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Randomized clinical trial of immunogenicity and safety of a recombinant H1N1/2009 pandemic influenza vaccine containing Advax™ polysaccharide adjuvant☆

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ABSTRACT

Background: Timely vaccine supply is critical during influenza pandemics. A recombinant hemagglutinin (rHA)-based vaccine could overcome production hurdles of egg-based vaccines but has never previously been tested in a real-life pandemic setting. The primary aim was to determine the efficacy of a recombinant pandemic vaccine and whether its immunogenicity could be enhanced by a novel polysaccharide adjuvant (Advax™).
Methods: 281 adults aged 18–70 years were recruited in a randomized, subject and observer blinded, parallel-group study of rHA H1N1/2009 vaccine with or without adjuvant. Immunizations were at 0 and 3 weeks with rHA 3, 11 or 45 μg. Serology and safety was followed for 6 months.
Results: At baseline, only 9.1% of subjects (95% CI: 6.0–13.2) had seroprotective H1N1/2009 titers. Seroconversion rates varied by rHA dose, presence of adjuvant, subject age and number of immunizations. Eighty percent (95% CI: 52–96) of 18–49 year olds who received rHA 45 μg with adjuvant were seroprotected at week 3, representing a 11.1-fold increase in antibody titers from baseline. Advax™ adjuvant increased seroprotection rates by 1.9 times after the first, and 2.5 times after the second, immunization when compared to rHA alone. Seroprotection was sustained at 26 weeks and the vaccine was well tolerated with no safety issues.
Conclusions: The study confirmed the ability to design, manufacture, and release a recombinant vaccine within a short time from the start of an actual influenza pandemic. Advax™ adjuvant significantly enhanced rHA immunogenicity.

1. Introduction

The 2009 H1N1 influenza pandemic was associated with a rapid upsurge in hospital and intensive care unit admissions for severe respiratory illness, characterized by hypoxemia, multi-organ failure and prolonged mechanical ventilation requirements [1–5]. This pandemic, the first in over 30 years, highlighted the need for faster and more efficient pandemic vaccine production. Traditional vaccines which rely on cultivation of adapted influenza virus in eggs take 3–4 months to establish, with yields dependent on the selected seed strain [6]. Early in the 2009 pandemic, the initial influenza A/H1N1/California/04/2009 strain distributed by Centre for Disease Control (CDC) provided unsatisfactory yields, requiring selection of a higher-yield strain (A/H1N1/California/07/2009), thereby delaying vaccine availability [7,8]. Furthermore, egg supply is vulnerable to supply disruptions and such vaccines may not be suitable for children with severe egg allergies [9,10]. While this problem has been addressed by the recent development of large-scale facilities for mammalian cell culture of influenza virus and several cell-culture inactivated vaccines now are licensed [11,12], an alternative vaccine substrate is recombinant hemagglutinin (rHA). HA is

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the dominant target of protective neutralizing antibodies after natural infection or vaccination [13,14]. The predictability and speed of recombinant protein production makes this an attractive technology for pandemic vaccines, and in response to the Public Health Emergency Medical Countermeasures Enterprise Review the US government has awarded large contracts to several companies to produce recombinant influenza vaccines [15]. Insect cell-derived rHA produced using the baculovirus expression system has been in clinical testing for a number of years as an alternative to inactivated influenza virus vaccines. The efficacy of rHA protection against seasonal influenza was confirmed in a study of 4648 subjects [16,17].

In pandemic studies, rHA protected birds against lethal infection with H5 or H7 strains [18] although only low levels of seroprotection were achieved in humans administered rHA5 [19], indicating the need for an adjuvant. Furthermore, given that antigen manufacture is a major limiting factor in vaccine supply, adjuvant-based dose-sparing strategies are a major pandemic priority. Advax™ is a novel polysaccharide adjuvant based on particles of semi-crystalline delta inulin [20], which was developed through the Adjuvant Development Program of the National Institutes of Health. Advax™ enhances vaccine immunogenicity and protection in a range of animal models including Japanese encephalitis [21], HIV [22] and avian H5N1 influenza [23]. Although its exact mechanism of action has yet to be determined, Advax™ particles bind directly to human monocytes and enhance their co-stimulatory function [20]. In an influenza challenge study, Advax™ adjuvant significantly enhanced H5N1 vaccine protection, with 100% survival of ferrets receiving adjuvanted vaccine versus only 66% survival with standard H5N1 vaccine [23]. Advax™ adjuvant significantly reduced neurological disease and H5N1 viral shedding while providing over 3-fold antigen dose-sparing [23].

The H1N1/2009 outbreak provided the first opportunity to test the speed and utility of the rHA approach in a real pandemic setting. We report here the findings of a clinical study performed on the first rHA vaccine to be developed during an actual pandemic. The study addressed two main questions: first, whether it was possible to design, manufacture and release a recombinant vaccine within 12 weeks of identification of a new pandemic influenza strain and, second, whether Advax™ adjuvant would improve the immunogenicity of the recombinant antigen.

2. Methods

2.1. Vaccine composition

Recombinant HA cloned from H1N1/A/California/04/2009 (Source: CDC ID number 2009712047; Passage 1 MDCK cells) was supplied by Protein Sciences Corporation (PSC), Meriden, USA. The HA gene was cloned from influenza viral RNA as a template in reverse transcriptase PCRs (RT-PCR) to generate cDNA which was cloned into a baculovirus transfer vector and then used to transfect Spodoptera frugiperda Sf9 insect cells using calcium phosphate precipitation with linearized Autographa californica nucleopolyhedrovirus (AcMNPV) genomic DNA and the baculovirus transfer plasmid containing the HA gene [24]. Recombinant virus stock was expanded and added to a bioreactor at a concentration of 1.0 plaque forming unit (PFU)/cell equivalent to 2% (v/v) and incubated at 28 °C for 48–72 h. Infected cells were then removed from the bioreactor(s), separated from the culture media by centrifugation, solubilized using non-ionic detergent and rHA purified by depth filtration, ion-exchange, cation exchange, hydrophobic interaction column, Q-membrane and finally ultrafiltration [24]. Single-dose vials of Advax™ adjuvant containing delta inulin 20 mg in 0.2 mL of bicarbonate buffer were supplied by Vaxine Pty Ltd., Adelaide, Australia. Advax™ adjuvant was mixed with the antigen at the bedside immediately prior to injection.

