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Original Article

Characterization of a whole, inactivated influenza (H5N1) vaccine

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Objectives Effective vaccines against the highly pathogenic influenza A/H5N1 virus are being developed worldwide. In Japan, two adjuvanted, inactivated, whole-virion influenza vaccines were recently developed and licensed as mock-up, pre-pandemic vaccine formulations by the Ministry of Health and Labor Welfare of Japan. During the vaccine design and development process, various obstacles were overcome and, in this report, we introduce the non clinical production, immunogenicity data in human and development process that was associated with egg-derived adjuvanted, inactivated, whole-virion influenza A (H5N1) vaccine.

Design Pilot lots of H5N1 vaccine were produced using the avirulent H5N1 reference strain A/Vietnam/1194/2004 (H5N1) NIBRG-14 and administered following adsorption with aluminum hydroxide as an adjuvant. Quality control and formulation stability tests were performed before clinical trials were initiated (phase I-III).
The research foundation for microbial diseases of Osaka University (BIKEN) carried out vaccine production, quality control, stability testing and the phase I clinical trial in addition to overseeing the licensing of this vaccine. Mitsubishi Chemical Safety Institute Ltd. carried out the non clinical pharmacological toxicity and safety studies and the Japanese medical association carried out the phase II/III trials. Phase I-III trials took place in 2006.

Results The production processes were well controlled by established tests and validations. Vaccine quality was confirmed by quality control, stability and pre-clinical tests, and the vaccine was approved as a mock-up, pre-pandemic vaccine by the Ministry of Health and Labor Welfare of Japan.

Conclusions Numerous safety and efficacy procedures were carried out prior to the approval of the described vaccine formulation. Some of these procedures were of particular importance e.g., vaccine development, validation, and quality control tests that included strict monitoring of the hemagglutinin (HA) content of the vaccine formulations.
Improving vaccine productivity, shortening the production period and improving antigen yield of the avirulent vaccine strains were also considered important vaccine development criteria.

Keywords Aluminum hydroxide adjuvant, inactivated whole virion, influenza, pandemic, reverse genetics, vaccine.

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Introduction

Since the first human infection with highly pathogenic avian influenza A (H5N1) virus was reported in Hong Kong in 1997, influenza A (H5N1) viruses have been identified in poultry, migratory birds, various mammals and human beings worldwide.[1] As of 19 July 2008, 385 human cases had been reported to the World Health Organization (WHO), including 243 deaths (63% mortality).[1] Although virus transmission from person to person is as yet minimal,[2] human viral infections have the potential of developing into the source of the next influenza pandemic, therefore the development of effective vaccines against the influenza A (H5N1) virus is a matter of considerable urgency. Previous work suggests that, in naïve humans, whole-virion vaccine formulations are more immunogenic than subunit formulations and the use of adjuvants improves immunogenicity.[3–9]

Development of an H5N1 mock-up vaccine in Japan was initiated as a government-supported national project in collaboration with the Ministry of Health and Labor Welfare (MHLW), the National Institute of Infectious Diseases (NIID), the Japanese Medical Association and the manufacturer's task force (BIKEN, Kitasato, Kaketsuken and Denka-Seiken) in 2004. This H5N1-based formulation contains inactivated whole-virion (A/Viet Nam/1194/2004 [H5N1] [NIBRG-14], hereafter referred to as NIBRG-14) adsorbed with aluminum hydroxide adjuvant as an active ingredient.[10]

Materials and methods

The vaccine strain developed for this study was the influenza NIBRG-14 reference strain prepared by the NIBSC. The NIBRG-14 strain had a 2:6 reassortment gene segment ratio between the A/Viet Nam/11904/2004 (H5N1) and A/PR/8/34 (H1N1) strains and was derived by reverse genetics (RG). The H5 hemagglutinin (HA) from the highly pathogenic avian strain, A/Viet Nam/1194/2004, was modified by replacing the polybasic amino acids at the cleavage site to render the virus avirulent.[11] The NIBSC strain was tested for antigenicity and virulence in animal models in addition to testing for hemagglutination inhibition in relation to the reference strain.

