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Advax™, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses

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Abstract

Advax™ adjuvant is derived from inulin, a natural plant-derived polysaccharide that when crystallized in the delta polymorphic form, becomes immunologically active. This study was performed to assess the ability of Advax™ adjuvant to enhance influenza vaccine immunogenicity and protection. Mice were immunized with influenza vaccine alone or combined with Advax™ adjuvant. Immuno-phenotyping of the anti-influenza response was performed including antibody isotypes, B-cell ELISPOT, CD4 and CD8 T-cell proliferation, influenza-stimulated cytokine secretion, DTH skin tests and challenge with live influenza virus. Advax™ adjuvant increased neutralizing antibody and memory B-cell responses to influenza. It similarly enhanced CD4 and CD8 T-cell proliferation and increased influenza-stimulated IL-2, IFN- γ , IL-5, IL-6, and GM-CSF responses. This translated into enhanced protection against mortality and morbidity in mice. Advax™ adjuvant provided significant antigen dose-sparing compared to influenza antigen alone. Protection could be transferred from mice that had received Advax™-adjuvanted vaccine to naïve mice by immune serum. Enhanced humoral and T-cell responses induced by Advax™-formulated vaccine were sustained 12 months post-immunization. Advax™ adjuvant had low reactogenicity and no adverse events were identified. This suggests Advax™ adjuvant could be a useful influenza vaccine adjuvant.

Keywords

vaccine; adjuvant; influenza; immunity

Introduction

The goal of influenza vaccination is to generate an immune response of sufficient strength and duration to prevent clinical disease caused by circulating influenza strains. Trivalent inactivated influenza virus (TIV) vaccines have efficacy against laboratory-confirmed influenza illness of 20–80% [1]. Unfortunately, high-risk groups for influenza-related

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morbidity, namely the elderly, the young and those with chronic disease, often have poor vaccine responses, creating a need for more potent influenza vaccines [1–3].

Strategies to enhance influenza vaccine immunogenicity include use of higher antigen dose vaccines or inclusion of an appropriate adjuvant. Benefits of adjuvants include enhanced immunogenicity, antigen-sparing and greater duration of protection [4]. However, adjuvants can increase vaccine reactogenicity and may adversely impact on vaccine safety and hence both risks and benefits need to be carefully considered when adding adjuvants to vaccines [5].

Advax™ adjuvant is a novel polysaccharide adjuvant derived from polyfructofuranosyl-D-glucose (delta inulin) that was developed through the National Institutes of Health's Adjuvant Development Program [6]. Advax™ adjuvant has been shown to enhance immunogenicity and provide antigen-sparing in vaccines against Japanese encephalitis in mice and horses [7], HIV in mice [8], avian (H5N1) influenza in ferrets [9], and African Horse Sickness and Glanders in camels [10], amongst others. Its attributes of immune enhancement, antigen-sparing and low reactogenicity suggested Advax™ adjuvant could be a useful adjuvant for influenza vaccines. The aim of this current study was to characterize the immunogenicity and reactogenicity of influenza vaccine formulated with Advax™ adjuvant.

Materials and Methods

Animals

Female BALB/c mice, 6 to 8 weeks of age, bred under specific pathogen-free conditions were supplied by the Flinders University animal facility. All procedures were performed in accordance with the Animal Experimentation Guidelines of the National Health and Medical Research Council of Australia and approved by the Flinders Animal Welfare Committee. For influenza challenges, a sickness scoring system based on coat condition, posture and activity was used to assess the extent of clinical disease. Mice were evaluated daily and scored for individual symptoms. Ruffled fur (absent = 0; slightly present = 1; present = 2), hunched back (absent = 0; slightly present = 1; present = 2) and activity (normal = 0; reduced = 1; severely reduced = 2) were evaluated. The final score was the addition of each individual symptom score (e.g. an animal showing slightly ruffled fur (1), slightly hunched back (1) and reduced activity (1) was scored as 3). The minimum score was 0 for a healthy mouse and maximum score 6 for an extremely unwell mouse.

