No evidence of a link between influenza vaccines and Guillain–Barre syndrome–associated antiganglioside antibodies

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Background Guillain–Barre syndrome (GBS) is a rare autoimmune disease characterized by acute, progressive peripheral neuropathy and is commonly associated with the presence of antiganglioside antibodies. Previously, influenza vaccination was linked with the increased incidence of GBS; however, whether antiganglioside antibodies are subsequently induced remains unresolved.

Methods Sera from human subjects vaccinated with seasonal influenza vaccines from the 2007–2008, 2008–2009, or 1976–1977 influenza seasons were screened for the induction of immunity to influenza and the presence of antiganglioside antibodies pre- and post-vaccination. Likewise, sera from mice vaccinated with seasonal influenza vaccines (1988–1989, 2007–2008) or “swine flu” pandemic vaccines (1976, 2009) were assessed in the same manner. Viruses were also screened for cross-reacting ganglioside epitopes.

Results Antiganglioside antibodies were found to recognize influenza viruses; this reactivity correlated with virus glycosylation. Antibodies to influenza viruses were detected in human and mouse sera, but the prevalence of antiganglioside antibodies was extremely low.

Conclusions Although the correlation between antiganglioside antibody cross-reactivity and glycosylation of viruses suggests the role of shared carbohydrate epitopes, no correlation was observed between hemagglutinin-inhibition titers and the induction of antiganglioside antibodies after influenza vaccination.

Keywords Antibodies, gangliosides, Guillain–Barre syndrome, influenza, vaccines.

Introduction

In 1976, the United States National Influenza Immunization Program resulted in the vaccination of approximately 45 million persons in 10 weeks. However, the program was stopped when the H1N1 virus failed to spread, and the usage of the vaccine at Fort Dix, a US Army base in New Jersey, was associated with Guillain–Barre syndrome (GBS). Guillain–Barre syndrome is a rare, acute autoimmune disease of the peripheral nervous system that is characterized by rapidly advancing, bilateral, ascending motor neuron paralysis that usually occurs after an acute respiratory or gastrointestinal infection. On rare occasions, GBS manifests after vaccination. It is the leading cause of acute paralysis in developed countries and remains the most reported serious adverse event after trivalent influenza vaccination in the Vaccine Adverse Event Reporting System database. This database has a report rate of 0.70 per 1 million vaccinations.

The incidence rate of GBS in the general population is 0.6–4.0 cases per 100 000 persons per year; the typical rate of GBS in recipients of any vaccine is 0.07–0.46 cases per 100 000 persons. During the 2009 H1N1 pandemic, the excess case rate of GBS was estimated to be 0.8 cases per 1 million vaccinations. Retrospective studies after the 1976 Fort Dix event found the vaccine-attributive risk, 6–8 weeks post-vaccination, to be 4.0–7.6. Despite multiple studies that have failed to show any association between influenza vaccination and GBS, the association between GBS and influenza vaccines continues to be an unresolved debate that was, in part, responsible for the concerns about the safety of the 2009 H1N1 vaccine.
Antianganglioside antibodies potentially play an important role in the pathogenesis of GBS, and approximately 60% of patients with GBS have these antibodies in their serum during the acute phase of the disorder.4,19–21 Guillain–Barre syndrome has been linked to a number of pathogenic agents, including Campylobacter jejuni, Cytomegalovirus, Epstein–Barr virus, Mycoplasma pneumoniae, and Haemophilus influenzae.4 However, whether GBS after influenza vaccination is associated with antianganglioside antibodies remains less clear. Anecdotal reports have been made about the presence of antianganglioside antibodies in patients in whom GBS and Miller Fisher syndrome developed after influenza vaccination.22 To our knowledge, the current study is the first to screen serum for the induction of antianganglioside antibodies in humans after influenza vaccination.

