Human Vaccines & Immunotherapeutics

ISSN: 2164-5515 (Print) 2164-554X (Online) Journal homepage: http://www.tandfonline.com/loi/khvi20

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To cite this article: John S Tregoning, Ryan F Russell & Ekaterina Kinnear (2017): Adjuvanted influenza vaccines, Human Vaccines & Immunotherapeutics, DOI: 10.1080/21645515.2017.1415684

To link to this article: https://doi.org/10.1080/21645515.2017.1415684

Accepted author version posted online: 12 Dec 2017.

Publisher: Taylor & Francis

Journal: *Human Vaccines & Immunotherapeutics*

DOI: https://doi.org/10.1080/21645515.2017.1415684

Adjuvanted influenza vaccines

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Running title: Use of adjuvants in human influenza vaccines

Abstract

In spite of current influenza vaccines being immunogenic, evolution of the influenza virus can reduce efficacy and so influenza remains a major threat to public health. One approach to improve influenza vaccines is to include adjuvants; substances that boost the immune response. Adjuvants are particularly beneficial for influenza vaccines administered during a pandemic when a rapid response is required or for use in patients with impaired immune responses, such as infants and the elderly. This review outlines the current use of adjuvants in human influenza vaccines, including what they are, why they are used and what is known of their mechanism of action. To date, six adjuvants have been used in licensed human vaccines: Alum, MF59, AS03, AF03, virosomes and heat labile enterotoxin (LT). In general these adjuvants are safe and well tolerated, but there have been some rare adverse events when adjuvanted vaccines are used at a population level that may discourage the inclusion of adjuvants in influenza vaccines, for example the association of LT with Bell's Palsy. Improved understanding about the mechanisms of the immune response to vaccination and infection has led to advances in adjuvant technology and we describe the experimental adjuvants that have been tested in clinical trials for influenza but have not yet progressed to licensure. Adjuvants alone are not sufficient to improve influenza vaccine efficacy because they do not address the underlying problem of mismatches between circulating virus and the vaccine. However, they

may contribute to improved efficacy of next-generation influenza vaccines and will most likely play a role in the development of effective universal influenza vaccines, though what that role will be remains to be seen.

Introduction

Very broadly, adjuvants are substances added to vaccines to boost immune response to the antigen. The first adjuvant used was an aluminium salt, Potassium Aluminium Sulphate ($KAl(SO_4)_2.12H_2O$) often called Alum [1]. When it was used in guinea pigs in 1926, it led to higher antibody titres to diphtheria toxoid; interestingly the beneficial effects were unexpected – Alum was used to precipitate the diphtheria toxoid component. Since the first use of Alum as an adjuvant, a huge array of substances have been tested as potential adjuvants; a small number of these have progressed into clinical trials and an even smaller number (six) have been included as part of licensed influenza vaccines. An important point to note is that adjuvants themselves are not licensed, but are licensed as part of the vaccine formulation.

In this review we cover which influenza vaccines include adjuvants, why they are included, their mechanisms of action and their effects on vaccine immunogenicity and safety; focussing on clinical studies. We also evaluate some experimental adjuvants that have been tested in clinical trials but have not yet progressed to licensure.

Influenza the basics

Before focussing on adjuvants, we will quickly recap some basics about influenza virus and disease as they pertain to vaccination. In spite of a vaccine being available, influenza is a significant cause of morbidity and mortality worldwide; the WHO estimates that there are 3 – 5 million severe influenza cases every year, leading to 250,000 – 500,000 deaths globally [2]. There is also a considerable economic burden associated with influenza epidemics, which can cost the European economy approximately €6 to €14 billion and the US economy $87.1 billion annually [3, 4]. Infections follow a seasonal pattern, with separate waves in the northern and southern hemispheres.

There are four types of influenza virus: A, B, C and D. Of these, the majority of human infections come from types A and B. Type A can be divided into 18 antigenic subtypes based on the haemagglutinin molecule, though of these only H1, H2, H3, H5 and H7 can infect humans and H5 and H7 do not currently transmit between humans. The subtypes themselves can be further subdivided into strains based on whether they are recognised by antibodies. These strains evolve over time, with small changes (antigenic drift) leading to epidemic spread and major changes (antigenic shift) leading to pandemic spread. These strain changes have an impact on influenza vaccines. Firstly, to cover the different concurrently circulating strains, influenza vaccines do not just contain a single flu strain they are either trivalent with two A strains and a B strain, or quadrivalent with two A strains and two B strains. Secondly, viral coat changes necessitate new influenza vaccines each season and though there are standardised processes by which the viruses in the vaccine are selected, there are

sometimes mismatches. Finally and most seriously, new strains of influenza with little antigenic overlap to existing strains emerge with extremely rapid global transmission.

Vaccines for influenza

Currently there are 26 licensed inactivated vaccines for influenza, of which 13 are routinely manufactured for each influenza season (Table 1). The vaccine manufacturers reflect a range of big pharma (GSK, Sanofi, Pfizer and Abbott) and smaller product focussed companies (Protein Sciences, Mylan, Microgen, Sinovac, Seqirus). The majority of the licensed vaccines are egg derived, and there are three manufacturing processes to recover and inactivate the virus: whole virus, split (where the virus has been disrupted by a detergent) and subunit (where the haemagglutinin and neuraminidase proteins have been further purified, removing other viral proteins). One manufacturer (Protein sciences) uses recombinant protein technology, expressing only the haemagglutinin protein from an insect cell line. Strikingly meta-analyses reveal very little difference in the safety or efficacy of these different approaches [5]. In addition to the inactivated vaccines there are also three live attenuated vaccines with slightly different backbones: Fluenz/Flumist (AstraZeneca) uses the Ann-Arbor backbone whilst Ultravac (Microgen) and Nasovac (Serum institute of India) use the Leningrad backbone..

