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EC MICROBIOLOGY
Research Article

Evaluation of Antigen Source and Neuraminidase Inhibition in the Influenza Hemagglutination Inhibition Assay

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Received: June 07, 2017; **Published:** August 29, 2017

Abstract

Influenza vaccines produced by recombinant technology provide an exact genetic match to the hemagglutinin of wild-type strains (grown solely in cell culture) selected for seasonal vaccines, whereas the decades-old technology producing inactivated subunit vaccine from virus grown in eggs results in genetic variations that may cause antigenic differences. We hypothesized that the source, and therefore the structure, of reference influenza hemagglutinin antigen used in the hemagglutination inhibition assay to measure influenza antibody levels may impact the results of the test, depending on the source of the vaccine administered to clinical trial participants. Agglutination by influenza neuraminidase has also been suggested to be a confounder of the assay for antibodies to influenza A (H3N2).

Thirty post-vaccination serum samples selected randomly from participants in a clinical trial comparing immunogenicity of quadrivalent recombinant hemagglutinin versus egg-derived quadrivalent inactivated influenza vaccine were tested for hemagglutination inhibition antibody titers using both cell-culture-derived and egg-derived reference antigens. Resulting antibody titers were compared as geometric means for all participants and for each vaccine group versus reference antigen from each source. The potential role of influenza neuraminidase in influenza A/H1N1 antigen was tested by adding the neuraminidase inhibitor, zanamivir, to assays.

The geometric mean titers for all 30 serum samples, for 12 serum samples from recombinant hemagglutinin vaccine recipients and for 18 serum samples from egg-derived vaccine recipients yielded higher titers to the three strains of influenza when measured by cell-derived antigen versus egg-derived antigen. The differences were of comparable magnitude (~20 – 25% lower with egg-derived antigen), regardless of influenza strain or vaccine received. Similarly, the additional of zanamivir to the assays reduced measured titers of anti-influenza A/Texas (H3N2) by approximately the same magnitude, regardless of the source of reference antigen or vaccine received.

We conclude that while cell-derived reference antigens in the hemagglutination inhibition assay yield a modestly higher antibody titer, the effect is similar, regardless of the vaccine received. Thus, the use of the validated egg-derived reference antigen is reasonable. Similarly, the addition of neuraminidase inhibitor reduced antibody titers modestly, with a similar effect regardless of source of reference antigen or vaccine received.

Keywords: *Influenza Vaccine; Hemagglutination Inhibition Antibody Assay; Recombinant Influenza Vaccine; Influenza Vaccine Immunogenicity; Neuraminidase*

Abbreviations

B.E.S.T.: Baculovirus Expression System Technology; CBER: Centers for Biologics Evaluation and Review (US Food and Drug Administration); DNA: Deoxyribonucleic Acid; FDA: United States Food and Drug Administration; HA: Hemagglutinin; HAI: Hemagglutination inhibition (assay for antibodies); IIV: Inactivated Influenza Vaccine (4 denotes quadrivalent; 3 denotes trivalent); NA: Neuraminidase; NIBSC: National Institute for Biological Standards and Control (source of reference reagents); rHA: Recombinant hemagglutinin; RBC: Red Blood Cells; RIV: Recombinant Influenza Vaccine (4 denotes quadrivalent; 3 denotes trivalent)

Introduction

In recent years, influenza vaccines produced using novel technologies have been introduced to compete with the decades-old process of growing infectious virus in embryonated hen's eggs, the latter of which are then inactivated and purified to produce the conventional inactivated split virus or sub-unit vaccine. The application of modern technologies includes two processes: 1) growing infectious virus in mammalian cell culture (which then follows a process of inactivation and purification like the egg process) or 2) the production of purified recombinant hemagglutinin (rHA) protein using recombinant DNA clones of the gene of interest selected from wild-type virus and expressed in insect cell culture (Baculovirus Expression System Technology, B.E.S.T.) [1,2].

These newer vaccines may yield a different tertiary molecular structure of HA from that produced in eggs, due to absence of amino acid mutations in the receptor site of the HA introduced in the process of adapting the virus to growth in eggs [3]. The structural difference is related to the amino acid mutations and the consequent difference in glycosylation and tertiary folding [4,5]. In several recent years of seasonal influenza, it has been shown that the differences between egg-produced HA and wild-type HA resulted in sufficient variation in antibody responses that the egg-produced vaccine lost clinical effectiveness [6]. Because of these differences, we hypothesized that hemagglutination inhibition (HAI) assays used for testing the immunogenicity of the new vaccines might report out different results depending on the source of the references antigen reagents used in the assay, and that egg-based reference antigens might yield less reliable antibody titers among recipients of recombinant HA vaccine [7].