We obtained the NIBRG-14 strain from the NIID, prepared master and working seeds, produced bulk material and vaccination preparations were completed by adding aluminum hydroxide. Quality controls tests were carried out during each step of the process. The research foundation for microbial diseases of Osaka University (BIKEN) carried out vaccine production, quality control, stability testing and the phase I clinical trial in addition to overseeing the licensing of this vaccine. The Mitsubishi Chemical Safety Institute Ltd. carried out the pre-clinical pharmacological toxicity and safety studies.

After the vaccine successfully met test criteria, it was submitted for licensing to the MHLW of Japan before it was certified for use in pre-clinical tests and clinical trials.

The Japanese Medical Association carried out the phase II/III trials, which took place in 2006.

A single-center, open-labeled phase I clinical trial was carried out in 120 male volunteers aged 20–40 years. Groups of 20 volunteers were allocated to receive two doses of inactivated whole-virion influenza A (H5N1) vaccines with 1·7, 5 or 15 μg HA/dose containing aluminum hydroxide adjuvant on days 0 and 21 by either the intramuscular (IM) or subcutaneous (SC) route. Serum samples were obtained on days 0, 21 and 42 for hemagglutination inhibition and virus neutralization assays. The hemagglutination inhibition assay was carried out according to established procedures with equine red blood cells and the vaccine strain antigen, NIBRG-14 at Kaketuken. The virus neutralization assay was carried out according to an established microneutralization procedure (NIID method) by BIKEN.

At the direction of the Pharmaceutical and Medical Devices Agency (PMDA), based on the results of phase I trial, Japanese vaccine manufacturers were allocated either the SC or IM route for a confirmatory, randomized double-blind, parallel-group phase II/III clinical trial. BIKEN was allocated to the SC route.

The trial was carried out in 300 volunteers aged 20–64 years. Two groups each of 150 volunteers were allocated to receive two doses of vaccine containing 5 or 15 μg HA/dose. The schedule for immunization, sampling and observations were as for the phase I trial.

The PMDA reviewed the files and carried out confirmatory and reliability assessments of the submitted documents. This report describes the vaccine-development process including development, testing procedures and immunogenicity data in humans.

Vaccine design

Vaccine production

Bulk materials were produced at large scale using embryonated hens' eggs at the licensed manufacturing facility used for the production of the seasonal vaccine and adapted for production of the avian strain in accordance with the WHO's biosafety guidelines for the production and quality control of pandemic human influenza vaccines.[12] Master and working seed viruses were prepared from the NIBRG-14 virus using specific pathogen-free embryonated hens' eggs. The seed virus was grown to high titer in the allantoic cavity of embryonated hens' eggs prior to harvesting. Harvested virus was clarified, concentrated and purified using filters and ultracentrifugation and then inactivated with formalin and filtered through a 0·2 μm membrane filter for bulk material preparation. The vaccine was mixed with 0·3 mg/ml aluminum hydroxide and 8 μg/ml thimerosal (as a preservative). One- or ten-milliliter vials were filled with 15 μg HA/dose and stored at 6–10°C until use. The aluminum hydroxide gel was carefully emulsified with the viral particles since stirring was required to prevent the sedimentation of inactivated whole virus adsorbed with aluminum hydroxide and to maintain the consistency and concentration of active ingredients in every vial during filling. Validation was also required for this process. The HA content of the bulk vaccine was determined by means of SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) of the purified virus before inactivation as single radial immunodiffusion (SRID) tests were unavailable.

Removal of impurities

The ovalbumin and endotoxin content were measured as indicators of impurity. These contaminants were removed using sucrose density gradient ultracentrifugation and ultrafiltration techniques.

In the formulation described here, the ovalbumin concentrations were below 1·2 ng/ml (0·6 ng/single human dose), which is significantly lower than the WHO recommended allowance, i.e. not more than 5 μg/single human dose.[13] In addition, bacterial endotoxin in the bulk material was not more than 7·46 EU/ml (3·73 EU/single human dose), which was also significantly less than European

requirements, i.e. not more than 100 EU/dose.[14] A critical control measure for maintaining reduced endotoxin levels was close inspection of the health practices in place at the manufacturing facility where the eggs were being collected for study purposes, including sanitation practices and chicken vaccination status.