2.2. Study design, subjects and study procedures

A randomized, subject and observer blinded, parallel-group trial was commenced in July 2009 in Adelaide, Australia, to assess safety and immunogenicity of a novel pandemic rHA vaccine in adults aged 18–70 years. The study was approved by the Flinders Clinical Research Ethics Committee. Exclusions included pregnancy, immuno-suppressive therapy, oral corticosteroids, HIV infection, or a history of drug or alcohol abuse. Subjects were randomized to 1 of 6 groups to receive rHA (3, 11 or 45 μg) ± Advax™ adjuvant. Randomization was stratified by age; with 18–45 and 46–70 year old age groups. On day 0 signed informed consent was obtained, venesection performed for baseline serology and the first vaccine dose administered by intramuscular injection of ~0.5 mL into the non-dominant deltoid muscle. At 3 weeks, follow-up bloods were obtained and the second vaccine dose administered. Follow up bloods were taken at 3 weeks and 5 months post the second immunization.

2.3. Safety assessments

Solicited local and systemic reactions were collected with a 7-day memory aid. Serious adverse events were collected throughout the study period. Causality of adverse events was assessed by a blinded Investigator (DLG).

2.4. Hemagglutination inhibition assay

Antibody titers were measured by hemagglutination inhibition (HI) assay with guinea pig RBC, as previously described [25], using HA from a South Australian H1N1/2009 virus isolate (SF3). The titer was expressed as the reciprocal of the highest serum dilution inhibiting hemagglutination. Assays included reference ferret hyperimmune sera to A/California/07/2009 (BEI No. NR-15429, NIH).

2.5. Statistical analysis

The three co-primary efficacy endpoints were seroprotection (HI titer≥40), seroconversion (≥4-fold increase and HI titer≥40), and fold increase in geometric mean titer (GMT). Data analysis was performed with Stata software (StataCorp, version 11.0). Baseline characteristics were compared using *t*-tests, chi-square, or ANOVA. Exact binomial confidence intervals were reported for all proportional end points. Reported *p*-values are two-sided, with no adjustment for multiple testing; $p \leq 0.05$ was considered significant. Geometric mean (GMT) and 95% confidence intervals were computed by taking the exponent of the mean and of the lower and upper limits of the 95% confidence intervals of the $\log_e$-transformed titers. Generalized linear models assessed effects of dose, adjuvant and age on seroprotection and seroconversion rates and GMT. The identity link function was used to assess GMT changes after transformation of HI titer. The logit link function was used to assess effects on seroprotection and seroconversion between visits.

3. Results

3.1. Study population

281 subjects were enrolled, randomized, had baseline serology and received the first vaccine dose. This group was included in the safety analysis. All subjects in whom follow-up blood samples were obtained at relevant time points after immunization were included in the efficacy analyses. Baseline characteristics of the efficacy population who completed the first immunization and 3 week immunogenicity testing ($n = 274$) are shown in Table 1. Subjects

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Table 1
Baseline subject characteristics (efficacy population) according to vaccine dose and adjuvant.

	All subjects	H1N1/2009 dose						Between group comparison
	n = 274	rHA – 3 μg		rHA – 11 μg		rHA – 45 μg		*p*-Value[a]
		Non-adjuvanted *n* = 46	Adjuvanted *n* = 47	Non-adjuvanted *n* = 46	Adjuvanted *n* = 43	Non-adjuvanted *n* = 46	Adjuvanted *n* = 46	
Age, median (IQR)	52(44–60)	51.5 (42–60)	50(39–59)	51(44–62)	50(45–60)	53(43–60)	54(45–63)	0.33
Gender								
Males, *n* (%)	126(46.0)	20(43.5)	25(53.2)	21(45.7)	21(48.8)	21(45.7)	18(39.1)	0.83
Females, *n* (%)	148(54.0)	26(56.5)	22(46.8)	25(54.3)	22(51.2)	25(54.3)	28(60.9)	
Received seasonal flu vaccine, *n* (%)								
In 2009	195(71.2)	33(71.7)	32(68.1)	29(63.0)	31(72.1)	35(76.1)	35(76.1)	0.73
Within last 3 years	223(81.4)	38(82.6)	37(78.7)	36(78.3)	36(83.7)	38(82.6)	38(82.6)	0.98
BMI, *n* (%)								
<25	75(27.4)	15(32.6)	12(25.5)	11(23.9)	13(30.2)	13(28.3)	11(23.9)	0.96
25–30	98(35.8)	14(30.4)	19(40.4)	15(32.6)	14(32.6)	16(34.8)	20(43.5)	
Above 30	101(36.9)	17(37.0)	16(34.0)	20(43.5)	16(37.2)	17(37.0)	15(32.6)	
Race, *n* (%)								
Caucasian	264(96.4)	45(97.8)	45(95.7)	44(95.6)	41(95.4)	44(95.6)	45(95.6)	0.97
Other (Asian/Lebanese)	10(3.6)	1(2.2)	2(4.3)	2(4.4)	2(4.6)	2(4.4)	1(2.2)	
Chronic disease,[b] *n* (%)	150(54.7)	23(50.0)	27(57.4)	23(50.0)	24(55.8)	22(47.8)	31(57.4)	0.42

[a] Difference between groups using ANOVA, median test or chi-square test as appropriate.
[b] Receiving medication for long-term chronic disease (excludes hay fever).

were predominantly Caucasian (96.4%), median age was 52 years, 73% were overweight or obese, 54.7% had chronic disease and 71.2% had received the 2009 seasonal trivalent influenza vaccine. There were no significant differences in baseline characteristics between study groups. A total of 25 subjects (8.9%) withdrew or were lost to follow-up during the study, the majority ($n = 15$) between Visit 3 and Visit 4 (Fig. 1). Attempts were made to contact all subjects to ascertain the reason for withdrawal, with the majority stating lack of time to attend the scheduled follow-up visit as the reason. No withdrawals were reported due to adverse events.

3.2. Serology at baseline

At baseline, just 9.1% (95% CI: 6.0–13.2) of subjects had H1N1/2009 HI titers ≥40, suggesting low prevailing levels H1N1/2009 of virus exposure at the time of study commencement