Methods

Vaccines
Seasonal trivalent influenza vaccines for the 1988–1989 (A/Taiwan/1/86, A/Sichuan/2/87, and B/Victoria/2/87-like) and the 2007–2008 (A/Solomon Islands/3/2006, A/Wisconsin/67/2005, and B/Malaysia/2506/2004-like) influenza seasons were provided by Biodefense & Emerging Infections Resources (Manassas, VA, USA). Monovalent subunit vaccine to the novel influenza A (H1N1) pandemic strain (A/Calfornia/04/09), which was manufactured by Sanofi Pasteur (Swiftwater, PA, USA), was provided by the National Institutes of Health. Additionally, for comparison to the commercially produced novel influenza A (H1N1) subunit vaccine, BPL-inactivated A/TN/1-560/09 (H1N1) virus was purified, concentrated, and administered to mice. HANAflu monovalent subunit influenza vaccine for the 1976 swine influenza pandemic was prepared, sealed, and stored at St. Jude Children’s Research Hospital (St. Jude) at 4°C for 34 years before the study. The HANAflu vaccine was standardized to 400 chick cell agglutinating units (CCA) and contained the high-yielding recombinant X-53A, a 6+2 reassortment containing two genes, hemagglutinin (HA) and neuraminidase (NA), from A/NJ/11/76 and six genes from the high-yielding parent strain A/PR/8/34. All vaccine dilutions were prepared in sterile phosphate-buffered saline (PBS).

Animals
Six- to 8-week-old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME, USA) and C3H/HeN mice (Charles River Laboratories International, Inc., Wilmington, MA, USA) were immunized as previously described23 with vaccines containing one of the following antigens: A/TN/1-560/09; 2009 Pandemic H1N1 (A/California-like); A/NJ/1976 (X-53A); A/Taiwan/1/86, A/Sichuan/2/87, and B/Victoria/2/87; or A/Solomon Islands/3/2006, A/Wisconsin/67/2005, and B/Malaysia/2506/2004. All experiments were conducted with the approval of the St. Jude Institutional Animal Control and Use Committee. Each cohort of mice, with the exception of a group of C3H/HeN mice vaccinated with 2009 pandemic H1N1 vaccine (15.6 µg HA/ml, 7.8 µg HA/ml), included 30 mice; 10 mice were used per vaccine dilution. Mice that received vaccine formulations containing an antigen to A/NJ/1976 were given dilutions based on 400 CCA, 120 CCA, or 12 CCA. All other mice immunized with BPL-inactivated A/TN/1-560/09 and A/NJ/76 received vaccine dilutions at 15 µg HA/ml, 7.5 µg HA/ml, or 0.75 µg HA/ml. Mice that were immunized with the 1988–1989 or the 2007–2008 trivalent influenza seasonal vaccines received vaccine doses rated at 90 µg HA/ml, 24 µg HA/ml, or 4.5 µg HA/ml. Blood for serum antibodies was collected retro-orbitally under anesthesia at 3 weeks post–primary injection and at 3 weeks post–boost injection.

Clinical serum samples
Human serum samples were from a prospective study involving 612 adult subjects from the Greater Vancouver Area of British Columbia, Canada, or the Greater Hartford Area of Connecticut during the 2007–2008 or 2008–2009 influenza seasons. Participants’ ages ranged from 20 to 40 years (median 29 years) and from 60 to 93 years (median, 74 years). Approval for this study was obtained from the Institutional Review Boards of those institutions involved in the study, and informed consent was obtained from each subject. Each subject received the recommended dose of commercial seasonal trivalent influenza vaccine, i.e., Fluvirin (Novartis, Basel, Switzerland), Flulaval (GlaxoSmithKline, Research Triangle Park, NC, USA), and Vaxigrip (Sanofi Pasteur) for the 2007–2008 and 2008–2009 influenza seasons. Commercial vaccines administered during the study contained purified HA antigen from A/Solomon Islands/2/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004-like strains for the 2007–2008 influenza season, while commercial vaccines administered during the 2008–2009 influenza season contained purified HA antigen from A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Florida/4/2006-like viruses. Serum samples were taken from each subject prior to vaccination and 4 weeks post-vaccination.

