

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Ehrlich HJ, Müller M, Oh HML, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N Engl J Med* 2008;358:2573-84.

Supplementary Appendix

METHODS

Vaccine

The wild type strain used (A/Vietnam/1203/2004) was an egg-adapted isolate. The original clinical material was isolated in embryonated hens' eggs and had 2 additional passages in eggs. This material was then passaged 3 times in Baxter's serum protein-free Vero cells for production of a seed virus bank which was used for production of the vaccine bulk material.

In total, the virus used for vaccine production had 3 passages in embryonated hens' eggs and 4 passages in serum protein-free Vero cells.

HA antigen content of the vaccine is determined by single-radial-immunodiffusion (SRD).

The SRD assay allows precise hemagglutinin (HA) antigen quantification in any type of influenza vaccine i.e. whole virus, split (subvirion), subunit, or virosome^{1,2,3,4,5}. Lysis of vaccine preparations with detergent (e.g. Zwittergent) enables HA molecules to diffuse into agarose gel plates and to react with antibodies to form complexes (visible as zones) which are stained to allow quantification. Thus, 15 µg HA in the presented whole virus vaccine is the same as 15 µg HA in a subunit vaccine.

Randomization, Cohort Allocation and Follow-Up

Subject assignment to a particular cohort was in order of enrollment. The staff involved in the conduct of the study was blinded to study group and clinical safety data remained blinded until the database had been cleaned and closed. Investigators were partially blinded, meaning that they were blinded within the respective dosing cohorts. Unblinded safety data from Cohorts 1a, 1b, 2 and 3 were provided to the independent Data Safety Monitoring Board (DSMB) while it was ensured that study monitors, data managers and other staff involved in the conduct of the study as well as laboratory personnel remained blinded.

The lowest dose of 3.75 µg HA antigen was included in the study to investigate its immunogenicity rather than for safety reasons. Therefore, the 3.75 µg dose and the 7.5 µg doses (with and without adjuvant) were administered in parallel to subjects in the first cohort, as even the 7.5 µg dose contains a considerably lower level of antigen than conventional trivalent interpandemic influenza vaccines (45 µg total HA antigen content).

Cohort 1 consisted of approximately 135 subjects randomized 1:1:1 to receive either 3.75 µg or 7.5 µg of alum adjuvanted H5N1 HA antigen, or 7.5 µg in a non-adjuvanted formulation. It was deemed acceptable to proceed with the two lowest doses simultaneously in Cohort 1 as each contains a considerably lower level of antigen than conventional trivalent interpandemic

influenza vaccines (45 µg total HA antigen content). Cohort 1 was further subdivided into two sub-cohorts identified as Cohorts 1a and 1b for safety monitoring purposes.

Cohort 1a consisted of approximately 30 subjects randomized in equal numbers to one of the three arms in Cohort 1. Unblinded safety data obtained at Day 3 after the first vaccination in Cohort 1a were reviewed by the independent DSMB and permission obtained to (i) recruit the remaining approximately 105 subjects into Cohort 1b and (ii) administer the second vaccination to Cohort 1a at Day 21. Seven days after the first vaccination in Cohort 1b, the unblinded safety data were again reviewed by the DSMB and permission obtained to proceed to Cohort 2.

Approximately 90 subjects (Cohort 2) were randomized 1:1 to receive 2 injections of 15 µg H5N1 HA antigen formulated with alum adjuvant or the same dose of antigen without adjuvant. Unblinded safety data from Cohort 2 through the seventh day after the first vaccination were reviewed and evaluated by the DSMB. Upon review of these data the DSMB raised no objection to the study continuing for enrollment of the third and final cohort.

Approximately 45 subjects were enrolled into Cohort 3 and received the first vaccination containing 30 µg of HA antigen in combination with an alum adjuvant. Safety data from Cohort 3 (through the seventh day after the first vaccination) were reviewed and evaluated by

the DSMB. Upon review of these data the DSMB raised no objection to the study continuing with the second vaccination.