Additionally, some researchers have found that variations in neuraminidase may be another confounding factor in the HAI assay [8]. Neuraminidase is known to contribute to protection against influenza infection [9] and if the neuraminidase enzymatic activity affects the HA structure or the NA antigen contributes to RBC agglutination, it may result in a poorly defined and uninterpretable agglutination inhibition. These researchers showed that incorporation of a neuraminidase inhibitor in the assay eliminated this artifact.

This pilot study was conducted to assess whether the optimal antigens for the HAI assay used for testing immunogenicity in rHA (Flublok) clinical trials would be conventional egg-based reagents or cell-grown antigens with wild-type HA sequence. Additionally, we tested whether the addition of a neuraminidase inhibitor provided important clarification of results.

Methods

Thirty post-vaccination serum samples from a randomized, comparative clinical trial were selected randomly from subjects who received either RIV4 (Flublok Quadrivalent®, Protein Sciences Corporation, Meriden, CT) or IIV4 (Fluarix Quadrivalent®, GlaxoSmith Kline, Research Triangle Park, NC). The vaccines contained the influenza HAs selected for the North American seasonal quadrivalent vaccine in 2014-2015: A/California/7/2009 (H1N1), A/Texas/50/2012 (H3N2), B/Massachusetts/2/2012 and B/Brisbane/60/2008. Samples were maintained in a frozen state until the time of assay. HAI assays were performed at Focus Diagnostics (subsidiary of Quest Diagnostics, Cypress, CA) using turkey red blood cells (Lampire Associates, PA) per their standard assay procedure [10]. Reference antigen reagents, both egg-based and cell-based for each of the A/H1N1, A/H3N2 and B/Massachusetts HAs were obtained from National Institute for Biological Standards and Control (NIBSC). The antigens were inactivated and partially purified viruses prepared in a process like that used for production of inactivated influenza vaccines. Cell-based reference antigens for B/Brisbane were not available commercially at the time of this study.

Using their standard procedure, the HAI assays were performed in triplicate for each antigen. In addition, the A/H3 titers were determined with and without added 10 µg zanamivir to assess the effect of inhibition of neuraminidase-mediated agglutination.

Results and Discussion

Egg vs. Cell-based Antigen

All Vaccine Recipients

Geometric mean titers (GMT) of all serum samples tested using egg-derived or cell-derived HA antigens shown a consistently higher HAI titer with the use of cell-based antigens (Table 1).

Post-Vaccination Geometric Mean HAI Titers measured with Egg-based and Cell-based Antigens				
Strain	Egg Reagent N=30	Cell Reagent N=30	GMT Ratio[2]	95% CI
A H1/California[1]	905.1 (615.5,1331.0)	1838.3 (1190.5, 2838.5)	0.492	0.438, 0.554
A H3/Texas[1]	926.3 (666.2, 1287.8)	1270.2 (941.6, 1713.3)	0.729	0.622, 0.855
B/Massachusetts[1]	125.1 (81.3, 192.3)	228.0 (144.5, 359.9)	0.549	0.476, 0.632
[1]Data pooled from Flublok and IIV4 recipients				
[2]GMT = (GMT Egg/GMT Cell). 95% CI for GMT Ratio is based on back transformation of 95% confidence limits calculated using a PAIRED t statistic for difference of log transformed HAI titers				

Table 1: *Geometric Mean HAI titers measured with Egg-based or Cell-based Antigens.*

The ratios of GMTs indicate that the CBER criterion for non-inferiority of two sets of HAI titers assessed by GMT ratio (an upper limit of the 95% confidence interval less than 1.5) [11] were met, confirming that the titers measured using the cell-based antigen are non-inferior by this criterion.

By Vaccine group

The titers yielded by the HAI assay using cell-based antigens were also higher regardless of vaccine received by the study subjects (Table 2).

HAI GMTs Measured by Each Antigen in Each Vaccine Group					
Vaccine	Strain	HAI GMT Titers[1]			
		Egg Reagent	Cell Reagent	Ratio	95% CI
Flublok n = 12	A H1/California	996.4 (542.4, 1831.1)	2237.2 (1162.2, 4306.5)	0.445	0.390, 0.509
Flublok n = 12	A H3/Texas	1185.1 (606.9, 2314.4)	1493.2 (773.7, 2881.8)	0.794	0.560, 1.125
Flublok n = 12	B/Massachusetts	124.6 (51.8, 299.5)	244.4 (92.5, 645.6)	0.510	0.412, 0.630
IIV4 n = 18	A H1/California	848.8 (490.6, 1468.7)	1612.7 (863.8, 3011.0)	0.526	0.440, 0.629
IIV4 n = 18	A H3/Texas	785.9 (545.5, 1132.4)	1140.4 (842.0, 1544.5)	0.689	0.585, 0.811
IIV4 n = 18	B/Massachusetts	125.4 (75.6, 207.8)	217.7 (131.44, 360.7)	0.576	0.469, 0.707
[1]GMT = (GMT Egg/GMT Cell). 95% CI for GMT Ratio is based on back transformation of 95% confidence limits calculated using a PAIRED t statistic for difference of log transformed HAI titers					

