

Increased antibodies against unfolded viral antigens in the elderly after influenza vaccination

Upma Gulati,^a Wendy A. Keitel,^b Gillian M. Air^a

^aDepartment of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA. ^bDepartments of Molecular Virology & Microbiology and Medicine, Baylor College of Medicine, Houston, TX, USA.

Correspondence: Gillian M. Air, Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA. Email: gillian-air@ouhsc.edu

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Objective Our studies aimed to measure the quality of antibody response to influenza vaccines in the elderly. The frequency of significant rise in hemagglutination inhibition (HAI) titer in the elderly is low and although annual vaccination reduces morbidity and mortality, better correlates of vaccine efficacy in the elderly are needed.

Methods We measured the amount and avidity of serum antibodies against native H3N2 influenza glycoproteins or denatured virus (unfoldons) in pre- and post-vaccinated sera of 36 elderly subjects.

Results Eighty percent of subjects had high pre-immunization antibody levels and only 13% showed ≥ 2 fold increase after vaccination, but 33% showed ≥ 2 fold increase in avidity. With increasing dosage there was a significant increase in avidity against unfoldons with 50% of subjects showing ≥ 2 fold increase at the highest dose. Elderly subjects given subunit vaccine showed higher

reactivity with unfoldons (78% of native) than younger subjects studied earlier who were given inactivated whole virus vaccine (19% of native).

Conclusion The clear inverse relationship between pre-immunization antibody levels and antibody increase after vaccination implies that a major reason for the low frequency of antibody responses in elderly subjects is simply because they have high pre-immunization antibody levels. Only low reactivity was observed with earlier viruses. The increased proportion and avidity of antibodies against unfoldons is of concern, as these are not protective, and vaccine developers need to be aware of the role of age or vaccine formulation in inducing anti-unfoldon antibodies.

Keywords Elderly, HA inhibition, influenza virus, native and denatured antigen, serum antibodies.

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Introduction

Influenza vaccine is recommended annually for prevention of influenza in people at high risk of complications and their contacts. Subunit influenza vaccines in the USA contain 15 μg of HA of each of three viruses per dose, but in elderly people this dose often fails to elicit significant antibody rise as measured by hemagglutination inhibition (HAI) titer.¹ Protection against influenza is thought to be correlated with serum antibody level and so an increase in HAI titer is considered a measure of response to vaccine. However, clinical data show a greater benefit of inactivated influenza vaccine to elderly individuals than is apparent from the low increase in HAI titer.^{2,3} The standard HAI assay has a number of uncertainties, including underestimation of the true amounts of antibody.⁴ The HAI test has been reported to be relatively insensitive for H5N1 avian influenza viruses^{5–7} and to date there is not a good correla-

tion between HAI, virus neutralization, and protection.⁸ de Jong *et al.*⁹ concluded that the HAI test is not 'a functional test for measuring immunity and suffers from a number of drawbacks.' The HAI test uses sialic acids on red blood cells as mimics of cellular receptors, and the problem seems to be that red cell sialic acids are not good receptor mimics for some viral strains. The HAI test for anti-H5 antibodies is more sensitive when horse red cells are used instead of turkey or chicken cells, but the chemical differences in the sialic acids expressed are largely unknown.¹⁰ Recent human H3N2 viruses have shown changes in binding specificity to red blood cells.¹¹ Both the H5 and H3 examples show that, although the basic sialic acid linkage specificity of NeuAc α 2-3Gal in avian viruses and NeuAc α 2-6Gal in human viruses is usually maintained, finer specificity differences in receptors are not mimicked by red cells and confound attempts to accurately measure neutralizing antibodies by HAI.

To investigate the utility of a more direct method of assessing vaccine response, we developed a sandwich ELISA method to separately measure antibodies against native glycoproteins, which are potentially protective, and antibodies against denatured glycoproteins or internal viral proteins ('unfoldons'),¹² which are not protective.^{13–18} Direct coating of virus onto microtiter plate wells results in virus disruption and denaturing some proportion of surface glycoproteins. Thus, direct virus coating will capture all anti-influenza antibodies against native, denatured, and internal proteins. We think that specific measurement of antibodies against native glycoproteins (the 'good' antibodies) might be a better predictor of vaccine efficacy and/or protection than HAI titers, but as yet there are no data. Comparison of HA inhibiting antibodies with total binding antibodies may be helpful in resolving the lack of correlation between HAI and immune response. Measurements of bulk avidity of antibodies in elderly subjects might also be useful in finding a better correlate of vaccine efficacy.

It has often been noted that antibody response to vaccine in the elderly is considerably lower than in younger adults.¹⁹ Keitel *et al.*²⁰ showed that a higher dosage of vaccine given to elderly subjects resulted in significantly higher serum HAI titers and a higher frequency of HAI increase. For our study we obtained pre- and post-vaccination serum from 36 subjects from this trial to segregate and quantitate the antibody response. We determined the relative amounts of antibodies against native versus denatured viral particles, the level of cross-reactivity with earlier strains and the overall avidity of anti-influenza antibodies in the serum of each subject.

