Journal of Microbiology, Immunology and Infection (2019) 52, 685–692

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

The safety and immunogenicity of a cell-derived adjuvanted H5N1 vaccine — A phase I randomized clinical trial

Aristine Cheng [a,1], Szu-Min Hsieh [a,1], Sung-Ching Pan [a], Yu-Han Li [b], Erh-Fang Hsieh [b], Hsiang-Chi Lee [b], Ting-Wan Lin [b], Kuan-Lang Lai [c], Charles Chen [b], Stanley Shi-Chung Chang [c], Shan-Chwen Chang [a,*]

[a] *Department of Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan*
[b] *Medigen Vaccine Biologics Corporation, Hsinchu, Taiwan*
[c] *Medigen Biotechnology Corporation, Taipei, Taiwan*

Received 13 June 2018; received in revised form 9 February 2019; accepted 25 March 2019
Available online 18 May 2019

KEYWORDS
Influenza A;
Avian influenza;
H5N1;
Vaccination;
Immune response

Abstract *Background:* Development of an efficacious egg-free mock-up H5N1 vaccine is key to our preparedness against pandemic avian flu.
Methods: This is a single-center, randomized, observer-blinded phase I clinical trial evaluating the safety and immunogenicity of an alum-adjuvanted Madin–Darby canine kidney (MDCK)-derived inactivated whole-virion H5N1 influenza vaccine in healthy adults. Hemagglutination inhibition (HAI) and neutralizing antibody titers were measured using horse and turkey red blood cells (RBCs).
Results: Thirty-six adult subjects were randomized to receive two doses of 0.5 mL of the MDCK-derived H5N1 alum-adjuvanted vaccine containing 7.5, 15, or 30 μg of hemagglutinin (HA) 21 days apart. The candidate vaccine was well tolerated and safe across the three dosing groups. The most frequent adverse event was injection site pain (46.5%). Both HAI and neutralizing antibody titers increased after each vaccination in all three dosing groups. The best HAI responses, namely a seroconversion rate of 91.7% and a geometric mean ratio of 9.51 were achieved with the HA dose of 30 μg assayed using horse RBCs at day 42. HAI titers against H5N1 avian influenza virus was significantly higher when measured using horse RBCs compared with turkey RBCs.
Conclusions: This Phase I trial showed the MDCK-derived H5N1 candidate vaccine is safe and immunogenic. The source of RBCs has a significant impact on the measurement of HAI titers

* Corresponding author. Fax: +886 2 2370 7772.
E-mail address: changsc@ntu.edu.tw (S.-C. Chang).
[1] A. Cheng and S.M. Hsieh contributed equally to this study.

https://doi.org/10.1016/j.jmii.2019.03.009

(ClinicalTrials.gov number: NCT01675284.).

Introduction

At the forefront of influenza prevention strategies is effective vaccination.[1] Of the avian influenza viruses that have caused infections in man, H5N1 is notable for several reasons.[2,3] H5N1 mutates rapidly and has a documented propensity to acquire genes from influenza viruses infecting other animal species.[4,5] Birds and humans infected with H5N1 are able to excrete virus via oral and fecal routes for at least 10 days and thus contribute to further spread of the virus to other birds in the wild or in live poultry farms and markets.[2] Since the first documented H5N1 bird-to-human transmission in 1997, the World Health Organization has confirmed 860 human infections and 454 deaths due to H5N1 in 16 countries across Asia, Europe, Africa and North America between 2003 and 2017.[6] Unlike avian H7N9 influenza, which affects predominantly the elderly and pre-morbid, H5N1 infects younger children and healthy adults and is associated with high mortality.[7–9]

Although no widespread human-to-human transmission has occurred,[6] ongoing evolution by drift and shift mechanisms that could potentially alter the protein-binding specificity of H5N1 from avian α2,3-linked sialic acid receptors to human α2,6-linked sialic acid receptors. It means that H5N1 continues to possess pandemic potential.[10] To address this pandemic threat, several H5N1 mock-up vaccine candidates have been prepared and evaluated in animal models and clinical trials.[11–13] In a previous phase I clinical study evaluating the safety and immunogenicity of a Madin–Darby canine kidney (MDCK)-derived H5N1 candidate containing 3 μg hemagglutinin (HA) or 6 μg HA, with or without 300 μg aluminum phosphate (AlPO4) adjuvant, the primary endpoint of safety was demonstrated in sixty healthy adult Taiwanese volunteers.[14] However, in that study, the immune responses generated were disappointing with only a significant neutralizing antibody response being observed for the group receiving the adjuvanted 6 μg HA vaccine.[14] Hence in this clinical study, we will evaluate the safety and immunogenicity of the AlPO4-adjuvanted MDCK-derived H5N1 candidate vaccine containing higher HA antigenic doses (7.5 μg, 15 μg, 30 μg) with the aim of improving the immunogenicity profile. When this trial was conducted, unlike for the H7N9 vaccine, standardized assays for determining serum antibody responses to vaccination against avian influenza were lacking and were adopted from vaccination studies against seasonal influenza.[15] Hence, we measured the hemagglutination-inhibition (HAI) titers using both horse and turkey erythrocytes.