Table 2: *Comparison of HAI GMTs Measured by Egg- vs. Cell-based Antigen in each Vaccine Group.*

Again, the upper limit of the 95% confidence interval around the ratio of HAI antibody titers using egg-based-to-cell-based antigens were < 1.5 for each strain tested, confirming the non-inferiority of results of the two assays, regardless of the vaccine used to induce antibodies.

The assays utilizing the cell-derived reagents consistently yielded somewhat higher titers to all influenza HAs in recipients of either rHA or IIV vaccine. The difference was of similar magnitude for each HA, regardless of which vaccine the subject had received. Given that the HAI titers are determined in biological assays by testing the agglutination inhibition properties of two-fold dilutions of subjects' sera, the limits of sensitivity of the assay are within a two-fold dilution. Thus, the results may be less different than they appear. Nevertheless, the discrepancy was consistent in direction and magnitude across all three influenza strains and both vaccine groups.

The reason for the higher antibody titers measured by the cell-based antigens is not immediately clear. Furthermore, the similarity of these finding, regardless of vaccine administered is not consistent with the hypothesis that antibodies induced by recombinant HA would be more highly reactive with wild-type HA in cell-grown antigen reagents, whereas HAI antibodies induced by egg-grown vaccine would be more reactive with egg-derived antigen reagents. Instead, uniformly modestly higher HAI titers for all three strains in recipients of both vaccines were observed with cell-grown antigens vs. egg-grown reagents.

Flublok vs. IIV HAI GMTs

GMTs by Antigen Reagent Source

The HAI GMTs for each strain by vaccine group showed higher HAI GMT for Flublok recipients than IIV recipients, differences that are more notable when sera were tested using cell-based antigens (Table 3).

Comparison of IIV vs. Flublok GMTs for each Influenza Strain by Egg- or Cell-Based Reagent						
Source of Reagent	IIV4 N=18		Flublok N=12		Comparison	
	GMT	95% CIs*	GMT	95% CIs*	GMT Ratio	95% CIs*
A H1/California						
Egg	848.83	490.57, 1468.72	996.56	542.37, 1831.12	0.85	0.38, 1.90
Cell	1612.70	863.76, 3011.01	2237.21	1162.22, 4306.51	0.72	0.29, 1.76
A H3/Texas						
Egg	785.91	545.45, 1132.38	1185.12	606.85, 2314.44	0.66	0.34, 1.29
Cell	1140.35	841.97, 1544.48	1493.16	773.66, 2881.77	0.76	0.41, 1.41
B/Massachusetts						
Egg	125.37	75.63, 207.83	124.57	51.81, 299.54	1.01	0.41, 2.46
Cell	217.73	131.44, 360.66	244.39	92.51, 645.60	0.89	0.35, 2.30

Table 3: *Comparison of IIV vs. Flublok GMTs for each Influenza Strain by Egg- or Cell-Based Reagent.*

**95% CI for GMT Ratio is based on back transformation of 95% confidence limits calculated using a t statistic for difference of log transformed HAI titers*

Although titers reported by the cell-based antigen reagents always appeared to be higher than those reported from the egg-based reagents, the HAI titers to the influenza A subtypes, especially A/H3, in Flublok recipients were consistently higher than those in IIV recipients. The HAI response to B/Massachusetts was notably lower in both assays than HAI titers to the A subtypes, with discrimination between Flublok and IIV recipients somewhat more apparent in the cell-based HAI assay.

Cell-derived hemagglutinins are known to be free of mutations induced by growth of the influenza virus in eggs, and therefore to maintain antigenic fidelity to the wild-type strains, as do the recombinant HAs Thus, one might expect to see a more robust antibody response to cell-based antigens among RIV recipients. The absence of such a differential response between vaccine recipients may suggest a more subtle difference between the antigen sources or may simply reflect the relative crudeness of the HAI assay. In larger, adequately powered clinical trials it is very possible that the higher titers measured by cell-derived reference antigens would result in greater ability to discriminate between vaccine effects. An assay with greater sensitivity to discriminate between antibody levels may ultimately support a distinction between egg-derived and cell-derived reference antigens.

