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# Towards a universal influenza vaccine: volunteer virus challenge studies in quarantine to speed the development and subsequent licensing

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There are now more than 5 experimental vaccine formulations which induce T and B cell immunity towards the internally situated virus proteins matrix (M1 and M2e) and nucleoprotein (NP), and towards stem and stalk regions of the HA which have a shared antigenic structure amongst many of the 17 influenza A virus sub types. Such 'universal vaccines' could be used, at least in theory, as a prophylactic stockpile vaccine for newly emerged epidemic and novel pandemic influenza A viruses or as a supplement to conventional HA/NA vaccines. My own laboratory has approached the problem from the clinical viewpoint by identifying $CD4^+$ cells which are present in influenza infected volunteers who resist influenza infection. We have established precisely which peptides in M and NP proteins react with these immune CD4 cells. These experimental vaccines induce immunity in animal models but with a single exception no data have been published on protection against influenza virus infection in humans. The efficacy of the latter vaccine is based on vaccinia virus (MVA) as a carrier and was analyzed in a quarantine unit. Given the absence of induced HI antibody in the new universal vaccines a possible licensing strategy is a virus challenge model in quarantine whereby healthy volunteers can be immunized with the new vaccine and thereafter deliberately infected and clinical signs recorded alongside quantities of virus excreted and compared with unvaccinated controls.

## Introduction

Osterhaus *et al*. [1], Ebrahimi & Tebianian [2] and Du *et al*. [3] have reviewed in excellent detail aspects of the universal influenza vaccine approach and have focused particularly on matrix (M) and nucleoprotein (NP) based vaccines. In contrast the present review will not emphasize, analyze or compare the various genetic constructs of M, M2e and NP which have been formulated. Rather this is a more general overview and places the established efficacy of killed influenza vaccines and the potential of new 'universal' influenza vaccines in context whilst examining a role of the virus challenge model in a quarantine unit both for discovery and later licensing of universal influenza vaccines. More recent research advances using the stem or stalk portion of the HA as potential vaccines are discussed.

## Are current HA/NA vaccines limited in efficacy and is there a need for a universal influenza vaccine?

The first formalin inactivated whole virus particle influenza vaccines were developed over six decades ago. The antibody based immunity and protection induced, both in animal models and in humans, is focused tightly

towards the HA and NA of the vaccine virus and its close relationship to the natural field virus (reviewed in [4]). Hence the scientific concept is one of close antigenic matching of the surface HA protein of community virus and vaccine virus which amounts to pre-estimating which influenza A (H3N2) and H1N1 and B virus will be dominating in the community and preparing ahead of time a correspondingly matched vaccine virus. This has been aided by the surveillance network of 120 or so WHO laboratories around the world which analyze, along with the five central laboratories in London, Atlanta, Melbourne, Tokyo and Beijing, around 30 000 human influenza viruses each year.

The current influenza HA/NA vaccines are safe and effective but have some limitations as reviewed in [4]. For example adults, post-vaccination, may produce a broad cross reacting (CR) series of HI antibodies and may indeed even recall an antibody response to the first member of the virus subtype encountered during a lifetime ('Original Antigenic Sin') whereas what is really required is a more tight response of strain specific (SS) antibody to the vaccine virus [5]. Therefore immunizing a 70-year-old with a current influenza vaccine in 2013 may induce CR and SS antibodies to A/HK/1/68 (H3N2) virus circulating four decades ago as well as antibodies to the current vaccine virus A/Victoria/30/2011 (H3N2). The former CR antibodies may even predominate whereas the strain specific antibody is estimated to be more protective. Nevertheless the most recent data with naturally contracted influenza [6, 7] show a good correlation between vaccine induced HI antibody to the HA (with an HI titre in excess 1/110 for children and 1/30 for adults) and protection against infection and disease. This confirms the much earlier data of Hobson *et al*. [8] who described the association of protection against disease both in quarantine and in natural infection, with increasing level of HI antibody and particularly an HI titre of 1/40 or above.