Materials and methods

Subjects and sera

The serum samples were obtained from a clinical trial at Baylor College of Medicine that was designed to evaluate the effects of increasing dosage levels of inactivated influenza vaccine among elderly subjects. Ambulatory individuals 65 years and older received a single intramuscular injection of the 2001–2002 formulation of trivalent inactivated subviral influenza vaccine containing 15, 30, or 60 µg of hemagglutinin per strain or placebo.²⁰ The vaccine components were A/Panama/99 (H3N2), A/NewCaledonia/99 (H1N1), and B/Victoria/2000. Our studies focused only on antibodies against H3N2 viruses in sera of 36 subjects collected before and 1 month after vaccination. These sera were from 10 subjects in each vaccine dosage group, with varied HAI responses, and six subjects from the placebo/control group. The samples were blinded and coded before they left Baylor.

Viruses and cells

Influenza H3N2 viruses included in this study were vaccine strains A/Sydney/5/97, A/Panama/2007/99, and A/Wyo-

ming/03/03 obtained from CDC, and A/Udorn/307/72 obtained from Dr. Ming Luo. The viruses were grown in embryonated chicken eggs and purified by sucrose gradient centrifugation.²¹ Hemagglutinin titrations were made at 4°C, using human red blood cells. Viral protein was determined by the Bradford method (Bio-Rad Protein Assay kit, Bio-Rad, Hercules, CA, USA).

Preparation of red cell membrane extract

Packed human red cells were washed five times in phosphate-buffered saline (PBS) and lysed in cold water for 1 hour at 4°C. The resulting red cell ghosts were pelleted at 26 700 g for 20 minutes. Ghosts were washed three times or until washes were clear, in water with one drop of PBS added each time. The pellet was resuspended in water, aliquoted and stored at –20°C. For coating ELISA plates, aliquots were allowed to thaw and OG (n-octyl-β-D-glucopyranoside) was added to a concentration of 34 mM. After standing for 10 minutes at room temperature, the solubilized membranes were diluted 1:10 in PBS for coating the plates (~200 µg protein per well).

ELISA to quantitate antibodies against native glycoproteins

Wells of a microtiter plate were coated with OG-solubilized red cell membranes and incubated overnight at 4°C. Wells were washed four times with PBS, then virus (~64 hemagglutinating units (HAU) per 50 µl in CaMg-saline) was adsorbed to the coated wells overnight at 4°C or 1 hour at RT. After washing four times with PBS, non-specific binding sites were blocked with 20% calf serum in PBS. The human serum samples were diluted 1:16 or 1:32 in 20% calf serum in PBS, and serial twofold dilutions of this were added to the wells and incubated at room temperature for 1 h. After washing in PBS, alkaline-phosphatase-conjugated goat anti-human polyvalent immunoglobulin (α, γ and μ-chain specific) secondary antibody (Sigma no. 3313, Sigma-Aldrich, St. Louis, MO, USA) was bound for an hour, the wells washed free of unbound conjugate and p-nitrophenyl phosphate substrate (Sigma 104) added. The color was developed at room temperature for 1 hour and the absorbance was read at 405 nm. We titrated all the reagents to maximize the signal to noise ratio of the assay (specific versus non-specific binding) but we used high concentrations of both red cell membranes and virus to ensure maximum capture.

ELISA to measure antibodies against denatured proteins (unfoldons)

On a duplicate plate, virus was adsorbed to the red cell membrane-coated wells as above so that the starting amount of virus was the same as for the native protein ELISA. After

washing, the wells were treated with methanol for 45 minutes at 37°C and dried before adding serum samples to quantify antibodies against unfoldons. No native protein structures remain after the methanol treatment. The assay was validated for native and denatured states using a monoclonal antibody against native neuraminidase (NA) and an anti-peptide antibody against denatured NA as described earlier.¹³

Pre-vaccination serum antibodies

The relative levels of pre-immunization antibodies against Panama/99 and Udorn/72 were measured by adding a single dilution (1:32) of each of the 36 pre-vaccine sera, in duplicate, to wells containing red cell membrane-bound virus. Anti-human secondary antibody was used as described above to quantify the pre-existing serum antibodies.

Data analysis

Binding curves (absorbance at 405 nm versus μl serum in 50 μl binding mix) were subjected to curve fitting using Kaleidagraph software as described earlier.¹³ The equation used for curve fitting is:

$$A_{405} = P_1 + \frac{(A_{\max}P_0)}{[1/K_{a(\text{app})}] + P_0}$$

where A_{405} is the absorbance reading, A_{\max} is the relative concentration of antibody, P_0 is the total antibody paratope concentration and $K_{a(\text{app})}$ is the apparent association constant.