Vaccines

Trivalent human seasonal influenza vaccine (TIV) containing inactivated virus from H1N1, H3N2 and B strains was from CSL (Parkville, Australia) and Kitasato Institute (Saitama, Japan). Beta-propiolactone-inactivated A/Puerto Rico/08/34 (PR8) antigen was from Advanced Biotechnologies Inc. (Columbia, MD, USA). PR8 antigen contained 3×10^{11} virus particles/ml before inactivation. Mouse-adapted PR8 virus was a gift of Dr. Darren Miller, (IMVS, Adelaide, Australia). Virus stocks were propagated in the allantoic fluid of 10- to 11-day-old fertile chicken eggs and purified by sucrose gradient ultracentrifugation as previously described [11] and stored at -80°C until use. Vaccines were administered intramuscularly into the mouse hind limb in 0.1ml volume.

Adjuvants

Advax™ adjuvant, which is a standard delta inulin formulation Ad1 in a bicarbonate buffer, was from Vaxine (Adelaide, Australia). Advax™ adjuvant was combined with influenza antigen by simple admixture prior to immunization. MF59 was prepared by homogenization

of 5% squalene, 0.5% Tween 80 and 0.5% Span 85 in water, as described elsewhere [12]. Montanide ISA720 adjuvant was a gift of Seppic Inc. (Fairfield, NJ, USA). Complete Freund's adjuvant (CFA) was from Millipore (Temecula, CA, USA) and Quil-A was from Accurate Chemical (Westbury, NY, USA).

Collection of mouse sera and virus challenges

After immunization, blood was obtained under anesthesia from the retro-orbital plexus and serum separated by centrifugation then stored at -20°C . For virus challenge, mice were anaesthetized and intranasally administered $25\mu\text{l}$ of PR8 virus into each nostril. One LD_{50} corresponded to $1250\text{ TCID}_{50}/\text{ml}$ on MDCK cells. Bodyweight and clinical signs were monitored daily and mice were euthanized when they became moribund.

ELISA for detection of anti-influenza antibodies

Influenza specific IgG, IgG1 and IgG2a were determined by ELISA. $100\mu\text{l}$ of PR8 $1\mu\text{g}/\text{ml}$ or 1:200 dilution of Fluvax were absorbed overnight at 4°C to ELISA plates in 0.1M sodium hydrogen carbonate buffer, pH 9.6. Wells were blocked with 1% BSA/PBS and $100\mu\text{l}$ of serum diluted in 1% BSA/PBS (1:1000 for total IgG or IgG1 antibodies and 1:200 for IgG2a) were added and incubated for 2 hours at RT. After washing, HRP-conjugated anti-mouse IgG (Millipore), IgG1 (BD Bioscience) and IgG2a (BD Bioscience) were added and incubated for 1 hour at RT. After final wash, plates were incubated with $100\mu\text{l}$ of freshly prepared TMB for 10 minutes and then the reaction stopped by 1M Phosphoric Acid and the optical density measured at 450nm ($\text{OD}_{450\text{nm}}$) using VersaMax ELISA microplate reader (Molecular Devices) and data analyzed by SoftMax Pro Software.

Hemagglutination inhibition (HI) assay

HI antibody titers were determined as described previously [13]. Briefly, serum was treated with receptor destroying enzyme (RDE (II), Denka Seiken, Japan at 37°C for 18 hours and then the RDE was inactivated by incubation at 56°C for 30 minutes. HI assays were performed in 96-well plates with 4 hemagglutination (HA) units of antigen and 1% guinea pig red blood cells (gRBC). HI titers were determined as the reciprocal of the last dilution of serum that completely inhibited HA. Samples negative at a dilution of 1:10 were assigned a titer of 5.