Materials and methods

Vaccine

The MDCK-derived H5N1 candidate vaccine used in this study was an inactivated, purified, whole virus adjuvanted vaccine that was developed by Medigen Vaccine Biologics Corporation (Nangang District, Taipei, Taiwan). The vaccine was produced via mammalian cell techniques using MDCK cells. The seed virus NIBRG-14 was a reassortant H5N1 strain derived from clade 1 (A/Vietnam/1194/2004) virus, and this seed virus was obtained from the UK National Institute for Biological Standards and Control (NIBSC; Hertfordshire, U.K.) which has been approved by the WHO as the reference H5N1 vaccine strain for use in the production of commercial H5N1 vaccines. In production, the viruses are propagated in a serum-free medium and then harvested, filtrated, and concentrated via ultracentrifugation. The concentrated suspension is purified to achieve high purity and then inactivated using formaldehyde. The concentration of the inactivated antigens was determined by single radial immunodiffusion (SRID). The investigational vaccines were formulated at three HA dose levels: 7.5, 15, and 30 μg using aluminum hydroxide ($Al(OH)_3$) in a phosphate buffer. Each dose of the 0.5 mL vaccine contained 300 μg of $Al(OH)_3$.

Study design

This prospective, randomized, open label, observer-blinded, single-center study with three parallel arms was conducted between August 2012 and March 2013 at one medical center (the National Taiwan University Hospital in Taipei, Taiwan; ClinicalTrials.gov identifier: NCT01675284). Thirty-six healthy subjects aged between 20 and 60 years were enrolled in this study. The detailed eligibility and exclusion criteria are provided (in the Supplement). The subjects were randomized into three arms corresponding to the HA antigen dose: group 1 (7.5 μg), group 2 (15 μg), and group 3 (30 μg). All of the subjects were administered two intramuscular 0.5-mL doses of the MDCK-derived H5N1 candidate vaccine on day 0 and 21 via the deltoid muscle. Blood samples for serological assays were collected prior to two vaccinations (day 0 and day 21), as well as 21 days after the second vaccination (day 42). All of the subjects were monitored for up to 180 days. The study was conducted according to the principles of the Declaration of Helsinki, Good Clinical Practice (as defined by the International Conference on Harmonisation) and approved by both the Taiwan Food and Drug Administration and the hospital research ethics committee. Written informed consent was

obtained from all subjects. The primary endpoint for this phase I study was to demonstrate safety and immunogenicity by seroconversion rate using hemagglutination inhibition (HAI) assay.

Safety

Solicited local and systemic signs and symptoms were recorded in the participants' diary card for 7 days after the first and second vaccine doses (namely, on each day of vaccination and the subsequent 6 days). Unsolicited adverse events (AEs) were recorded during a 21-day follow-up period after the first and second vaccine doses (namely, on each day of vaccination and the subsequent 20 days). The proportion of participants who had AEs, the intensity of these events, and the relationship of these events to the vaccine were determined. The occurrence of overall AEs and serious AEs (SAEs) during the study period were also recorded.

Immunogenicity

Antibody responses were evaluated on days 0, 21, and 42 using the HAI and neutralization assays on sera collected before vaccination and at 21 days after each vaccination. For both assays, the serum samples were tested separately and in duplicate under blinded conditions and the seed virus NIBRG-14 was used. HAI were conducted according to established procedures with turkey erythrocytes.[9] A modified HAI was performed using horse erythrocytes because of their increased sensitivity in detecting human antibodies against H5.[16] The turkey and horse erythrocytes were obtained from the Agriculture Technology Research Institute (Hsinchu County, Taiwan) and the National Pingtung University of Science and Technology (Pingtung County, Taiwan), respectively. The reference antiserum to A/California/7/2009 was obtained from the NIBSC. According to the guidelines on reporting of immunogenicity results of influenza vaccines by the European Committee for Medicinal Products for Human Use (CHMP) (1) the seroconversion rates (SCR, the proportion of subjects with a pre-vaccination HAI antibody titer $<$1:10 and a post-vaccination titer $\geq$1:40, or a pre-vaccination titer $\geq$1:10 and a post-vaccination titer that has increased fourfold or more) and (2) the geometric mean ratios (GMRs, the fold increase of serum anti-HA antibody titers from post-to pre-vaccination) were calculated. The neutralization assay was performed using MDCK cells and 100 $TCID_{50}$ of influenza virus as previously described.[17]