Problems with the current licensed influenza vaccines

There are two important considerations for an influenza vaccine, immunogenicity – its ability to induce an immune response and efficacy – its ability to reduce influenza disease in vaccinated individuals. In healthy adults, inactivated influenza vaccines are mostly immunogenic (for example [6-9]). Indeed until 2015, in the EU, influenza vaccines were evaluated by serological tests alone and licensed on >70% of individuals achieving a haemagglutination-inhibition (HAI) titre of >1:40 and a four-fold increase in HAI titre in >40% of individuals. The haemagglutination-inhibition (HAI) titre is a functional assay which assesses the ability of the antibody to prevent the haemagglutinin protein from binding sialic acid. HAI>40 is a surrogate of protection defined in the 1970's by a series of human influenza challenge studies [10].

However, the ability of a vaccine to induce HAI titres against a specific virus does not necessarily lead to protection against the circulating strain in the subsequent flu season. Influenza vaccines have highly variable rates of efficacy, ranging from 10% in 2004-5 [11] to 60% in 2010-11 [12]; the biggest factor being the match or mismatch between the vaccine strains and the circulating strains [13]. Between 2000 and 2011, influenza B vaccine strains did not match circulating strains in six influenza seasons [14]. In the autumn of 2014 increased rates of influenza activity were observed in the United States and this was attributed to poor vaccine effectiveness as a result of a mismatch of the H3

component of the current influenza vaccine to circulating strains [13]. The overall effectiveness of the 2014-15 influenza vaccine for preventing medically attended laboratory confirmed influenza virus was 23% [15]. Early studies of influenza infections during the 2014/2015 season found that 100% of lab confirmed influenza A infections were A/H3N2 and of those 67% were antigenically drifted from A/Texas/50/2012, the reference strain used for the 2014/2015 vaccine in the northern hemisphere and more closely related to A/Switzerland/9715293/2013, the reference strain used for the southern hemisphere [15]. A similar report from Canada found that of the laboratory confirmed cases of influenza, the majority were influenza A infections (95%), and where subtype information was available, 99% were found to be A/H3N2. Sequencing data available showed that 91% of the isolates were found to be genetically and antigenically distinct from the A/Texas/50/2012 vaccine strain [16].

Why Adjuvants

One potential approach to improve influenza vaccines is to include adjuvants. There are a number of reasons adjuvants might be included in a vaccine:

1. **Populations with poor immune responses**

Adjuvants are used to boost responses in populations with poor immune responses; this includes patients who are immunosuppressed due to either primary immunodeficiencies, transplant treatment or infection – particularly HIV. For example, the inclusion of the adjuvant AS03 improves the anti-influenza antibody quality in HIV positive patients [17]. Likewise, the inclusion of AS03 improved influenza vaccine responses in haemodialysis patients [18]. Vaccination is also less effective in individuals at the extremes of age – the very young and the very old [19] – and adjuvants can help in these situations. Influenza causes the most severe disease in these age groups; infants (under 2 years) and elderly patients (≥65 years) have higher influenza attack rates, more frequent influenza related hospitalisations and greater rates of influenza related mortality [4]. Globally, influenza infection results in approximately 374,000 hospitalisations in 1 year old children [20]. The addition of MF59 to an influenza vaccine induced substantially faster and higher antibody titres in children than a non-adjuvanted vaccine [21]. There is no global estimate for influenza infection in the elderly, but estimates from the USA put the rate of influenza hospitalisation in elderly patients (≥65 years) at nearly twice that of infants [22]. The addition of AS03 improved responses in young and elderly adults [23], the addition of MF59 improved responses in subjects older than 65 [24] and virosomes increased the response in geriatric patients [25].

2. **Boosting the immunogenicity of an antigen**

As well as some individuals being poor at making immune responses, some antigens are less immunogenic than others. Many of the longstanding vaccine antigens are pathogen derived, for example diphtheria toxoid, tetanus toxoid and haemagglutinin. Newer vaccines often contain recombinant proteins and these can be less immunogenic than pathogen derived antigens. This is probably because the pathogen derived antigens contain trace levels of inflammatory material from the pathogen and some of the classical vaccine antigens may also have some inherent self-adjuvanting property. It is of note that Flublok – the only licensed recombinant influenza vaccine – does not contain an adjuvant, but it does contain three times as much of each haemagglutinin (45µg) as Aggripal (15µg) which is the Seqirus (formerly CSL/Novartis) unadjuvanted egg-derived inactivated virus influenza vaccine.

3. Accelerating responses to a vaccine

Another advantage is that adjuvants can accelerate responses to the vaccine, for example during a pandemic. Most vaccines require more than one administration to reach protective levels in recipients; the addition of an adjuvant can elevate the response to the first dose and push it over the protective threshold. For example HAI titres to an H1N1 vaccine were above the US and European licensure criteria after a single dose only when MF59 was included in both pre-clinical [26] and clinical studies [27]. For an experimental H9N2 vaccine, antibody titres after the administration of a single dose of MF59 adjuvanted vaccine were similar to those after two doses of nonadjuvanted vaccine [28]. Whilst accelerating the response may not lead to enduring immunity, it may be sufficient to protect individuals during the main wave of a pandemic.

4. Dose sparing

The inclusion of adjuvants can enable dose sparing, both for routine and pandemic vaccines. Vaccine antigens are expensive to manufacture and there are limited manufacturing facilities for making vaccines to the required good manufacturing practice (GMP) standards. The addition of AS03 led to a similar response to a lower dose of H5N1 antigen (3.75µg) compared to the standard 15µg dose [29]. When alum was included as an adjuvant, equivalent responses were seen when doses of influenza antigen were reduced from 15µg to 6µg in both young and elderly adults [30]. In a phase I study investigating the unlicensed adjuvant Advax (a polysaccharide particulate adjuvant derived from inulin) responses were equivalent between the adjuvant group that used a third of the antigen (15µg) and the unadjuvanted group that received 45µg influenza antigen [31].