Additionally, serum samples were obtained in July of 2009 under an IRB-approved protocol from St. Jude employees who had previously been vaccinated against the A/NJ/1976 (H1N1) “swine flu” strain in 1976.24 Ages of the 46 participants at the time of serum collection ranged from 55 to 77 years (median, 60.5 years).
Hemagglutination-inhibition antibody titers
To determine whether seroconversion was induced after vaccination, we performed hemagglutination-inhibition (HI) assays. We treated all sera with receptor-destroying enzyme (Denka Seiken Co., Ltd, Tokyo, Japan) overnight. The serum samples were then serially diluted twofold with PBS and mixed with an equal volume of wild-type viral stocks expressing HA from A/Solomon Islands/3/06 (H1N1), A/Brisbane/59/07 (H1N1), A/NJ/76 (H1N1), or 2009 Pandemic H1N1 (A/California-like) adjusted to 4 HA units/50 µl. The plates were covered and incubated at room temperature for 30 minutes. Turkey red blood cells (RBCs) or chicken RBCs were then used to determine HI antibody titers. To account for differences in the receptor specificity of the seasonal vaccine viruses (α2-6 sialic acid receptors) and the 1976 swine flu vaccine virus (α2-3 sialic acid receptors), turkey RBCs that express more α2-6 sialic acids were prepared to a working solution of 0.5% RBCs in PBS and added to the serum of mice incubated with seasonal vaccine viruses. Chicken RBCs that express more α2-3 sialic acids were added to serum of mice incubated with the 1976 swine flu virus.

The plates were mixed by agitation, covered, and allowed to set for 30 minutes at room temperature. The HI titers were determined by the reciprocal of the last dilution that contained non-agglutinated turkey RBCs. A similar method was used to measure the cross-reactivity of commercially available anti-GM-1, anti-GM-2, or anti-GD1α ganglioside antibodies with H1N1 and H3N2 influenza A viruses.

Detection of antiganglioside antibodies
To screen for the presence of antiganglioside antibodies, we used an enzyme-linked immunosorbent assay (ELISA) that was similar to others described elsewhere. Each sample was diluted 1:100 in a solution of 1% bovine serum albumin (BSA), PBS, and 0.05% Tween and tested in duplicate with a corresponding negative control well. Immulon 2 HB, 96-well polystyrene plates (Thermo Scientific, Milford, MA, USA) were coated with 200 ng GM-1, GM-2, or GD1α gangliosides from bovine brain (Sigma, St. Louis, MO, USA) reconstituted in a 1:1 solution of methanol and chloroform. Additionally, commercially purchased antiganglioside IgG antibodies generated in rabbits with purified bovine brain ganglioside – GM-1, GM-2, and GD1α – antibodies (EMD Chemicals, Gibbstown, NJ, USA; Millipore, Billerica, MA, USA) were used as positive plate controls, and commercially purchased human serum (Sigma) was used as the negative plate control. The plates were left at 4°C overnight. The following morning, they were blocked for non-specific binding (2 hours at 4°C) with 3% BSA–PBS. Following incubation and the addition of 100 µl horseradish peroxidase-conjugated anti-human or anti-mouse IgG (Sigma) diluted 1:5000 in dilution buffer (1% BSA–PBS/0.05% Tween), plates were washed three times with ice-cold 0.05% Tween–PBS in a Biotek ELx405 (Biotek, Winooski, VT, USA) automated microplate washer. Afterward, the plates were developed by adding 100 µl premixed TMB substrate (3,3′,5,5′-tetramethylbenzidine) (Sigma) and allowed to incubate in the dark for 10 minutes. The peroxidase reaction was stopped with 100 µl 1N H2SO4, and the plates were read with a Biotek Synergy II automated microplate reader (Biotek) at 450 nm with an optical density (OD) threshold of 0.1. Final OD values were calculated by averaging duplicates and subtracting from the corresponding negative control wells. All confirmed positives were retested to further ensure the accuracy and viability of the assay.