In Kaleidagraph this equation was entered as:

$$y = m_1 + \frac{(m_2m_0)}{(1/m_3) + m_0}$$

where $y = A_{405}$, $m_0 = P_0 = \mu\text{l}$ serum in 50 μl binding mix, $m_1 = \text{no serum blank}$, $m_2 = A_{\max}$ and $m_3 = 1/K_{d(\text{app})} = K_{a(\text{app})}$. A_{\max} is a relative measure of total antibody concentration and $K_{a(\text{app})}$ gives the overall avidity.

The 72 serum samples (36 pre- and post-immunization) were titrated with four viruses (A/Panama/2007/99, A/Sydney/5/97, A/Wyoming/03/03, and A/Udorn/307/72) and A_{\max} and $K_{a(\text{app})}$ were obtained for each serum that showed significant reactivity under native and denatured conditions for each virus. The fold differences between the pre- and post-vaccination sample were calculated for each subject. We also compared single point A_{405} values between all subjects for a direct comparison of pre-immunization antibody levels, native and denatured. The samples from the 36 subjects were processed together and repeated at least once, on a different day, and the results averaged.

Results

Pre-immunization antibody levels

Individuals had been selected into the vaccine trial regardless of their immunization or influenza infection

history, but 82% of participants reported receiving vaccine in the previous year.²⁰ As might be expected for elderly people who have lived through many influenza epidemics and who have been vaccinated in recent years, we found high pre-immunization antibody levels ($A_{405} \geq 0.4$) against native A/Panama/99 in 80% of subjects (Table 1). No subject had this level of antibodies against Udorn/72. The average anti-Udorn/72 antibody level was $43 \pm 25\%$ of the average antibody against Panama/99.

Pre-immunization antibody levels against denatured Panama/99 were higher (average $78 \pm 23\%$ of native) compared with Udorn/72 ($43\% \pm 34\%$ of Panama/99), suggesting either an increased response toward unfoldons as the subjects aged, or that infection yields higher antibody levels against native proteins than the subunit vaccine. Healthy people were unlikely to have been vaccinated in the 1970s and the anti-Udorn antibodies seen here likely resulted from infection.

Increase in antibody levels and overall avidity against native glycoproteins after vaccination

Response

Response toward native glycoproteins was classified as 'good,' 'intermediate' or 'none' according to the increase in A_{\max} as shown in Figure 1. A 'good response' was defined as more than twofold increase in A_{\max} together with $A_{405} \geq 0.4$ after vaccination, mostly observed when pre-immunization serum antibody levels were low (Figure 1A). 'No response' was defined as low pre- and low post-vaccination levels (Figure 1C), while 'intermediate response' was defined by high pre- and high post-vaccination A_{\max} , indicating a capacity to respond (Figure 1B). Most of these elderly subjects showed intermediate responses and only 13% of all subjects showed a good response. However, the good response rate was 57% among those subjects with low pre-immunization antibody levels ($A_{405} < 0.4$) (Table 1).

Rise in antibody levels and avidities

A clear inverse relationship between pre-immunization antibody levels and fold increase in A_{\max} for the vaccinating virus was observed (Figure 2A), as was found in our earlier study.¹³ No clear correlation was observed between pre-immunization levels and increase in overall avidity but the increases in avidity were higher than those in A_{\max} (Figure 2B). Results were very similar for anti-unfoldon antibody levels (Figure 2C,D). Comparing these results to the younger population studied earlier,¹³ the average fold increase in anti-native antibody against the vaccine strain (subunit vaccine) is 1.5-fold in the elderly averaging over the 15, 30, and 60 μg dosages compared with 2.4-fold in

Table 1. Serum response (as fold difference) in terms of hemagglutination inhibition (HAI) and to native and denatured red cell-bound virus after vaccination, compared with the corresponding pre-vaccination sample

