Original Article

Effect of Aluminum Hydroxide Adjuvant on the Immunogenicity of the 2009 Pandemic Influenza A/H1N1 Vaccine: Multi-level Modeling of Data with Repeated Measures*

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Abstract

Objective To evaluate the effect of the aluminum hydroxide (Al-OH) adjuvant on the 2009 pandemic influenza A/H1N1 (pH1N1) vaccine.

Methods In a multicenter, double-blind, randomized, placebo-controlled trial, participants received two doses of split-virion formulation containing 15 μg hemagglutinin antigen, with or without aluminum hydroxide (Al-OH). We classified the participants into six age categories (>61 years, 41-60 years, 19-40 years, 13-18 years, 8-12 years, and 3-7 years) and obtained four blood samples from each participant on days 0, 21, 35, and 42 following the first dose of immunization. We assessed vaccine immunogenicity by measuring the geometric mean titer (GMT) of hemagglutination inhibiting antibody. We used a two-level model to evaluate the fixed effect of aluminum Al-OH and other factors, accounting for repeated measures.

Results The predictions of repeated measurement on GMTs of formulations with or without Al-OH, were 80.35 and 112.72, respectively. Al-OH significantly reduced immunogenicity after controlling for time post immunization, age-group and gender.

Conclusion The Al-OH adjuvant does not increase but actually reduces the immunogenicity of the split-virion pH1N1 vaccine.

Key words: Aluminum hydroxide; Adjuvant; Immunogenicity; 2009 pandemic influenza A/H1N1 vaccine; Multi-level model

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INTRODUCTION

In response to the 2009 H1N1 influenza pandemic (pH1N1)[1], the Chinese Center for Disease Control and Prevention undertook a multicenter, double-blind, randomized, placebo-controlled trial to assess the safety and immunogenicity of different formulations of the pH1N1 vaccine. The study concluded that one dose of split-virion vaccine containing 7.5 μg hemagglutinin with no aluminum hydroxide adjuvant (Al-OH) could be promoted as the formulation of

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choice against pH1N1[1]. However, a subset of study participants received an initial dose of split-virion vaccine containing 15 μg hemagglutinin antigen, with and without Al-OH, and 4 GMT measurements were taken from these individuals at days 0, 21, 35, and 42 after vaccination. These data were analyzed using a multilevel model to evaluate the effect of the Al-OH adjuvant on the immunogenicity of the pH1N1 vaccine[2].

MATERIALS AND METHODS

Study Subjects

The multi-center, double-blind, randomized, placebo-controlled trial has been described in detail previously[1]. Briefly, this multicenter trial was undertaken in ten centers in China. Healthy people aged 3 years or older were recruited for the study. In the present study, we select the participants in Jiangsu Province center, who received two doses of the split-virion formulation containing 15 μg of hemagglutinin, with or without Al-OH. Subjects were classified into six age bands: 61 years of age or older and 41-60, 19-40, 13-18, 8-12, and 3-7 years of age. The general rationale for the age division was the weak immune response of the young and the elderly in the early report[1]. Four blood samples were obtained from each participant on days 0 (immediately before the first dose), 21 (immediately before the second dose), 35 (14 days after second dose), and 42 (21 days after second dose) following the initial dose of immunization.

Vaccine and Titre

The influenza A/H1N1 2009 monovalent, split-virion vaccine was developed by Hualan Biological Bacterin Company. The seed virus was prepared from reassortant vaccine virus A/California/7/2009 NYMC X-179A (New York Medical College, New York). The two vaccines were split-virus products containing 15 μg hemagglutinin antigen with or without Al-OH.

The titer of antibodies against the vaccine strain was measured in all samples by means of hemagglutination-inhibition assays, which were performed in accordance with established procedures[3-4] and with the use of turkey erythrocytes. In brief, samples were treated with cholera filtrate at 36 °C for 16 h and were then tested at dilutions of 1:10 and 1:20. Titers of anti–hemagglutinin antigen antibodies that were below the detection limit (i.e., <1:10) were assigned a value of 1:5.

Statistical Analysis

Multilevel models can disentangle total variance into subcomponents at each level of the data structure[2,5-6]. As our data present a two-level structure with time occasions nested within individual, we used multilevel models to analyze the data. Model analysis was carried out using MLwin2.18 software[2].