Virus inactivation

Viruses used for vaccine development were inactivated by formalin. However, as inactivation efficacy can be affected by the viral and formalin concentrations and the inactivation temperature, validation procedures must be in place before large-scale vaccine production occurs to insure product consistency.

Inactivation efficacy and consistency were determined by passaging formalin-treated virus samples through embryonated eggs. Inactivation was confirmed if hemagglutination activity was negative after three passages.

Viral antigen yield

Previously, we produced bulk vaccine formulations derived from the Vietnam (NIBRG-14) strain (clade 1), the Indonesia/5/05 strain (clade 2-1) and the Anhui/1/05 (clade 2-3) strain;[10] however, modification of each strain by RG manipulations resulted in strains with increased growth rates but differing antigen yields. As part of the success of any pre-pandemic formulation is product availability, utilization of RG methodologies may have the potential of providing a vaccine virus that retains its antigenicity while giving good growth, thereby providing sufficient vaccine material.

Table 1. Control tests for aluminum hydroxide gel

Name of test	Criteria	Source
Description of material	Whitish gel, odorless	BIKEN
Identification test	Qualitative reactions for aluminum salt should be shown	BIKEN
pH analysis	pH 4·5–8·0	JMR*
Test for aluminum content	1·8–2·2 mg/ml	
Purity test		
Heavy metals	No more than 10 ppm	JP**
Arsenic	No more than 5 ppm	
Sulfate	No more than 0·480%	
Test for particle distribution	Particle size of 2–24 μm: not less than 99% Distribution order of particles: 2–4 μm > 5–9 μm > 10–24 μm > not less than 25 μm	
Protein absorbtion confirmation	31·4–42·2%	BIKEN
Bacterial endotoxin tests	Less than 0·25 EU/ml	JMR
Sterility test	No evidence of microbial growth was observed	JMR
Test for abnormal toxicities	No animals showed any abnormal signs	JMR

*The test was derived from the 'Minimum Requirements for Biological Products' of Japan (Japanese Minimum Requirements).
**Test was derived from the Japanese Pharmacopeia.

Preparation of the aluminum hydroxide gel

Aluminum hydroxide gel made from aluminum potassium sulfate and sodium phosphate (Alum) was selected as the adjuvant for the influenza vaccine described. We have established 10 different control tests for the validation of aluminum hydroxide efficacy (Table 1). Five of these tests were derived from the Minimum Requirements for Biological Products Guidelines of Japan (hereafter Japanese Minimum Requirements or JMR),[15] one of these tests was derived from the Japanese pharmacopoeia,[16] and the other four tests were established by our laboratory (BIKEN). Criteria for the tests (e.g. tests for aluminum content and protein adsorption) were established by trend analysis and are important to maintain the lot-to-lot consistency on the quality of aluminum hydroxide gel.

Adsorption

Before aluminum hydroxide was used as an adjuvant, its capacity to absorb whole virus particles had to be determined. We demonstrated that 0·3 mg/ml of aluminum hydroxide gel had the capacity of absorbing more than 15 μg HA/0·5 ml, which was the dose selected for this vaccine formulation.

Control tests

Tests for HA content

We have established two kinds of tests for defining the HA content (Tables 2 and 3): SRID and calculating the HA content of bulk material by defining the ratio of purified virus suspension (before inactivation) to the bulk protein content by SDS-PAGE. In most cases, we were unable to obtain SRID data in a timely manner, therefore we determined the HA content by SDS-PAGE.

We have compared the results of SDS-PAGE and SRID testing between Japanese manufacturers and the NIID and found inconsistent results in SDS-PAGE. Therefore, harmonization of SDS-PAGE analysis for the HA content ratio being carried out was recommended. In addition, quick preparation of reagents and determination of the standard antigen titers for SRID by the National Control Laboratories and/or the WHO collaborating centers are needed.