B- and T-cell Assays

Bones, spleens and lymph nodes were collected and bone marrow isolated from femur by flushing 3% FBS/PBS, red blood cells were removed by osmotic shock and after washing, cells were resuspended in RPMI 1640 complete medium supplemented with 10% heat-inactivated FBS. PR8-specific IgG- or IgM-antibody secreting cells (ASC) were evaluated by ELISPOT. Multiscreen filtration plates were coated with $10\mu\text{g}/\text{ml}$ PR8 antigen in PBS then blocked with RPMI 1640 complete medium. Serial dilutions of bone marrow or spleen cells were incubated in coated wells overnight at 37°C with 5% CO_2 . Plates were then washed and incubated for 3 hours with either $1\mu\text{g}/\text{ml}$ biotinylated goat anti-mouse IgG antibody (Abcam) or $0.25\mu\text{g}/\text{ml}$ biotinylated goat anti-mouse IgM antibody (Abcam) diluted in 10% FBS/PBS. After washing, streptavidin-HRP (BD Bioscience, 1:500) was added and incubated for 1 hour. Plates were washed, incubated with AEC substrate (BD Bioscience) then stopped by extensive washing. For T-cell proliferation studies, splenocytes were labeled with $2.5\mu\text{M}$ carboxyfluorescein succinimidyl ester (CFSE, Invitrogen Life Technologies) and cultured in 24-well plates at 10^6 cells/ml/well, as previously described [14]. Positive and negative controls were stimulated with $1\mu\text{g}/\text{ml}$ pokeweed mitogen or medium, respectively. To measure memory T-cell responses influenza antigen was added at $20\text{ng}/\text{ml}$. After 5 days incubation at $37^{\circ}\text{C}/5\% \text{CO}_2$, cells were washed with BSA/PBS then

treated with Mouse BD FcBlock (BD Bioscience) for 5 minutes before staining with anti-mouse CD4-PE-Cy5 and CD8-PE-Cy7 (BD Bioscience) for 30 minutes at 4°C. FACS was performed with a FACSCanto II analytical flow cytometer and analyzed on FACSDiva software. Proliferation was expressed as the percentage of divided cells.

Adoptive transfer experiments

Lymphoid cells and serum were collected from immunized mice 2 weeks after the second immunization. Recipient mice received 200µl of immune sera containing 800 HI units of anti-PR8 antibody intravenously. B cells were enriched from bone marrow cells by EasySep Mouse B-cell enrichment kit and T cells from splenocytes and draining lymph nodes by EasySep Mouse T-cell enrichment kit according to manufacturer's instruction (STEMCELL Technologies, Melbourne, Australia). Percentages of B- (average purity 85%) and T- (average purity 88%) cell populations were determined by FACS with anti-mouse CD3 and CD19 antibodies (BD Bioscience). Enriched B cells (1.3×10^7 cells in 0.2 ml) or T cells (8.0×10^6 cells in 0.2ml) were injected intravenously into recipient mice. Recipient mice were challenged intranasally with PR8 virus (2 or $10 \times \text{LD}_{50}$) 18 hours after the adoptive transfer.

Cytometric Bead Array (CBA)

Cytokines and chemokines from splenocytes stimulated with influenza antigen were measured by Mouse Th1/Th2 10plex and Mouse Chemokine 6plex FlowCytomix Multiplex and analyzed by FlowCytomix Pro Software (eBioscience, CA, USA) according to the manufacturer's instructions.

Footpad swelling test

Groups of 3 BALB/c mice were injected with 50µl of various adjuvants into the left footpad and the same volume of saline injected into right footpads as a control. Advax™ (0.1mg/mouse), MF59 (1:1 vol/vol), Montanide ISA720 (3:7 vol/vol), CFA (1:1 vol/vol) and Quil-A (0.1mg/mouse) were tested. Thickness of footpads was measured by a thickness gage 24 hours later. Footpad swelling was calculated by (thickness of left footpad – right footpad).

Delayed-type hypersensitivity (DTH)

DTH reactions were measured as previously described [15]. In brief, inactivated PR8 antigen was diluted in saline (0.1mg/ml) and 50µl was injected into the right footpad using a 30G needle. The same volume of saline was injected into the left footpad as a control. The extent of inflammation in both footpads was measured at 72 hours after injection using a spring gauge caliper (Mitutoyo, Japan). Three measurements were taken on each footpad. Antigen-specific DTH was evaluated as the mean of the difference of thickness between the left (antigen) and right (saline) footpad.