Subject	Dose (μg)	Fold HAI (\log_2)* Panama/99	Native			Denatured		
			Pre-vac A_{405}	Fold A_{max} Panama/99	Fold K_a Panama/99	Pre-vac A_{405}	Fold A_{max} Panama/99	Fold K_a Panama/99
D	Placebo	1	0.8	1.0	0.9	0.6	1.1	0.8
H	Placebo	1	0.4	0.9	1.1	0.4	1.0	0.8
L	Placebo	1	0.7	1.1	1.4	0.5	1.0	0.8
P	Placebo	1.8	0.4	1.0	0.9	0.3	0.8	1.1
T	Placebo	1	0.7	1.1	1.2	0.5	1.1	1.1
W	Placebo	1	0.4	1.0	0.8	0.4	1.1	1.2
A	15	1	0.8	1.0	1.2	0.8	1.2	1.0
E	15	1.3	0.9	1.2	1.9	0.7	1.2	2.1
I	15	1.3	0.7	1.1	1.1	0.7	1.1	1.1
M	15	1.5	0.4	1.5	0.6	0.3	2.0	0.5
Q	15	1	0.7	1.1	0.7	0.4	1.0	0.9
U	15	1.5	0.5	1.1	1.2	0.5	0.8	2.5
X	15	1	0.4	1.1	1.4	0.3	1.2	1.4
AA	15	2	0.9	1.2	2.2	0.9	1.2	2.3
DD	15	3.5	0.2	3.6	1.4	0.1	2.2	5.0
GG	15	1.3	0.4	1.4	2.6	0.3	1.8	2.5
B	30	1	0.4	1.1	1.0	0.3	1.4	0.9
F	30	1	0.3	2.3	0.4	0.1	1.7	0.9
J	30	1	0.4	1.2	1.0	0.2	1.8	0.7
N	30	1.5	0.6	1.2	1.5	0.6	0.8	1.9
R	30	1	0.7	1.0	2.1	0.6	0.9	3.6
Y	30	1	1.0	0.8	2.2	0.7	1.1	1.2
BB	30	1.4	0.6	1.1	1.3	0.5	1.1	1.5
EE	30	2.3	0.3	1.6	5.2	0.1	1.6	5.0
HH	30	1.6	0.2	1.6	3.4	0.1	2.7	3.2
II	30	2.3	0.3	1.5	1.1	0.2	1.4	2.2
C	60	0.9	0.8	1.0	1.1	0.5	1.4	0.8
G	60	1	0.5	1.3	2.0	0.3	1.0	2.7
K	60	1	0.4	1.1	1.4	0.4	1.0	1.5
O	60	1.3	0.6	1.0	2.4	0.6	0.9	2.8
S	60	2	0.5	1.5	3.2	0.5	1.2	3.9
V	60	1.3	0.6	1.3	2.9	0.4	1.0	4.0
Z	60	1.4	0.7	0.9	1.1	0.5	1.0	1.6
CC	60	1	0.9	0.9	0.9	0.8	1.0	1.3
FF	60	3	0.2	5.1	0.5	0.1	5.0	2.3
JJ	60	3	0.2	4.3	1.4	0.0	5.0	2.4

*The HAI responses in this vaccine trial²⁰ and HAI for a younger population²³ were reported earlier.

the younger group after the first vaccination with 15 μg (whole virus vaccine). An increase in K_a was sometimes observed in the absence of increased antibody level (Figure 2A,B). Subjects whose pre-immunization antibody levels were ≥ 0.4 absorbance units averaged 1.1-fold increase in A_{max} but a significantly higher 1.6-fold increase in K_a ($P = 0.003$).

Antibody levels and apparent avidity against unfolds

Elderly subjects exhibited higher pre-immunization serum antibody levels against denatured determinants than were observed in younger subjects.¹³ The amount of anti-

unfolds antibody in elderly subjects given subunit vaccine ($78 \pm 23\%$ of anti-native antibody for Panama/99) was almost four times that of the younger population given inactivated whole virus ($19 \pm 10\%$) but only slightly increased over younger subjects given subunit vaccine in a separate study (64%; J-Q. Feng and G. M. Air, unpublished results). Antibodies against unfolds increased an average 1.9-fold for Panama/99 in the elderly group after vaccination (Figure 2C). As with antibodies against native proteins, avidity increase was higher (1.9-fold) than increase in A_{max} (1.1-fold, $P = 0.004$) for those subjects with pre-immunization levels of antibody