Statistical analysis

All analyses were performed using Statistical Analysis System (SAS) version 9.3. (SAS Institute, Cary, NC, USA). Descriptive statistics were summarized as point estimates with 2-sided 95% CIs. The analysis of immunogenicity was based on a per-protocol cohort, who met all eligibility criteria, complied with the procedures defined in the protocol, and did not fulfill any elimination criteria during the study and for whom the data concerning immunogenicity endpoint measures were available. Analysis of safety included all participants who received at least one dose of the study vaccine according to their random assignment and had sufficient data to perform an analysis of safety (at least one safety follow-up).

Results

Study subjects

Between August 29, 2012 and March 28, 2013, a total of 53 subjects were screened (Fig. 1). Fourteen of these subjects were excluded after screening, and 3 subjects withdrew their consent prior to randomization. Thirty-six subjects were randomized and immunogenicity data on days 21 and 42 were available for all 35 subjects who received two doses of vaccine (11 subjects in group 1 and 12 subjects in each of groups 2 and 3). Post-vaccination immunogenicity data was not available for the single subject who withdrew his/her consent after receiving only the first dose of vaccine. Data from all of the vaccinated subjects were included in the safety analysis, but two subjects were subsequently lost to follow-up, leaving 33 subjects in total who were followed to the end of the 180-day study period. No subject terminated the study prematurely because of AEs or protocol deviation/violations. The demographic characteristics of all of the vaccinated subjects are summarized in Table 1. The median age of participants in groups 1, 2, and 3 were 34, 38 and 36 years, respectively. More female subjects were randomly assigned to group 1 (75%) than in the other two groups (58% in group 2 and 50% in group 3). All subjects were of Chinese ethnicity.

Safety

Solicited

The solicited local and systemic AEs that occurred during the 7 days after each vaccination are summarized in Table 2. A total of 52 solicited local symptoms and 52 solicited systemic symptoms were reported during the 7-day follow-up period after each vaccination during the study; these symptoms occurred equally across the three treatment groups. Symptoms were slightly more frequently reported after the first dose of vaccination in all of the three groups. Injection site pain was the most common solicited local AE (63.9% after the first injection decreasing to 29% after the second injection). The majority of the reactions were mild (grade 1) to moderate (grade 2), resolving within 48 h, and did not affect the subjects' daily activities nor require medical attention. One grade 3 pain was recorded by a group 1 subject after the first dose, and was probably related to the vaccine. All other local symptoms were mild (Table 2).

Fatigue was the most common solicited systemic adverse effects (36.1% after the first injection, 37.1% after the second injection) followed by headache and myalgia. Only one subject in group 1 reported grade 3 fatigue and headache after the first dose, with both events probably related to study vaccine and resolved within 7-day period (Table 2).

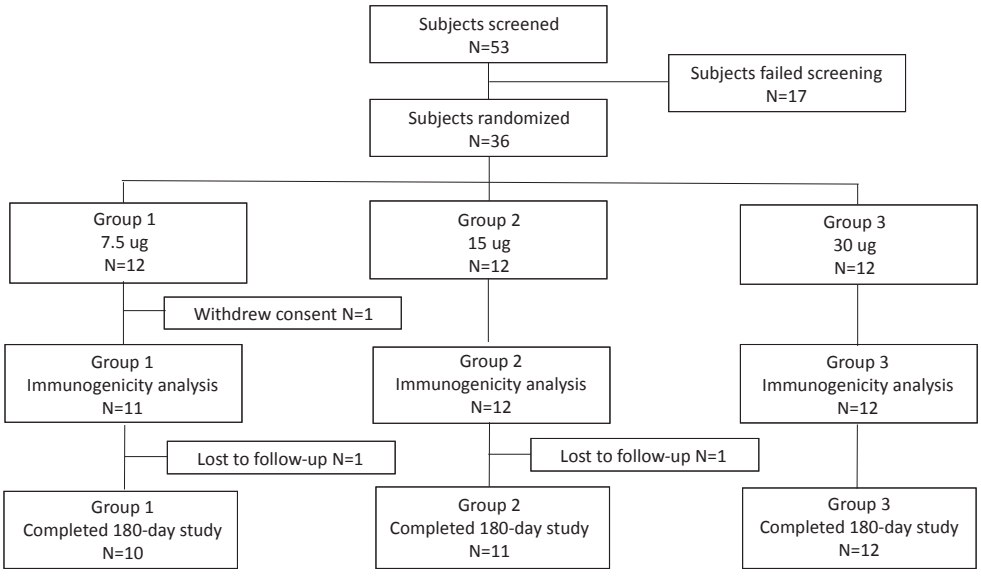

Figure 1. Enrollment and follow-up of the study subjects.