5. Immunomodulation

Adjuvants can also change the quality of the immune response to antigen. The use of different adjuvants can change the pattern of cytokines and chemokines released, leading to the recruitment

of different cells [32]. This is particularly noticeable with T cell responses where the combination of different adjuvants with the same antigen can lead to very different outcomes. In a recent study the combination of influenza antigen with MF59 or Alum gave a strong IgG1 antibody responses associated with IL-5 producing T cells, whilst combination of haemagglutinin with the cationic liposomal adjuvant CAF01 led to a more Th1 and Th17 biased cellular response [33]. Since current influenza vaccines primarily confer immunity through antibody, shaping the CD4 T helper response may not be necessary to improve protective efficacy. However, the addition of adjuvant can also potentially improve the quality of the B cell response and CD8 T cell responses both of which may be important for the development of a universal flu vaccine. Additionally, there may be subtle effects if the adjuvant leads to a switch in antibody subtype, when non-neutralising antibody functions, such as antibody dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), are important.

6. Mucosal Vaccine delivery

A final use is to enable mucosal delivery of vaccines. Through the induction of local immunity at sites of infection, mucosal vaccination may be more appropriate than systemic vaccination. However, mucosal surfaces are much harder to vaccinate for a number of reasons – they are broadly tolerogenic and they also have mechanical (cilia, gap junctions), chemical (mucus) and biochemical (proteolytic enzymes) barriers to antigen. Specific adjuvants may be required to protect the antigen in this environment and to induce a local immune response. One adjuvant that was licensed for this purpose was the heat labile enterotoxin (LT) of *Escherichia coli*, which was included in Nasalflu [34].

How adjuvants work

Before describing what is known of the mechanism of the specific adjuvants included in licensed influenza vaccines, it is necessary to give a brief overview of their general mechanism of action (reviewed in depth elsewhere [35]). Fundamentally, adjuvants improve the ability of the host immune system to recognise the administered antigen as foreign and respond to it; beyond this simple description they are extremely diverse in their molecular and cellular mechanisms of action.

The first requirement is for the vaccine antigen to be seen by the immune system. One problem is that soluble antigen is quickly cleared by the lymphatics and therefore is never seen by the immune system. Adsorbing (sticking) the antigen onto an insoluble complex (Alum) or in an oil-in-water emulsion (MF59/ AS03/ AF03) leads to the retention of antigen at the injection site. In theory, the antigen/adjuvant depot can then be sampled by antigen presenting cells, which then take antigen to the lymph nodes. However, recent studies in mice have shown that removing the site of the depot

even as early as two hours after immunisation had no negative effect on the immune response, suggesting that formation of a depot at the injection site is not essential [36].

Adjuvants can also increase antigen visibility by increasing the recruitment of cells to the site of injection [37]. The adjuvant can either recruit cells directly[38] or indirectly by inducing local sentinel cells to release cytokines and chemokines [39]. Another method of increasing antigen visibility to the immune system is to increase uptake by antigen presenting cells. This can also occur directly by acting on antigen presenting cells [40] or indirectly by inducing antigen shuttling to lymph nodes by other cell types [41].

However, seeing the antigen is not sufficient to induce an adaptive immune response, cells also need to be licensed to respond. Some adjuvants promote dendritic cell (DC) maturation, via increased expression of MHCII and the co-activation markers CD80 and CD86 [42]. This effect is not limited only to DCs, as Alum and MF59 have both been shown to upregulate MHCII and CD86 expression on other antigen presenting cells including monocytes and macrophages [43].

Underpinning the recruitment and activation of antigen presenting cells is the ability of adjuvants to stimulate the innate response, with a particular focus on triggering pattern recognition receptors (PRR). PRRs are expressed by innate cells and enable them to recognise infections. It covers a broad range of families including the Toll like receptors (TLR), Rig-like receptors (RLR) and the inflammasomes. Some adjuvants act directly by engaging these receptors, for example the TLR5 ligand flagellin [44]. However other adjuvants, particularly particulate adjuvants, act more indirectly by inducing local damage which is in turn detected by inflammasome complexes [45], though the exact pathway by which this occurs is not fully characterised.

For the effective induction of an antibody response, there needs to be an interaction between T and B cells. B cells do not directly interact with antigen presenting cells, but they do respond to some of the same signals [46], so it may be that adjuvants activate them in this way. Alternatively, improving the T cell quality with adjuvant, for example increasing the number of T follicular helper cells [47], may lead to improvement in the antibody response.

Whilst many of the adjuvants that are used have been developed empirically, greater insight about the induction of the innate immune response and how that shapes the adaptive immune response has led to immunologically designed adjuvants, for example MPLA targeting TLR4 which is incorporated into AS04. However, for many of the adjuvants in wide use, mechanistic knowledge is incomplete, but this doesn't prevent vaccine licensure; provided a vaccine works and is safe, the mechanism of action is a secondary consideration.

Adjuvants in licensed influenza vaccines: characterisation and mechanism

Alum is the most commonly included adjuvant in influenza vaccines, but even then is only included in five vaccines. The other adjuvants used are virosomes (Inflexal V), MF59 (FluAd), AS03 (Pandemrix). AF03 was licensed for as part of Humenza, but this product was never marketed. Heat labile enterotoxin (LT) was licensed as part of Nasalflu, but this has been withdrawn.

Alum

Alum is the oldest and most widely used adjuvant. Though it should be noted that the description Alum, which strictly refers to $KAl(SO_4)_2$ only, often covers a broad range of Aluminium salts, including aluminium phosphate and aluminium hydroxide. Strikingly the immunological mechanism of action of Alum is still not entirely understood [48-50]. Recent studies have suggested that the formation of an antigen depot is not sufficient to explain the mechanism of alum[36]. Sensing of alum appears to be inflammasome mediated via uric acid crystals leading to the release of interleukin-1β (IL-1β) [45], this was supported by studies where treatment with uricase reduced alum induced inflammation [51]. However patients receiving the anti-IL-1β monoclonal antibody Canakinumab had no difference in their response to adjuvanted influenza vaccination, suggesting that the effect of Alum is partially IL-1β independent [52]. Other studies have identified a role for DNA release following alum induced necrosis of local cells, this DNA is then sensed by the STING pathway [53]. However it is sensed, alum leads to local inflammation of neutrophils via the chemokines CXCL2 and CXCL1 [51] and macrophages via CCL2 and CCL4 [54]. The cells that reach the vaccination site either shuttle antigen to antigen presenting cells or are capable of acting as antigen presenting cells in their own right, with in vitro data suggesting that Alum improves antigen uptake [55]. Alum activated antigen presenting cells tend to shift the response towards a T helper 2 phenotype [56], though it is not clear how.