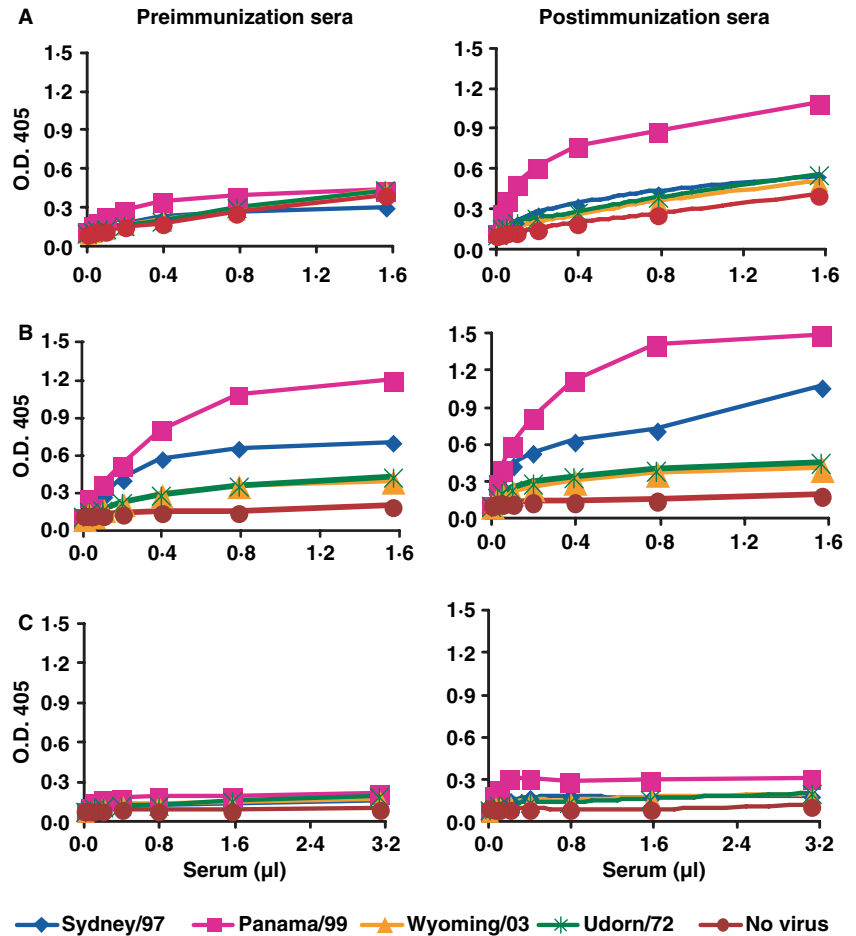


Figure 1. Examples of binding curves showing variable response toward native glycoproteins. The pre-vaccination and post-vaccination serum samples from three subjects are shown. In panel (A) the subject has very low pre-immunization antibody levels and shows a good rise after vaccination. Panel (B) shows a subject with high levels of pre-immunization antibodies and little increase on vaccination. The subject in panel (C) is a true non-responder, with a very low level of pre-immunization antibody and no increase after vaccination.

≥0.4 absorbance units (Figure 2D). Younger subjects did not show any significant increase in antibodies against denatured virus, even after repeated vaccination with inactivated whole virus vaccine¹³ or when given subunit vaccine.

Cross-reactivity

Various cross-reactivity patterns with earlier and later viruses were observed in pre-immunization sera. In most cases the reactivity with Sydney/97 mirrored that with Panama/99. There was no significant reactivity of pre-immunization sera with the later strain Wyoming/03. Reactivity with native Udorn/72 averaged 43% of the antibody level against native Panama/99. An increase in antibodies against Panama/99, the vaccine strain, was often accompanied by an increase against Sydney/97, but there was almost no increase in antibodies against Wyoming/03, a later strain. Ten percent of subjects showed a twofold increase in antibodies against Udorn/72 after vaccination with Panama/99, but in these the antibody level remained low, resulting in large errors in the fold increase, and there was a much greater increase in antibodies against Panama/99.

We interpret this as no significant increase in antibodies against Udorn/72.

Dose response

Increases in average A_{max} and $K_{a(app)}$ of anti-native antibodies were observed with increasing dosage, but these were not significant ($P = 0.46$ and 0.48 , respectively) because so few subjects showed any increase. Both A_{max} and avidity increased with increasing dosage more against unfoldons than against native proteins (Figure 3) although again not statistically significant ($P = 0.48$ and 0.16 , respectively, comparing $15 \mu\text{g}$ with $60 \mu\text{g}$ dosage). There was an increase in avidity of antibodies against unfoldons ($P = 0.10$) at the highest dosage ($60 \mu\text{g}$) when compared with the regular $15 \mu\text{g}$ dosage level; for A_{max} the difference was not significant ($P = 0.25$).

Discussion

Rationale and standardization

The sandwich ELISA method was developed using fetuin as a capturing agent,¹³ but recent H3N2 influenza viruses