Unsolicited

A total of 18 unsolicited AEs occurred during the 21-day period after the vaccinations (11 after the first dose and 7 after the second dose) (Supplementary Table). The number of these unsolicited AEs was similar across the three treatment groups. Each individual AE occurred in fewer than 10% subjects (≤1 subject for each AE) for each dosing group after each vaccination. The most frequently reported unsolicited AEs included atypical injection—site reactions such as pruritis (3 subjects), coughing, dermatitis and dysmenorrhea (2 subjects each). All of the unsolicited AEs were grade 1 (16) or grade 2 (2) and not related (12) or probably related (6) to the study vaccine. No subject withdrew from the study due to AEs, and no SAE was reported over the course of the entire study period.

Immunogenicity

The per-protocol analysis of immunogenicity included 35 subjects (11 subjects in group 1 (7.5 μg HA), 12 subjects in group 2 (15 μg HA), and 12 subjects in group 3 (30 μg HA) (Table 3). With the turkey RBCs and horse RBCs HAI assays, all of the subjects had low titers (<1:40) against A/H5N1 prior to the vaccination, as expected for a naïve population. After two vaccinations, the Geometric Mean Titers (GMTs) of anti-HA antibodies measured using either turkey or horse RBCs increased for all three groups. A higher anti-HA antibody titer increase was noted after two doses on day 42. With the turkey RBC assay, the GMTs ranged from 5.61 to 7.94 on day 21 and 7.07—10.59 on day 42. The GMTs from the horse RBC assay demonstrated higher values (16.80—20.00 on day 21 and 27.40—59.90 on day 42) than the turkey RBCs assay.

The GMRs and seroconversion rates were reported using the HAI assay.[15,18,19] With the horse RBC HAI assay, the highest antibody responses were observed in group 3 after two vaccinations yielding a GMR of 9.51 and a seroconversion rate of 91.7%. Furthermore, all three groups elicited a GMR of more than 2.5 after one vaccination and a dose—response effect was also observed following the second vaccination dose by using horse RBCs. In contrast, using the turkey RBC HAI assay, no antigen dose—response effect was observed.

By neutralization assay the GMTs ranged from 2.29 to 2.81 on day 21 and 2.52—3.77 on day 42. Group 3 exhibited the highest GMT after two vaccination doses by neutralization assay (Table 4).

Table 1 Demographic characteristics of all of the vaccinated subjects.

	Group 1 7.5 μg	Group 2 15 μg	Group 3 30 μg
	n = 12	n = 12	n = 12
Gender — no. (%)			
Male	3 (25.0)	5 (41.7)	6 (50.0)
Female	9 (75.0)	7 (58.3)	6 (50.0)
Age — years			
Median (range)	34 (20—60)	38 (21—59)	36 (22—60)
Race — no. (%)			
Chinese	12 (100.0)	12 (100.0)	12 (100.0)

Discussion

This study showed that the candidate mock-up MDCK cell-derived inactivated H5N1 influenza virus vaccine adjuvanted with aluminum hydroxide was well tolerated and fairly immunogenic in healthy adults. No serious AE was recorded, nor did the frequency or severity of either local or systemic symptoms appear to be greater after the second dose than after the first. The humoral immune response measured by the HAI and neutralization assays, was greatest in the 30 μg HA group wherein a seroconversion of 91.7% and geometric mean ratio (GMR) of 9.51 were observed

Table 2 Proportion of subjects with a solicited local or systemic event within 7 days after each vaccination.