MF59

MF59 is an oil-in-water emulsion which was originally designed by Chiron to meet the need for an adjuvant which could induce good immunogenicity to purified antigen vaccines [57]. At the time of MF59 development, Alum remained the gold standard adjuvant, but it was ineffective as an adjuvant for new recombinant technologies. MF59 was designed using the principles of Freund's incomplete adjuvant, a mineral oil–in-water emulsion which although tested in human influenza vaccination [58], was deemed too reactogenic for regular use [59]. MF59 contains squalene, polysorbate 80 and sorbitan trioleate. Squalene was chosen as the oil component as it is a naturally occurring oil found in large quantities in human tissues.

The mechanism of MF59 action has been well studied [60]. Whilst antigen can form complexes with MF59, there is no evidence that depot formation is required for MF59 function as it is quickly cleared

from the site of immunisation [61]. As with alum, the innate sensing pathways involved in the detection of MF59 have not been fully identified: it appears to act independently of NLRP3 [62, 63], though MyD88 [62] and CARD [63] appear to play a role. At a molecular level, MF59 induces a specific gene signature that is distinct to Alum, with enrichment in four KEGG categories: cytokine-cytokine receptor interaction, host–pathogen interaction, defense immunity protein activity and the type I IFN response [37]. MF59 induces a distinctive pattern of cytokines after immunisation [64] including the monocyte chemoattractant CCL2, and the neutrophil chemoattractants CCL3 and CXCL8 [43]. These are associated with the recruitment of neutrophils to the site of immunisation that transport the antigen to the lymph nodes [41]. Interestingly, the mechanism of action of MF59 requires the whole formulation; none of the individual components induce an adjuvant effect [65]. MF59 has also been shown to activate DCs [55, 66] and other antigen presenting cells including monocytes and macrophages [43]. MF59 also induces a shift in the T cell response towards Th2. Both IL-4 and STAT-6 signalling are required for its mechanism and there is a shift towards IL-5, evident even after infection of MF-59 immunised animals [64]. How MF59 administration leads to the release of these specific chemokines and cytokines is not known.

AS03

AS03 is an oil-in-water adjuvant, developed by GSK as part of a broader Adjuvant System which has multiple members [67]. AS03 contains squalene, DL-α-tocopherol and polysorbate 80. Variants of AS03 have been produced, based on the amounts of squalene, DL-α-tocopherol and polysorbate 80: $AS03_A$ has 0.86 mg polysorbate 80, 10.69 mg squalene and 11.86 mg α-tocopherol, whilst $AS03_B$ has half the quantities of these components. The inclusion of α-tocopherol, a bioavailable form of vitamin E, has been argued to boost the immunogenicity of AS03. In order to exert an adjuvant effect, AS03 needs to be administered at the same time as the antigen [68]. AS03 works in a similar fashion to MF59, by engaging the innate immune system leading to cellular recruitment and antigen uptake at the site of immunisation [69]. At a molecular level AS03 administration led to the upregulation of MX1 and STAT1 gene expression [70]. Following AS03 delivery, both neutrophil and monocyte chemoattractants are induced [68]. The administration of AS03 leads to the upregulation of CD4 T cell responses and IFNγ release [71].

AF03

AF03 (Sanofi Pasteur) is an oil-in-water adjuvant containing squalene, montane 80 and eumulgin b1 ph. The manufacture of AF03 is slightly different to MF59 and AS03, using phase inversion temperature emulsification process [72]. But since it is also an oil-in-water adjuvant it is likely to have a

similar mechanism of action to other oil-in-water adjuvants. It is included in the Humenza vaccine but this has never been marketed. The mechanism of AF03 has not been characterised.

Virosomes

Inflexal V uses virosomes in its formulation. Virosomes, sometimes referred to as liposomes, are based on lipid droplets [73], most commonly using phospholipids. Lipids in aqueous solution can spontaneously form bilayers generating a vesicle that encapsulates a volume of aqueous solution inside. Influenza virosomes incorporate influenza antigen onto the surface of the vesicle and so mimic a virus; as such virosomes can be considered as a type of viral like particle (VLP). The physical properties of these particles are critical in their efficacy [74]. By mimicking a virus, virosomes can assist with antigen uptake into antigen presenting cells, cell activation and trafficking within the lymph system. Virosomes with surface exposed antigen can also boost antibody responses by improving the 3D structure of the antigen, increasing antigen density, which leads to greater cross linking of B cell receptors. Whilst the virosomes used do not have influenza genetic material incorporated, there is scope to incorporate this or other PAMPs and therefore deliver immune activators directly to the B cells [46].

Impact of adjuvants on the immune response to flu

The inclusion of an adjuvant increases anti-influenza antibody responses. When compared against unadjuvanted vaccines, virosome adjuvanted vaccines were more immunogenic in both children [75] and the elderly [25]. In children, the addition of MF59 induced greater antibody [76] and cellular [77] responses than vaccine without adjuvant. MF59 also induced a better response in immune naïve individuals [78] to a potential pandemic antigen. Likewise the inclusion of the AF03 adjuvant boosted responses compared to unadjuvanted vaccine in 6-35 month old children [79]. H5N1 influenza vaccine formulated with AS03 induces stronger B and T-cell responses than vaccine alone [80]. When MF59 was compared directly against virosomes, it led to a significantly greater number of elderly patients seroconverting (fourfold increase in antibody titre) [24], but both have been shown to have efficacy against influenza infection in the elderly [81]. Comparisons have also been performed between AS03, MF59 and unadjuvanted H7N9 antigen; both the adjuvants induced seroconversion in significantly more patients than no adjuvant [82], in this study AS03 inclusion led to a higher antibody titre. A couple of meta-analyses indicated that inclusion of MF59 increased Haemagglutination inhibition (HI) titres by 1.14-1.4 fold [5, 83], but it was argued that this would not have a big impact on efficacy, as based on human challenge studies [10], this difference in HI titre is not large enough to have an effect.