Statistical analysis

GraphPad Prism 4 for Windows was used for drawing graphs and statistical analysis (GraphPad Software, San Diego, CA, USA). Significant differences between experimental and control groups were analyzed by t-test or one-way ANOVA, using Turkey's post-test. For animal survival data, survival curves were created using the Kaplan-Meier method, and statistical analyses of survival curves used a log-rank test. Differences were considered statistically significant when *p*-value was less than 0.05.

RESULTS

Advax™ adjuvant enhances antibody responses to influenza vaccine

To investigate whether influenza vaccine immunogenicity was enhanced by Advax™ adjuvant, antibodies were measured in mice after two immunizations 2 weeks apart with either trivalent seasonal influenza vaccine (TIV) alone (40ng/mouse), or together with Advax™ adjuvant (1mg/mouse). The 1mg dose of Advax™ adjuvant was found to be the optimal mouse dose when dose titrations were performed (data not shown). Mice were bled two weeks later and anti-influenza IgG responses measured by ELISA. Formulation of TIV with Advax™ significantly increased the IgG response to influenza (Fig. 1A), with increases in both IgG1 ($p < 0.001$) (Fig. 1B) and IgG2a subtypes ($p < 0.05$) (Fig. 1C). This translated into significantly higher hemagglutinin inhibition titers in mice receiving Advax™-adjuvanted TIV ($p < 0.01$) (Fig. 1D) when compared to TIV alone.

Advax™ adjuvant increases antibody secreting B cells

To assess whether higher antibody responses correlated with a higher frequency of antibody secreting cells (ASC), influenza-specific antibody secreting cells were measured by ELISPOT in bone marrow and spleen from PR8-immunized mice. Mice immunized with PR8 formulated with Advax™ adjuvant had significantly higher frequencies of influenza-specific B cells secreting either IgG or IgM in bone marrow (Fig. 1E) and spleen (Fig. 1F) when compared to mice immunized with PR8 alone.

Advax™ adjuvant increases T-cell proliferative responses to influenza

T-cell help is required for generation of isotype-switched B cells. To assess whether influenza antigen formulated with Advax™ adjuvant increased T-cell recall responses, splenocytes from mice immunized with influenza antigen with or without Advax™ adjuvant were labeled with CFSE and then cultured with influenza antigen for 5 days. Mice that had received vaccine formulated with Advax™ adjuvant had significantly higher CD4 ($p < 0.01$) and CD8 ($p < 0.001$) T-cell proliferation in response to influenza antigen when compared to mice that received influenza antigen alone (Fig. 1G).

Advax™-adjuvanted vaccine induces a mixed Th1 and Th2 cytokine profile

Given the increased T-cell proliferation in response to influenza antigen observed in mice immunized with influenza antigen plus Advax™ adjuvant, we asked whether Advax™ might have imparted a skew towards either a Th1 or Th2 response. Splenocytes from immunized mice were re-stimulated *in vitro* for 3 days with influenza antigen and culture supernatants harvested for cytokine measurement. Splenocytes from mice that received Advax™-adjuvanted vaccine produced significantly higher IL-2, IL-5, IL-6, IFN- γ and GM-CSF, no change in IL-4 and a non-significant trend towards lower IL-1 and TNF (Fig. 2), when compared to cytokines produced by splenocytes from mice immunized with influenza antigen alone.

Advax™ adjuvant enhances vaccine protection against influenza infection

To assess the influenza antigen-sparing capability of Advax™ adjuvant, BALB/c mice ($n=5$) were immunized twice at a 3-week interval with PR8 antigen (10ng, 100ng or 1000ng) with or without Advax™ adjuvant. For each dose level of PR8 antigen, the addition of Advax™ adjuvant to the influenza antigen significantly enhanced antiinfluenza antibody titers by IgG ELISA (Fig. 3A) and by microneutralization assay (Fig. 3B). There was no significant difference in influenza IgG and microneutralization titers between mice that received the highest 1000ng dose of PR8 without adjuvant and those that received the lowest 10ng dose

of PR8 with Advax™ adjuvant (Fig. 3B), consistent with at least 100-fold antigen-sparing by Advax™ adjuvant.