To perform the multilevel analysis, participants were considered to be Level 2 units, and the observed time occasions Level 1 units. In this hierarchical structure, units at one level were recognized as being grouped, or nested within units at the next higher level. We set up the two-level model to explain the fixed effect of Al-OH and other factors. Meanwhile, variation of immunogenicity due to individual differences was examined using random effects parameters in the model. A logarithmic transformation of GMTs was performed to make the distribution of this outcome at each occasion closer to a normal distribution. Throughout this paper, we will denote the logarithm of the GMTs measurement by the variable Y. We used the z-test to test the difference between any two coefficients of different vaccine formulations. Alpha was set at 0.05 for a 2-tailed test. Explanatory variables were left in the model when there was a significant reduction in the likelihood-radio statistic (LRS) using Chi-square tests due to the inclusion of those variables.

As part of the modeling strategy, we first set up a simple two-level model to analyze the effect of Al-OH without controlling for time occasion, age group and gender factors as following:

$$Y_{ij} = \beta_0+\beta_1 \text{Al-OH}_j+\mu_{0j}+\varepsilon_{0ij} \quad \text{(Model 1)}$$

In Model 1, β_0 indicates the lg(titer) mean value of non-Al-OH group, and ($\beta_0+\beta_1$) indicates the mean value of Al-OH group. The term u_{0j} denotes the random effects at the individual level or between-individual variation, with expected mean $E(\mu_{0j})=0$ and variance $var(\mu_{0j})=\sigma_{u0}^2$. The term ε_{0ij} indicates random effects at observation occasion level or within-individual variation, with $E(\varepsilon_{0ij})=0$ and $var(\varepsilon_{0ij})=\sigma_{e0}^2$.

To estimate effects on immunogenicity that were independent of time and interactive effects between time and Al-OH, we added time and the interactive terms to set up Model 2 as following:

$$Y_{ij} = \beta_0+\beta_1 Al\text{-}OH_j+\beta_2 Time\_2_{ij}+\beta_3 Time\_3_{ij} + \beta_4 Time\_4_{ij} +\beta_{1\text{-}2} Al\text{-}OH*Time\_2_{ij}+ \beta_{1\text{-}3} Al\text{-}OH*Time\_3_{ij} +\beta_{1\text{-}4} Al\text{-}OH*Time\_4_{ij} + \mu_{0j}+\varepsilon_{0ij} \quad \text{(Model 2)}$$

where β_2, β_3, and β_4 indicates the increase of lg(GMT) on days 21, 35, and 42 following the first immunization, respectively, compared with day 0 (reference subgroup). Parameters β_{12}, β_{13}, and β_{14} denote the interactive effect of Al-OH with each of the time occasions respectively (days 21, 35, and 42). Based on Model 2, the mean titer of each subgroup can be estimated. For example, the mean GMT for those with Al-OH and on day 21 subgroup should be $10^{(\beta 0+\beta 1+\beta 2+\beta 12)}$.

To estimate effects on immunogenicity that were independent of age and interactive effects between age and Al-OH, we added age and the interactive terms to Model 1 to set up Model 3 as following:

$$Y_{ij} = \beta_0+\beta_1 Al\text{-}OH_j+\beta_5 Agegroup\_2_{ij}+\beta_6 Agegroup\_3_{ij}+ \beta_7 Agegroup\_4_{ij}+\beta_8 Agegroup\_5_{ij}+\beta_9 Agegroup\_6_{ij}+ \beta_{1\text{-}5} Al\text{-}OH*Agegroup\_2_{ij}+\beta_{1\text{-}6} Al\text{-}OH*Agegroup\_3_{ij}+ \beta_{1\text{-}7} Al\text{-}OH*Agegroup\_4_{ij}+\beta_{1\text{-}8} Al\text{-}OH*Agegroup\_5_{ij}+ \beta_{1\text{-}9} Al\text{-}OH*Agegroup\_6_{ij} +\mu_{0j}+\varepsilon_{0ij} \quad \text{(Model 3)}$$

where β_5, β_6 and β_7 denote the increase of lg(GMT) for age sub-groups 8-11 y, 12-17 y, 18-40 y, 41-60 y, and over 60 y, respectively, compared with the 3-7 y age group (reference subgroup). Based on Model 3, the mean titer of each age group can be estimated. For example, the mean titer for those with Al-OH and 8-11 years old should be $10^{(\beta 0+\beta 1+\beta 5)}$.