Safety/ Tolerability of adjuvanted flu vaccines

In general adjuvants are well tolerated, though they may increase some local site symptoms, particularly injection site pain. Two Phase III studies of AS03 adjuvanted H5N1 have been performed covering 10,000 adults [84, 85]. In these studies, the adjuvanted vaccine solicited local and general symptoms more frequently, including pain, fatigue, headache and myalgia. Immunisation of children with an AS03 adjuvanted vaccine was also associated with transient injection site pain [86]. Similar results were seen with an AS03 adjuvanted H1N1 vaccine, the most frequently reported symptom was injection site pain [87], and local and general symptoms were reported more frequently for AS03-adjuvanted H1N1 vaccine recipients than for controls [88, 89]. In children, the incidence of some reactions, especially fever (axillary temperature ≥37.5°C), increased after the second dose [90]. A meta-analysis of MF59 usage in clinical trials in elderly adults suggested that local reactions were slightly more common for vaccine with adjuvant, but fever was very uncommon in either group [91]. A retrospective review over the lifespan of the virosome adjuvanted vaccine, [92] Inflexal V [93], suggest that virosomes are well tolerated.

When adjuvants didn't work:

However, there have been notable cases where adjuvanted influenza vaccines have had to be withdrawn (Nasalflu) or the recommended usage altered (Pandemrix). Separating the specific contribution of adjuvant to the adverse effect is complicated as they are always administered in combination with the antigen. However, it is likely that the adjuvant played a role in the adverse effects seen. There are a range of possible mechanisms by which the inclusion of an adjuvant might have increased the incidence of severe adverse effects including increased inflammation caused by the adjuvant, as seen with LT adjuvanted Nasalflu, or altered responses to the antigen including increased immunogenicity of sub-dominant epitopes, as possibly seen with AS03 and narcolepsy.

AS03 adjuvanted H1N1 vaccine and narcolepsy.

One of the vaccines produced in response to the emergence of the 2009 H1N1 pandemic virus was an AS03 adjuvanted H1N1 vaccine, marketed by GSK as Pandemrix. Approximately 90 million doses of AS03-adjuvanted H1N1 vaccine were administered worldwide during the 2009–2010 H1N1 pandemic. After the vaccination campaign had been completed, cases of the rare sleeping disorder, narcolepsy, were reported in Sweden and Finland [94]; this was particularly seen in individuals with the HLA-DQB1*0602 haplotype. A retrospective study in the UK also reported an increased risk of narcolepsy in ASO3 adjuvanted pandemic A/H1N1 2009 immunised children [95]. The cause of vaccine associated narcolepsy is uncertain, but one suggestion is that there was an increased frequency of antibodies to hypocretin receptor 2 in the sera of immunised patients [96]. Since the antibodies were cross reactive with a fragment of the influenza nucleoprotein, one suggestion that it was a

combination of HLA haplotype and nucleoprotein rather than AS03 *per se* [97]. Subsequently the use of Pandemrix has been restricted to people over 20 years of age.

Heat labile enterotoxin adjuvanted vaccine and Bell's Palsy

Nasalflu (Berna Biotech) was an intranasally delivered virosomal influenza vaccine adjuvanted with the heat labile enterotoxin of *E. coli*. In the pre-licensure trials covering 1,218 volunteers no adverse effects were reported. However, in the first seven months after licensure, 46 cases of Bell's Palsy were reported. In a subsequent matched case-control study, the risk of Bell's Palsy was 19 times the risk of controls, or 13 excess cases per 10,000 vaccinees [98]. This appears to have been driven by the inclusion of heat-labile enterotoxin as an adjuvant; a study using a genetically detoxified mutant also led to transient Bell's Palsy [99]. One suggested mechanism is that LT undergoes retrograde neuronal uptake [100] via the olfactory nerve leading to uptake of the adjuvant and possibly the vaccine by the nerve cell [101], which may then lead to inflammation of the nerve.

Future of flu vaccines and adjuvants

Clinical trials of adjuvanted flu vaccine studies in humans have a long history, with one of the earliest studies being performed by one of the founders of modern vaccinology, Maurice Hilleman, who used a stabilised water-in-oil formulation in 1967 [102]. The appetite for new adjuvants has ebbed and flowed, at times they are heralded as the next big thing that will change vaccinology, but at other times they are seen as a red herring. The number of experimental adjuvants that have been used pre-clinically is too large for the scope of this review. Whilst there is a huge range of pre-clinical vaccine adjuvants in development, a smaller number have made it into clinical trials (Table 2). There are a number of reasons why the pre-clinical adjuvants have not moved forwards: some of them simply do not work, some have limited efficacy in animal models that fails to translate into human responses, some would be too expensive to manufacture for a mass market and some are just too weird and wonderful to have a pathway to commercial and clinical development. There have been cycles of development of the substances used, from empirical approaches to immunological design based on better understanding of immune sensing. The adjuvants that have been tested clinically fall into four broad categories: toll like receptors (TLR) ligands, formulation, cytokines and immunostimulators with unknown mechanism. Where results are reported, experimental adjuvants have mostly increased the antibody response to influenza, though in some cases the increases have been marginal.