Effect of Anti-Neuraminidase on A/H3N2 HAI Results

Pooled A/H3N2 HAI GMTs with Zanamivir

It has been demonstrated that a mutation in the receptor site of the neuraminidase of A/H3N2 subtypes of influenza causes hemagglutination that may result in higher titers in the HAI assay [8]. The authors further demonstrated that adding a neuraminidase inhibitor to the HAI assay at a concentration that approximates the level of enzymatic activity reduced the titers reported out. To evaluate the impact of neuraminidase in the HAI assays of this study, the NA inhibitor zanamivir, was added to the assay of anti-H3N2 titers (Table 4).

Geometric Mean A/H3N2 HAI titers measured with Egg-based and Cell-based Antigens							
Egg Reagent		GMT Ratio	95% CI	Cell Reagent		GMT Ratio	95% CI
-Zanamivir	+ Zanamivir			- Zanamivir	+ Zanamivir		
926.3 (666.2, 1287.8)	740.9 (554.5, 989.8)	0.800	0.691, 0.926	1270.2 (941.6, 1713.3)	1055.8 (787.1, 1416.3)	0.831	0.743, 0.930
	740.9 (554.5, 989.8)				1055.8 (787.1, 1416.3)	0.702	0.633, 0.778
* Data pooled from Flublok and IIV4 recipients							

Table 4: *Comparison of A/H3 HAI GMTs by Egg- or Cell-based Reagents with and without NA Inhibition*.

The HAI GMTs were affected to the same degree (approximately 20% reduction) by the neuraminidase inhibitor, zanamivir, regardless of the source of antigen used in the assay. Furthermore, the GMT ratio from egg-based to cell-based reagent assays is similar to that observed in the absence of neuraminidase inhibitor (See Table 2).

Effect of Zanamivir on A/H3N2 HAI GMTs for Each Vaccine Group

H3 HAI titers among subjects in each vaccine group yielded similar results to the pooled data: higher titers with cell-based antigens, regardless of vaccine group, and somewhat lower titers with added zanamivir, regardless of vaccine (Table 5).

A/H3N2 HAI GMTs by Reagent Source and Assay Conditions -- Vaccine Groups										
Vaccine	Egg Reagent	Egg + Zanamivir	GMT Ratio	95% CI		Cell Reagent	Cell + Zanamivir	GMT Ratio	95% CI	
Flublok	1185.12 (606.85, 2314.44)	887.8 (528.9, 1490.3)	0.749	0.551	1.019	1493.16 (773.66, 2881.77)	1255.6 (692.3, 2277.2)	0.841	0.696	1.016
		887.8 (528.9, 1490.3)					1255.6 (692.3, 2277.2)	0.707	0.596	0.839
IIV4	785.91 (545.45, 1132.38)	656.6 (452.9, 952.1)	0.835	0.712	0.981	1140.35 (841.97, 1544.48)	940.6 (673.3, 1314.2)	0.825	0.707	0.962
		656.6 (452.9, 952.1)					940.6 (673.3, 1314.2)	0.698	0.605	0.806

Table 5: *Comparison of Flublok vs. IIV A/H3N2 HAI GMTs by Reagent Source with and without NA Inhibition.*

Confidence intervals for these GMTs were quite wide due to the relatively small number of samples tested in each group. However, the trends are consistent, demonstrating a reduction in measured HAI titers when the neuraminidase inhibitor is added to the assay.

Regardless of vaccine administered to the subjects in this pilot group, post-vaccination HAI titers were consistently lower when measured using egg-derived antigen as compared to those measured using cell-derived antigen. The ratio of egg-derived titers vs. cell-derived titers for each influenza strain was similar when comparing the two vaccine groups. Furthermore, addition of zanamivir to the assays for H3N2 A/Texas reduced the measured HAI titers to a similar degree, regardless of the vaccine group.

Conclusions

As the differences between antigen source and neuraminidase inhibition do not differentially impact the HAI titers from recipients of either vaccine, we conclude that the conventional HAI assay, using either egg-derived or cell-derived reference antigens without a neuraminidase inhibitor is appropriate for testing sera from recipients of either egg-based vaccine or rHA vaccine in clinical trials.

Acknowledgements

The authors acknowledge the contributions of Wayne Hogrefe, PhD, Focus Diagnostics, in whose laboratory the HAI titers were performed. The study was supported by a contract from the Biomedical Advanced Research and Development Authority (BARDA), Contract # HSSO 100200900106C.

Conflict of Interest

All authors are employees of Protein Sciences Corporation and are shareholders of the Company.

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Volume 11 Issue 4 August 2017
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