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# A High Dosage Influenza Vaccine Induced Significantly More Neuraminidase Antibody than Standard Vaccine among Elderly Subjects

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## Abstract

Antibody to the neuraminidase (NA) antigen of influenza viruses has been shown to correlate with immunity to influenza in humans and animal models. In a previous report, we showed that an inactivated influenza vaccine containing 60 μg of the hemagglutinin of each strain induced significantly more serum anti-HA antibody among elderly persons than did the standard vaccine containing 15 ug of the HA of each component. We developed a lectin-based assay for anti-NA antibody and used it to measure anti-NA antibody responses among subjects who had participated

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in that study. The high dosage vaccine contained eight times as much NA activity as the standard vaccine and induced a significantly higher frequency of antibody responses and higher mean postvaccination anti-NA titers to the N1 and N2 of the A/H1N1 and A/H3N2 viruses in the vaccines than did the standard vaccine. Ensuring an increased antibody response to the NA antigen in inactivated influenza virus vaccines should increase the protection against influenza. An increased quantity of the NA antigen in the vaccine will ensure an increased response.

### Keywords

Influenza vaccine; neuraminidase; antibody

## 1. Introduction

Antibody to the hemagglutinin (HA) of influenza viruses in serum and respiratory secretions was shown to be a correlate of immunity to influenza soon after the virus was first isolated and has been repeatedly confirmed over the decades since (1,2). This demonstration forms the basis for a focus on inducing anti-HA antibody as the goal of vaccination for prevention of influenza (3). The HA is the dominant surface glycoprotein on virus particles and attaches to the cell surface receptor, N-acetyl neuraminic acid (NANA), as the initial step in cell infection (4). A second viral surface glycoprotein, the neuraminidase (NA), promotes release and spread of virus particles newly budded from the surface of infected cells by destroying cell surface NANA and disrupting aggregates of particles formed after budding has occurred (4,5). Still other functions of NA, such as promoting virus penetration through respiratory secretions, are likely. Antibody to the NA in humans has also been shown to be a correlate of immunity to influenza (6,7). Although the primary role of anti-NA antibody is to reduce the intensity of an influenza virus infection and associated illness, inhibition of the NA can also prevent infection as has been shown for both antibody and the NA inhibitor antivirals (7,8). Thus, it is appropriate to ensure that influenza vaccines induce anti-NA antibody as well as anti-HA antibody so as to enhance their ability to induce protection against influenza virus infections and illnesses.

We reported previously that a trivalent influenza vaccine manufactured by the Sanofi Pasteur Corp. containing 60 μg of the HA of each component induced significantly more serum anti-HA antibody than did their standard vaccine containing 15 μg of the HA of each component (9). In the present manuscript, we report that the higher dosage vaccine also contained more NA antigen (as increased NA activity) and induced significantly more anti-NA antibody than did standard vaccine.

## 2. Materials and Methods

### 2.1 Previous Study

The study design, participating subjects, vaccine procedures and anti-HA serologic tests were described in the earlier report (9). Briefly, the study was multi-site and involved vaccination of 414 medically stable ambulatory subjects ≥65 years old with a standard or high dosage influenza vaccine. The study was conducted in the spring with separate randomizations for those given vaccine for the previous influenza season (2-5 months earlier) and those not vaccinated the previous season. Vaccines were the licensed sanofi pasteur (sp) 2004-2005 trivalent vaccine containing 15 μg of the HA of A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2003 (H3N2), (A/Wyoming is an A/Fujian/411/2002-like virus) and B/Jiangsu/10/2003 and a high dosage vaccine prepared in the same manner except that it contained 60 ug of the HA of each of the same strains and lacked gelatin and thimerosal. Blood specimens were obtained before and 28 days after vaccination. Tests for serum anti-HA antibody were hemagglutination-inhibition (HAI) and neutralization tests as described (9).