TLR Ligands

Increased understanding of the events initiating the immune response have led to more targeted adjuvant approaches [103]. The TLRs are a family of evolutionarily conserved pattern recognition

receptors that recognise conserved biochemical motifs that are common in pathogens. Over recent years, the TLRs have been the focus of immunopotentiator development for use as both prophylactic and therapeutic vaccine adjuvants [104, 105]. The most widely studied Toll like receptor, TLR4, recognises lipopolysaccharide (LPS), a major component of the outer membrane of gram negative bacteria. In its native form LPS is too inflammatory to be used as part of a vaccine, but a number of modified versions have been used, including monophosphoryl lipid A (MPLA) and Glucopyranosyl Lipid Adjuvant (GLA). MPLA is present in two adjuvants that are part of licensed vaccines (AS01 and AS04). GLA has been successfully tested in a clinical trial for a potentially pandemic H5 strain of influenza [106]. Many of the other TLRs have also been targeted for adjuvants to boost influenza responses, for example, topical application of imiquimod, a TLR7 agonist [107] which has already been licensed for the treatment of genital warts. Likewise, agonists of TLR3 (rintatolimod) [108] and TLR9 (CpG oligodeoxynucleotides) [109] have also been tried. Fusions of antigens and the TLR5 agonist flagellin have also been developed [44, 110].

Formulation

The second class of adjuvants in development are those that broadly effect vaccine formulation. They are either oil-in-water variants, with similarities to MF59/AS03 or liposomal, with similarities to virosomes. Formulations adjuvants work in part by delivering the vaccine antigen to the correct cell types and in part by causing some local inflammation. Often the formulation incorporates directly inflammatory material.

Cytokines

Cytokines are cell signalling molecules used by the immune system to program the response of other cells. Cytokine induction is a key mechanism of action of many adjuvants and so some studies have looked at directly incorporating cytokines into vaccines to improve responses. These have included the T cell activator IL-2 [111], the dendritic cell activator GM-CSF [112] or type I interferon [113]. These approaches only had a modest effect and the cost of generating a second protein for inclusion in a vaccine makes these unlikely candidates for any onward development. One interesting variant on this is DNA vaccines, where DNA encoding antigen is used as the immunogen [114]. In these vaccines, DNA encoding cytokines has been included to boost the immune response for example IL-12 [115] and GMCSF [116]. A number of clinical trials of DNA encoded influenza vaccines have been performed, but the immune response to them has been modest. An alternative nucleic acid based approach is to deliver the immunogen as RNA. Both DNA and RNA vaccines will have some inherent adjuvant qualities, which will boost the immune response to the expressed antigen, but may limit antigen expression in the first place.

Immunostimulators with unknown mechanism

This covers a diverse range of substances that can boost the immune response to antigen, but without a clear understanding of the immunological mechanism. The likelihood is that they cause some local disruption of cell membranes leading to the release of 'danger signals' triggering a local innate immune response. The clinical studies for these compounds have reported limited increases in immune response.

Of the adjuvants in development, we would speculate that the most likely to progress forward are the TLR based adjuvants. This is because the mechanistic understanding here is the greatest, they are relatively cheap to manufacture and the research on them is the most mature. Indeed a TLR4 ligand (MPLA) has already been included in licensed vaccine adjuvants – AS01 and AS04.

Adjuvants in licensed vaccines other than influenza

In addition to those that have been tested in early phase clinical trials, there are adjuvants that have been included in licensed vaccines that may be included in influenza vaccines in the future. GSK has two other adjuvant formulations that are used in licensed vaccines. AS01 is a liposomal adjuvant containing the TLR4 ligand monophosphoryl lipid A (MPLA) and the saponin QS-21 and is part of the anti-malaria vaccine Mosquirix. AS01 was designed specifically to boost cell-mediated immunity, with a particular focus on CD8 T cells [117]. A vaccine containing this adjuvant has now completed phase III clinical trials [118, 119], conferring medium-term moderate protection to malarial disease. AS04, which contains Alum and MPLA, is used in Cervarix (human papilloma virus) and Fendrix (Hepatitis B). AS04 was first used in Fendrix in 2005 and is currently licensed in Europe [120].

Future of adjuvanted flu vaccines

The biggest question is whether any of the adjuvants in development will be included in a licensed commercial product. There are two hurdles to overcome – the cost of manufacturing the adjuvant to GMP standard at a scale required for influenza vaccine and the risk of an unforeseeable adverse effect occurring when the vaccine is deployed at a population level. Realistically for the current generation of influenza vaccines, particularly in the face of existing adjuvants from the major vaccine manufacturers, in our opinion it is unlikely that a new adjuvant will be included in a seasonal influenza vaccine. However, there is still scope for research into adjuvants to support the next generation of influenza vaccines. Speculatively this could focus on the following areas:

1. **Stabilising the haemagglutinin stem region.** The holy grail of influenza vaccine research is the 'universal flu vaccine'. This would be one vaccine that covers all current and future strain variations. One speculative approach to achieve this extremely difficult goal has been to

target the stem region of the haemagglutinin antigen [121]. Very approximately speaking, haemagglutinin is shaped like a mushroom, with a head and a stem. The head is immunologically dominant, but is also the region that changes the most, the stem is more conserved across different flu stains [122]. Potentially antibodies raised against this region may be able to cross neutralise different strains of virus. Whilst natural infection does raise some anti-stem antibodies [123], raising them with a vaccine has proved tricky. One application of adjuvants could be to stabilise structures that expose the stem region without the head domain.

2. **Universal T cell vaccines.** Whilst most influenza vaccine research has focussed on vaccines that can induce antibody, an alternative might be to induce T cells. Adjuvants that stimulate T cell responses e.g. CAF09 [124] or IC31 [125] may potentiate stronger CD8 responses which may be beneficial. Because they often recognise conserved regions of influenza, T cells can possibly offer better cross neutralisation [126, 127]. There is a history of pre-clinical studies indicating that cross protection can be achieved with CD8 T cells [128] and in human challenge studies T cells correlated with viral shedding [129]. More recently, studies have shown that individuals with elevated T cell responses experienced less severe disease on exposure to pandemic influenza [130]. Interestingly CD4 T cells have also been shown to correlate with protection against challenge [131].