Based on models 2 and 3, we set up Model 4 to estimate effects of Al-OH, age and time occasion factors as follows:

$$Y_{ij} = \beta_0+\beta_1 Al\text{-}OH_j+\beta_2 Time\_2_{ij}+\beta_3 Time\_3_{ij} +\beta_4 Time\_4_{ij} + \beta_{1\text{-}2} Al\text{-}OH*Time\_2_{ij}+\beta_{1\text{-}3} Al\text{-}OH*Time\_3_{ij} + \beta_{1\text{-}4} Al\text{-}OH*Time\_4_{ij} +\beta_5 Agegroup\_2_j+ \beta_6 Agegroup\_3_j+\beta_7 Agegroup\_4_j+\beta_8 Agegroup\_5_j+ \beta_9 Agegroup\_6_j+\beta_{1\text{-}5} Al\text{-}OH*Agegroup\_2_{ij}+ \beta_{1\text{-}6} Al\text{-}OH*Agegroup\_3_{ij}+ \beta_{1\text{-}7} Al\text{-}OH*Agegroup\_4_{ij}+ \beta_{1\text{-}8} Al\text{-}OH*Agegroup\_5_{ij}+ \beta_{1\text{-}9} Al\text{-}OH*Agegroup\_6_{ij}+\mu_{0j}+\varepsilon_{0ij} \quad \text{(Model 4)}$$

RESULTS

Data for 3 520 participants were included in this analysis. The mean age of the participants was 32 years (range: 3 to 76 years) (Table 1). At baseline, various vaccine groups did not differ significantly in age- and gender-distributions.

Table 1. Demographical Characteristics of Participants

Age Group	Split*			Split+Al*		
	n	Age#	F (%)##	*n*	Age#	F (%)##
3-7 y	268	5.3±1.6	128 (47.8)	236	4.9±1.4	124 (52.5)
8-12 y	260	10.6±1.3	128 (49.2)	252	10.2±1.3	112 (44.4)
13-18 y	360	14.7±1.4	184 (51.1)	420	14.7±1.4	208 (49.5)
19-40 y	212	30.7±6.2	112 (52.8)	196	31.3±6.6	108 (55.1)
41-60 y	220	52.1±6.0	108 (49.1)	216	51.7±6.3	108 (50.0)
>61 y	440	65.0±3.2	220 (50.0)	440	64.8±2.9	220 (50.0)
Total	1 760	31.8±23.9	880 (50.0)	1 760	31.7±23.8	880 (50.0)

Note. *Split: Non-adjuvanted split-virion vaccine of 15 μg hemagglutinin antigen; Split+Al: Aluminum-adjuvanted split-virion vaccine of 15 μg hemagglutinin antigen. #Age: Mean±Standard Deviation; ##F(%): Proportion female.

In the basic two-level Model 1, only the effect of Al-OH factor was analyzed; time occasion, age-group and gender were not included in this model. The predictions of repeated measurement on GMTs of participants who received two doses of split-virion formulation containing 15 μg hemagglutinin with or without Al-OH, were 80.35 and 112.72, respectively. Al-OH was found to lower immunogenicity (P<0.001) (Table 2).

All fixed factors (including time occasions and age-groups) had significant effects on immunogenicity. After accounting for these factors, a significant reduction in the IGLS was observed for the effect of Al-OH on immunogenicity, as shown in Models 2, 3, and 4 (Table 2).

When considering the interactive effects between time and Al-OH on immunogenicity in Model 2, the predictions of repeated measurement of GMTs at days 0, 21, 35, and 42 following first dose of immunization without

adjuvant were 6.49, 299.92, 325.09, and 309.74 respectively, and the those with adjuvant are 6.52, 166.72, 224.91, and 212.81 respectively. A significant negative effect by Al-OH was found at each time occasion (Table 2). A significant negative effect by Al-OH was also found at each time occasion based on raw data (Table 3).

Among each age-group, the immunogenicity observed in the subgroup without Al-OH was significantly higher than that in the subgroup with Al-OH in Model 3 (Table 2). The lower immunogenicity was also found at each time occasion (except day 0) and sub-age group, using the raw data analysis (Table 3).

Table 2. Two-level Model of Immunogenicity of Pandemic 2009 A/H1N1 Clinical Trial with Repeated Measurement

Effect	Parameter		Coefficient (Standard Error)			
			Model 1	Model 2	Model 3	Model 4
Fixed Effect	β0	constant	2.052 (0.045)	0.812 (0.053)	1.880 (0.053)	0.412 (0.110)
	β1	Al-OH	-0.147 (0.028)*	0.002 (0.033)	-0.158 (0.027)*	0.141 (0.071)
Time Difference		time_1 (0 day, ref)		--	--	--
	β2	time_2 (21 days)		1.665 (0.051)*		1.666 (0.051)*
	β3	time_3 (35 days)		1.700 (0.052)*		1.702 (0.052)*
	β4	time_4 (42 days)		1.679 (0.053)*		1.682 (0.053)*
Interactive Factor	β12	Al-OH*time_2		-0.257 (0.032)*		-0.257 (0.032)*
	β13	Al-OH*time_3		-0.162 (0.033)*		-0.163 (0.033)*
	β14	Al-OH*time_4		-0.165 (0.033)*		-0.166 (0.033)*
Age Difference		3--7 y, ref		--	--	--
	β5	8--12 y			0.168 (0.050)*	0.433 (0.150)*
	β6	13--18 y			0.415 (0.046)*	0.747 (0.138)*
	β7	19--40 y			0.216 (0.054)*	0.515 (0.158)*
	β8	41--60 y			0.179 (0.053)*	0.498 (0.156)*
	β9	>61 y			0.114(0.045)*	0.273(0.133)*
Random Effect						
Level 2 (Individual)	σ_{u0}^2		0.013 (0.009)	0.135 (0.008)*	0.000 (0.000)	0.114 (0.007)*
Level 1 (Time Occasion)	σ_{e0}^2		0.618 (0.018)*	0.112 (0.003)*	0.613 (0.015)*	0.112 (0.003)*
-2log-likelihood (IGLS)			7866.311	3629.048*	7774.000*	3520.733*