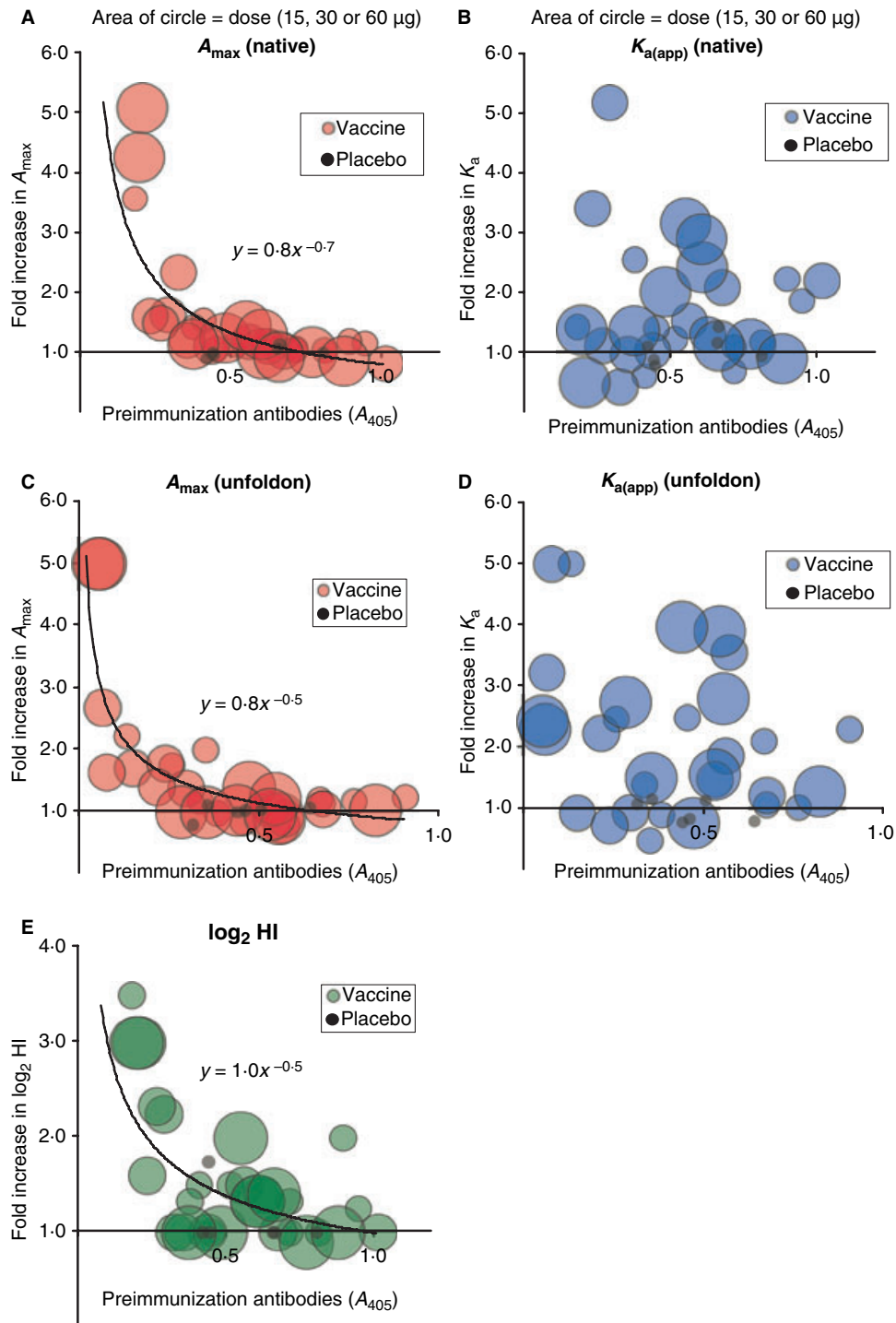


Figure 2. Relationships between pre-immunization antibody levels, fold increase in antibodies, and vaccine dose. Panel (A) shows the fold increase in A_{max} (post-/pre-immunization) determined by fitting the binding curve, plotted against initial antibody level (A_{405}) obtained by running all samples together for comparison, where the diameter of the circles reflects the dose of vaccine. Panel (B) shows fold increase in $K_{a(\text{app})}$ plotted against pre-immunization levels. In panels (C) and (D), the fold increase A_{max} and K_a of antibodies against denatured proteins are plotted against the pre-immunization levels of anti-unfoldon antibodies. Panel (E) shows the fold increase in hemagglutination inhibition determined previously²⁰ plotted against the pre-immunization A_{405} determined in this study.

