

# Long-term persistence of IgE anti-influenza A H1N1 virus antibodies in serum of children and adults following influenza A vaccination with subsequent H1N1 infection: a case study

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**Background and methods:** The role of immunoglobulin (Ig) E in immunity against influenza A H1N1 has not been studied. Total serum IgE and specific IgE and IgG anti-H1N1 virus responses were studied in children and adults (n = 2) who received influenza virus vaccination (Flumist® or Fluzone®) in autumn 2008 and 2009, and then subsequently became infected with the H1N1 virus in spring 2009. Twelve months after infection, antibodies in their serum were compared with those in the serum of subjects who were either vaccinated but not infected (n = 4) or nonvaccinated and noninfected subjects (n = 2), using UniCAP total IgE fluoroenzyme immunoassay, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blotting. Band sizes for the influenza virus (58, 56, 40, 30, 25, and 17 kDa) and H1N1 viral proteins (58, 56, 25, and 17 kDa) were determined, using sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie brilliant blue.

**Results:** We found that the serum of vaccinated and subsequently infected children and adults contained IgE and IgG antibodies to both H1N1 and influenza virus, with a strong IgE and IgG band intensity at 56 kDa. Interestingly, in subjects who were vaccinated but not infected, band intensity at 56 kDa was lowered by approximately two-fold. Serum of nonvaccinated and noninfected subjects had no detectable IgE or IgG antibodies to influenza virus or H1N1.

**Conclusion:** This is the first description of IgE anti-influenza A H1N1 antibodies in human serum and the first demonstration of their long-term persistence. The decreased intensity of the 56 kDa band in vaccinated noninfected subjects compared with vaccinated infected subjects suggests augmented IgE and IgG antibody responses to influenza A H1N1.

**Keywords:** influenza A H1N1, immunoglobulin E, vaccination

## Introduction

Previous studies in our laboratory have established that immunoglobulin (Ig) E plays a role in the immune response to various viruses, including human immunodeficiency virus-1 (HIV-1) seropositive nonprogressing pediatric patients with decreased numbers of peripheral blood CD4+ T cells,<sup>1,2</sup> parvovirus B19 in children,<sup>3</sup> and varicella zoster virus<sup>4,5</sup> in both children and adults with a past history of chicken pox infection or varicella zoster virus vaccination.<sup>6</sup> Recent studies in the laboratory have described the presence and long-term persistence of IgE anti-influenza virus antibodies in the serum of IgE positive and negative vaccinated pediatric and adult subjects, approaching 2 years since vaccination.<sup>7</sup> The presence of IgE anti-influenza virus antibodies several months

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following vaccination may have biological significance. However, the exact role of IgE in influenza virus infection remains to be elucidated.

Outbreaks of annual influenza A virus are normally reported in the winter months, and cause fever, cough, and fatigue.<sup>8</sup> However, the Centers for Disease Control and Prevention identified two cases of human infection with a swine-origin influenza A H1N1 virus on April 15 and April 17, 2009, which was characterized by a combination of gene segments not previously identified among human or swine influenza A viruses.<sup>9</sup> By May 2009, the new H1N1 virus infected humans in Mexico, Canada, and elsewhere in the US,<sup>9</sup> and spread to other parts of the world, resulting in the World Health Organization declaring the infection a global pandemic.<sup>10</sup>

The aim of this study was to assess for the presence of IgE anti-influenza A H1N1 antibodies in human serum. We found lower intensity of the 56 kDa band only in serum from vaccinated subjects compared with vaccinated subjects who were subsequently infected with H1N1, suggesting augmented IgE and IgG antibody responses to influenza A H1N1.

## Materials and methods

### Characterization of patients

Peripheral blood (3 mL total) was obtained from both pediatric (male and female, aged 1–18 years) and adult (male and female, aged 40–59 years) Caucasian or Hispanic subjects from an outpatient pediatric practice in Brooklyn, NY, and from adults who worked in the same practice, as previously described.<sup>7</sup> Briefly, one child and one adult subject ( $n = 2$ ) received influenza virus vaccination (Flumist® or Fluzone®) in the autumn of 2008, and then subsequently became infected with H1N1 virus in the spring of 2009 (H1N1 influenza pandemic 2009). At 12 months after immunization, IgG and IgE anti-H1N1 antibodies in their serum were compared with those in subjects who were either vaccinated and infected ( $n = 4$ ) or nonvaccinated and noninfected ( $n = 2$ ). Subjects were both atopic and nonatopic, with normal ( $<100$  IU/mL) or elevated ( $>100$  IU/mL) serum IgE levels. The study was approved by the institutional review board of the SUNY Downstate Medical Center, Brooklyn, NY.