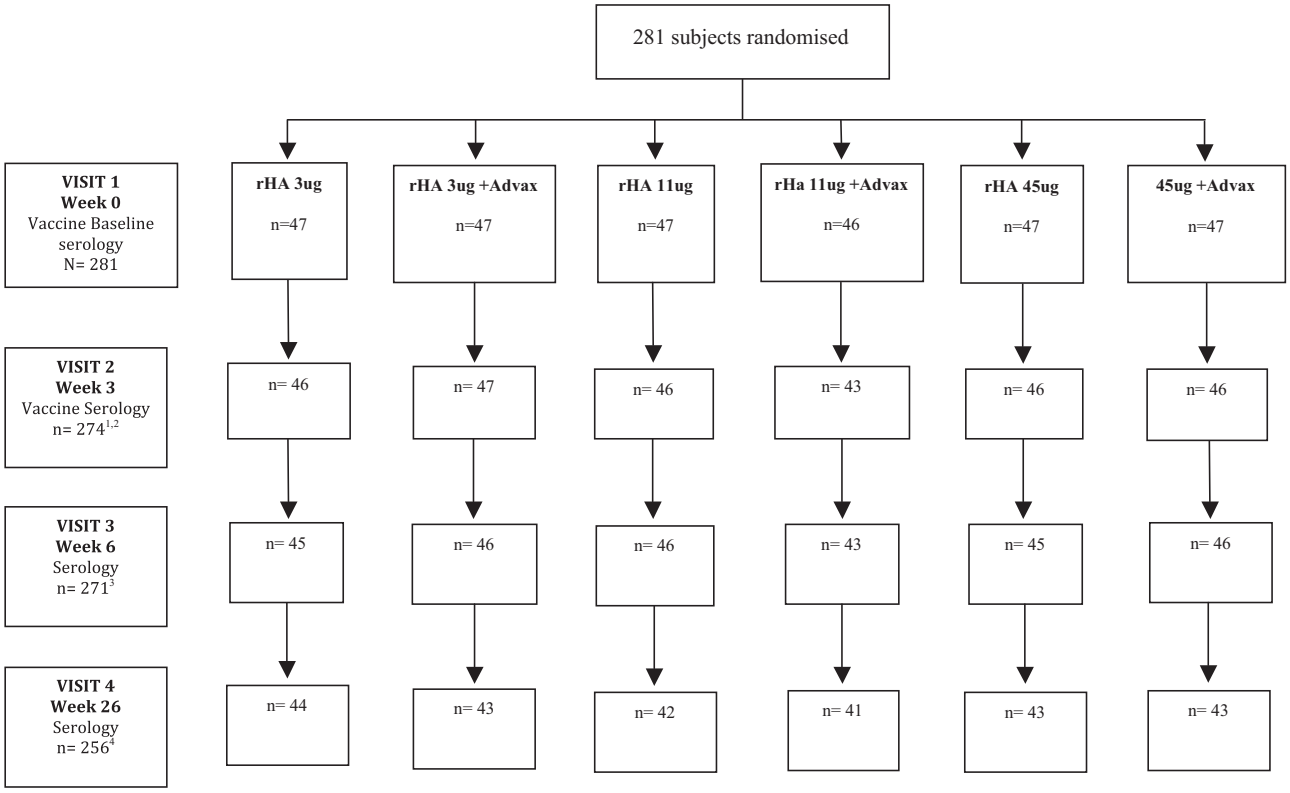

Fig. 1. Subject randomization schedule. [1]Number of subjects providing blood samples at baseline and 3 weeks post-vaccination. [2]Total of 7 subjects did not have week 3 bloods taken. Reasons for missing samples (number of individuals giving these reasons in brackets): work/other commitments (4); non study-related health issue (1); personal reasons (1): problems giving blood (1). [3]Total of 3 subjects did not have week 6 bloods taken: work/other commitments (1), non study-related health issue (1), lost to follow up (1). [4]Total of 15 subjects did not have week 26 bloods taken: work/other commitments (2), personal reasons (2), non study-related health issue (1), deceased (1), withdrawal consent (2), lost to follow up (7).

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(Table 2A). There was no difference in baseline H1N1/2009 seropositivity or GMT between groups and there was no evidence of an effect of age on baseline GMT values.

3.3. Timelines of vaccine production

The development of a rHA vaccine against the newly identified 2009 H1N1 strain commenced on April 29, 2009 when Protein Sciences Corporation received an isolate of A/California/04/2009 from the Center for Disease Control in Atlanta. By mid-June 2009, just six weeks later, the first batch of rHA was ready for release testing. GMP rHA was supplied to the site in single dose vials, with recoverable antigen in the vials of 3, 11 or 45 μg rHA as determined by bicinchoninic acid (BCA) assay, adjusted for product purity. The first clinical dose of vaccine was administered on 18 July 2009. Production of rHA was undertaken in a 600 L fermenter with a working volume of 460 L and a run rate of one batch per 5 days. This yielded 15 mg/L of purified rHA protein. At the current scale, 150,000 rHA vaccine doses (of 45 μg) can be produced every 5 days. Plans are ongoing to enable scale up of the process to a 10,000 L bioreactor that would allow production of >2 million rHA doses of 45 μg every 5 days.

3.4. Serological response to the first immunization

Three weeks after a single rHA immunization, the highest responders were the 18–49 year old group who received rHA 45 μg with Advax™ adjuvant; with a seroprotection rate of 80.0%; (95% CI: 51.9–95.7), seroconversion 73.3%; (95% CI: 44.9–92.2), and GMT fold increase from baseline of 11.1 (95% CI: 4.6–26.4). At the other end of the spectrum, the lowest responding group were subjects aged over 50 years who received rHA 3 μg without adjuvant; with a seroprotection rate of 25.0% (95% CI: 9.8–46.7); seroconversion 20.8% (7.1–42.2); and GMT fold increase 1.5 (1.1–2.0). Hence, response rates varied according to rHA dose, presence of adjuvant and subject age (Tables 2A and 2B). The lower responses in older subjects after the first immunization were significantly improved by higher antigen dose and addition of adjuvant (Tables 2A and 2B).

The data was analyzed in accordance with the European Union Committee for Medicinal Products for Human Use (CHMP) criteria for influenza vaccines; these require the HI response for adults 18–60 years old to achieve seroprotection ≥ 70%, seroconversion ≥ 40%, and GMT fold rise ≥ 2.5, and for those over 60 years seroprotection ≥ 60%, seroconversion ≥ 30%, and GMT fold rise ≥ 2.0. By these criteria, for the group aged 18–60 years, 11 μg rHA + Advax™ passed on 1/3 CHMP criteria, and both 45 μg rHA alone and 45 μg rHA + Advax™ passed on 2/3 CHMP criteria (Table 2C). For the group aged >60 years, only 45 μg rHA + Advax™ passed on 2/3 CHMP criteria (Table 2D).

3.5. Serological response to the second immunization

Three weeks after the second immunization there was an overall 1.2-fold (95% CI: 1.1–1.3) increase in GMT over the response to the first immunization (Table 3A). The antibody response to the second immunization was again dependent on rHA dose, adjuvant and age (Tables 3A and 3B). Overall, subjects receiving Advax™ adjuvant had 2.5-fold higher odds of achieving seroprotection (95% CI: 1.5–4.2, $p = 0.001$) and 2.3-fold higher odds of seroconversion (95% CI: 1.4–3.8, $p = 0.002$) after the second immunization, when compared to subjects receiving rHA alone (Table 3B).