### 2.2. NA Activity and Anti-NA Procedures

The procedure used for measuring anti-NA antibody is a modification of a lectin-based procedure described by Lambre, et al. (10). This method uses peroxidase-labeled peanut agglutinin (the lectin) for detection of sites where NA has acted on fetuin in coated 96 well plates. Assay antigens were N1 and N2 prepared from A/New Caledonia (H1N1) and A/Wyoming (H3N2) viruses. For NA antigen preparations, egg allantoic fluid containing virus was treated with Triton X-100 to disrupt virus particles; triton was then removed from the solution using SM-2 BioBeads. After centrifugation, the NA containing supernate was centrifuged in Amicon Ultra 15 tubes to remove residual triton and concentrate the NA. The retentate was removed from the filter and constituted the NA used in serological assays. Calcium chloride was added to aid enzyme stability.

For assays, microtiter plates were coated with fetuin and stored at 4°C until used. For NA activity assays, the virus or vaccine was serially diluted, added in duplicate to fetuin-coated plates and subsequently processed as described for antibody assays. For antibody assays, two-fold serum dilutions in duplicate starting at a dilution of 1:16 were incubated for four hours at 37°C with the N1 or N2 antigen preparations, transferred to fetuin-coated plates and incubated overnight at 37°C. After washing, peroxidase-labeled peanut agglutinin was added and incubated for two hours at room temperature. After washing again, the peroxidase substrate TMB (trimethylbenzidine) was added and the reaction stopped with 0.3 M phosphoric acid. An O.D. for each well was obtained with a microplate reader. The serum titer causing a 50% reduction compared to controls was calculated using O.D. for sequential dilutions and the GraphPad Instat program. The assay was shown to yield serum titers of <1:4 in sera from some young children and differences between sera were shown to be significant if they were ≥2-fold.

### 2.3 Statistics

Frequency of antibody responses was compared in 2 × 2 contingency tests; mean titers were compared in both paired t and Wilcoxon tests.

## 3.0 Results

### 3.1 Vaccine NA

The relative concentrations of neuraminidase in each vaccine were estimated by measuring enzyme activity in each vaccine. Shown in Table 1 are results for four different comparisons. The mean ratio of NA activity for the standard to high dosage vaccine was 1:7.678. Dilutions of each vaccine were made using the mean titer of enzyme activity for each calculated to yield an O.D. of 2.0. These dilutions were then tested against sheep antiserum prepared against purified NA from each of the viruses in the trivalent vaccines [obtained from National Institute for Biological Standards and Control (NIBSC), United Kingdom] along with the NA preparation of each virus. A representative result of three different tests is shown in Table 2. As shown, the NIBSC sera were specific for each NA and each vaccine contained enzyme activity of each virus, (N1, N2 and B). Moreover, these tests confirmed the relative enzyme ratio of the two vaccines as about 1:7.7.

### 3.2 Immunogenicity

The majority of subjects possessed measureable antibody in the prevaccination blood specimen; only 5.6% of subjects had a titer <1:16 against the N1 antigen and only 2.7% against the N2 antigen. The frequencies of both the serum anti-HA and anti-NA antibody responses among the vaccinated subjects are shown in Table 3. The frequencies of subjects with an increase in anti-HA and NA was significant for all vaccine dosage groups ($p = 0.04$ for H1N1 NA in no previous vaccine group given standard vaccine, $p < .01$ for all other groups). For each