Assays

Hemagglutination inhibition assays were done according to established procedures, using horse erythrocytes and Madin-Darby canine kidney (MDCK) cell derived antigen from the A/Vietnam/1203/2004 reverse genetics derived attenuated virus RG VN1203⁶. These were carried out at AnDiaTec, Stuttgart, Germany. Virus neutralization assays were performed using wild type H5N1 influenza strains A/Vietnam/1203/2004 (vaccine strain), A/Hong Kong/156/1997 (clade 3) and A/Indonesia/05/2005 (clade 2). Serum samples were initially diluted 1:5 and then serially diluted in two fold steps with cell culture medium. The dilutions were then mixed at a ratio of 1:1 with the appropriate virus strain (100 TCID₅₀ per well) and transferred to a microtiter plate with a Vero cell monolayer. After 5 days incubation at 37°C, the cultures were inspected for cytopathic effect. The neutralizing titer, expressed as the reciprocal of antiserum dilution at which virus growth is inhibited by 50%, was calculated by the number of virus negative wells and the serum dilution according to the method of Reed and Muench⁷. On this basis, negative sera were assigned a nominal value of 3.9. Single radial

hemolysis (SRH) was carried out at the Laboratory of Molecular Epidemiology, University of Siena, Italy according to published methods⁸.

Statistical Analysis

The number of subjects with local reactions as well as the number of subjects with systemic reactions within 7 days after vaccination were compared between the different vaccine formulations by likelihood ratio chi-square test. The analyses were carried out separately for the 1st and 2nd vaccinations.

Changes from baseline in the log-transformed neutralization (NT) antibody titers and SRH values were analyzed by a mixed model accounting for the effects of vaccine group, time, age, sex, vaccine formulation - day interaction and baseline neutralization antibody titer. A compound symmetry covariance structure was assumed and the Kenwood-Roger correction for the degrees of freedom was applied.⁹

The geometric mean of the NT titer and SRH fold increases (GMI) from baseline and their 95% confidence intervals were computed for each strain, vaccine group and time point separately (back-computed by exponentiation from the mean differences of the log-transformed values), within the same mixed model analysis framework. Results are presented

in Table 2. The model-adjusted point estimates and 95% confidence intervals are very close to the empirical ones presented in the manuscript (see Manuscript Table 3).

Non-adjuvanted vaccine formulations were contrasted to the adjuvanted ones within the same model. P-value is presented in the manuscript (see Manuscript Table 4). The 7.5 µg and 15 µg non-adjuvanted vaccine formulations were also compared. P-values are presented in the manuscript (see Manuscript Table 4). The ratios of geometric means corresponding to these contrasts are presented in Table 3.

Results of the pairwise comparisons between adjuvanted vaccine formulations (using virus neutralization antibody titer measurements) are presented in Table 4.

Results of the analyses of SRH values are presented in Tables 5A and 5B. Dose groups within the adjuvanted and non-adjuvanted preparations were also contrasted. Comparing the adjuvanted formulations' multiplicity for the 6 pairwise comparisons was considered.

The rate of subjects with NT titer of $\geq 1:20$ and the rate of subjects with SRH results $\geq 25 \text{ mm}^2$ were analyzed by generalized linear model with repeated measurements. Log link function was used and the General Estimating Equations (GEE) method was applied, using proc genmod of SAS statistical package. The explanatory variables were: vaccine group,

baseline seroprotection, sex, age and day. An autoregressive correlation structure was assumed. Differences between vaccine groups and their 95% CIs were computed within this model. GEE estimates were then back-transformed to the original scale by exponentiation.

Results are presented in Tables 6 and 7.

RESULTS

Demographic Characteristics

A total of 284 subjects were enrolled in the study; 275 subjects received the first vaccination (9 subjects were enrolled but not vaccinated). The Austrian study site (Vienna) enrolled the highest number of subjects (160/275). The two study sites in Singapore (Singapore NUH and Singapore CGH) enrolled 47/275 and 68/275 subjects, respectively (see Table 1).

Seventeen subjects did not come back for the Day 21 blood draw; these subjects were included in the safety (275 subjects) but excluded from the immunogenicity dataset (258 subjects) for the first vaccination. A total of 257 subjects received the second vaccination.

Eight subjects did not come back for the Day 42 blood draw. Therefore the immunogenicity dataset for the second vaccination comprises 249 subjects.