### Vaccine

Adults received the influenza virus vaccine, Fluzone (inactivated influenza virus vaccine, 2008–2009 formula, Sanofi Pasteur Inc, Swiftwater, PA) and children were

vaccinated with Flumist (live attenuated influenza virus vaccine, intranasal, 2008–2009 formula, MedImmune, Gaithersburg, MD). Each 0.25 mL dose of Fluzone vaccine contains 7.5 µg of influenza virus hemagglutinin and each 0.5 mL dose contains 15 µg hemagglutinin from each of the following three viruses: A/Brisbane/59/2007, IVR-148 (H1N1), A/Uruguay/716/2007, NYMC X-175C (H3 N2, an A/Brisbane/10/2007-like virus), and B/Brisbane/60/2008. Each 0.2 mL dose of Flumist intranasal spray contains 10 fluorescent focus units of live attenuated influenza virus reassortants of each of the three strains for the 2008–2009 season: A/California/7/2009, A/Perth/16/2009, and B/Brisbane/60/2008. Time since vaccination for subjects was up to 24 months. Past history of vaccination was confirmed by positive immunoblot for IgG anti-influenza virus.

The influenza A (H1N1) 2009 monovalent vaccine (Sanofi Pasteur Inc) was used for our sodium dodecyl sulfate polyacrylamide gel electrophoresis study, and is defined as follows: H1N1 2009 monovalent vaccine formulated to contain 15 µg hemagglutinin of influenza A/California/07/2009 (H1N1 v-like) virus per 0.5 mL dose. Gelatin 0.05% is added as a stabilizer. Each 0.5 mL dose may contain residual amounts of formaldehyde (not more than 100 µg), polyethylene glycol p-isooctylphenyl ether (not more than 0.02%), and sucrose (not more than 2.0%).

### Influenza A H1N1 infection

The diagnosis of influenza in the context of the influenza A H1N1 pandemic (May 2009) was confirmed by an infectious disease specialist based on clinical symptoms (history of flu-like symptoms, fever and/or cough, malaise) according to the case definition provided by public health authorities.<sup>10,11</sup> Diagnostic modalities for influenza virus A testing varied (direct fluorescence antibody testing or viral culture). Patients did not require hospital inpatient care and were defined as mild to moderate H1N1 infection, according to accepted Centers for Disease Control and Prevention guidelines.<sup>11</sup> It should be noted that, as of October 3, 2009, data from the Centers for Disease Control and Prevention reported that 99% of circulating influenza viruses in the US were H1N1.<sup>11</sup>

### Total serum IgE determination

Blood was collected and IgE levels were detected in serum (Quest Diagnostics Inc, Teterboro, NJ) according to the manufacturer's recommendation. The reference range for IgE in healthy adult or child serum is 20–100 IU/mL.

## Detection of IgG or IgE anti-influenza A H1N1

Serum from subjects vaccinated with influenza virus vaccine and subsequently infected with wild-type H1N1 infection had a positive cross-reaction with nitrocellulose blots coated with H1N1 influenza vaccine, because part of the vaccine contains the regular influenza virus vaccine. In order to differentiate between the two specific viral protein fractions, the influenza vaccine (0.9 µg protein) and H1N1 vaccine (0.3 µg protein, 20 µL/well) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 1), as previously described by Laemmli.<sup>12</sup> The broad range molecular weight marker (Bio-Rad Laboratories, Hercules, CA) used was 210, 125, 101, 56.2, 35.8, 29, 21, and 6.9 kDa (Figure 1). The gel was run at 70 V through stacking and then 150 V through resolving. The gel was stained overnight with Coomassie brilliant blue G (Sigma, St Louis, MO), and destained with a methanol-acetic acid-water mixture (Figure 1).