	Group 1	Group 2	Group 3	Overall
	7.5 μg	15 μg	30 μg	
Solicited Local Events n (%)				
First Vaccination	**n = 12**	**n = 12**	**n = 12**	**n = 36**
Pain	6 (50.0)	9 (75.0)	8 (66.7)	23 (63.9)
Swelling	1 (8.3)	2 (16.7)	1 (8.3)	4 (11.1)
Redness	4 (33.3)	3 (25.0)	1 (8.3)	8 (22.2)
Hematoma	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Second Vaccination	**n = 11**	**n = 12**	**n = 12**	**n = 35**
Pain	3 (27.3)	3 (25.0)	4 (33.3)	10 (28.6)
Swelling	0 (0.0)	1 (8.3)	1 (8.3)	2 (5.7)
Redness	3 (27.3)	1 (8.3)	1 (8.3)	5 (14.3)
Hematoma	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
SUMMARY OF LOCAL EVENTS	**17**	**19**	**16**	**52**
Solicited Systemic Events n (%)				
First Vaccination	**n = 12**	**n = 12**	**n = 12**	**n = 36**
Fever	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fatigue	4 (33.3)	3 (25.0)	6 (50.0)	13 (36.1)
Headache	3 (25.0)	0 (0.0)	2 (16.7)	5 (13.9)
Myalgia	2 (16.7)	1 (8.3)	0 (0.0)	3 (8.3)
Joint Pain	1 (8.3)	0 (0.0)	1 (8.3)	2 (5.6)
Rigors	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Second Vaccination	**n = 11**	**n = 12**	**n = 12**	**n = 35**
Fever	0 (0.0)	0 (0.0)	1 (8.3)	1 (2.9)
Fatigue	2 (18.2)	7 (58.3)	4 (33.3)	13 (3.7)
Headache	3 (27.3)	1 (8.3)	2 (16.7)	6 (17.1)
Myalgia	1 (9.1)	2 (16.7)	2 (16.7)	5 (14.3)
Joint Pain	1 (9.1)	1 (8.3)	1 (8.3)	3 (8.6)
Rigors	0 (0.0)	0 (0.0)	1 (8.3)	1 (2.9)
SUMMARY OF SYSTEMIC EVENTS	**17**	**15**	**20**	**52**

Table 3 Comparing the immunogenicity as determined by the hemagglutination inhibition assay (HAI) using turkey versus horse red blood cells (RBCs).

RBCs	Group 1 7.5 μg n = 11		Group 2 15 μg n = 12		Group 3 30 μg n = 12	
	Turkey	Horse	Turkey	Horse	Turkey	Horse
Geometric Mean Titers (95% CI)						
Day 0	5.00	6.90	5.00	6.30	5.00	6.30
	NA	(4.20–11.10)	NA	(4.22–9.94)	NA	(4.22–9.94)
Day 21	6.85	20.00	5.61	16.80	7.94	18.90
	(4.23–11.10)	(9.20–43.60)	(4.35–7.24)	(9.50–29.70)	(4.47–14.09)	(8.20–43.20)
Day 42	8.82	27.40	7.07	27.40	10.59	59.90
	(4.44–17.49)	(14.00–53.60)	(4.75–10.53)	(15.50–48.40)	(4.63–24.26)	(29.30–122.40)
Geometric Mean Ratios						
Day 21	1.32	**2.90**	1.12	**2.67**	1.59	**3.00**
Day 42	1.76	**3.97**	1.41	**4.35**	2.12	**9.51**
Seroconversion Rate (%)						
Day 0	0	0	0	0	0	0
Day 21	9.1	27.3	0	25.0	8.3	50.0
Day 42	18.2	36.4	0	**41.7**	16.7	**91.7**

Seroconversion Rate is defined who have either a pre-vaccination titer $< 1{:}10$ and a post-vaccination titer $\geq 1{:}40$ or a pre-vaccination titer $\geq 1{:}10$ and at least a 4-fold increase in post-vaccination titer.
The Geometric Mean Ratios (GMRs) is defined as the ratio serum anti-HA antibody GMTs post-vaccination at Day 21 and 42 compared to Day 0.

Table 4 Summary of Geometric Mean Titers determined by the neutralization assay.

Assay Timing	Group 1 7.5 μg n = 11	Group 2 15 μg n = 12	Group 3 30 μg n = 12
Geometric Mean Titers (95% CI)			
Day 0	2.00	2.00	2.00
	NA	NA	NA
Day 21	2.81 (1.69 −4.69)	2.31 (1.68 −3.17)	2.29 (1.70 −3.08)
Day 42	2.52 (1.71 −3.70)	2.75 (1.71 −4.41)	3.77 (1.66 −8.57)

based on HAI assayed by using horse erythrocytes. However, the lower antigenic doses (7.5 μg and 15 μg HA) elicited only modest responses (seroconversion rates $< 50\%$ and GMRs < 5) after two doses and did not offer the advantage of decreased adverse effects either.

While a key challenge with pandemic influenza vaccine development is that clinical efficacy and immunity correlates of protection are unknowable before a pandemic has started, the safety and immunogenicity profile of this cell-culture derived alum-adjuvanted H5N1 whole virion vaccine is comparable to that of the first and only US FDA approved pandemic H5N1 vaccine by Sanofi-Pasteur.[20] This licensed inactivated, non-adjuvanted, egg-cultured, split-virion vaccine administered as two intramuscular doses containing 90 μg HA dosed 28 days apart was able to induce seroprotection rates (HAI >1:40) of only 57% in healthy adults.[21] The frequencies of pain and local tenderness at the injection site was similar despite the lack of adjuvant in the earlier study (58% and 57% versus 67% and 33% in our study after the first and second doses, respectively). Systemic adverse effects of headache (30% versus 14% after the first dose) were more commonly reported for the 90 μg HA licensed vaccine than our candidate vaccine with 30 μg HA.[21]