Table 2. Local (injection-site) and systemic reactions in phase I clinical trial (%)

	Hemagglutinin dose						
	1·7 μg (*n* = 40)		5 μg (*n* = 40)		15 μg (*n* = 40)		Total (*n* = 120) SC + IM
	SC	IM	SC	IM	SC	IM	
Local (injection-site) reactions							
Redness	20	15	25	15	55	30	26·7
Pain	10	25	30	25	45	65	33·3
Itching	5	0	0	0	5	0	1·7
Swelling	10	10	10	0	20	20	11·7
Injection-site warmth	5	0	0	0	0	5	1·7
Systemic reactions							
Weariness	10	10	25	5	15	15	13·3
Headache	15	15	15	5	10	15	12·5
Nasal drainage	5	0	5	0	0	5	2·5
Fever	0	5	15	5	20	0	7·5

SC, subcutaneous injection; IM, intramuscular injection.

The process of assessing the HA content of adsorbed vaccines could not be determined using conventional SRID; however in our laboratory, we have measured HA content using a novel method developed by Kaketsuken (Kumamoto, Japan). This method consisted of eluting viral HA adsorbed to aluminum hydroxide with 0·5 m citrate–phosphate buffer (pH 6·5) and then examining using SRID. Specifically, 140 μl of citrate–phosphate buffer and 60 μl of 10% Zwittergent (CALBIOCHEM, La Jolla, CA, USA) were added to 400 μl of inactivated adjuvanted whole-virion vaccine containing aluminum phosphate and incubated in a water bath at 37°C for 2 hours with agitation (10 seconds) every 30 minutes. The mixture was then centrifuged at 2500 × g for 5 minutes at 4°C and the supernatant carefully applied to a standard SRID gel after making appropriate dilutions with phosphate-buffered saline.

Pyrogen control

The presence of pyrogenic substances was controlled by two different tests: bacterial endotoxin tests and the pyrogen test using rabbits.[15] Our bulk materials were pyrogen-free, and heat-inactivation for heat labile pyrogenic factors was not necessary.

Table 3. Antibody responses in phase I clinical trial

	CHMP acceptance criteria	Hemagglutinin dose					
		1·7 μg		5 μg		15 μg	
		SC (*n* = 19)	IM (*n* = 20)	SC (*n* = 20)	IM (*n* = 20)	SC (*n* = 19)	IM (*n* = 20)
Day 21 (one vaccination)							
GMT	No standard	6·6	5·7	7·8	12·7	14·1	16·8
Mean geometric increase (ratio day 21/day 0 GMT)	>2·5×	1·3×	1·1×	1·6×	**2·5**×	**2·6**×	**3·4**×
Significant increase in titers (≥4×) (%)	>40	5	0	5	25	25	**40**
Seroprotection rate (titer: ≥40) (%)	>70	5	0	5	25	30	40
Day 42 (two vaccinations)							
GMT	No standard	7·3	6·8	10·7	14·6	15·7	20·7
Mean geometric increase (ratio day 42/day 0 GMT)	>2·5×	1·5×	1·4×	2·1×	**2·9**×	**2·9**×	**4·1**×
Significant increase in titers (≥4×) (%)	>40	11	0	15	30	21	**45**
Seroprotection rate (titer: ≥40) (%)	>70	11	0	15	30	27	45

SC, subcutaneous injection; IM, intramuscular injection; GMT, geometric mean titer; × means times, significance level of bold value: 0.05.

Toxicity assays

Tests for abnormal toxicity are typically carried out using the final bulk or filling lot materials and not unprocessed bulk materials. As some Japanese manufacturers have found that some inactivated whole virion unprocessed bulk samples inoculated into guinea pigs resulted in significant weight loss, it has been recommended that bulk materials be tested as part of the development process.[15]

Production requirements

The JMR for influenza vaccine (whole virion) has been including a leukopenic toxicity test and mouse weight gain test, neither of which is now used in vaccine testing. However, these control parameters are important readouts for vaccine safety and should be included in the testing and design process.[15]

Stability testing and pharmacologic studies

Long-term stability

Long-term stability tests were carried out for both bulk materials and filling lots, including photostability tests for the filling lots. We have confirmed long-term stability of the bulk and filling lot preparations to be 26 and 15 months respectively.[17]

Electron microscopic analysis and fractionation tests were also used to confirm vaccine formulation stability and virus particle integrity.