Slightly more male subjects (143 for the first vaccination and 137 for the second vaccination) than female subjects (115 for the first vaccination and 112 for the second vaccination) were

included in the immunogenicity dataset. Also for the safety dataset, slightly more male subjects (156 and 143 for the first and second vaccination, respectively) than female subjects (119 and 114, respectively) were included in the evaluation.

The largest number of subjects in both immunogenicity and safety datasets were aged 18 to 25 years; the second largest number of subjects were aged 26 to 30 years. The majority of subjects weighed between ≤ 60 kg to 70 kg and were between 161 and 180 cm tall.

Distributions of age, height and weight were well balanced between the three cohorts.

DISCUSSION

Comments on Whole Virus Seasonal Influenza Vaccine

Historically, use of whole virus seasonal influenza vaccines was discontinued largely due to their reactogenicity profile in children. A seasonal whole virus vaccine which was developed using the technology described here was also significantly more reactogenic in adult populations than the H5N1 vaccine described here. The lower reactogenicity of the H5N1 vaccine may be due the absence of an Influenza B strain in the vaccine. Pro-inflammatory cytokine studies (data unpublished) indicated that the B strain in the trivalent seasonal vaccine was largely responsible for inducing such cytokine responses. This hypothesis is also

supported by reports that an egg-derived monovalent B vaccine induced much higher rates of systemic reactions than a monovalent A vaccine in human volunteers.¹⁰

References

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Table 1. Number of subjects enrolled and vaccinated (first vaccination) in each study center

Study group	Study center						Total
	Vienna		Singapore NUH		Singapore CGH		
	N	%	N	%	N	%	
3.75µg adjuvanted	31	(68.9%)	6	(13.3%)	8	(17.8%)	45
7.5µg adjuvanted	28	(62.2%)	7	(15.6%)	10	(22.2%)	45
7.5µg non-adjuvanted	34	(75.6%)	2	(4.4%)	9	(20.0%)	45
15µg adjuvanted	27	(58.7%)	8	(17.4%)	11	(23.9%)	46
15µg non-adjuvanted	23	(51.1%)	7	(15.6%)	15	(33.3%)	45
30µg adjuvanted	17	(34.7%)	17	(34.7%)	15	(30.6%)	49
Total	160	(58.2%)	47	(17.1%)	68	(24.7%)	275

Table 2. Virus neutralization antibody titers - geometric mean of the fold increase (GMI) from baseline estimated from the repeated mixed model ANCOVA*

Strain / Day	Study Group																		
	3.75µg adjuvanted			7.5µg adjuvanted			7.5µg non-adjuvanted			15µg adjuvanted			15µg non-adjuvanted			30µg adjuvanted			
	N	GMI	95% C.I.	N	GMI	95% C.I.	N	GMI	95% C.I.	N	GMI	95% C.I.	N	GMI	95% C.I.	N	GMI	95% C.I.	
A/Vietnam/1203/2004, clade 1																			
21	42	2.0	(1.6 ; 2.5)	42	2.2	(1.8 ; 2.7)	42	3.4	(2.7 ; 4.2)	43	1.9	(1.6 ; 2.4)	43	3.1	(2.5 ; 3.9)	46	2.0	(1.6 ; 2.5)	
42	42	4.5	(3.6 ; 5.6)	39	4.3	(3.4 ; 5.3)	42	5.6	(4.5 ; 7.0)	41	4.0	(3.3 ; 5.0)	41	5.6	(4.5 ; 7.0)	44	4.4	(3.6 ; 5.4)	
A/Indonesia/05/2005, clade 2																			
21	42	1.7	(1.4 ; 2.0)	42	1.6	(1.4 ; 2.0)	42	2.4	(2.0 ; 2.8)	43	1.4	(1.1 ; 1.6)	43	2.2	(1.9 ; 2.7)	46	1.8	(1.5 ; 2.1)	
42	42	2.8	(2.3 ; 3.4)	39	2.7	(2.3 ; 3.3)	42	3.3	(2.8 ; 4.0)	41	2.4	(2.0 ; 2.9)	41	3.5	(2.9 ; 4.2)	44	3.0	(2.5 ; 3.5)	
A/Hong Kong/156/1997, clade 3																			
21	42	2.1	(1.7 ; 2.7)	42	2.3	(1.8 ; 3.0)	42	3.6	(2.8 ; 4.7)	43	2.1	(1.6 ; 2.6)	43	3.3	(2.6 ; 4.3)	46	2.0	(1.6 ; 2.5)	
42	42	5.4	(4.2 ; 6.9)	39	5.4	(4.2 ; 7.0)	42	6.3	(4.9 ; 8.1)	41	5.1	(3.9 ; 6.5)	41	7.8	(6.1 ; 10.0)	44	5.9	(4.6 ; 7.5)	