We can justifiably ask whether we need a new series of influenza vaccines which might also necessitate total reorganization of licensing requirements or whether the two approaches viz. anti HA and an M/NP antigens could be combined to produce both B cell HI antibody and T cell responses? Undoubtedly the unsettling problem at the centre of discussion is the sudden emergence of new pandemic influenza A/California/6/2009 (H1N1) virus, probably having evolved for 17 years in S.E. Asia but unexpectedly coming to attention in Mexico. Evolutionists have identified S.E. Asia as the place for the emergence of epidemic and pandemic influenza [9]. Huge technical manufacturing and scientific expertise with cell substrates such as Vero cells and MDCK cells as well as embryonated hen's eggs enabled production of large quantities of vaccine, in excess of half a billion doses including whole virion, HA/NA sub unit, split virion and finally live attenuated vaccines [10]. However the delay from initiation of vaccine production to clinical use was still 4–6 months. Within that period the virus had circulated the globe and probably infected at least one billion persons in both hemispheres. Fortunately, at least compared with the three pandemics of the 20$^{th}$ century, there were 500 000 deaths rather than millions but still up to 9 million years of life were lost [11].

## What is the scientific basis that a new vaccine composed of internal virus structural proteins such as M1, NP and M2e shared by many influenza A viruses would induce broadly protective immunity?

That a post infection immune response exists which gives broad protective immunity was first shown in an important series of animal experiments published four decades ago. Both Werner [12] and Schulman & Kilbourne [13] noted that mice could be infected with a sub lethal dose of influenza of one subtype A (H1N1) virus and after recovering would retain solid immunity to super infection with an otherwise lethal dose of another antigenically unrelated (at least with HA and NA) influenza virus subtype such as influenza A (H3N2) or *vice versa* (Table 1). However a viewing of the data in Table 1 shows that heterotypic cross immunity is less long lived than homologous immunity and did not extend to 36 weeks post-infection in the mouse model. In the ferret heterotypic immunity was exemplified by a reduction of titre of excreted virus. Kreijtz *et al*. [14] have also demonstrated this phenomenon more recently with antigenically unrelated influenza A (H3N2) and influenza A (H5N1) viruses.

These two early animal model papers stimulated a whole research field just at the time when the chemist Laver in Australia had developed electrophoretic methods to separate the external HA, NA proteins, from the internal NP and M proteins of the influenza virus in biologically active form [15]. However the first experiments to separate out NP and M and use them as vaccines *per se* failed to induce solid protection in mouse or ferret models as compared with HA [16]. Later experiments on transfer of specific antibody to M1 and M2 proteins showed both *in vivo* and *in vitro* virus neutralizing activity [17].

Of course currently produced whole virus killed vaccines will also have internal M1, M2, M2e and NP proteins and may also induce a broader immunity than supposed or detected to date. The so called subunit HA/NA detergent or/split virion vaccines also have low quantities of M, M2e and NP protein which are difficult to remove during subunit purification but these internal proteins may not be presented to the immune system in an optimum manner, allowing anti HA responses to dominate.

Finally we have to bring into the practical equation the important live attenuated virus vaccines where

**Table 1**

Heterotypic (cross sub type) immunity in mouse and ferret models

| Model | Animal initially infected with: | Number of animals infected (ferrets) or dying (mice) at (weeks) after challenge with A/PR/8/34 (H1N1) virus: 3 | 12 | 36 | Titre of virus excreted post-infection ($log_{10}$ $EID_{50}$ $ml^{-1}$) |
|---|---|---|---|---|---|
| CBA mouse | A/HK/1/68 (H3N2) | 1/10* | 2/17 | 19/20 | ND |
| | B/LEE | 19/20 | 12/18 | 12/14 | ND |
| | A/PR/8/34 (H1N1) | 1/15 | 2/19 | 1/10 | ND |
| | None | 20/20 | | | ND |
| Ferret | A/PC/1/73 (H3N2) | 12/12 | – | – | 2.4±0.5 |
| | None | 7/7 | – | – | 6.2±0.8 |

J.S. Oxford, previously unpublished data.

investigations started as early as 1947 in the USA and Russia and were recently (reviewed in [18]) both cold adapted (ca) master strains have been licensed in the USA, EU, UK, Russia and India. The ca vaccines do give a broader immune response across the subtype [19] but not to date, between subtypes but further study is required.