3.6. Persistence of vaccine response

Pandemic strains may recirculate for several years, making the durability of vaccine-induced protection an important

Table 2A
Effects of dose, adjuvant and age on GMT, seropositivity and seroconversion at week 3 post-immunization.

rHA dose	Adjuvant	Age	*n*	Baseline GMT (%, 95% CI)	Baseline seroprotection (%, 95% CI)	Week 3 GMT (95% CI)	Week 3 seroprotection (%, 95% CI)	Week 3 seroconversion (%, 95% CI)	Week 3 GMT (fold change)
3 μg	No	18–49	22	15.1 (10.9–20.8)	18.2 (5.2–40.3)	24.2 (15.9–36.6)	31.8 (13.9–54.9)	18.2 (5.2–40.3)	1.6 (1.2–2.2)
	No	50+	24	11.2 (9.7–12.9)	4.2 (0.1–21.1)	16.3 (11.4–23.4)	25.0 (9.8–46.7)	20.8 (7.1–42.2)	1.5 (1.1–2.0)
	No	All ages	46	12.9 (10.9–15.3)	10.9 (3.6–23.6)	19.7 (15.1–25.8)	28.3 (16.0–43.5)	19.6 (9.4–33.9)	1.5 (1.2–1.9)
	Yes	18–49	22	12.9 (10.5–15.8)	9.1 (1.1–29.2)	26.6 (18.7–37.7)	54.5 (32.2–75.6)	36.4 (17.2–59.3)	2.1 (1.6–2.7)
	Yes	50+	25	12.8 (10.2–16.2)	12.0 (2.5–31.2)	22.3 (14.7–34.0)	40.0 (21.1–61.3)	24.0 (9.4–45.1)	1.7 (1.2–2.5)
	Yes	All ages	47	12.8 (11.1–14.9)	10.6 (3.5–23.1)	24.2 (18.5–31.7)	46.8 (32.1–61.9)	29.8 (17.3–44.9)	1.9 (1.5–2.4)
11 μg	No	18–49	19	14.9 (9.9–22.4)	10.5 (1.3–33.1)	44.6 (24.7–80.7)	63.2 (38.4–83.7)	52.6 (28.9–75.6)	3.0 (1.8–5.0)
	No	50+	27	11.7 (9.4–14.5)	3.7 (0.1–19.0)	18.0 (10.9–30.0)	11.1 (2.4–29.2)	11.1 (2.4–29.2)	1.5 (1.1–2.2)
	No	All ages	46	12.9 (10.5–15.9)	6.5 (1.4–17.9)	26.2 (17.7–38.9)	32.6 (19.5–48.0)	28.3 (16.0–43.5)	2.0 (1.5–2.8)
	Yes	18–49	21	11.8 (9.7–14.4)	9.5 (1.2–30.4)	25.2 (17.0–37.2)	38.1 (18.1–61.6)	23.8 (8.2–47.2)	2.1 (1.5–3.0)
	Yes	50+	22	12.9 (9.5–17.5)	13.6 (2.9–34.9)	25.7 (15.2–43.5)	40.9 (20.7–63.6)	27.3 (10.7–50.2)	2.0 (1.3–3.1)
	Yes	All ages	43	12.3 (10.3–14.7)	11.6 (3.9–25.1)	25.5 (18.6–34.9)	39.5 (25.0–55.6)	25.6 (13.5–41.2)	2.1 (1.6–2.7)
45 μg	No	18–49	19	14.4 (8.8–23.5)	15.8 (3.4–39.6)	48.0 (23.6–97.8)	57.9 (33.5–79.7)	42.1 (20.3–66.5)	3.3 (1.8–6.3)
	No	50+	27	10.8 (9.9–11.8)	0.0 (0.0–12.8)	23.3 (14.8–36.9)	29.6 (13.8–50.2)	29.6 (13.8–50.2)	2.2 (1.5–3.2)
	No	All ages	46	12.2 (9.9–14.9)	6.5 (1.4–17.9)	31.4 (21.2–46.7)	41.3 (27.0–56.8)	34.8 (21.4–50.2)	2.6 (1.8–3.7)
	Yes	18–49	15	11.5 (9.3–14.2)	6.7 (0.2–31.9)	127.0 (51.5–313.0)	80.0 (51.9–95.7)	73.3 (44.9–92.2)	11.1 (4.6–26.4)
	Yes	50+	31	12.2 (10.1–14.8)	9.7 (2.0–25.8)	37.4 (23.1–60.7)	48.4 (30.2–66.9)	45.2 (27.3–64.0)	3.1 (2.0–4.7)
	Yes	All ages	46	12.0 (10.4–13.8)	8.7 (2.4–20.8)	55.7 (35.4–87.6)	58.7 (43.2–73.0)	54.3 (39.0–69.1)	4.7 (3.0–7.1)
All subjects			274	12.5 (11.7–13.4)	9.1 (6.0–13.2)	28.7 (24.8–33.2)	41.2 (35.4–47.3)	32.1 (26.6–38.0)	2.3 (2–2.6)

Table 2B
Effects of dose, adjuvant and age on GMT, seropositivity and seroconversion at week 3 post-immunization.

Week 3 geometric mean titer	Ratio of predicted geometric means[a] (95% CI)	p-Value[b]
Dose		
3	1.00	
11	1.2 (0.9–1.6)	0.21
45	2.1 (1.6–2.9)	<0.001
Adjuvant		
No adjuvant	1.00	
Advax™	1.3 (1.0–1.7)	0.026
Age		
50 years or older	1.00	
Less than 50 years old	1.6 (1.2–2.0)	<0.001
Week 3 seroprotection	**Odds ratio (95% CI)**	**p-Value[a]**
Dose		
3	1.00	
11	0.9 (0.5–1.8)	0.84
45	2.0 (1.1–3.6)	0.03
Adjuvant		
No adjuvant	1.00	
Advax™	1.9 (1.2–3.2)	0.01
Age		
50 years or older	1.00	
Less than 50 years old	2.8 (1.7–4.6)	<0.001
Week 3 seroconversion	**Odds ratio (95% CI)**	**p-Value[a]**
Dose		
3	1.00	
11	1.1 (0.6–2.3)	0.70
45	2.8 (1.5–5.2)	0.002
Adjuvant		
No adjuvant	1.00	
Advax™	1.6 (0.9–2.7)	0.08
Age		0.003
50 years or older		0.003
Less than 50 years old	2.3 (1.3–3.9)	0.003

[a] After adjustment for baseline (week 0) titer.
[b] Using generalized linear models with main effects of dose, adjuvant and age.