*Mixed model accounting for the following fixed effects: vaccine group, day, age, sex, vaccine group-day interaction, baseline neutralization antibody titer and for random subject effect

Table 3. Ratio of Geometric Mean Fold Increases (GMI) from baseline of virus neutralization antibody titer values

Contrast	Strain	Ratio of GMIs	95% CI
Adjuvanted vs. non-adjuvanted	<i>A/Vietnam/1203/2004</i>	0.7	(0.6 ; 0.8)
	<i>A/Indonesia/05/2005</i>	0.7	(0.6 ; 0.9)
	<i>A/Hong Kong/156/1997</i>	0.7	(0.6 ; 0.8)
Non-adjuvanted 7.5 µg vs. 15 µg	<i>A/Vietnam/1203/2004</i>	1.0	(0.8 ; 1.4)
	<i>A/Indonesia/05/2005</i>	1.0	(0.8 ; 1.3)
	<i>A/Hong Kong/156/1997</i>	0.9	(0.7 ; 1.3)

Table 4. Pair wise comparisons between adjuvanted vaccine formulations (using virus neutralization antibody titer measurements)

Contrast	P-value*		
	A/Vietnam/1203/2004	A/Indonesia/05/2005	A/Hong Kong/156/1997
Adjuvanted 3.75 vs. 7.5 µg	0.957	0.784	0.794
Adjuvanted 3.75 vs. 15 µg	0.566	0.120	0.757
Adjuvanted 3.75 vs. 30 µg	0.894	0.672	0.964
Adjuvanted 7.5 vs. 15 µg	0.533	0.203	0.568
Adjuvanted 7.5 vs. 30 µg	0.853	0.485	0.825
Adjuvanted 15 vs. 30 µg	0.652	0.044	0.717

*Applying a Bonferroni-correction for multiplicity, p-values above 0.0028 (=0.05/18) have to be considered significant

Table 5. Results of the mixed model analysis performed on the log-transformed SRH changes from baseline **A)** P-values and **B)** Ratio of Geometric Mean Fold Increases (GMI)

A)

Effect / Contrast	A/Vietnam/1203/2004 P-value
Effects	
Vaccine formulation	<0.001
Day (d 21 vs d 42)	<0.001
Baseline	<0.001
Sex	0.094
Age	0.794
Vaccine formulation - day	0.062
Contrasts	
Adjuvanted vs. non-adjuvanted	<0.001
Non-adjuvanted 7.5 vs. 15 µg	0.052

B)

Contrasts	A/Vietnam/1203/2004 Ratio of GMIs (95% CI)	
Adjuvanted vs. non-adjuvanted	0.5	(0.4 ; 0.6)
Non-adjuvanted 7.5 vs. 15 µg	1.5	(1.0 ; 2.3)