## Examples of current experimental universal influenza vaccines formulated with virus structural M1, M2, M2e and NP proteins and a variety of adjuvants

The gene 7 of influenza codes for two proteins, M1 and M2, with a short overlapping region. The M1 structural protein gives rigidity to the virion and is the larger protein whilst the smaller M2 (96 amino acid) protein is an integral membrane protein serving as an ion channel for the early steps of virus uncoating. Virus infected cells may have more M2 molecules than HA on the surface but the virion itself has only 60 or so M2 molecules compared with 500 HAs. There has been particular interest in the extracellular region of M2 (M2e) which has 24 amino acids and 17 of these have 94% conservation between influenza A viruses. The N terminal epitope (residues 2–9, SLLEVET) has over 99% conservation between many influenza A viruses. Unfortunately the native form of M2e is poorly immunogenic (reviewed in [2]).

We should note that post-infection antibodies to M2 are rare [20] and that only 1–2% of persons in the community have antibodies to M2e. Anti HA antibodies are more neutralizing than antibodies to M2e but antibodies to the latter protein bind to virus infected cells and may thus mediate $CD^+8$ cell killing whilst such antibodies may also impede influenza virus exit.

Therefore to formulate a novel universal influenza vaccine the scientific strategy has been to construct fusion proteins of M2e, for example using hepatitis B core antigen [21, 22] or fused to the heat shock protein (HSP) 70 of *M. tuberculosis* [23] in attempts to enhance the immunogenicity of this small protein. Some of the formulations summarized in Table 2 [24–29] do induce antibody and/or T cell responses in animal and clinical models but to date only a single construct has been tested and the data published against a virus challenge in humans ( [30] see below).

## Cross reactive epitopes on the HA: a second variety of universal vaccine

Most antibodies, either from natural infection or from immunization with conventional HA/NA influenza vaccines, bind to the externally located globular head of the HA where the HA1 protein is located.

However there are regions in HA2, the second polypeptide of the HA, where antibodies bind and several of these antibodies are the subject of new investigations which have identified cross reactive antigenic regions in the HA stalk or stem [31–37]. One group of investigators has designed and produced an *E. coli* expressed antigen, called HA6, made of most of HA2 and two small regions of HA1. Immunization of mice gives protection against homologous influenza A (H3N2) viruses in a broader sense than using the whole HA. Similarly Steel *et al.* [32] have described an HA2 stalk antigen which gives broad immunity within the subtype also raising expectations of a truly universal vaccine against all 17 subtypes.

Babon *et al.* [35] have located a $CD4^+$ T cell epitope near the fusion peptide of the HA at the junction of HA1 and HA2 which is well conserved across at least 16 subtypes and, most interestingly, of influenza B.

**Table 2**

Stages of development of some universal influenza vaccines

| Company or scientific group | Construct | Animal *vs.* clinical data and references |
|---|---|---|
| A Vaccines of internal virus proteins | | |
| Acambis and Sanofi (USA) | M2e HBc Ag fusion protein: a particle coated with M2e | Immunogenic in phase I in trials adjuvanted with Q521 humans [21, 22] |
| VaxInnate (USA) | M2e flagellin fusion protein | Immunogenic in phase I trials [25] when co-administered with conventional flu vaccine [27] |
| Seek (UK) | Peptides of HA, NP and M1 | Immunogenic in phase I trials [24] |
| University of Oxford (UK) | M1 and NP cloned into modified Vaccinia Virus Ankara (MVA) | Immunogenic in phase I trial and induced moderate protection in volunteers in quarantine [30] |
| Cytos Biotechnology (Switzerland) | M2e is fused to the coat protein of RNA phage AP 205. VLP | Animal model [26] |
| Razi Institute (Iran) | 4 × M2e HSP70c fusion protein | Animal model [23] |
| Merck | M2e synthetic peptides conjugated to outer membrane protein complex | Immunogenic in animal models [29] and including primates |
| Biondvax | Concatamer of nine conserved sequences from HA, NP and NA | Immunogenic in phase I trials |
| Dynavax | M2e and NP fusion protein conjugated to CpG oligonucleotide TLR-9 ligand | Animal model and phase I |
| B Vaccines | | |
| Academic groups | Regions of HA2 | Animal models only to date 31–37 |

Note that this is not an exhaustive listing of current scientific endeavours.