3. **Mucosal protection.** It is becoming clear that local, mucosal immune responses may be more protective than systemic responses. For example, we have recently shown that local IgA is a correlate of protection against influenza challenge [132]. Likewise lung resident T cells (Trm) correlate with protection against challenge in both mouse [133] and human infection studies [134]. However, mucosal vaccination has to date been sub-optimal, the addition of an adjuvant may improve mucosal responses. The addition of an adjuvant to a mucosal influenza vaccine is challenging as the only licensed mucosal vaccine containing an adjuvant (LT/ NasalFlu) had to be withdrawn due to the association with Bell's Palsy [98].

4. **Protection in diverse age groups.** One of the major priorities for an adjuvanted influenza vaccine is the ability to induce a strong response in individuals who are most susceptible to infection – the elderly and the very young [19]. It is likely that since the reasons vaccines are less effective in these age groups are different, different approaches will be required. The use of GLA with an RSV antigen improved anti-RSV responses in adults over the age of 60, suggesting that it may also be effective with influenza [135, 136]. Studies in children are more complex to perform, with a greater risk of unforeseen complications, but this doesn't stop

there being a need for infant specific adjuvants. AS02 has been tested in infants (under 1 year of age) in the context of a malaria vaccine, increasing T cell responses [137].

5. **Pandemic protection.** Of all the viruses, influenza remains one of the most likely to cause a pandemic. This has occurred multiple times, most recently with the emergence of the 2009 H1N1 strain. Under these circumstances, the addition of an adjuvant would enable faster responses and dose sparing to achieve greater coverage. Any of the adjuvants described in this review could potentially perform this function and a number of clinical trials have been performed to pre-test adjuvanted vaccines in this capacity. There are also a number of pandemic vaccines that are pre-licensed to cover the emergence of new strains.

6. **Boosting recombinant and neoantigens.** The majority of the current influenza vaccines are egg derived and therefore contain some degree of other viral material which may boost the immune response to the antigens. However, recombinant antigens, especially those that have been specifically designed using structural vaccinology approaches, may need boosting by adjuvant [138]. This is particularly important for neoantigens from newly emerged pandemic influenza strains for which there is no pre-existing adaptive immunity, notably avian derived antigens have lower immunogenicity in humans.

7. **Resetting original antigenic sin.** An individual's influenza exposure history over life is complex with a mixture of vaccination and natural infection. This repeated exposure shapes the antibody and T cell responses, often focussing the response on immunodominant regions [139]. The concern is that original antigenic sin may reduce the ability to generate responses to novel antigens. It is possible that an adjuvant could reset the B cell response or present new antigens in such a way that B cell memory is altered.

8. **Altering isotype for maternal vaccination.** One usage of influenza vaccines is maternal immunisation, this protects both the mother and the offspring in early life by passive antibody transfer. During pregnancy, maternally derived antibodies are actively transported through placenta from the mother to the foetus, which can provide passive immunity for infants up to 6 months against infection [140]. There are four subclasses of human IgG (IgG1-4). Placental transfer of IgG depends on the subclass, IgG1 is best followed by IgG4, IgG3 and IgG2 [141]. Since adjuvants can alter the IgG subclass [33], potentially the inclusion of a minimally inflammatory adjuvant that preferentially boosts the IgG1 response could boost the amount of antibody transferred to the foetus.

Conclusion

In general, adjuvanted influenza vaccines have a good safety profile and improve the immune response to vaccine antigens. However the addition of an adjuvant may not address the problems with the current generation of influenza vaccines. The problem with these influenza vaccines is not immunogenicity, for the majority of healthy adults influenza vaccination is sufficiently immunogenic. The problem is that the influenza virus evolves away from the vaccine antigen and the induced response is protective against the wrong virus. Changing the magnitude of the response with adjuvant would not necessarily address the problem. Indeed, there are costs that argue against the routine incorporation of adjuvants in seasonal influenza vaccines. This includes the manufacturing cost of an extra component to the required good manufacturing practice (GMP) standard, the elevated frequency of low severity adverse effects after adjuvanted vaccination and finally the small risk of low frequency unexpected severe adverse effects, such as Bell's Palsy after LT adjuvanted vaccination or narcolepsy after AS03 adjuvanted vaccination. However, there are two current usages that warrant the addition of an adjuvant, firstly vaccination of elderly patients with sub-optimal immune responses and secondly pandemic vaccination where fast responses to smaller doses of a previously unseen antigen are required to maximise coverage. Looking forwards, novel adjuvants may also help in the drive for a universal influenza vaccine by stabilising antigens, boosting responses to recombinant antigens, or redirecting the immune response towards either a local or a cellular response.

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Table 1. Licensed vaccines for influenza. Trivalent vaccines contain three strains, usually two influenza A (currently a representative H1N1 and H3N2 strain) and an influenza B strain (either a Yamagata lineage or Victoria lineage depending on what is currently circulating). Quadrivalent vaccines contain four strains two A and two B. Pandemic vaccines are pre-licensed in anticipation of that strain becoming more prevalent. Apart from Flublok which is recombinant protein, the majority of vaccines are egg derived and either whole virion, split (where the virus has been disrupted by a detergent) and subunit (where the haemagglutinin and neuraminidase proteins have been further purified, removing other viral proteins).

Product Name	**Vaccine type**	**Manufacturer**	**Adjuvant**	**Currently i**
Influvac	Subunit, inactivated, Trivalent	Abbot Biologicals (Distributor Mylan)	None	Still in Use
Fluenz	live attenuated virus, Quadrivalent	AstraZeneca	None	Still in Use
Nasalfu	Subunit, inactivated Trivalent	Berna Biotech	Virosome Heat Labile enterotoxin (LT)	Not in use
Inflexal V	Subunit, inactivated Trivalent	Crucell (formerly Berna Biotech)	Virosome	Unclear
Panvax/ Panvax Junior	Split virion, inactivated Pandemic H1N1	CSL Ltd	None	Not in use
Fluvax/ Fluvax Junior	Split virion, inactivated	CSL Ltd	Non	Still in use