Note. *$P \leqslant 0.05$.

Table 3. Means of GMT with or without Al-OH by time following First dose of Immunization and Age-group Based on Raw Data

Age Group(y)	0 Day		21 Days		35 Days		42 Days*	
	Non-adjuvant	Adjuvant	Non-adjuvant	Adjuvant	Non-adjuvant	Adjuvant	Non-adjuvant	Adjuvant
3-7	5.27	5.20	##	##	##	##	166.33	172.57
8-12	5.27	5.77	##	##	##	##	242.17*	134.05
13-18	6.96	8.26	317.85*	109.43	##	##	451.10*	215.04
19-40	6.32	7.07	248.33*	136.56	168.43	##	249.19*	141.51
41-60	6.15	6.10	250.91*	144.45	204.19	##	284.50*	160.00
>61	6.23	6.71	##	160.00	##	##	186.38*	111.69
Total	6.14	6.68	282.64*	123.96	481.15	##	272.54*	162.56

Note. *$P \leqslant 0.05$. ##No blood samples collected.

DISCUSSION

In our study, Al-OH was found to lower immunogenicity. This negative effect was also observed when age and interactive effects between time and Al-OH on immunogenicity were considered.

The Al-OH adjuvant is intended to augment immune responses to vaccine antigens. However, the Al-OH adjuvant is not widely used in inactivated influenza vaccines. In groups in which antibody responses have been inadequate, such as the elderly and young children, there is increased interest as to whether the addition of Al-OH may enhance immunogenicity. One study showed that influenza A(H5N1) vaccine with the Al-OH adjuvant was well tolerated and immunogenic in children and infants[7]. However, in other studies of subvirion inactivated influenza A/H5N1 vaccine, no meaningful benefit of Al-OH was observed[8-9].

As with the present study, other studies have found influenza A/H1N1 vaccine formulations without adjuvant to be more immunogenic than formulations with adjuvant[1,10], which may be the result of immunological memory of previous influenza infection from other virus subgroups. In our study, using the raw data analysis, the peak of immunogenicity was achieved on day 35 in the non-adjuvant subgroup, and the peak GMT of antigen together with Al-OH was later than this time. The depot mechanism postulates that the adsorbed antigen remains at the site of injection and the antigen is released more slowly to stimulate the production of antibodies. This may explain the later peak of immunogenicity of adjuvanted vaccine in our study and in other studies[8-9].

However, when considering the interactive effects between time and Al-OH on the immunogenicity in Model 2, the peak of prediction of repeated measurement of GMTs was achieved at day 35. Furthermore, for the 3-7 y participants, the GMT of day 42 day does differ significantly with or without Al-OH. More observations may be needed to verify the depot mechanism of Al-OH in A/H1N1 vaccines.

Novel adjuvants have been invented and used with influenza vaccines. For example, JVRS-100, composed of cationic liposome-DNA complexes, has been shown to elicit robust immune responses compared to Al-OH adjuvants, and to efficiently enhance both humoral and cellular immune responses[11]. Also, MF59-adjuvanted monovalent 2009 influenza A/H1N1 vaccine has been observed to generate stronger antibody responses than that of non-adjuvanted vaccines[12-13]. A growing body of evidence seems to support the potential use of MF59-adjuvanted influenza vaccine as a safe and highly immunogenic influenza vaccine[14].

We used multilevel modeling to account for clustering effects derived from repeated measurements of individuals. In general, ignoring clustering effects would have led to biased estimates in standard errors of regression coefficients. Correct standard errors could be estimated only if variation at occasion level were allowed for in the analysis, and multilevel modeling provides an efficient way of doing this. It also makes it possible to model and investigate the relative sizes and effects of age and gender on GMT[15].

In conclusion, our data showed that Al-OH adjuvant does not increase but actually reduces the immunogenicity of split-virion pH1N1 vaccine.

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