Zetasizer 3000 to determine z-average diameter respectively (Table 1).

Formulations were prepared in triplicate, two vials for dosing (prime and boost) and one vial for % entrapment calculations based on radiolabeled tracer (HA and OVA, DNA and protein) added to entrapped materials and freeze-dried overnight as described [4, 5].

Liposomal Formulation	% Entrapment		Size (nm)
	DNA	Protein	
1.1	70µg ^a	4.2µg ^a	679
2.1	70µg ^b	4.2µg ^a	689
3.1	70µg ^a	4.2µg ^b	774
4.1	70µg ^a	-	624
5.1	-	4.2µg ^a	586

^a = HA protein / encoding DNA, ^b = Ovalbumin

Table 1: Characterisation (% entrapment efficiency and size) of DNA and protein liposomal compositions

Immunisation studies

Female Balb/c mice 6-12 weeks old (Harlan, UK) were immunised by subcutaneous injection administered in 0.2 ml dose volume (Table 2, also Figure 1). Final dose quantities were calculated based on % material (DNA, protein or both) entrapped (from radioactivity count vials) Negative control mice received doses of PBS. Mice received two doses at days 0 and 28, with sample bleeds collected from the tail vein at days 16, 28, 42, 56 and 68 with respect to the first injection.

Sample	Dose (µg / animal (0.2ml S/C))		
	formulation	DNA	Protein
1.1	Liposome co-delivery	HA (10)	HA (0.6)
2.1	Liposome co-delivery	OVA (11)	HA (0.6)
3.1	Liposome co-delivery	HA (10)	OVA (0.76)
4.1	Liposome	HA (10)	nil
5.1	Liposome	nil	HA (0.6)
6.1	Liposome co-delivery (admix 4.1 & 5.1)	HA (10)	HA (0.6)
7	DNA and protein admix	HA (10)	OVA (0.76)
8	DNA and protein admix	OVA (11)	HA (0.6)
9	DNA and protein admix	HA (10)	HA (0.6)
10	DNA alone	HA (10)	nil
11	Protein alone	nil	HA (0.6)
12	Control (PBS)	nil	nil

Table 2. Permutations of homologous (“=”) & non-homologous (“≠”) DNA & protein used to immunise mice

Anti Influenza response analysis

Sera obtained from sample bleeds were diluted 20-fold in PBS, kept at -40°C until assayed by indirect ELISA for total anti influenza antibody responses. The ELISA plate antigen used was the same influenza antigen used in the immunisation formulations.

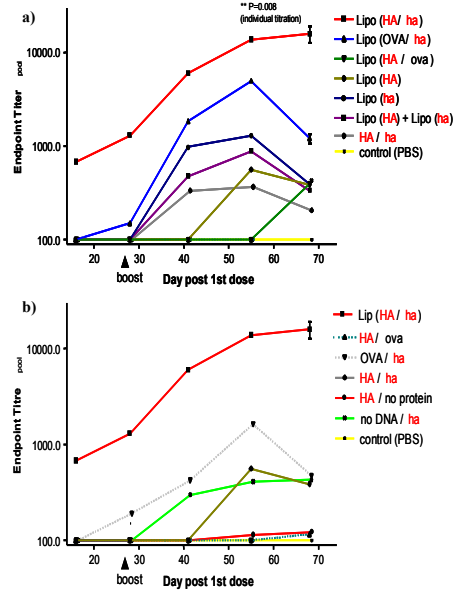
Results and Discussion

Liposomal product characterisation

Liposomal product characterisation results (Table 1), indicate that ImuXen™ liposomal formulations efficiently package both DNA and protein, either separately or together, with no evidence of competition between the two payloads for packaging.

Antibody response to influenza

The experimental results of the DNA and protein co-entrapment are shown on figures 2a) and b). Note that the protein component is indicated in capitals (i.e. “HA”) whilst the DNA component is indicated in lowercase (i.e. “ha”).



Figures 2a) and b). Anti influenza total Ig response following immunisation with samples (Table 2) used to test the “co-delivery” concept (shown as two graphs for clarity).

Figure 2 demonstrates that the ‘co-delivery’ hypothesis formulation (i.e. cell 1 of Figure 1) elicited a greater response than all other formulations at each time point in the series, and elicited by far the strongest response after a single dose. This formulation comprised both HA protein and its corresponding DNA in the same liposomal vehicle. The response for this co-delivered formulation was greatest in terms of magnitude (titre) whether expressed as ‘titre’ or as the number of animals deemed sero-positive at each bleed point.

Notably, the formulation ‘Lip (OVA/ha)’, which controls for the CpG adjuvant effect of plasmid DNA [6], gave a response which was much lower than that of ‘co-delivery’ with the appropriate ‘homologous pair’ of HA DNA and protein. Likewise, Lip (HA/ova) (an inappropriate pairing according to the hypothesis), gave a markedly weaker response.

Figure 2 also demonstrates that separately entrapped HA DNA and protein (in neighbouring vesicles) gives rise to an inferior response, supporting the hypothesis that delivery of both payloads to the same cell (which is best achieved by co-entrapment in the same liposome) is important in achieving the optimal antibody response.

It is also remarkable from these data, with the modest DNA dose and number of immunisations used, that several formulations completely failed to generate an anti-HA response. These included HA DNA alone, and liposomally entrapped HA DNA. These findings serve to emphasize the striking degree of superiority of ‘co-delivery’ over previous methods of DNA-based immunisation against influenza virus.

Conclusion

These studies demonstrate, using influenza virus, that very small doses of protein as an additive in DNA immunisation can dramatically improve the antibody response to the target protein, provided that the protein and DNA are homologous to one-another (i.e. that the DNA can express the protein) and that the payloads are delivered in the same individual liposomal vehicle. The simplest hypothesis to explain these data is that the synergy observed between the appropriately delivered ‘homologous pair’ of protein and DNA involves delivery of both payloads to the same antigen-presenting cell. The application of the co-delivery concept to alternative delivery systems; niosomes, proteosomes, dendrimers, PLA/PLGA, chitosans, alginates and microspheres awaits investigation.

The new ‘co-delivery’ principle may lead to better DNA-based vaccines for prophylactic and therapeutic use, particularly where these vaccines require the elicitation of antibody responses (e.g. influenza vaccines).