Immunized mice then received intranasal challenge with live PR8 virus. The mice that received PR8 formulated with Advax™ adjuvant had significantly lower clinical disease as measured by sickness scores (Fig. 3F) than mice that received the same dose of PR8 antigen alone (Fig. 3D). Mice that had received Advax™ adjuvant with either 100ng or 1000ng of PR8 had minimal weight loss (Fig. 3E) and 100% survival (Fig. 3H) whereas even mice that received the highest 1000ng dose of PR8 antigen without adjuvant lost ~10% body weight (Fig. 3C) and suffered 20% mortality (Fig. 3G). Mice injected with Advax™ adjuvant alone without PR8 antigen did not show any protection against influenza infection or mortality (Figs. 3E, 3F and 3H).

Advax™ adjuvant provides long-term enhancement of vaccine-induced immunity

To assess the durability of the immunity induced by influenza vaccine formulated with or without adjuvant, mice were immunized twice, 2 weeks apart, with 200ng PR8 with or without Advax™ adjuvant or MF59 squalene emulsion adjuvant. At 49 weeks post-immunization, mice that received PR8 antigen formulated with Advax™ or the comparator MF59 adjuvant had significantly higher IgG (Fig. 4A) and DTH responses (Fig. 4B) to PR8 antigen compared to mice immunized with PR8 antigen alone. Similarly, CD4 and CD8 T-cell proliferative responses to PR8 antigen were significantly higher at 49 weeks post-immunization in mice that received vaccine formulated with Advax™ adjuvant or MF59 adjuvant compared to PR8 alone (Fig. 4C). When challenged with PR8 virus, mice immunized 49 weeks previously with saline, or PR8 antigen alone, exhibited high sickness scores, greater than 30% weight loss and 100% mortality, whereas mice immunized with PR8 together with either Advax™ adjuvant or MF59 adjuvant had significantly less weight loss and clinical disease (Figs. 4D and 4E) with 33% (3/9) and 50% (5/10) survival, respectively, in these groups (Fig. 4F). Weight loss, clinical disease scores and mortality were not significantly different between Advax™ adjuvant and MF59 adjuvant groups (Figs. 4D, 4E and 4F).

Protection induced by influenza vaccine formulated with Advax™ adjuvant can be transferred by immune sera

To dissect out the contribution of serum antibody versus T- and B-cells to influenza protection induced by vaccine formulated with Advax™ adjuvant, immune sera or enriched T- or B-cells from immunized mice were transferred into naïve recipient mice, which were then challenged with either high ($10 \times \text{LD}_{50}$) or low dose ($2 \times \text{LD}_{50}$) PR8 virus. An aliquot of sera containing 800 HI units of anti-PR8 antibody from mice immunized with PR8 vaccine formulated with Advax™ adjuvant, provided complete protection of naïve mice against weight loss or mortality after high (Fig. 5A) or low dose (Fig. 5B), PR8 virus challenge. Naïve recipients were also protected from influenza-related mortality, but not from weight loss, by T cells from the immunized mice, but only after low, but not high, dose PR8 virus challenge. B cells from immunized mice did not transfer protection against either low or high dose PR8 challenge (Fig. 5).

Advax™ adjuvant has low reactogenicity

A major barrier to adoption of new influenza adjuvants is the propensity for local or systemic adverse reactions. The local reactogenicity of Advax™ was compared using a mouse footpad swelling test to a panel of adjuvants including Montanide ISA720, MF59, CFA and Quil-A. Twenty four hours after injection, Advax™ adjuvant induced significantly less footpad swelling than the oil emulsion adjuvants Montanide ISA720, MF59, or CFA or the saponin adjuvant, Quil-A (Fig. 6A). When a comparison was made of muscle injection

site histology, Advax™ adjuvant injection exhibited only a mononuclear cell infiltrate (Fig. 6B) whereas at the other end of the scale CFA injection was associated with muscle necrosis with extensive inflammatory changes (Fig. 6C). Injection of Advax™ adjuvant was not associated with any significant change in footpad temperature at 24 hours post-injection as measured by infrared camera (data not shown), consistent with minimal local inflammation induced by Advax™ adjuvant.