## Infection of immunized volunteers with influenza virus in quarantine may allow licensure of universal influenza vaccines which do not induce HI antibody

The approaches to universal influenza vaccines described above contain either the HA2 stalk region of the HA, or internal proteins MP, M2e and NP, none of which will induce HI antibody. As a key part of the current licensing requirements new influenza vaccines are tested for HA content (10–15 μg per dose) using single radial diffusion [38]. How then can such HA free vaccines be licensed and would they, like the current inactivated HA vaccines, be required to be relicensed yearly or less frequently? One solution for licensing would be the establishment of a precise and quantitative level of cellular $CD4^+$ and $CD8^+$ cells or an antibody response to virion M and NP peptides which have been shown to correlate with protection in quarantine experiments. An alternative licensing procedure could be an actual virus challenge experiment in a quarantine unit whenever the vaccine is formulated. Typically 70–80% of volunteers who are HI antibody negative are infected after challenge with influenza A/Wisconsin/67/05 (H3N2) virus [39] and 79% of these develop symptoms.

Our own quarantine work in London [39–41] has drawn heavily on the prior experience of the MRC Common Cold Unit, Harvard Hospital in Salisbury, where between 1948 and 1988 many thousands of quarantined volunteers were infected with respiratory viruses [42].

At the first screening visit the volunteer is helped through the informed consent, a very important document, and once signed, a medical history is taken and inclusion criteria for the particular trial are checked. Entry criteria are most strict and half the young 20–40-year-old volunteers fail the screen on medical criteria. Next the volunteers are screened for HI antibody to the challenge virus [commonly influenza A (H3N2)] and again more than half the healthy volunteers are rejected for possessing HI antibody which would prevent infection.

For the 9 day quarantine the volunteers are housed in individual rooms with an en suite bathroom, with negative air pressure and with no opportunity to exchange viruses with each other or with the medical and nursing staff who are protected with modern personal protective equipment (PPE) including a small HEPA half suite. Nasal and throat swabs are harvested thereafter each day post-infection for laboratory quantification of virus whilst quantitative assessment of respiratory symptoms are constructed from diary and temperature charts, tissue counts and weight of tissues and with additional precise clinical observational scoring of sore throats.

There is intense daily clinical monitoring of the volunteers by ECG and spirometry. Haematology and blood chemistry investigations are carried out before, throughout the trial and afterwards. Several blood samples can be taken daily to analyze for human gene activity in the PBMCs and for relative immune $CD4^+$ and $CD8^+$ T cell activity.

## The first example of a candidate universal influenza vaccine to show efficacy in quarantine

A group at the Jenner Institute in Oxford [30] formulated an experimental universal influenza vaccine using vaccinia virus (MVA) into which was cloned influenza genes for internal proteins NP and M1.

Following intranasal influenza challenge in quarantine, two out of 11 vaccinees and five out of 11 control subjects developed laboratory confirmed influenza (symptoms plus virus shedding). Additionally clinical symptoms of influenza were less pronounced in the vaccinees than in control subjects. Furthermore a more activated profile of specific T cells was detected in vaccinees compared with control subjects, with an increase in perforin, granzyme A and CD38 expression. This study provided the first evidence of clinical efficacy of a specifically designed universal influenza vaccine.

## A quarantine experiment has identified pre-existing memory $CD4^+$ cells reacting with internal influenza M and NP peptides which correlate with disease protection

McMichael and colleagues [43] described protective effects of T cells directed towards internal influenza proteins in volunteers infected with influenza at the MRC Quarantine Unit in Salisbury. Recently we performed a similar experiment but used HI antibody negative volunteers and two separate viruses, namely influenza A (H3N2) and influenza A (H1N1) for challenge [39] alongside modern techniques to search for $CD4^+$ *vs.* $CD8^+$ T cell activity and for the biological activity of these cells.