	Trivalent			
Pandemrix	Split virion, inactivated, Pandemic H1N1	GSK	AS03	Not in use
Daronrix	Whole virion, inactivated, Pandemic H5N1	GSK	$AlPO_4$ & $Al(OH)_3$	Not in use
Prepandrix	Split virion, inactivated, Pandemic H5N1	GSK	AS03	Still in Use
Arepanrix	Split virion, inactivated, Pandemic H1N1	GSK	AS03	Not in use
Fluarix	Split virion, inactivated, Quadrivalent	GSK	None	Still in use
Q-Pan H5N1	Split virion, inactivated, Pandemic H5N1	GSK	AS03	Still in use
Orniflu	Subunit, inactivated, Pandemic H5N1	Microgen Russia	$Al(OH)_3$	Still in use
Imuvac	Subunit, inactivated, Trivalent	Mylan	None	Still in Use
Celtura	Subunit, inactivated, Pandemic H1N1	Novartis	MF59	Pandemic c
Focetria	Subunit, inactivated, Pandemic H1N1	Novartis	MF59C.1	Not in use
Fluval-AB/Fluval-P/Fluval-K	Whole virion, inactivated, Trivalent	Omnivest	$AlPO_4$ gel	Still in Use
Enzira	Split virion, inactivated, Trivalent	Pfizer	None	Still in Use
Flublok	Recombinant protein, Trivalent	Protein Sciences	None	Still in Use
Emerflu	Split virion, inactivated, Pandemic H5N1	Sanofi Pasteur	$AlPO_4$	Not in use
Humenza	Split virion, inactivated, Pandemic H1N1	Sanofi Pasteur	AFO3	Not in use
Fluzone Quadrivalent	Split virion, inactivated, Quadrivalent	Sanofi Pasteur	None	Still in Use
Intanza	Split virion, inactivated, Trivalent	Sanofi Pasteur	None	Still in Use
Agrippal	Subunit, inactivated, Trivalent	Seqirus	None	Still in Use
Optaflu	Subunit, inactivated, Trivalent	Seqirus	None	Not in use
FluAd	Subunit, inactivated, Trivalent	Seqirus	MF59C.1	Still in Use
Panflu	Whole virion, inactivated, Pandemic H5N1	Sinovac	$Al(OH)_3$	Still in Use

Table 2. Human clinical trials with experimental adjuvants (Pubmed influenza vaccine adjuvant: Clinicaltrials.gov condition influenza, other terms adjuvant)

Adjuvant Name	**Adjuvant Class**	**Adjuvant Description**	**Associated CT.gov ref**	**Sponsor/ Associated**	**St D**
GLA	TLR	TLR4 ligand	NCT01147068.	IDRI	20

Imiquimod ointment	TLR	TLR7 agonist	NCT02103023	University of Hong Kong	20
Aldara/ Imiquimod	TLR	TLR7 topical agonist	NCT02960815	University of Lausanne	20
Rintatolimod	TLR	TLR3 agonist (plus LAIV)	N/A	University Alabama/ Hemispherx	20
Vax128	TLR	Haemagluttinin-flagellin fusion (TLR 5 agonist)	NCT01172054	Vaxinnate	
VAX102	TLR	Matrix protein-flagellin fusion	N/A	Vaxinnate	20
CpG	TLR	TLR9 agonist	N/A	Coley Pharmaceutical Group	20
IC31	TLR	TLR9 agonist + uptake peptide	N/A	Intercell/ Novartis	20
ISS	TLR	TLR9 agonist	N/A	Dynavax	20
JVRS-100	Formulation	Cationic lipid/ DNA complex	NCT00662272, NCT00936468	Colby Pharmaceutical	20
MAS-1	Formulation	Nanoparticular, emulsion	NCT02500680 NCT01623232	Nova Immunotherapeutics/ Mercia	20
Vaxisome	Formulation	Cholesterol liposome	NCT00915187	NasVax	20
PAL	Formulation	Papaya mosaic virus nanoparticle	NCT02188810	Folia Biotech	20
Matrix-M1	Formulation	Saponin, cholesterol and phospholipid (ISCOM)	NCT01897701 NCT01444482	Novavax	20
Montantide	Formulation	Water in oil	NCT00877448	BiondVax Ltd	20
Proteosome	Formulation	Bacterial hydrophobic outer membrane proteins	NCT02522754	hVIVO	20
W805EC	Formulation	Nanoemulsion delivered intranasally with Fluzone	N/A	NanoBio Corporation	20
ISCOM	Formulation	Immune stimulating complexes	N/A	Erasmus	20
Liposome	Formulation	Oligolamellar phospholipid	N/A	St Louis University	19
NanoStat, NB1008	Formulation	Emulsion	NCT01333462/ NCT01354379	NanoBio	20
Type I Interferon	Cytokine	Cytokine delivered mucosally	NCT00436046	Baylor College	20
IL-2	Cytokine	Cytokine	N/A	Hebrew University, Jerusalem	20
GM-CSF	Cytokine	Cytokine	N/A	Emory University	20
LT Patch	Immunostimulator	Heat labile enterotoxin	NCT00908687	Intercell	20
BCG	Immunostimulator	Nonspecific immunity, delivered 14 days before vaccine	N/A	Radboud Institute for Health Sciences	20
Advax	Immunostimulator	Polysaccharide/ delta Inulin	ACTRN12612000709842	Vaxine	20

OM-85 BV	Immunostimulator	Mixed Bacterial lysate	N/A	Fondazione IRCCS	20
OMP-TIV	Formulation/ Immunostimulator	Meningococcal outer membrane proteins	N/A	GSK	20
Mimopath/ FluGEM	Formulation/ Immunostimulator	Bacterium Like Particles	N/A	Mucosis	20
Nasalflu/ LT	Formulation/ Immunostimulator	Virosomal-Subunit adjuvanted LT	N/A	Berna Biotech	20
sLAG-3 (IMP321)	Immunostimulator	MHC II ligand	N/A	Immutep	20
QS21	Immunostimulator	Saponin	N/A	Baylor	20
DHEAS	Immunostimulator	Dehydroepiandrosterone sulphate	N/A	Paradigm Biosciences	19