Table 6. Rate of subjects with virus neutralization antibody titer $\geq 1:20$

Strain	Comparison	Ratio of Seroprotection Rates	95% CI	P-value
Vietnam/1203/2004	3.75 μg + Al vs. 30 μg + Al	1.02	(0.77 ; 1.35)	0.915
	7.5 μg + Al vs. 30 μg + Al	0.94	(0.69 ; 1.26)	0.660
	7.5 μg vs. 30 μg + Al	1.15	(0.89 ; 1.49)	0.293
	15 μg + Al vs. 30 μg + Al	0.90	(0.66 ; 1.23)	0.522
	15 μg vs. 30 μg + Al	1.09	(0.83 ; 1.44)	0.527
	Baseline seroprotection (Yes vs. No)	1.77	(1.42 ; 2.20)	<0.001
	Sex (Male vs. Female)	0.85	(0.72 ; 1.00)	0.053
	Age	0.99	(0.98 ; 1.00)	0.074
	Day (42 vs. 21)	2.53	(2.09 ; 3.06)	<0.001
	Adjuvanted vs. non-adjuvanted	0.86	(0.73 ; 1.01)	0.074
	Non-adjuvanted 7.5 μg vs. 15 μg	1.05	(0.82 ; 1.35)	0.693
Indonesia/05/2005	3.75 μg + Al vs. 30 μg + Al	0.80	(0.41 ; 1.56)	0.509
	7.5 μg + Al vs. 30 μg + Al	1.07	(0.57 ; 1.99)	0.840
	7.5 μg vs. 30 μg + Al	1.55	(0.89 ; 2.72)	0.124
	15 μg + Al vs. 30 μg + Al	0.31	(0.10 ; 0.94)	0.039
	15 μg vs. 30 μg + Al	1.31	(0.72 ; 2.38)	0.383
	Baseline seroprotection (Yes vs. No)	3.04	(1.91 ; 4.84)	<0.001
	Sex (Male vs. Female)	0.61	(0.42 ; 0.89)	0.009
	Age	0.99	(0.97 ; 1.02)	0.555
	Day (42 vs. 21)	2.28	(1.71 ; 3.03)	<0.001
	Adjuvanted vs. non-adjuvanted	0.50	(0.34 ; 0.75)	<0.001
	Non-adjuvanted 7.5 μg vs. 15 μg	1.19	(0.73 ; 1.92)	0.482

Strain	Comparison	Ratio of Seroprotection Rates	95% CI	P-value
Hong Kong /156/1997	3.75 µg + Al vs. 30 µg + Al	0.87	(0.67 ; 1.12)	0.284
	7.5 µg + Al vs. 30 µg + Al	0.84	(0.66 ; 1.09)	0.188
	7.5 µg vs. 30 µg + Al	0.98	(0.78 ; 1.23)	0.857
	15 µg + Al vs. 30 µg + Al	0.83	(0.64 ; 1.08)	0.169
	15 µg vs. 30 µg + Al	1.04	(0.83 ; 1.29)	0.754
	Baseline seroprotection (Yes vs. No)	1.55	(1.33 ; 1.80)	<0.001
	Sex (Male vs. Female)	0.89	(0.77 ; 1.04)	0.136
	Age	0.99	(0.98 ; 1.00)	0.025
	Day (42 vs. 21)	2.28	(1.93 ; 2.70)	<0.001
	Adjuvanted vs. non-adjuvanted	0.88	(0.76 ; 1.02)	0.082
	Non-adjuvanted 7.5 µg vs. 15 µg	0.95	(0.75 ; 1.19)	0.631

Table 7. Rate of subjects with Single Radial Hemolysis (SRH) area $\geq 25 \text{ mm}^2$

A/Vietnam/1203/2004			
Comparison	Ratio of sero-protection rates	95% CI	P-value
3.75 μg + Al vs. 30 μg + Al	0.90	(0.62 ; 1.30)	0.563
7.5 μg + Al vs. 30 μg + Al	0.71	(0.45 ; 1.11)	0.133
7.5 μg vs. 30 μg + Al	1.40	(1.04 ; 1.89)	0.024
15 μg + Al vs. 30 μg + Al	0.72	(0.47 ; 1.09)	0.118
15 μg vs. 30 μg + Al	1.12	(0.79 ; 1.58)	0.537
Baseline seroprotection (Yes vs. No)	1.82	(1.48 ; 2.23)	<0.001
Sex (Male vs. Female)	0.92	(0.76 ; 1.13)	0.442
Age	1.00	(0.99 ; 1.02)	0.659
Day (42 vs. 21)	1.48	(1.32 ; 1.66)	<0.001
Adjuvanted vs. non-adjuvanted	0.66	(0.53 ; 0.81)	<0.001
Non-adjuvanted 7.5 μg vs. 15 μg	1.26	(0.95 ; 1.67)	0.111