consideration. Seroconversion and seroprotection rates were not significantly reduced at final follow up (Table 4A). Seroprotection rates remained highest (80.0%; 95% CI: 51.9–95.7) in the 18–49 year old group who had received rHA 45 μg with Advax™ adjuvant (Table 4A). Groups who received Advax™ adjuvant had a 1.5-fold greater GMT increase (95% CI: 1.2–1.8, $p < 0.001$), 3-fold higher seroprotection (95% CI: 1.7–5.2, $p < 0.001$) and 2.7-fold higher seroconversion rate (95% CI: 1.6–4.6, $p < 0.001$) at 5 months, when compared to subjects who received rHA alone (Table 4B). Late seroconversion may be useful as a marker of clinical virus exposure. Late seroconversion occurring between 6 week and 26 was therefore assessed as a surrogate measure of H1N1/2009 infection. Overall, a low rate of late serocoversion (10/256; 3.9%) was observed across all study groups; rHA 3 μg (0/42; 0.0%), rHA 11 μg (1/43; 2.3%), rHA 45 μg (2/44; 4.5%), rHA 3 μg + Advax™ (4/43; 9.3%), rHA 11 μg + Advax™ (1/43; 2.3%), and rHA 45 μg + Advax™ (2/41; 4.9%) (Table 4A).

3.7. Effect of H1N1/2009 immunization on immunity to seasonal H1N1 strains

The effect of immunization on development of cross-protective antibodies against the co-circulating seasonal influenza strain A/Brisbane/59/2007 was assessed. At baseline 114 (42%) of subjects were seronegative to A/Brisbane/59/2007 Table 5. Administration of the H1N1/2009 vaccine induced seroconversion (6/114; 5%) in a small proportion of subjects initially seronegative to A/Brisbane/59/2007. In univariate analysis, there was a significant effect of age on the A/Brisbane/59/2007 response to H1N1/2009 immunization (χ^2; $p = 0.006$).

3.8. Vaccine tolerability and safety

Frequencies of solicited local and systemic reactions in the 7 days following each immunization were compared between groups. Overall, rHA alone or with Advax™ adjuvant was well tolerated. Solicited local reactions in the first 7 days following the first immunization were reported by 26.1% of subjects. The most common local reaction was injection site discomfort (pain and/or tenderness). Injection site discomfort was reported as grade 1 by 68 subjects (25%), grade 2 by two subjects (<0.1%) and no grade 3 reactions were reported. Other local reactions (bruising, redness, swelling) had frequencies of less than 3%. The majority of local reactions were grade 1, with only 3 subjects experiencing grade 2 reactions. With the exception of grade 1 injection site discomfort, there were no significant differences in frequencies of local reactions between vaccine groups. The most common solicited systemic reaction following the first immunization was headache in 19 subjects (6.9%), followed by fatigue (4.8%), myalgia (3.3%), nausea/vomiting (1.8%) and fever (1.1%). The frequency of headache was significantly lower ($p < 0.05$ by Fishers exact test) in subjects receiving Advax™ adjuvant (4/137: 2.9%), compared to rHA alone (15/137: 10.9%). There were no

Table 2C
Vaccine HI responses in 18–60 year olds by CHMP criteria.

18–60 years	Seroprotection	>70%	Seroconversion	>40%	Fold GMT increase	>2.5
3 μg	35.1	N	24.3 (11.8–41.2)	N	1.7 (1.3–2.2)	N
3 μg + Advax	47.5	N	32.5 (18.6–49.1)	N	2.0 (1.6–2.6)	N
11 μg	40.6	N	34.4 (18.6–53.2)	N	2.1 (1.5–3.0)	N
11 μg + Advax	45.5	N	33.3 (18.0–51.8)	N	2.5 (1.8–3.4)	Y
45 μg	48.6	N	40.0 (23.9–57.9)	Y	2.9 (1.8–4.5)	Y
45 μg + Advax	63.6	N	57.6 (39.2–74.5)	Y	5.8 (3.4–10.0)	Y

Table 2D
Vaccine HI responses in >60 year olds by CHMP criteria.

60+ years	Seroprotection	>60%	Seroconversion	>30%	Fold GMT increase	>2.0
3 μg	0	N	0	N	1	N
3 μg + Advax	42.9	N	14.3	N	1.3	N
11 μg	14.3	N	14.3	N	1.9	N
11 μg + Advax	20	N	0	N	1.1	N
45 μg	18.2	N	18.2	N	1.9	N
45 μg + Advax	46.2	N	46.2	Y	2.6	Y

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Table 3A
Study week 6 response (3 weeks post-2nd immunization). Study week 3 GMT responses are included for comparison.

rHA dose	Adjuvant	Age	*n*	Week 3 (1 dose) GMT value (95% CI)	Week 6 (2 dose) GMT value (95% CI)	Week 6 seroprotection %, (95% CI)	Week 6 seroconversion %, (95% CI)	Week 6 to week 3 GMT fold change
3 μg	No	18–49	22	24.2 (15.9–36.6)	22.0 (15.8–30.7)	31.8 (13.9–54.9)	22.7 (7.8–45.4)	0.9 (0.6–1.3)
	No	50+	23	16.3 (11.4–23.4)	15.7 (10.5–23.4)	12.5 (2.7–32.4)	12.5 (2.7–32.4)	1.0 (0.8–1.2)
	No	All ages	45	19.7 (15.1–25.8)	18.5 (14.3–23.9)	22.2 (11.2–37.1)	17.8 (8–32.1)	0.9 (0.8–1.1)
	Yes	18–49	21	26.0 (18.1–37.6)	36.2 (22.9–57.4)	59.1 (36.4–79.3)	54.5 (32.2–75.6)	1.4 (1.0–1.8)
	Yes	50+	25	22.3 (14.7–34.0)	28.7 (18.5–44.4)	48.0 (27.8–68.7)	36.0 (18.0–57.5)	1.3 (1.0–1.6)
	Yes	All ages	46	24.0 (18.2–31.5)	31.9 (23.5–43.4)	54.3 (39.0–69.1)	45.7 (30.9–61.0)	1.3 (1.1–1.6)
11 μg	No	18–49	19	44.6 (24.7–80.7)	48.0 (26.4–87.3)	73.7 (48.8–90.9)	57.9 (33.5–79.7)	1.1 (0.8–1.4)
	No	50+	27	18.0 (10.9–30.0)	25.9 (15.3–43.6)	33.3 (16.5–54.0)	33.3 (16.5–54.0)	1.4 (1.1–1.8)
	No	All ages	46	26.2 (17.7–38.9)	33.4 (22.6–49.3)	50.0 (34.9–65.1)	43.5 (28.9–58.9)	1.3 (1.1–1.5)
	Yes	18–49	21	25.2 (17–37.2)	44.2 (29.9–65.2)	71.4 (47.8–88.7)	61.9 (38.4–81.9)	1.8 (1.4–2.2)
	Yes	50+	22	25.2 (14.5–43.7)	28.3 (17.6–45.3)	45.5 (24.4–67.8)	36.4 (17.2–59.3)	1.1 (0.9–1.4)
	Yes	All ages	43	25.2 (18.3–34.8)	35.2 (26.0–47.6)	58.1 (42.1–73.0)	48.8 (33.3–64.5)	1.4 (1.1–1.7)
45 μg	No	18–49	18	48.5 (22.8–103.2)	68.6 (36.1–130.4)	63.2 (38.4–83.7)	47.4 (24.4–71.1)	1.4 (1.1–1.8)
	No	50+	27	23.3 (14.8–36.9)	25.2 (16.6–38.2)	37.0 (19.4–57.6)	37.0 (19.4–57.6)	1.1 (0.9–1.3)
	No	All ages	45	31.3 (20.9–46.9)	37.6 (25.9–54.6)	48.9 (33.7–64.2)	42.2 (27.7–57.8)	1.2 (1–1.4)
	Yes	18–49	15	127.0 (51.5–313)	139.3 (60.4–321.3)	80.0 (51.9–95.7)	73.3 (44.9–92.2)	1.1 (0.7–1.6)
	Yes	50+	31	37.4 (23.1–60.7)	46.8 (29.3–74.7)	61.3 (42.2–78.2)	58.1 (39.1–75.5)	1.3 (1.0–1.5)
	Yes	All ages	46	55.7 (35.4–87.6)	66.8 (43.6–102.2)	67.4 (52.0–80.5)	63.0 (47.5–76.8)	1.2 (1.0–1.4)
All subjects			271	28.6 (24.7–33.2)	34.8 (30.1–40.2)	50.2 (44.1–56.3)	43.5 (37.6–49.7)	1.2 (1.1–1.3)