Since the global demand would far outstrip the manufacturing capacity of the above Sanofi-Pasteur vaccine during a pandemic (even with stockpiling), dose-sparing and lead-time sparing strategies, including the use of adjuvants, egg-independent manufacturing and whole-virion instead of subunit or split-virion vaccines were incorporated in the design of our candidate and other subsequent vaccines.[22–25] Of the four European Union approved "mock-up" H5N1 pandemic vaccines, our candidate vaccine is most similar to the egg-cultured, whole-virion alum-adjuvanted H5N1 vaccine (Daronrix™), given as two 15 μg intramuscular doses 21 days apart, that provided 70.6% seroconversion rates and a GMR of 12.4 in healthy adults.[26] However, like Vero or EB55 cell derived inactivated whole-cell vaccines which have also demonstrated good immunogenicity and safety, our MDCK-passaged candidate vaccine does not need to rely on the supply of hens and eggs as Daronrix™ during a pandemic.[27–29] Other advantages of cell culture technology over egg-based manufacturing is that it allows the vaccines to be produced in an aseptic environment and as both avian and human receptor are present on MDCK (α2,6 and α2,3 sialoglycans), there is no need for human strains to adapt their hemagglutinins to grow in eggs by mutating potential surface antigens.

Newer adjuvants to alum have substantially altered the vaccine landscape, by demonstrating even better antigen-sparing and immunogenicity properties. For example, an AS03 (oil-in-water)-adjuvanted H5N1 vaccine (Prepandrix™) was licensed by the EU since the lowest antigen dose of 3.8 μg HA elicited a neutralizing seroconversion rate of 86% in healthy adults and protected ferrets against homologous and heterologous (cross-clade) lethal challenge.[30,31] MF59, another oil-in-water adjuvant containing squalene, was also able to lower the required antigen dose to 3.75 μg HA and generate cross-clade protection.[23] However, due to its wide use in vaccines and excellent safety profile, alum remains an attractive, affordable and easy-to-obtain adjuvant in most countries although paradoxically inferior HAI antibody responses to alum-adjuvanted vaccines compared with their non-adjuvanted counterpart and increased local tenderness have been described.[27,32]

In addition, our results confirmed that the type of erythrocytes used largely determines the sensitivity of the HAI assay. As previously shown, the measurement of HAI titers against H5N1 avian influenza virus was significantly improved by the use of horse erythrocytes compared with turkey erythrocytes.[33] Since the efficiency of the binding of the influenza virus is dependent on the specificity of the sialic acid cellular receptor, the agglutination of virus and RBC from different species will be influenced by the receptor specificity of influenza viruses. Human influenza viruses preferentially bind to oligosaccharides containing *N*-acetylneuraminic acid α2,6-galactose (NeuAcα2,6Gal); avian and equine influenza virus strains bind to NeuAcα2,3Gal. Many animal species, including horses and cows, have large numbers of NeuAcα 2,3Gal receptors but virtually no NeuAc2,6Gal receptors in their erythrocytes. Chicken RBCs have less NeuAcα2,6Gal and more NeuAcα2,3Gal; turkey RBCs have more NeuAα2,6Gal than chicken RBCs.[34] Therefore, seasonal H1 and H3 influenza viruses preferentially agglutinate chicken or turkey but not horse or bovine RBCs; avian influenza viruses agglutinate RBCs preferentially from horses or cows. The previous use of turkey or chicken RBCs may possibly have led to the relative insensitivity of HAI for detecting H5 antibodies and the "negative" results reported in our earlier Phase I study using the same cell-cultured vaccine at lower antigenic doses of 6 μg.[14]

The shortcomings of this study include the lack of a non-adjuvanted or placebo group. However, in our previous study with non-adjuvanted and adjuvanted vaccines, no increase in side effects including local injection site tenderness was reported in the adjuvanted group.[14] We also did not evaluate the cross-neutralizing capacity and long-term persistence of these antibodies, nor did we perform functional assays of cellular immunity since antibody levels by themselves provide a narrow view of the immune response to vaccination. However, we did achieve the primary aim of this phase I trial which was to demonstrate that the alum-adjuvanted whole-H5N1 vaccines with different antigenic strengths are characterized by an acceptable safety profile.