Discussion

This study confirms that Advax™, an adjuvant produced from the polysaccharide delta inulin, increases the immunogenicity of influenza vaccines and thereby enhances protection against influenza infection. Advax™ adjuvant increased vaccine-associated humoral responses with increases in influenza-specific IgM, IgG1 and IgG2a. This translated into higher serum neutralizing antibody titers and correlated with an increase in the frequency of influenza antibody-secreting B cells in the bone marrow and spleen in mice that had received Advax™-adjuvanted vaccine. Advax™ adjuvant increased T-cell responses to influenza antigen as measured by DTH skin tests, influenza-stimulated splenocyte cytokine secretion and T-cell proliferation assays. Increased immunogenicity obtained with influenza vaccine formulation with Advax™ adjuvant translated into enhanced protection against clinical disease and mortality after influenza virus challenge. The extent of immune enhancement and increased protection afforded by Advax™ adjuvant was not significantly different to the enhancement obtained with MF59, a squalene emulsion adjuvant [16].

From a reactogenicity perspective, Advax™ adjuvant caused significantly less local swelling and inflammation than oil emulsion adjuvants (Montanide ISA720, MF59 and CFA) or a saponin adjuvant (Quil-A) when tested in a footpad swelling test. Furthermore, immunization with Advax™ was not associated with any local or systemic adverse reactions. This supports low reactogenicity findings in other studies, including a recent vaccine adjuvant tolerability study in camels, where Advax™ adjuvant combined with either Glanders or African Horse Sickness antigens was associated with lower injection site reactogenicity than a panel of commercial veterinary adjuvants [10].

Rather than skewing the immune response to influenza vaccine in either a Th1 or Th2 direction, formulation of influenza vaccine with Advax™ adjuvant increased both Th1 (IgG2a) and Th2 (IgG1) antibody responses. Similarly, mice immunized with vaccine containing Advax™ adjuvant demonstrated increased production of a mix of both Th1 (IL-2, IFN- γ) and Th2 (IL-5, IL-6) cytokines by influenza-stimulated splenocytes. By contrast, aluminum adjuvants skew towards Th2 immune responses [17] whereas toll-like receptor (TLR) agonists typically skew towards Th1 responses [18]. Given the absence of Th1 or Th2 polarization, Advax™ may best be described as a Th0 adjuvant. MF59 has similarly been described as a Th0 adjuvant [16]. Despite their very different chemistries, Advax™ adjuvant being a polysaccharide particle and MF59 being a squalene oil emulsion, the effects of Advax™ adjuvant and MF59 adjuvant on influenza vaccine immunogenicity were similar in respect of the ability to increase influenza IgG titers, T-cell proliferation and protection one year post-immunization. The major distinguishing feature was that MF59, as with the other oil emulsion adjuvants, was associated with greater local injection site reactogenicity than Advax™ adjuvant, as assessed by footpad swelling test. Advax™ adjuvant provided significant influenza antigen-sparing of the order of 10–100 fold, comparable to published antigen-sparing data on MF59 [19] and AS03 [20], squalene adjuvants. A potential advantage of Advax™ adjuvant over other candidate influenza adjuvants is its simple polysaccharide composition, which may help reduce concerns regarding the safety of adjuvants in influenza vaccines. There are recent reports of narcolepsy in Scandinavian children immunized with a H1N1/2009 pandemic influenza

vaccine (Pandemrix®) containing AS03 squalene adjuvant [21, 22]. Although the mechanism underlying this association is not yet understood, Advax™ adjuvant shares no chemical similarities with squalene, making it a viable alternative candidate should future investigation implicate squalene adjuvant in the pathogenesis of narcolepsy.