Table 3B
Effects of dose, adjuvant and age on GMT, seropositivity and seroconversion at Week 6 (3 weeks post 2nd immunization).

Week 6 geometric mean titer	Ratio of predicted geometric means[a] (95% CI)	*p*-Value[b]
Dose		
3	1.00	
11	1.4 (1.0–1.9)	0.02
45	2.3 (1.7–3.1)	<0.001
Adjuvant		
No adjuvant	1.00	
Advax™	1.5 (1.2–1.9)	0.001
Age		
50 years or older	1.00	
Less than 50 years old	1.7 (1.3–2.2)	<0.001
Week 6 seroprotection	**Odds ratio (95% CI)**	***p*-Value[a]**
Dose		
3	1.00	
11	2.1 (1.1–3.9)	0.02
45	2.9 (1.5–5.4)	0.001
Adjuvant		
No adjuvant	1.00	
Advax™	2.5 (1.5–4.2)	0.001
Age		
50 years or older	1.00	
Less than 50 years old	3.2 (1.9–5.5)	<0.001
Week 6 seroconversion	**Odds ratio (95% CI)**	***p*-Value[a]**
Dose		
3	1.00	
11	2.0 (1.0–3.7)	0.04
45	2.9 (1.5–5.5)	0.001
Adjuvant		
No adjuvant	1.00	
Advax™	2.3 (1.4–3.8)	0.002
Age		
50 years or older	1.00	
Less than 50 years old	2.5 (1.5–4.2)	0.001

[a] After adjustment for baseline (week 0) titer.
[b] Using generalized linear models with main effects of dose, age and adjuvant.

other significant differences in frequencies of systemic reactions between vaccine groups and no grade 3 systemic reactions were reported.

After the second immunization the pattern of solicited local reactions was similar to the first, with grade 1 injection site discomfort again being the most common local reaction. Only 5 subjects (1.8%) experienced grade 2 local reactions, with 4/5 (80%) being injection site discomfort. No grade 3 local reactions were reported. The most common systemic reaction was again headache in 10 (3.7%) subjects, followed by fatigue (1.8%) and fever (1.8%). There was again a trend ($p = 0.06$) to less post-immunization headaches in groups receiving Advax™ adjuvant (2/135: 1.5%) compared to rHA alone (8/137: 5.8%). There were no significant differences in frequencies of other systemic reactions between vaccine groups. Frequencies of unsolicited AE collected throughout the whole study period were not significantly different between vaccine groups. A total of 18 SAE were reported involving 17 individual subjects with none being classed as vaccine-related by the Study Investigator.

4. Discussion

The 2009 H1N1 pandemic highlighted the need for rapidly scalable influenza vaccine production and presented a unique opportunity to test a new vaccine approach in a real-life pandemic setting. The rHA vaccine was produced and released in less than 12 weeks from initial virus identification. The vaccine was well tolerated and responses were influenced positively by antigen dose and adjuvant and negatively by subject age. This study included a

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Table 4A
Durability of response at study week 26, 23 weeks after 2nd immunization.