In conclusion, this MDCK-derived whole-virion, inactivated, alum-adjuvanted H5N1 influenza vaccine was safe and the 30 μg HA dose was immunogenic in healthy Asian adults. Similar to the recent study of the Phase I H7N9 vaccine trial, these results demonstrate our ability to prepare such a 'mock-up' pre-pandemic vaccine locally.[35] Although newer antigen-sparing adjuvants such as ASO3 or MF59 and strategies such as prime-boosting or use of live-attenuated viruses to generate broader, longer-lasting cellular immune protection have been developed to mitigate the estimated need during a pandemic to produce 13 billion vaccine doses against a yet uncharacterized virus in a manner of weeks, the widespread availability of the alum-adjuvant and the egg-free design of this vaccine as currently formulated, enables local communities to prepare and respond to a pandemic.[36,37]

Funding

This study was sponsored by Medigen Vaccine Biologics Corporation.

Authorship contributions

A. Cheng and S.M. Hsieh prepared the manuscript. S.M. Hsieh, S.C. Pan, and S.C. Chang designed and conducted the study. Y.H. Li, E.F. Hsieh, H.C. Lee, T.W. Lin, K.L. Lai, C. Chen, S.S.C. Chang coordinated and organized the study. S.M. Hsieh collected and measured immunogenicity of sera samples and analysed the data. A. Cheng, S.M. Hsieh, S.C. Pan, S.C. Chang interpreted the results independently of Medigen Vaccine Biologics Corporation and Medigen Biotechnology Corporation.

Conflicts of interest

The authors at National Taiwan University Hospital have no conflicts of interest, and analysed the data and interpreted the results independently of Medigen Vaccine Biologics Corporation and Medigen Biotechnology Corporation.

References

1. Harfoot R, Webby RJ. H5 influenza, a global update. *J Microbiol* 2017;**55**(3):196—203.
2. Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. *Clin Microbiol Rev* 2007;**20**(2):243—67.
3. Leroux-Roels I, Leroux-Roels G. Current status and progress of prepandemic and pandemic influenza vaccine development. *Expert Rev Vaccines* 2009;**8**(4):401—23.
4. Hui DSC, Lee N, Chan PKS. A clinical approach to the threat of emerging influenza viruses in the Asia-Pacific region. *Respirology* 2017;**22**(7):1300—12.
5. Duan L, Campitelli L, Fan XH, Leung YH, Vijaykrishna D, Zhang JX, et al. Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. *J Virol* 2007;**81**(14):7529—39.
6. World Health Organization. *Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO*. 2018. http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/. accessed 21 February 2018 25 January 2018.
7. Cowling BJ, Jin L, Lau EH, Liao Q, Wu P, Jiang H, et al. Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. *Lancet* 2013;**382**(9887):129—37.
8. Sha J, Dong W, Liu S, Chen X, Zhao N, Luo M, et al. Differences in the epidemiology of childhood infections with avian influenza a H7N9 and H5N1 viruses. *PLoS One* 2016;**11**(10):e0161925.
9. World Health Organization. *Manual for the laboratory diagnosis and virological surveillance of influenza*. 2011. http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf. Accessed 31 Dec 2018.
10. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 2004;**101**(13):4620—4.
11. Song JY, Choi MJ, Noh JY, Choi WS, Cheong HJ, Wie SH, et al. Randomized, double-blind, multi-center, phase III clinical trial to evaluate the immunogenicity and safety of MG1109 (egg-based pre-pandemic influenza A/H5N1 vaccine) in healthy adults. *Hum Vaccines Immunother* 2017;**13**(5):1190—7.
12. Chada KE, Forshee R, Golding H, Anderson S, Yang H. A systematic review and meta-analysis of cross-reactivity of antibodies induced by oil-in-water emulsion adjuvanted influenza H5N1 virus monovalent vaccines. *Vaccine* 2017;**35**(24):3162—70.
13. Guo Q, Liu Z, Gao J, Zhou J, Hu W, Cun Y, et al. Immunogenicity and safety of pandemic influenza H5N1 vaccines in healthy adults through meta-analysis. *Cell Physiol Biochem* 2016;**40**(5):921—32.
14. Pan SC, Kung HC, Kao TM, Wu H, Dong SX, Hu MH, et al. The Madin-Darby canine kidney cell culture derived influenza A/H5N1 vaccine: a phase I trial in Taiwan. *J Microbiol Immunol Infect* 2013;**46**(6):448—55.
15. Noah DL, Hill H, Hines D, White EL, Wolff MC. Qualification of the hemagglutination inhibition assay in support of pandemic influenza vaccine licensure. *Clin Vaccine Immunol* 2009;**16**(4):558—66.
16. World Health Organization. *Laboratory Procedures: Serological detection of avian influenza A(H7N9) virus infections by modified horse red blood cells haemagglutination-inhibition assay*. http://www.who.int/influenza/gisrs_laboratory/cnic_serological_diagnosis_hai_a_h7n9_20131220.pdf. Accessed 30 Dec 2018.
17. Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminum-adjuvanted influenza whole virus vaccine for pandemic use. *Virus Res* 2004;**103**(1—2):163—71.
18. Khurana S, Suguitan Jr AL, Rivera Y, Simmons CP, Lanzavecchia A, Sallusto F, et al. Antigenic fingerprinting of H5N1 avian influenza using convalescent sera and monoclonal antibodies reveals potential vaccine and diagnostic targets. *PLoS Med* 2009;**6**(4):e1000049.
19. European Medicines Agency (EMA). *Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application (revision)*. CHMP); 2008.
20. Food and Drug Administration (FDA) of the United States of America. *Influenza virus vaccine, H5N1 (for National Stockpile)*. 2007.
21. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* 2006;**354**(13):1343—51.
22. Leroux-Roels I, Van der Wielen M, Kafeja F, Vandermeulen C, Lazarus R, Snape MD, et al. Humoral and cellular immune