vaccine and vaccination group, the frequency of subjects developing an increase in anti-HA antibody is greater than the frequency developing an anti-NA antibody increase. For both anti-HA and anti-NA, the frequencies were significantly higher for the high dosage vaccine than for the standard dosage vaccine ($p = 0.02$ for H3N2 NA in no previous vaccine group, $p < 0.01$ for all other groups). The increased frequency for the high dosage vaccine was greater in each vaccine group for an anti-NA response than for an anti-HA response (1.5-2.3 fold for anti-HA and 1.9-4.9 fold for anti-NA). As shown in Table 4, the proportion of subjects with an increase in titer was greater among those with lower prevaccination titers in both serum HAI and neutralization tests for HA antibody and the lectin-based test for NA antibody for both vaccines and both NA antigens (for anti-NA in the two vaccine groups, $p = 0.04$ for N2 in the standard vaccine comparison, $p < 0.01$ for all other comparisons; for HAI and neut antibodies in the standard vaccine groups, $p = 0.06$ for N2 in the HAI comparison but $p > 0.10$ for all other comparisons; for HAI and neut in the high dosage groups, $p = 0.07$ for N2 in the neut comparison, $p \leq 0.01$ for all other comparisons).

Geometric mean antibody titers for the N1 and N2 antigens before and after vaccination are shown in Table 5. The prevaccination mean for the N1 antigen was greater for those given standard vaccine previously than among those not vaccinated previously (131 vs. 92, $p = 0.05$); all other prevaccination mean titers were similar. The mean post vaccination titers were significantly greater than prevaccination means for all vaccine groups for both antigens except for the N1 antigen in the previously vaccinated group given standard dosage vaccine ($p > 0.05$ for N1 previously vaccinated standard dosage; $p = 0.03$ for N1 in the standard vaccine, no prior vaccine and total groups; $p \leq 0.01$ for all other groups). The mean titer post vaccination for the high dosage vaccine group was significantly greater than the standard dosage group for all six comparisons, including for the total, previous, no previous vaccine groups, and for both the N1 and N2 antigens ($p = 0.04$ for N1 and N2 in the previous vaccine group; $p < 0.01$ for all other comparisons).

## 4. Discussion

For the present study, an assay procedure for measuring antibody to the neuraminidase antigen of an A/H1N1 and A/H3N2 virus was developed, standardized, and used for measuring serum anti-NA antibody responses to a standard dosage and high dosage trivalent influenza vaccine among elderly subjects. The N1 and N2 antigen preparations were obtained from triton split virus and used in a micro lectin-based neuraminidase-inhibition assay. This assay method was chosen to avoid the requirement for enhanced biological safety when infectious influenza viruses containing an avian HA are used; the traditional assay for measuring antibody to the neuraminidase uses reassortant infectious virus containing an avian influenza virus HA (11). An additional reason for resorting to the NA preparation was that a set of comparison assays indicated that anti-HA antibody interfered with measurements of anti-NA antibody when infectious A/H1N1 of A/H3N2 virus were used in the enzyme inhibition assay (data not shown).

The vaccine evaluation reported here was conducted in persons ≥65 years old, most of whom have received influenza vaccine annually. Not surprisingly, only a few subjects lacked antibody to the NA antigens and was the reason for using a starting serum test dilution of 1:16. Both the standard and high dosage vaccines used in this study were shown to contain N1 and N2 enzyme activity and both were increased in quantity to about the same degree in the vaccine with increased HA concentration. However, while the single radial immunodiffusion assay for HA had indicated a 4-fold increase in antigen concentration in the high dosage vaccine, the enzyme activity comparisons indicated an increase of almost 8-fold in NA activity. The increase in anti-NA antibody frequencies and mean titers detected after vaccination among those persons given the standard influenza vaccine was low but significant, whereas increases in antibody were common and significantly greater among those given the high dosage vaccine. It seems

reasonable to suggest that the almost 8-fold increase in enzyme activity corresponded to a similar increase in the NA antigen. The reason for the 8-fold increase in our enzyme activity assay and a 4-fold increase in the sp HA assay is not known. While the optimal anti-NA antibody responses and amount of NA antigen necessary for inducing those responses are uncertain, it seems clear that the NA dosage needs to be greater than that contained in the standard 15 μg HA vaccine used in the current study.