We found a significant inverse relationship between pre-existing T cell responses to NP and M1 protein and severe illness and this was most closely associated with the number of pre-existing $CD4^+$ T cells rather than $CD8^+$ T cells, although both made a contribution. Furthermore we were able to identify a number of particular peptides to NP and M which reacted with $CD4^+$ T cells in the volunteers with reduced symptoms after challenge [39].

We concluded that identification, for the first time, of particular 'protective' epitopes in the virus structural proteins M1 and NP would provide a more satisfactory basis for the logical construction of a T cell epitope vaccine than has been possible to date.

## Discussion

A broadly reacting universal influenza vaccine inducing immunity to newly emerging pandemic viruses would complement the existing stockpile of anti (H5N1) vaccine which, of course, would only be effective against the homologous virus. This latter preventative public health measure is recognized by many governments especially since the pandemic (H1N1) vaccine was so effective [44] and bird influenza is recognized as the most likely influenza virus to spread and cause a pandemic. The UK has a strategic stockpile of some 17 million influenza A (H5N1) vaccine doses. We have noted above that other influenza A viruses could also emerge, such as the virus of a relatively recent pandemic in 1957, namely influenza A (H2N2), and a universal influenza vaccine would be expected, although not guaranteed, to provide cross immunity.

At present the potentially pandemic virus influenza A (H5N1), 'bird flu', causes human mortality each month in SE Asia and Egypt and the most recent analyses show human infection to be five times more common than previous estimates. Furthermore a retrospective analysis of an influenza A (H5N1) outbreak in Abbotsbad on the Pakistan/Afghanistan border in 2007 [45] showed person to person spread. Finally, the recent studies of Imai *et al*. [46] and Herfst *et al*. [47] show that as few as four mutations in the genome can convert a purely avian influenza A virus such as (H5N1) into one able to spread between mammals and, by deduction, humans. Given the extraordinary high mutation rate of influenza whereby 10% of the virus population can be mutants (reviewed in [18]), the number of potentially infected and carrier migrating ducks and geese (over 100 million) and 21 billion domesticated chickens and given that each infected bird can easily harbour $10^9$ influenza viruses, it is not difficult to hypothesize that such mutants are existing already in nature and constitute a public health threat.

Approximately 500 million doses of conventional influenza A and B vaccines are used yearly to protect especially vulnerable groups in the community against epidemic and reformulation and relicensing is carried out yearly unlike any other vaccine either for adults and children. However at present most persons in the community, including children, are not offered vaccine although with the licensing of live attenuated vaccines this rather unsatisfying situation could change [18]. It should be noted that the strict licensing requirements for influenza vaccines have taken over 30 years to develop and refine using SRD and HI testing. Should new vaccines such as those described in the present review reach the stage of phase II testing in the community, licensing would not be straightforward and could impede the speed of clinical use. Here we raise the possibility for the first time that precise and quantitative virus challenge in a quarantine unit could provide data for clinical and virological efficacy in vaccinees who have received a novel anti HA-2, M or NP vaccine, and provide a basis for licensing.

Our own studies [39] have provided a new and stronger scientific basis for the design of a universal influenza vaccine for humans. We have identified particular virus

peptides of M and NP proteins which could be formulated into a vaccine to target and stimulate memory $CD4^+$ T cells which in turn would be expected to ablate influenza symptoms and virus excretion regardless of the influenza A subtype causing the infection.

## Competing Interests

The author has completed the Unified Competing Interest form at http://www.retroscreen.com (available on request from the corresponding author) and declares 1) no support from any organization for the submitted work, 2) JSO is Scientific Director and a Shareholder of Retroscreen Virology Ltd in the previous 3 years and 3) JSO receives lecture fees, travel expenses and consultancies from pharmaceutical groups including Roche, Novartis, GSK, Baxter, The EU, the Wellcome Trust and a number of scientific societies around the world.

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