Statistical analysis
To assess the effect of HI titers (and thus vaccination) on the induction of antiganglioside antibodies, we stratified the data into two age-groups (20–40 years and 60 years or older) and performed regression analyses using InStat 3 (GraphPad Software, Inc., La Jolla, CA, USA). Individual analyses were performed for each antiganglioside and age-group.

Results
Cross-reactivity of antiganglioside antibodies with influenza viruses
To determine whether influenza viruses possess epitopes recognized by antiganglioside antibodies, representative H1N1 and H3N2 subtype influenza viruses circulating during the last 40 years were assessed. Using an HI assay to test the reactivity of commercially available antiganglioside antibodies with these strains, we found that both GM-1 and GM-2 antiganglioside polyclonal antibodies cross-reacted with multiple H1N1 and H3N2 influenza strains, thereby preventing agglutination of chicken RBCs (Table 1). This inhibition varied between influenza virus subtypes, i.e., H3N2 viruses reacted better than H1N1 strains; however, this reactivity directly associated with the glycosylation of HA globular heads. As the number of potential glycosylation sites increased on the HA, the reactivity of the virus with the antiganglioside antibody also increased. Our data suggest that influenza viruses possess epitopes recognized by antiganglioside antibodies and that the extent of reactivity associated with the extent of glycosylation of the virus. Vaccines containing influenza strains with high amounts of ganglioside cross-reactivity were chosen from the human vaccine studies and used for vaccinating mice, with the hypothesis that these viruses would most likely induce antiganglioside antibodies in the sera of mice.
Detection of antiganglioside antibodies in human serum

To determine whether humans immunized with influenza vaccine had elevated levels of antiganglioside antibodies, we screened pre- and post-vaccination human serum samples for anti-GM-1, anti-GM-2, and anti-GD1a antibodies using ELISA. Hemagglutination-inhibition assays were conducted to confirm seroconversion after immunization with seasonal trivalent influenza vaccines from the 2007–2008 influenza seasons (Table 2). We found that serum samples from St. Jude employees (1976) and from all subjects younger than 40 were negative for antiganglioside antibodies (Figure 1A–B). Of the serum samples screened from subjects older than 60 years, 20 (n = 15 patients; average age, 75.5 years) had OD values exceeding the 0.1 threshold (positive OD) (Figure 1B); most of these samples were positive for GD1a. Positive OD values in post-vaccination serum potentially indicate influenza vaccine–induced production of antiganglioside antibodies. However, only four subjects displayed positive values after vaccination alone (Table 3); the rest were immunopositive either before vaccination or positive before and after. We found positive OD values only in the sera of those patients who were 60 years or older; thus, we performed multiple regression analyses on that subset of data. We found no correlation between the production of HI titers and the induction of antiganglioside antibodies during the 2007–2008 or the 2008–2009 influenza seasons (results not shown).

Induction of antiganglioside antibodies in mice

A previous study has shown that antiganglioside antibodies are induced in mice after vaccination with influenza vaccine. In an effort to support or refute our human data, we vaccinated groups of 6- to 8-week-old mice (n = 30 mice per group) with influenza vaccine preparations from the 1988–1989, 2007–2008, 2009 pandemic, or 1976 pandemic influenza seasons (Table 4). Vaccines were administered at different concentrations of HA to determine whether higher concentrations of influenza vaccines were more likely to induce antiganglioside antibodies in mice. Mice vaccinated with human seasonal trivalent vaccines from the 1988–1989 and 2007–2008 influenza seasons did not develop antiganglioside antibodies associated with the testing of human serum. Similar results were observed when C57/BL6 and C3H/HeN mice were vaccinated with the 2009 pandemic H1N1 vaccine. We detected the induction of antiganglioside antibodies only in two C57/BL6 mice, which were vaccinated with vaccine preparations containing the antigen from A/TN/1-560/09 (H1N1) or
that from A/NJ/1976 (H1N1) human swine influenza viruses (Table 4). Interestingly, these mice did not come from groups administered with the highest vaccine dilutions. Owing to technical difficulties, we were not able to perform HI assays on all groups of mice. Of the groups tested, the C57BL6 mice that received the whole-virion preparation had the greatest increase in HI titer from their primary to boost vaccination. Additionally, C3H/HeN mice appeared to respond better to the A/NJ/1976 HANA flu monovalent vaccine than did the C57BL6 mice.