A protective value for anti-NA antibody has been established in both humans and animal models; these demonstrations have concluded that the antibody is a correlate as well as an independent effector of immunity to influenza (6,7,12-15). Additionally, a number of vaccine evaluations have shown that inactivated influenza vaccines can induce anti-NA antibody in humans (7,10,16-19). There is, however, uncertainty as to whether this antibody is induced regularly by different vaccine preparations and whether adequate amounts are induced.

Current inactivated influenza vaccines induce protective immunity to influenza and are the primary means for effecting control of influenza. While effective in most individuals, these vaccines need to be improved so as to increase the immunity they convey, particularly among elderly persons (3). One option for improving protective immune responses is to ensure induction of anti-NA antibody responses in most vaccinated persons. In addition to a capacity for contributing to the immunity conveyed by the anti-HA antibody induced by vaccination, antibody to the NA should be more durable as the NA antigen has been shown to vary at a slower rate than does the HA antigen (20). Ensuring induction of this antibody in protective amounts by inactivated influenza vaccines should further ensure protection against influenza and its complications among vaccinated persons.

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## Table 1

**Comparison of Neuraminidase Activity in the High Dosage and Standard Dosage Influenza Vaccines**

| | Vaccine NA Titer[1] | |
|---|---|---|
| **Test** | **Standard** | **High Dosage** |
| 1 | 23,367 | 181,829 |
| 2 | 24,087 | 167,767 |
| 3 | 21,383 | 175,251 |
| 4 | 24,655 | 192,967 |
| Mean | 23,373 | 179,454 |
| Ratio | 1 | 7.678 |

[1] Titer calculated to yield O.D. reading of 2.0

## Table 2

**Specificity of the Neuraminidase (NA) Antigens Used in Serologic Tests and Confirmation of Differences in NA Activity in the Two Vaccines**

| | | Antibody Titer for Indicated Anti-NA Serum[1] | | |
|---|---|---|---|---|
| **Source of NA** | **Reciprocal Test Dilution** | **N1** | **N2** | **B** |
| A/New Caledonia (H1N1) Virus | 25 | 17,871 | <160 | <160 |
| A/Wyoming (H3N2) Virus | 25 | <160 | 2,284 | <160 |
| B/Jilin Virus | 300 | <160 | <160 | 68,866 |
| Standard Vaccine | 23,400 | 17,328 | 10,134 | 199,693 |
| High Dosage Vaccine | 179,500 | 17,259 | 19,069 | 196,289 |

[1] Specific NA antisera obtained from NIBSC, UK; N1: A/New Caledonia (H1N1), N2: A/Wyoming (H3N2), B: B/Jilin

**Table 3**

**Proportion (%) of Subjects Developing an Increase in Anti-hemagglutinin (HA) and Antineuraminidase (NA) Antibody Titers[1]**

| | | H1N1[3] | | H3N2[3] | |
|---|---|---|---|---|---|
| **Group and Dosage[2]** | **No.** | **HA[4]** | **NA[4]** | **HA[4]** | **NA[4]** |
| Total Group | | | | | |
| Standard | 208 | 29.3 | 5.8 | 32.2 | 13.0 |
| High | 206 | 60.7 | 24.3 | 55.8 | 30.6 |
| Previous Vaccine | | | | | |
| Standard | 162 | 23.5 | 4.9 | 25.9 | 9.3 |
| High | 159 | 54.1 | 18.9 | 48.4 | 25.2 |
| No Previous Vaccine | | | | | |
| Standard | 46 | 50.0 | 8.7 | 54.3 | 26.1 |
| High | 47 | 83.0 | 42.6 | 80.1 | 48.9 |

[1] Anti-HA = % with ≥4-fold increase in titer in hemagglutination inhibition and/or neutralization tests. Anti-NA = ≥2-fold increase in enzyme-linked lectin assay.