The protein content of filling lot supernatants was also measured to confirm antigen adsorption into the aluminum hydroxide gel during the stability testing period, and mouse immunogenicity tests were also carried out to confirm the integrity of the vaccine formulation over time.

Pharmacologic studies

Single dose toxicity (rats and dogs), local irritation toxicity (rabbits), repeated dose toxicity (4-week intervals, rats), reproductive and developmental toxicity (seg II and seg III, rats) and pharmacologic safety (rats and dog) tests were carried out as pre-clinical tests for the candidate vaccines according to PMDA recommendations.

In the local irritation and toxicity testing, the candidate-adsorbed vaccine formulation showed similar effects as the commercial-adsorbed DTaP vaccine suggesting that the test vaccine did not elicit any adverse cutaneous responses.

Clinical trials

Phase I clinical trial

In the results of the phase I clinical trial, all formulations were well tolerated, no severe adverse events were found and local reactions were much greater in the SC group than in the IM group. However, no differences were found in systemic reactions (Table 2), and the vaccine showed good neutralizing antibody responses between 5 and 15 μg HA/dose. There were no significant differences in neutralizing antibody responses between the SC and IM routes.[9]

In HA inhibition antibody responses, after the second vaccination, the 15-μg IM vaccine met two of three CHMP criteria: significant increase in titers (>40%) and mean geometric increase (>2·5 times). However, the 15 μg SC and 5 μg IM vaccines met one of three CHMP criteria (Table 3).

From these results, we have selected the 5- and 15-μg vaccines as vaccine doses for phase II/III trial using SC delivery.

Phase II/III clinical trial

All vaccine formulations were well tolerated with no serious adverse events reported between days 0 and 42. Most of the reported local and systemic reactions were graded as mild and transient. Fewer injection-site and systemic reactions arose after the second vaccination than after the first in both groups. The highest incidence of injection-site reactions in each vaccine group were redness, pain, itching, swelling and injection-site warmth. The incidence of induration was not so high (less than about 10%). The systemic reactions in each vaccine group were weariness, headache and others (e.g. fever: <3% in both vaccine group; data not shown).[9]

In HA inhibition antibody responses, after one and two vaccinations, the 15-μg vaccine group met two of three CHMP criteria: significant increase in titers (>40%) and mean geometric increase (>2·5 times), and after two vaccinations, the 5-μg vaccine group met one CHMP criteria: significant increase in titers (>40%) (data not shown).

Thus the safety and immunogenicity of a two-dose regimen of adjuvanted 15 μg inactivated whole-virion H5N1 vaccine given by the SC route in healthy adults were confirmed by this trial.

The vaccine was approved as a mock-up vaccine and pre-pandemic vaccine by the MHLW. Both immunization routes, IM and SC were approved.

Conclusion

Various non-clinical studies have been carried out as a way of gaining regulatory approval of egg-derived, adjuvanted, inactivated, whole-virion vaccines. Because electron microscopy and fractionation tests were so valuable in defining the long-term stability of the inactivated whole-virion influenza vaccine in this study (by confirming the integrity of the viral particles) and the immunogenicity and protein content evaluations of the supernatants (to confirm the

efficacy of adsorption and aluminum hydroxide gel respectively), we recommend that these safety and control regimens be part of the vaccine validation process. In addition, they will be important quality control criteria when discussing licensing with national agencies.

Consensus on the HA content ratio as determined by SDS-PAGE is also necessary. Reverse genetics is an important technology for the derivation of an avirulent vaccine strain; however, production time for pre-pandemic or pandemic vaccines will depend on the antigen yield of the vaccine strain derived in this way.

A two-dose regimen of an adjuvanted 15 μg inactivated whole-virion H5N1 vaccine was safe in humans and generated immunity consistent with European regulatory requirements for licensing of pandemic influenza vaccine in HA inhibition antibody responses. The vaccine was approved as mock-up vaccine and pre-pandemic vaccine by MHLW.

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