Whilst the ability of Advax™ adjuvant to enhance CD4 T-cell proliferation is consistent with it being a Th0 adjuvant, its effect on CD8 T-cell proliferative recall responses was less expected. Presentation of exogenous antigens to CD8 T cells requires antigen cross-presentation [23], normally a feature restricted to professional antigen presenting cells, i.e. dendritic cells [24]. Injection of Advax™ adjuvant alone attracts a mononuclear population to the injection site and therefore it may enhance CD8 responses through recruitment to the antigen injection site of dendritic cells which are then able to cross-present the antigen to CD8 T cells, with this possibility currently being investigated. Further studies will also examine the functional nature of the CD8 T cells generated by vaccine formulated with Advax™ adjuvant, to specifically address whether or not such CD8 T cells have features of cytotoxic T lymphocytes (CTL) able to kill influenza labeled targets or whether they are instead more consistent with memory CD8 T cells.

Advax™ adjuvant enhanced vaccine protection against lethal influenza challenge. Transfer of immune sera from mice immunized with Advax™-formulated vaccine protected naïve recipient mice against both mortality and clinical disease. This suggests that the major factor behind the enhanced protection provided by influenza vaccine formulated with Advax™ adjuvant is through enhanced antibody titers. T cells from mice immunized with Advax™-formulated influenza vaccine also provided some modest protection against mortality from low dose PR8 challenge, although not against clinical disease. This suggests that T-cell immunity may also have made a modest contribution to protection conferred by Advax™-formulated influenza vaccine, consistent with a T-cell contribution to influenza protection reported by many other studies [25–27].

Whilst the exact mechanism of action of Advax™ adjuvant is not yet fully known, a not uncommon feature in the adjuvant domain with ongoing debate regarding the mechanism of action of aluminum adjuvants 90 years after its discovery, progress has been made in this direction. Injection of Advax™ adjuvant alone without antigen does not provide any non-specific protection against influenza viruses or other pathogens such as Japanese encephalitis virus [7]. This contrasts to the toll-like receptor agonist adjuvants where injection of adjuvant alone may either provide protection [28, 29] or increase susceptibility to infection [30], through activation of the innate inflammatory response. The neutral effect of Advax™ adjuvant on susceptibility to infection when administered alone suggests its adjuvant effect does not involve activation of innate immune inflammation. This is further supported by the observation that Advax™ retains its adjuvant effect in MyD88 knockout mice (manuscript in preparation). Studies have not shown any direct effect of Advax™ adjuvant on T-cell activation, *in vitro*, as measured by proliferation or cytokine secretion (unpublished data). However, Advax™ adjuvant particles bind to mononuclear cells and upregulate antigen presentation and co-stimulatory molecules including MHC II, CD11c, CD83 and CD86 [31]. The current working hypothesis, therefore, is that Advax™ adjuvant acts primarily through enhancement of antigen presenting cell function with this leading to enhanced B- and T-cell activation.

In conclusion, the results show that co-administration of influenza vaccine with Advax™ adjuvant enhanced protection against morbidity and mortality, with low vaccine reactogenicity. Ongoing studies into the molecular mechanisms of action of Advax™ adjuvant should cast interesting insights into its ability to enhance vaccine immunity.

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Highlights

- Confirms delta inulin adjuvant (Advax) enhances influenza vaccine protection
- Enhanced protection is associated with increased neutralizing antibody and T cell responses
- Protection can be transferred using immune sera
- Confirms Advax confers 10–100 fold antigen sparing
- Shows that Advax, like MF59, works as a Th0 type adjuvant

Fig. 1.

Co-administration of AdvaxTM adjuvant with influenza vaccine enhances humoral and cellular responses. (A–D) Adult female BALB/c mice (n=5) were immunized intramuscularly twice at a 2-week interval with 40ng HA alone (white bars) or with AdvaxTM 1mg (black bars). Blood samples were collected 2 weeks after the second immunization and IgG (A), IgG1 (B) and IgG2a (C) measured by ELISA. The HI titer was read as the endpoint dilution of serum that completely inhibited hemagglutination and is presented as the log₂ titer plus standard error (D). (E–F) BM and spleen were collected from adult BALB/c mice two weeks following the second immunization with PR8 antigen alone (white bars) or with AdvaxTM (black bars). PR8-specific IgG or IgM ASC in BM (E) and spleen (F) were detected by ELISPOT assay using PR8-coated plates. Data show the average ASC frequencies from 11 mice/group. (G) Female BALB/c mice were immunized intramuscularly twice at a 2-week interval with 45ng HA of TIV antigen with or without AdvaxTM adjuvant. Spleens were collected 5 weeks after the second immunization and antigen-specific CD4 and CD8 T-cell proliferation measured by culturing CFSE-labeled splenocytes with TIV antigen for 5 days (n = 6, mean + SEM). Asterisks designate significant differences (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Fig. 2.