Dose	Adjuvant	Age group	*n*	Week 26 GMT value (95% CI)	Week 6 GMT value (95% CI)	Week 26 seroprotection % (95% CI)	Week 26 seroconversion % (95% CI)	Week 26 over week 6 GMT fold change
3 μg	No	18–49	22	22.1 (16.3–29.9)	22.0(15.8–30.7)	31.8 (13.9–54.9)	18.2 (5.2–40.3)	1.0 (0.8–1.2)
	No	50+	24	17.7 (12.5–25.1)	15.7(10.5–23.4)	8.3 (1–27)	8.3 (1–27)	1.1 (0.9–1.4)
	No	All ages	44	19.7 (15.7–24.7)	18.5(14.3–23.9)	20.5 (9.8–35.3)	13.0 (4.9–26.3)	1.1 (0.9–1.2)
	Yes	18–49	22	45.9 (32.1–65.8)	36.2(22.9–57.4)	68.2 (45.1–86.1)	59.1 (36.4–79.3)	1.3 (0.9–1.7)
	Yes	50+	25	25.5 (16.9–38.4)	28.7(18.5–44.4)	40.0 (21.1–61.3)	28.0 (12.1–49.4)	0.9 (0.7–1.2)
	Yes	All ages	43	33.5 (25.3–44.3)	31.9(23.5–43.4)	58.1 (42.1–73)	42.6 (28.3–57.8)	1.0 (0.8–1.3)
11 μg	No	18–49	19	41.7 (25.4–68.4)	48.0(26.4–87.3)	52.6 (28.9–75.6)	42.1 (20.3–66.5)	0.9 (0.6–1.2)
	No	50+	27	25.7 (16.9–39.1)	25.9(15.3–43.6)	33.3 (16.5–54)	33.3 (16.5–54)	1.0 (0.8–1.3)
	No	All ages	42	31.2 (22.8–42.8)	33.4(22.6–49.3)	45.2 (29.8–61.3)	37.0 (23.2–52.5)	0.9 (0.8–1.1)
	Yes	18–49	21	44.4 (31.2–63.2)	44.2(29.9–65.2)	71.4 (47.8–88.7)	66.7 (43–85.4)	1.0 (0.8–1.2)
	Yes	50+	22	28.8 (18.9–43.7)	28.3(17.6–45.3)	50.0 (28.2–71.8)	36.4 (17.2–59.3)	1.0 (0.9–1.2)
	Yes	All ages	41	35.5 (27.1–46.6)	35.2(26–47.6)	63.4 (46.9–77.9)	51.2 (35.5–66.7)	1.0 (0.9–1.2)
45 μg	No	18–49	19	56.6 (35.7–89.8)	68.6(36.1–130.4)	78.9 (54.4–93.9)	63.2 (38.4–83.7)	0.8 (0.6–1.2)
	No	50+	27	22.3 (15.9–31.3)	25.2(16.6–38.2)	25.9 (11.1–46.3)	25.9 (11.1–46.3)	0.9 (0.7–1.1)
	No	All ages	43	33.0 (24.4–44.5)	37.6(25.9–54.6)	51.2 (35.5–66.7)	41.3 (27–56.8)	0.9 (0.7–1.1)
	Yes	18–49	15	105.6 (57.2–194.7)	139.3(60.4–321.3)	80.0 (51.9–95.7)	73.3 (44.9–92.2)	0.8 (0.5–1.1)
	Yes	50+	31	38.1 (25.7–56.4)	46.8(29.3–74.7)	51.6 (33.1–69.8)	48.4 (30.2–66.9)	0.8 (0.7–1)
	Yes	All ages	43	54.3 (38.2–77.3)	66.8(43.6–102.2)	65.1 (49.1–79)	56.5 (41.1–71.1)	0.8 (0.7–1)
All			256	33.0 (29.2–37.3)	34.8(30.1–40.2)	50.4 (44.1–56.7)	43.0 (36.8–49.3)	1.0 (0.9–1)

Table 4B
Effects of dose, adjuvant and age on GMT, seropositivity, and seroconversion at week 26 (23 weeks post 2nd immunization).

Week 26 geometric mean titer	Ratio of predicted geometric means[a] (95% CI)	*p*-Value[b]
Dose		
3	1.00	
11	1.3 (1.0–1.7)	0.031
45	1.8 (1.4–2.3)	<0.001
Adjuvant		
No adjuvant	1.00	
Advax™	1.5 (1.2–1.8)	<0.001
Age		
50 years or older	1.00	
Less than 50 years old	1.7 (1.4–2.1)	<0.001
Week 26 seroprotection	**Odds ratio (95% CI)**	***p*-Value[a]**
Dose		
3	1.00	
11	2.1 (1.1–4.1)	0.03
45	2.9 (1.5–5.6)	0.002
Adjuvant		
No adjuvant	1.00	
Advax™	3.0 (1.7–5.2)	<0.001
Age		
50 years or older	1.00	
Less than 50 years old	4.1 (2.3–7.2)	<0.001
Week 26 seroconversion	**Odds ratio (95% CI)**	***p*-Value[a]**
Dose		
3	1.00	
11	2.3 (1.2–4.5)	0.015
45	3.3 (1.7–6.5)	0.001
Adjuvant		
No adjuvant	1.00	
Advax™	2.7 (1.6–4.6)	<0.001
Age		
50 years or older	1.00	
Less than 50 years old	3.3 (1.9–5.7)	<0.001

[a] After adjustment for baseline (week 0) titer.
[b] Using generalized linear models with main effects of dose, age and adjuvant.

high proportion of elderly, obese and chronic disease subjects, not well represented in other H1N1/2009 trials, which may have negatively impacted on the overall seroprotection rates achieved with the rHA vaccine. The lower vaccine response rates observed in subjects over the age of 50 years have similarly been reported by other H1N1/2009 vaccine studies [26,27]. The reduced responsiveness in older subjects in our study was improved by higher antigen doses, Advax™ adjuvant and a booster immunization. With all factors optimized (maximum rHA dose, Advax™ adjuvant, and two immunizations), subjects aged over 50 years achieved a seroprotection rate of 61.3%.

On their face, the HI results obtained with the rHA vaccine appear lower than results of studies performed with conventional egg-based H1N1/2009 pandemic vaccines, where in most cases a single immunization achieved over 80% seroprotection. For example, in a Chinese study a 15 μg dose of inactivated H1N1 in adults 18–60 years achieved 82.1% seroprotection [28] and in a US study a 15 or 30 μg dose of inactivated H1N1 vaccine in adults 18–64 year achieved >95% seroprotection [29]. Equally high seroprotection levels were seen in studies of either conventional egg-based or cell culture grown inactivated H1N1/2009 vaccines [26,28,30]. This raises the important question of why the rHA vaccine required at least three fold higher antigen doses plus an adjuvant to achieve seroprotection which, except at the highest 45 μg HA dose, did not generate HI titers sufficient to meet CHMP/FDA licensing requirements, and even at the highest 45 μg dose still only met 2/3 CHMP criteria? This low immunogenicity outcome is reflected in previous studies when recombinant and inactivated seasonal influenza

Table 5
Seroconversion to A/Brisbane/59/2007 seasonal influenza strain in response to immunization with recombinant H1N1/2009 pandemic vaccine. Number of subjects who seroconverted to A/Brisbane/59/2007 according to H1N1/2009 vaccine dose, adjuvant, baseline A/Brisbane/59/2007 titer and age group.

	rHA – 3 μg		rHA – 11 μg		rHA – 45 μg		All groups	Effect of age (χ^2, df)
	Non-adjuvanted $n=46$	Adjuvanted $n=47$	Non-adjuvanted $n=46$	Adjuvanted $n=43$	Non-adjuvanted $n=46$	Adjuvanted $n=46$		p-Value
Baseline HI < 40								
<50 years	1/5	2/10	0/8	0/9	0/5	2/4	5/41	(4.16, 1df)
>50 years	0/9	1/13	0/13	1/17	0/10	0/12	2/74	$p=0.04$
Baseline HI ≥ 40								
<50 years	0/17	0/12	1/11	1/12	2/14	2/11	6/77	(4.08, 1df)
>50 years	0/15	0/12	0/14	0/5	0/17	1/19	1/82	$p=0.043$
All subjects								
<50 years	1/22	1/21	1/17	1/20	2/18	4/11	11/118	(7.59, 1df)
>50 years	0/24	1/24	0/27	1/21	0/27	1/30	3/156	$p=0.006$

vaccines have been compared head to head, where 45 μg dose of rHA was required to produce the same HI titers as 15 μg of inactivated HA antigen [17]. Thus, an inherent property of rHA antigen appears to be that it is a third the immunogenicity of an equivalent dose of inactivated virus antigen. The reasons for this difference is not known but may reflect the fact that inactivated vaccines contain many other components including neuraminidase and nuclear proteins, plus viral RNA, that act as inbuilt adjuvants and thereby increase the immunogenicity of inactivated HA antigen [31,32]. Notably, inactivated influenza vaccines lose their immunogenicity when administered to toll-like receptor 7 or MyD88 knockout mice, in which RNA contained in inactivated vaccines is unable to activate the innate immune system and act as an adjuvant [33].