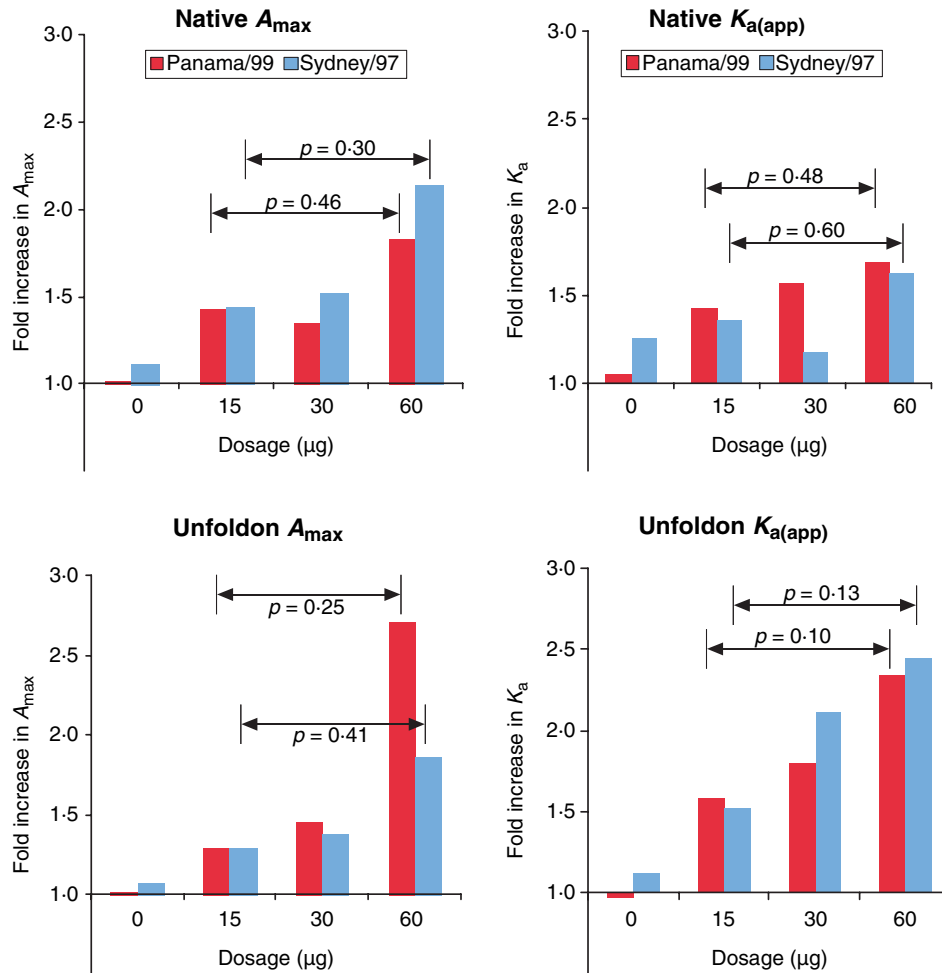


Figure 3. Effect of increasing dosage of vaccine on A_{max} and $K_{a(app)}$ of antibodies against Panama/99 and Sydney/97. The average fold increase in anti-native and anti-unfoldon serum antibody levels and avidity after vaccination with increasing dosages of vaccine is compared. The P -values from the Student's t -test comparing the 15 μg dosage with the highest dosage (60 μg) are shown for each virus. There are increasing trends with dosage for each parameter, but only the avidity of unfoldon antibodies significantly increases with dosage. Each vaccine dosage group has 10 subjects, and the control group has six subjects.

have changed receptor specificity and were not retained on fetuin. Recent viruses bind tightly to human red blood cells and we first designed a triple-layer ELISA in which lectin from *Sambucus nigra* is bound to the wells and used to capture glycoconjugates containing α 2-6 sialic acid linkages from a detergent extract of human red blood cell ghosts. Virus particles then bind to the exposed α 2-6 sialic acids. Unexpectedly, we found strong nonspecific binding between virus and lectin, which was not reduced after neuraminidase treatment of virus, so instead we coated the plates directly with the red cell membrane extract and this efficiently captured virus in the native state. Then each serum sample was titrated to obtain a binding curve to distinguish the effects of increased avidity from the effects of increased antibody levels. We wanted to compare the cross-reactivity of

serum antibodies against newer and older H3N2 viruses; however, they have differing receptor specificities resulting in different affinities for red cell membranes.¹¹ This might lead to different amounts of virus being captured. We coated with ~ 64 HAU per well to ensure that all viruses were maximally bound.

Pre-vaccinated serum antibody level determines antibody rise against native antigen after vaccination

Our earlier study showed a clear inverse relationship between pre-immunization antibody levels and increase after vaccination.¹³ This study of elderly subjects also shows this inverse correlation, but with 80% subjects exhibiting high pre-immunization levels against native proteins, far more than in the younger population studied earlier.¹³

The persistence of vaccine- or infection-induced antibody is consistent with other reports.^{2,22,23}

Thus, the well-known lack of antibody rise after vaccination of elderly subjects, with no statistically significant differences between elderly vaccinees and controls (placebo),^{19,24} is largely due to the high pre-immunization antibody levels in elderly subjects. In our study, only 13% subjects showed more than twofold increase in A_{\max} , and all of these had low pre-existing antibody levels (Figure 2A).

Conformation dependence: native versus denatured antigens

As neutralizing antibodies against influenza are conformation-dependent,¹⁵ the ratio of antibody reactivity to native versus denatured viral antigens should reflect the protective potential of antibodies. The elderly population studied here exhibited approximately four times higher antibodies against denatured antigens than the younger group¹³ and showed more increase in avidity than in amount after vaccination. Younger subjects given subunit vaccine had approximately three times higher antibodies against unfoldons than a similar group given whole virus vaccine, indicating that subunit vaccine induces more antibodies against unfoldons than whole virus vaccine. A high dosage of vaccine increased the avidity of antibodies against unfoldons more than those against native proteins in the elderly (Figure 3). The higher fold increase in antibodies against unfoldons in elderly subjects compared with the younger group given a similar subunit vaccine suggests that the elderly have an enhanced response to denatured protein. This might reflect a memory response to conserved linear epitopes, as conformational epitopes on HA and NA are variable and so would be expected to show less memory response. However, the subjects showed little cross-reactivity to denatured epitopes on Udorn/72, despite the sequence conservation. Therefore, it seems that there might be more processing of antigen in elderly people, leading to a biased response toward unfoldons that is specific to the vaccine and not due to recall.

Comparison with HAI

The HAI responses in this vaccine trial were reported earlier and the conclusion was that higher pre-immunization antibody levels in elderly subjects lead to a higher seroprotection rate and lower seroconversion rate. Overall 80–90% of subjects achieved protective antibody levels against the H3N2 virus.²⁰ A review by Goodwin *et al.*¹⁹ showed that for the H3 antigen, the young had a 62% seroconversion rate and 84% seroprotection rate (the percentage of subjects with HAI antibody titer ≥ 40 after vaccination), while institutionalized elderly had a 65% seroconversion rate and

80% seroprotection rate. In our subset of 36 subjects, 53% were seroprotected before vaccination (titers ≥ 32 HAU) and 44% subjects seroconverted after vaccination as indicated by HAI.