[2] Previous vaccine: Previously vaccinated (2-5 months earlier); No previous vaccine = Not previously vaccinated; Standard dosage: 15 μg HA of each component; High dosage: 60 μg HA of each component

[3] H1N1 = A/New Caledonia (H1N1): H3N2 = A/Wyoming (H3N2) for HAI and anti-NA, A/Fujian (H3N2) for neut

[4] Frequencies significantly higher for the high dosage groups in all comparisons

**Table 4**

**Proportion (%) of Subjects Developing an Increase in Serum Antineuraminidase Antibody Titer in Relation to Prevaccination Antibody Titer**

| | N1[2] | | | | N2[2] | | | |
|---|---|---|---|---|---|---|---|---|
| | Standard[3] | | High[3] | | Standard[3] | | High[3] | |
| Test & Titer[1] | No. | % ↑[4] | No. | % ↑[4] | No. | % ↑[4] | No. | % ↑[4] |
| HAI | | | | | | | | |
| <4-32 | 109 | 7.3 | 123 | 30.1 | 88 | 18.2 | 99 | 41.4 |
| 32-≥256 | 99 | 4.0 | 83 | 15.7 | 120 | 9.2 | 107 | 20.6 |
| Neut | | | | | | | | |
| <2-16 | 100 | 8.0 | 112 | 34.8 | | | | |
| 16-≥32 | 106 | 3.8 | 96 | 11.5 | | | | |
| <2-32 | | | | | 108 | 16.7 | 111 | 36.0 |
| 32-≥128 | | | | | 100 | 9.0 | 95 | 24.2 |
| NI | | | | | | | | |
| <16-128 | 120 | 10.0 | 109 | 34.9 | | | | |
| 128-≥512 | 88 | 0.0 | 97 | 12.4 | | | | |
| <16-64 | | | | | 124 | 16.9 | 116 | 44.8 |
| 64-≥512 | | | | | 84 | 7.1 | 90 | 12.2 |

[1] HAI = Hemagglutination inhibition titer (reciprocal); neut = neutralization titer (reciprocal); NI = neuraminidase inhibition titer (reciprocal)

[2] N1 = A/New Caledonia (H1N1), N2 = A/Wyoming (H3N2)

[3] Standard = Standard dosage, 15 μg HA of each component; high = high dosage, 60 μg HA of each component

[4] Lower % in higher pre-NI antibody groups significant for all four comparisons; lower % in higher pre HAI and neut groups significant for 3 of 4 comparisons in high dosage groups with p = 0.07 for N2 neut group; lower % in higher pre HAI and neut groups in standard dosage groups not significant for any comparison but p = 0.06 for N2 HAI comparison

**Table 5**

**Geometric Mean Serum Anti-Neuraminidase Antibody Titers Before (Pre) and After (Post) Immunization**

| | | N1[2] | | N2[2] | |
|---|---|---|---|---|---|
| **Group and Dosage[1]** | **No.** | **Pre** | **Post[4]** | **Pre** | **Post[4]** |
| Total Group | | | | | |
| Standard | 208 | 122 | 131 | 55 | 69 |
| High | 206 | 125 | 187 | 56 | 90 |
| Previous Vaccine | | | | | |
| Standard | 162 | 131[3] | 138[3] | 58 | 71 |
| High | 159 | 128 | 178 | 57 | 85 |
| No Previous Vaccine | | | | | |
| Standard | 46 | 92[3] | 110 | 47 | 63 |
| High | 47 | 116 | 220 | 51 | 112 |

[1] Previous vaccine: Previously vaccinated (2-5 months earlier); No previous vaccine = Not previously vaccinated; Standard dosage: 15 μg HA of each component; High dosage: 60 μg HA of each component

[2] N1 = A/New Caledonia (H1N1), N2 = A/Wyoming (H3N2)

[3] Mean titers for prevaccination higher for standard vaccine for previous than no previous vaccine; all other prevaccination titers similar. Post vaccination mean significantly greater than pre for all groups and antigens except for N1 in the previously vaccinated group given standard vaccine.

[4] Postvaccination mean titer greater for high dosage than standard dosage for all groups and both NA antigens.

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