Another surprising finding in our study was the limited boosting effect of the second dose of rHA vaccine. This may partly be because of the short time window between doses, as a longer dose interval of up to 12 months between boosters has previously been shown in the case of hepatitis B vaccines to improve vaccine responses [34]. Alternatively, given the inverse relationship between cellular (Th1) and humoral (Th2) immunity [35], it is possible that the low-responders on the HI assay were instead making a strong T-cell response to the vaccine, which in turn inhibited the antibody response [35]. In future vaccine studies, we plan to also measure anti-influenza T-cell responses to examine this more closely.

Another consideration when trying to compare the study responses to those obtained with inactivated H1N1/2009 vaccines, is that inter-study comparisons based on HI titers are problematic as they are highly dependent on factors such as the HA antigen and red blood cell species used, and HI assays remain poorly standardized between laboratories [36]. For example, another study reported a much higher estimate of baseline seroprotection to H1N1 2009 of 32% in the South Australian population compared to our HI assay based estimate of just 9.1% baseline seroprotection, despite the two studies being performed in the same population at the same time [26]. Our estimate of 9.1% baseline seroprotection appears more consistent with reported US, Chinese and European data obtained early in the H1N1/2009 pandemic [27,37,38]. Seroprotection rates measured by HI assay may understate the protection achieved with adjuvanted rHA vaccine as, for example, a single dose of Advax™-formulated H5N1 vaccine in a recent ferret study completely protected against H5N1 challenge even when there was no detectable HI titer prior to challenge [23]. Thus, HI titers alone are an imperfect measure of influenza protection. Advax™ adjuvant has been shown to enhance memory CD4 and CD8 T cell vaccine responses, and T cells make an important contribution to protection against influenza [39–42]. Although our study was not designed as an infection outcome study, it is notable that there was an extremely low rate of late seroconversions between weeks 6 and 26 (3.9%), consistent with a low rate of H1N1/2009 infection in the immunized study subjects during a period when the H1N1/2009 pandemic was at its peak.

There has been considerable debate regarding the benefits and risks of incorporating adjuvants into influenza vaccines. Aluminum adjuvants are unsuitable for influenza vaccines and paradoxically may even reduce immunogenicity [27]. Several squalene oil adjuvanted seasonal influenza vaccines are licensed in Europe for use in the elderly [43]. Following the commencement of our study, studies were undertaken of inactivated H1N1/2009 vaccines together with squalene adjuvants, MF59 or AS03. Squalene adjuvant provided high levels of seroprotection and dose-sparing; for example a 3.75 μg dose of inactivated HA with AS03A adjuvant achieved 94% seroprotection compared to 73% seroprotection with inactivated HA alone [44]. Overall, vaccines containing squalene adjuvants were well tolerated apart from a propensity to increase injection site pain and muscle aches [37]. However, the importance of developing alternative pandemic influenza vaccine adjuvants is highlighted by recent reports of an increased risk of narcolepsy in Scandinavian children immunized with the squalene-adjuvant formulated H1N1/2009 pandemic influenza vaccine (Pandemrix®) [45]. Although the mechanism underlying the association of Pandemrix® with narcolepsy is not understood, and may ultimately not implicate the squalene adjuvant used, this issue reinforces the importance of developing a broader range of adjuvants for use in future influenza pandemics.

Advax™ is a novel polysaccharide adjuvant made from particles of delta inulin [20]. In support of previous findings in animal models [21,23], Advax™ adjuvant significantly increased the immunogenicity of the rHA vaccine in this study. Given the difficulties of interpreting HI results from different studies we compared, in the same assay, HI titers from the H1N1/2009 vaccine study to convalescent H1N1/2009 patient sera [46]. This confirmed that subjects given the highest dose of rHA plus Advax™ adjuvant achieved comparable HI titers to patients recovered from clinical infection with H1N1/2009.

Enhanced vaccine immunogenicity should not be at the expense of tolerability or safety [47] and in this respect the tolerability and safety profile of Advax™ was reassuring. The lower rate of post-immunization headaches in subjects receiving Advax™ was unexpected, as studies of most adjuvants reveal an increase in headaches [48]. Reduced headaches may relate to Advax™ adjuvant-induced changes in cytokine production. Immunization headaches are likely to be mediated by inflammatory cytokines, in particular interleukin (IL)-1, as IL-1 serum levels are increased in cluster headaches and IL-1 gene polymorphisms (3953C/T) are associated with migraine headaches [49,50]. Unlike pro-inflammatory adjuvants, Advax™ adjuvant does not induce IL-1 gene expression (N. Petrovsky, unpublished data) and this might explain why it does not exacerbate post-immunization headaches.

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The H1N1/2009 pandemic reinforced the need for innovation in influenza vaccine design and manufacture. Our results confirm the utility and speed of a recombinant vaccine approach to pandemic vaccine production, although there remains a need to optimize the immunogenicity of the recombinant antigen. However, while the overall HI data obtained in the current study of recombinant hemagglutinin were modest by reference to CHMP/FDA licensing requirements, this does not necessarily mean that the recombinant vaccine was not effective. While high vaccine-induced HI titers are generally predictive of protection, the converse is not necessarily true, namely low vaccine-induced HI titers do not necessarily predict lack of protection. This fact was clearly demonstrated when ferrets immunized with a H5N1 vaccine formulated with Advax™ adjuvant were completely protected against lethal H5N1 infection despite having no detectable HI titers to H5N1 prior to challenge [23]. What this clearly demonstrates is the urgent need for better assays of immune correlates of influenza vaccine protection to replace traditional HI assays in the licensing of new influenza vaccines. Nevertheless, there is also scope for further enhancement of the rHA approach through further adjustments in antigen and adjuvant dose to help them better meet current CHMP/FDA licensing requirements, and the potential inclusion of recombinant neuraminidase protein to enhance heterotypic immunity [47]. With such strategies to improve immunogenicity in place, a rHA pandemic vaccine may overcome many of the problems of traditional inactivated influenza vaccines and thereby provide benefit in future pandemics.

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