As in our previous study,¹³ we did not see a direct correlation between fold increase in HAI titers and fold increase in serum antibody level or avidity. Among the elderly subjects, 13% had ≥ 2 fold increase in A_{\max} while 33% showed ≥ 2 fold increase in avidity. The increase in HAI (23% of our subset of subjects had ≥ 2 fold increase in \log_2 HAI titer, Figure 2E) therefore appears to be derived from a combination of increase in antibody amount and increase in avidity. Subjects with the highest increase in HAI titers had the lowest pre-immunization antibody levels, in accord with the increase in antibody amount (Figure 2).

Cross-reactivity and original antigenic sin

'Original antigenic sin' is the concept that the level of antibody which is specific to the first influenza strain experienced continues to rise throughout life through boosts from each subsequent infection or vaccination with viruses of the same subtype.²⁵ We reasoned that by 1972 most people would have been exposed to an H3N2 virus and therefore we used Udorn/72 to represent the early H3 strains rather than using the 1968 strain itself. There is a high degree of cross-reactivity between Aichi/68 and Udorn/72, while there was much less between these and the next drift strain, Victoria/3/75 (data from CDC). In our studies we saw increases in antibodies against Panama/99 and the closely-related Sydney/97 but only marginal increase, and in only 10% of subjects, against Udorn/72. We also observed higher pre-immunization antibody levels against Panama/99 and Sydney/97, compared with Udorn/72, in these elderly subjects, indicating recent vaccination history or influenza exposure. Thus, our data suggest that the serum antibodies are specific, cross-reactive only to closely related strains, with no sign of original antigenic sin. Brokstad *et al.*²⁶ observed a stronger immune response (in terms of HAI) to previous H3N2 strains in young healthy subjects than against vaccine virus, which they thought was a recall of H3N2 priming to common antigenic determinants.²⁷ These antibodies were non-neutralizing. We did not observe this phenomenon in our study, again emphasizing the difference in assay system. Haaheim has discussed evidence that antibodies generated under the mechanism of original antigenic sin may not have any major epidemiological relevance²⁸ and perhaps the HAI test over-emphasizes these.

Dose response

There have been several studies to determine whether increasing the dosage can increase the effectiveness of

vaccination in the elderly.^{1,20,29} Palache *et al.*³⁰ reported that higher vaccine dosages (20 and 60 µg) resulted in significant antibody response against influenza B but not against A/Taiwan/1/86 (H1N1) or A/Sichuan/2/87 (H3N2). Goodwin *et al.*¹⁹ concluded from other studies that there is no indication that increasing the dosage of antigen increases the response to the vaccine in the elderly, but the number of subjects was low. Increased dosage was reported to be more effective in case of H5 vaccine³¹ and for elderly subjects given baculovirus-expressed trivalent HA vaccine.³²

The samples we are using were from the study by Keitel *et al.*,²⁰ who found that increasing dosages of vaccine (0, 15, 30, 60 µg) in elderly subjects increased the geometric mean serum HAI titers against H3N2 virus to 43, 86, 91, and 125, respectively. Frequencies of antibody responses nearly doubled after the 60 µg dosage and the percentage of participants showing a fourfold increase in HAI increased with dosage, although only 10% of subjects had low initial HAI titers and showed a significant increase.

Our measurement of antibodies showed an increase in level and/or avidity of antibodies against native Panama/99 as the dosage of vaccine increased, but this was accompanied by a higher proportion of antibodies with higher avidities against unfoldons (Figure 3).

Conclusions

Subjects with low pre-immunization levels showed a significant increase in serum antibody levels after vaccination, while the 80% of subjects with high pre-immunization antibody levels showed little increase in amount but significantly greater increase in avidity (Figure 2). There was a significant trend toward an increased proportion of antibodies to denatured antigens with increasing dosage of vaccine (Figure 3), especially in avidity, even though there were only 10 subjects given each dose. In the context of a vaccine, antibodies against unfoldons represent wasted resources and vaccines that need to be given at high dosages may need to be adjuvanted in a way that minimizes denaturation or degradation. An additional purification step could also be added during vaccine manufacture to remove denatured antigen.

The sandwich ELISA method segregates the response in terms of antibodies against native glycoproteins versus unfoldons. The inverse correlation between pre-immunization serum antibody levels and fold increase in A_{\max} is maintained in the elderly, the relationship approximating $y = x^{-0.5}$ where y is fold increase in A_{\max} and x is pre-immunization antibody level.

The subjects in this study were not followed to assess protection, but we are participating in an ongoing study to determine if our direct measurement of antibodies against

native glycoproteins versus unfoldons is a better correlate of protection than HAI or neutralizing antibody titers.

Author contribution

All authors contributed to the design or data analysis of the study, the writing of the article, and approval of the final version.

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Conflict of interest

WAK has received funding for research from Protein Sciences, Inc.

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