Immunization with PR8 plus AdvaxTM adjuvant results in enhanced Th1 and Th2 cytokine secretion by PR8-stimulated splenocytes. Spleens ($n = 3$) were collected from mice that had received two immunizations of PR8 alone (white bars) or together with AdvaxTM (black bars), and cultured with PR8 antigen for 3 days. Cytokines in the supernatant were quantitated by cytokine bead array and presented as pictograms (pg)/ml. Means + SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS; not significant).

G **H** **Fig. 3.**

AdvaxTM adjuvant improves vaccine protection against influenza challenge. Adult female BALB/c mice ($n = 5$) were immunized i.m. twice 3-weeks apart with the indicated dose of inactivated PR8 antigen. Blood samples were collected 4 weeks after the second immunization and anti-PR8 antibodies measured by (A) ELISA or (B) microneutralization assay with results shown as means + SEM. Mice were given an intranasal challenge with PR8 virus 4 weeks after the second immunization. (C & E) Bodyweight changes, (D & F) sickness score and (G & H) survival after challenge for the PR8 alone immunized group (C, D and G) or PR8 with AdvaxTM immunized group (E, F and H) are shown. +; no survivor. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 4.

AdvaxTM adjuvant enhances the duration of influenza vaccine protection. Female BALB/c mice ($n = 22$ or 23) were immunized i.m. twice with a 2-week interval with 200 ng PR8 antigen alone or combined with either AdvaxTM or MF59 adjuvant. A control group received saline alone. (A) Blood samples were collected 49 weeks after the second immunization and anti-PR8 antibody measured by ELISA. (B) Ten mice were randomly selected from each group for measurement of PR8-specific delayed type hypersensitivity (DTH). (C) Antigen-specific CD4⁺ and CD8⁺ cell proliferation was measured by culturing CFSE-labeled splenocytes with PR8 antigen for 5 days ($n=3$, mean + SEM). (D) Bodyweight, (E) sickness

score and (F) survival post-challenge are also shown (n=9 or 10). +; no survivor. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS; not significant).

A **B** **Fig. 5.**

Immune sera from mice immunized with PR8 plus AdvaxTM adjuvant transfers protection. Bone marrow, spleens and sera were collected from PR8 plus AdvaxTM immunized mice. Immune sera (-○-), or B (-□-) or T-cells (-▲-) enriched by EasySep immunomagnetic beads were intravenously transferred into naïve BALB/c mice (n = 2) and 18 hours later the recipient mice were infected intranasally with PR8 virus at (A) a high dose (10 × LD₅₀) or (B) low dose (2 × LD₅₀). Average bodyweight changes are shown. +; no survivors.

A **B** **C** **Fig. 6.**

Advax™ adjuvant has low local reactivity. Advax™, Montanide ISA720, MF59, CFA or Quil-A were injected into the left footpad of mice with the same volume of saline injected into right footpads as a control (n=3). **(A)** Thickness of footpads were measured by a thickness gage 24 hours later and swelling was calculated by (thickness of left footpad - right footpad) with mean + SEM shown. Significant differences with Advax™ group were determined by a one-way ANOVA with Dunnet's multiple comparison test. **(B–C)** H & E stained muscle section of **(B)** Advax™ adjuvant injection site or **(C)** CFA injection site. The Advax™ injection site shows a predominantly mononuclear cell infiltrate with minimal

muscle disruption whereas the CFA site shows extensive inflammation and muscle cell necrosis. Scale bar = 50µm.