**Discussion**

The intent of this study was to assess whether influenza vaccination would induce antiganglioside antibodies in humans. We began by comparing influenza and ganglioside antibody cross-reactivity between several historical influenza viruses of the H1N1 and H3N2 subtypes in addition to those found in trivalent seasonal influenza vaccines. Recognition of influenza viruses by antiganglioside antibodies increased as the number of potential HA glycosylation sites increased. Hemagglutinin is a viral surface glycoprotein partially responsible for facilitating the entry of influenza into host cells by binding to terminal sialic acid residues on extracellular glycoproteins and gangliosides (viral receptors). After viral replication in the host cell, the virus buds are released from the host cell membrane following the cleavage of HA from cell surface viral receptors via NA. Extracellular sialic acid residues on host cells may remain partially attached to newly budded influenza viruses,
thereby forming a sialic acid–HA complex that mimics host cell gangliosides. This ganglioside mimicry may then inadvertently allow the host’s immune system to develop an immune response against its own cell surface glycoproteins or gangliosides.23 Of additional interest is the lack of activ-

### Table 4. ELISA screening of mouse serum for antiganglioside antibodies

| Vaccine               | Antigen type | Mouse strain | n | Mean HI titer (range)* | Immunopositive serum** |
|-----------------------|--------------|--------------|---|------------------------|------------------------|
|                       |              |              |   | Primary                | Boost                  |
| A/TN/1-560/09         | Whole virion | C57/BL6      | 30| 229 (0–640)            | 938 (320–640)          |
| 2009 Pandemic H1N1    | Subunit, purified HA | C57/BL6 | 30| N/A                    | 105 (0–640)           |
| A/NJ/1976             | Subunit, purified HA | C57/BL6 | 30| 10 (0–80)              | 41 (0–160)            |
| 1988–1989 seasonal trivalent | Subunit, purified HA | C57/BL6 | 30| 63 (0–160)           | 216 (20–1280)         |
| 2007–2008 Seasonal trivalent | Subunit, purified HA | C57/BL6 | 30| N/A                    | N/A                    |

CCA, chick cell agglutinating units; HA, hemagglutinin; HI, hemagglutination inhibition; N/A, not applicable.
*Owing to technical difficulties, HI titers were not obtainable from some groups of serum samples; those are noted as N/A.
**Serum was sampled after the primary vaccination and then again after a boost vaccination.
***Vaccine dose given to mice.

lar head.27 These results are further supported by those from our mouse model vaccine experiments in which we found no induction of antiganglioside antibodies after immunization with several seasonal influenza vaccines.

Although HI titers were not correlated with the induction of antiganglioside antibodies after influenza vaccination in the oldest cohort of subjects, the presence of very low levels of antiganglioside antibodies in their sera seemingly supports the idea that GBS risk increases with age.28 However, there has been no link to increased risk of GBS and receipt of influenza vaccination in older adults. Furthermore, because the majority of our sample population was in the older age bracket and we lacked vaccination and travel histories on these subjects, it is impossible to determine whether previous exposure to influenza strains or other insults on their immune system influenced our results. The presence of antiganglioside antibodies in both the pre- and post-vaccination serum samples from some subjects suggests that other prior factors led to the generation of those antibodies. However, despite our observations, the possibility of influenza vaccination–related GBS mediated by antiganglioside antibodies in rare instances cannot be discounted.