responses to split-virion H5N1 influenza vaccine in young and elderly adults. *Vaccine* 2009;**27**(49):6918—25.
23. Keitel W, Groth N, Lattanzi M, Praus M, Hilbert AK, Borkowski A, et al. Dose ranging of adjuvant and antigen in a cell culture H5N1 influenza vaccine: safety and immunogenicity of a phase 1/2 clinical trial. *Vaccine* 2010;**28**(3): 840—8.
24. Ehrlich HJ, Muller M, Fritsch S, Zeitlinger M, Berezuk G, Low-Baselli A, et al. A cell culture (Vero)-derived H5N1 whole-virus vaccine induces cross-reactive memory responses. *J Infect Dis* 2009;**200**(7):1113—8.
25. Pandey A, Singh N, Sambhara S, Mittal SK. Egg-independent vaccine strategies for highly pathogenic H5N1 influenza viruses. *Hum Vaccine* 2010;**6**(2):178—88.
26. (EMA) EMA. Daronrix. *Pandemic influenza vaccine (H5N1) (whole virion, inactivated, adsorbed)*. 2007.
27. Keitel WA, Dekker CL, Mink C, Campbell JD, Edwards KM, Patel SM, et al. Safety and immunogenicity of inactivated, Vero cell culture-derived whole virus influenza A/H5N1 vaccine given alone or with aluminum hydroxide adjuvant in healthy adults. *Vaccine* 2009;**27**(47):6642—8.
28. Naruse T, Fukuda T, Tanabe T, Ichikawa M, Oda Y, Tochihara S, et al. A clinical phase I study of an EB66 cell-derived H5N1 pandemic vaccine adjuvanted with AS03. *Vaccine* 2015;**33**(45): 6078—84.
29. Schuind A, Segall N, Drame M, Innis BL. Immunogenicity and safety of an EB66 cell-culture-derived influenza a/Indonesia/5/2005(H5N1) AS03-adjuvanted vaccine: a phase 1 randomized trial. *J Infect Dis* 2015;**212**(4):531—41.
30. Leroux-Roels G. Prepandemic H5N1 influenza vaccine adjuvanted with AS03: a review of the pre-clinical and clinical data. *Expert Opin Biol Ther* 2009;**9**(8):1057—71.
31. Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet* 2007; **370**(9587):580—9.
32. Manzoli L, Salanti G, De Vito C, Boccia A, Ioannidis JP, Villari P. Immunogenicity and adverse events of avian influenza A H5N1 vaccine in healthy adults: multiple-treatments meta-analysis. *Lancet Infect Dis* 2009;**9**(8):482—92.
33. Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/-Singapore/97 vaccine. *Virus Res* 2004;**103**(1—2):91—5.
34. Suzuki Y, Ito T, Suzuki T, Holland Jr RE, Chambers TM, Kiso M, et al. Sialic acid species as a determinant of the host range of influenza A viruses. *J Virol* 2000;**74**(24):11825—31.
35. Wu UI, Hsieh SM, Lee WS, Wang NC, Kung HC, Ou TY, et al. Safety and immunogenicity of an inactivated cell culture-derived H7N9 influenza vaccine in healthy adults: a phase I/II, prospective, randomized, open-label trial. *Vaccine* 2017; **35**(33):4099—104.
36. Talaat KR, Luke CJ, Khurana S, Manischewitz J, King LR, McMahon BA, et al. A live attenuated influenza A(H5N1) vaccine induces long-term immunity in the absence of a primary antibody response. *J Infect Dis* 2014;**209**(12):1860—9.
37. Langley JM, Frenette L, Jeanfreau R, Halperin SA, Kyle M, Chu L, et al. Immunogenicity of heterologous H5N1 influenza booster vaccination 6 or 18 months after primary vaccination in adults: a randomized controlled clinical trial. *Vaccine* 2015; **33**(4):559—67.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2019.03.009.