In order to detect H1N1 in our serum samples, the monovalent influenza A H1N1 vaccine was added (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 10%) and the gel was then transferred to 0.2 µm nitrocellulose membrane at 4°C for 4 hours. Membranes were then blocked overnight with 5% milk in the cold. Serum samples

were added and either IgG fraction goat anti-human IgG (heavy chain-specific and light chain-specific, ICN/Cappell, West Chester, PA) diluted 1:100 in TBS-Tween 20 and 1% milk in TBS-Tween 20 (1 mL) or IgE fraction goat anti-human IgE (epsilon chain, MP Biomedicals, Solon, OH), diluted 1:40 was added to the membranes and incubated for one hour on a shaker at room temperature. The membranes were then washed three times with TBS-Tween 20.

For detection and development of both IgG and IgE isotypes, nitrocellulose membranes were then incubated with rabbit anti-goat peroxidase-labeled antibody (whole molecule, Cappell), diluted 1:2000 in TBS-Tween 20 and 1% milk for one hour on a shaker, and washed three times with TBS-Tween 20. Bands were visualized using chemiluminescence (2 mL, ECL Detection Reagents RPN3004, GE Healthcare Biosciences, Pittsburgh, PA). Membranes were read, dried, and scanned (Gel Doc 2000 System with specific The Discovery Series: Quantity One software (Bio-Rad)).

## Results

### Subject characteristics

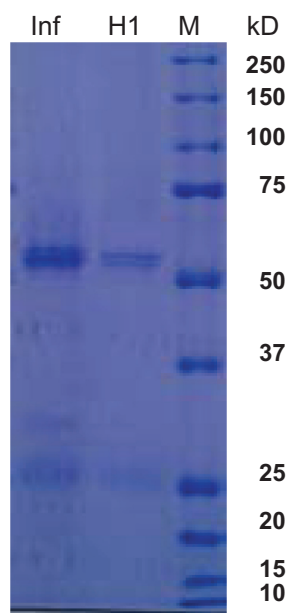
Total serum IgE levels and IgE anti-influenza A virus and H1N1 virus antibodies were studied in children (male/female, aged 1–18 years) and adults (male/female, aged 41–49 years) approaching 12 months since vaccination, as well as in noninfected and nonvaccinated children (controls,  $n = 2$ , males aged one year, Table 1). Total serum IgE levels were either normal or high in adults and children vaccinated for the influenza virus. Children with no history of either influenza virus infection or vaccination had low serum IgE levels (Table 1). Total serum IgE levels were low in subjects infected with the H1N1 virus (Table 1).

### IgG anti-influenza A H1N1 antibodies

Serum obtained from vaccinated and subsequently infected children and adults contained IgG anti-H1N1 antibodies to both H1N1 (Figure 2) and influenza virus (data not shown), with a prominent band at about 56 kDa. In subjects who were vaccinated against influenza virus, but not subsequently infected with H1N1, the band intensity at 56 kDa was lowered by about 50% (Figure 3A). In contrast, serum from nonvaccinated and noninfected (control) subjects did not contain IgG antibodies to either H1N1 or influenza virus (data not shown).

### IgE anti-influenza A H1N1 antibodies

Serum obtained from vaccinated and subsequently infected children and adults contained IgE antibodies to both H1N1



**Figure 1** Polyacrylamide gel electrophoresis of influenza A and H1N1 proteins. Lane 1, influenza A virus vaccine; lane 2, H1N1 virus vaccine; lane 3, broad weight molecular weight marker.

**Notes:** The gel was prepared as described in the Materials and methods section. The amount of protein loaded in each track was 0.9 µg/mL influenza A virus vaccine and 0.3 µg/mL H1N1 virus vaccine. Staining was with Coomassie brilliant blue G.

**Table 1** Subject characteristics

Patient	Gender/age (years)	Race	Influenza virus inoculation	Serum IgE levels (IU/mL)	IgE anti-influenza virus (+/-) <sup>#</sup>	IgE anti-vaccination infection H1N1 with influenza with virus A (month/year) H1N1 (+/-) <sup>#</sup> (month/year)
1	F (41)	C	Fluzone	15	+	+ 09/2008 May 2009
2	M (14)	C	Flumist	34	+	+ 10/2008 May 2009
3	M (16)	C	Flumist	132	–	– 10/ 2008 N/A
4	F (18)	C	Flumist	154	+	– 10/2008 N/A
5	M (44)	C	Fluzone	232	+	– 09/2008 N/A
6	F (49)	C	Fluzone	34	+	– 09/2008 N/A
7	M (1)	H	None	14	–	– N/A N/A
8	M (1)	H	None	15	–	– N/A N/A

**Notes:** Patients were inoculated with either Flumist® (live attenuated influenza virus vaccine) or Fluzone® (inactivated influenza virus vaccine) or not inoculated. Reference range for healthy adult or child serum: IgE 20–100 IU/mL. <sup>#</sup>Immunoblot/sodium dodecyl sulfate polyacrylamide gel electrophoresis (see Materials and methods section).