Traditional HA assay systems based on the agglutination of RBCs (e.g., CCA) provide varying results. Studies comparing single radial immunodiffusion and traditional HA assay systems using subunit A/New Jersey/8/76 (X-53A) vaccine have shown that traditional methods significantly underestimate the amount of microgram HA activity/ml in subunit and split-product vaccines.29,30 On the basis of this finding, we prepared multiple vaccine dilutions for each
round of vaccination in mice to examine the dose required to induce antiganglioside antibodies. Despite our usage of higher doses of vaccine and a previous study showing the induction of IgG and IgM antibodies to GM-1 in the C3H/HeN strain of mice, we found that antiganglioside antibodies were not readily produced or detected in either C57/BL6 or C3H/HeN strains vaccinated with seasonal influenza vaccines (1988–1989, 2007–2008) or pandemic influenza vaccines (1976, 2009). Additionally, all vials of vaccine used had HA activity (data not shown). We hypothesize that because all mice used were inbred, the absence of antibodies to gangliosides after vaccination would logically extend to all other mice in the cohort. Those mice whose sera contained antibodies after vaccination with low- to mid-range dilutions demonstrate the random nature of induction.

Detecting antiganglioside antibodies in serum by ELISA has several limitations. The clinical features of certain subtypes of GBS are composed of a myriad of pathologic subtypes, each of which is associated with specific antiganglioside antibodies; thus, the inclusion of three single gangliosides – GM-1, GM-2, and GD1a – and the omission of others reduced the range of detection of antiganglioside antibodies associated with GBS. Furthermore, sera from GBS-affected individuals react more readily to mixtures of gangliosides, known as ganglioside complexes, and not to their individual constituents. In addition, this study and others have utilized gangliosides of bovine brain origin for antibody detection in mouse and human sera with success. Across species, gangliosides are structurally similar; however, human gangliosides contain only N-acetylgalactosaminic acids, whereas bovine gangliosides contain N-acetylgalactosaminic and N-glycolylneuraminic acids. Although it remains unclear whether this difference would reduce antibody detection by ELISA, the use of bovine gangliosides may not accurately measure the true antibody reactivity of mouse and human antiganglioside antibodies. Lastly, the presence of antiganglioside antibodies in the sera of humans exposed to influenza vaccine does not indicate the likelihood that the subject has had or will experience GBS; further testing of other parameters and clinical signs are needed to make the assessment. To our knowledge, screening of antiganglioside antibodies has been performed only in persons presenting with clinical signs of GBS; therefore, the baseline levels of antiganglioside antibodies in the population remain unknown. Although antiganglioside antibodies involved with GBS cannot be treated as a definitive marker for the syndrome, they potentially play a key role in its pathophysiology, and their importance must not be underestimated.

Studies of the 2009 H1N1 pandemic vaccine uptake among various groups in different counties have shown a lower uptake relative to seasonal influenza vaccine. Despite strong governmental and institutional reassurance that pandemic vaccines are safe, the primary concerns associated with vaccine refusal were vaccine side effects and efficacy. Much of the negative light shed on the 2009 H1N1 pandemic vaccine may be attributed to the 1976 swine flu fiasco. However, influenza vaccines have improved substantially since the 1970s, with the introduction of zonal centrifugation, chromatographic purification strategies, and stringent quality control standards. The results in this study provide additional evidence that the triggering of GBS by influenza vaccination is an unlikely and rare event. Additionally, studies on the 2009 H1N1 vaccines in the USA and in China have shown that the rates of GBS following vaccinations are very low (<1 per 2 million doses of vaccine and 0.1 per million doses of vaccine, respectively). Thus, adverse events are very rare and probably less than background levels that occur in the general population.

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Conflicts of interest

All authors report that there are no conflicts of interest.

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