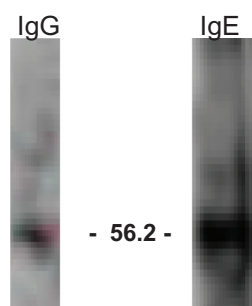
**Abbreviations:** C, Caucasian; H, Hispanic; N/A, not applicable.

(Figure 2) and influenza virus (data not shown), with strong IgE anti-H1N1 band intensity also at about 56 kDa. In subjects who were vaccinated against influenza virus, but not subsequently infected with H1N1, the band intensity at 56 kDa was lowered to about 35% (Figure 3B). In contrast, sera from nonvaccinated and noninfected (control) subjects did not contain IgE antibodies to either H1N1 (data not shown).

## Discussion

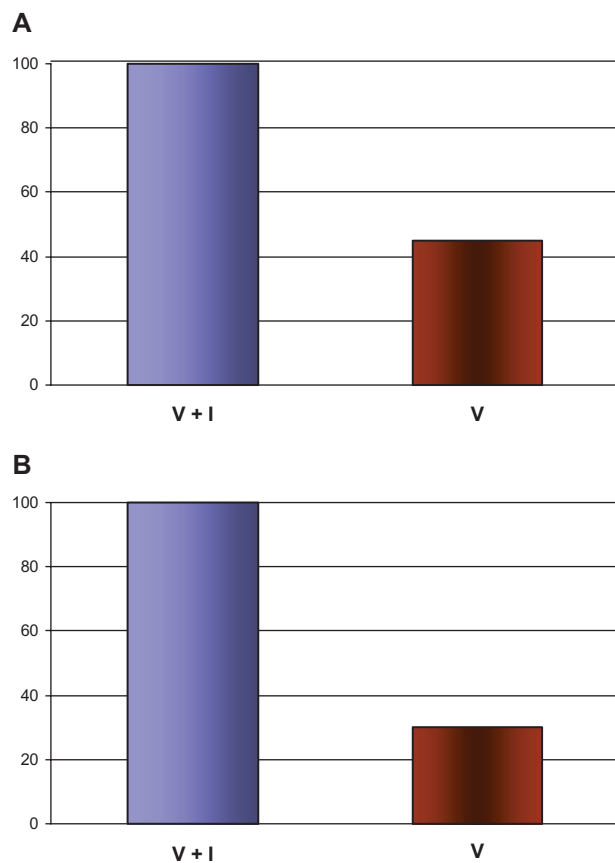
The present study is the first to describe the existence and persistence of IgE anti H1N1 influenza virus antibodies in human sera obtained during and after the pandemic influenza H1N1 phase. Here we report the presence of IgE anti-H1N1 antibodies (as well as IgG anti-H1N1) antibodies in the serum of patients infected with H1N1 during the 2009 pandemic

and in serum from the same patients up to 12 months following infection (Western blotting). Because our infected H1N1 patients were also previously vaccinated with influenza A virus vaccine, the H1N1 enzyme-linked immunosorbent assay which we prepared displayed pre-existing cross-reactive influenza A virus-specific responses with the H1N1 virus antigen, thus necessitating us to separate (and compare) the viral protein components from H1N1 influenza virus and seasonal influenza A virus on polyacrylamide gel. Earlier studies reported by other investigators<sup>13</sup> have used polyacrylamide gel electrophoresis to compare the structural proteins of the A/Havana/1292/78 national strain with the proteins of three international strains in relation to protein structure (surface antigens).<sup>13</sup> The authors reported that the most abundant protein in the four strains was M protein, while other differences between the Cuban strain and the three international strains were not observed.<sup>13</sup> In our study, we found that the serum of vaccinated and infected children and adults contained IgE and IgG antibodies to both H1N1 and influenza virus, with strong IgE and IgG band intensity at 56 kDa. Interestingly, in subjects who were vaccinated but not infected, band intensity at 56 kDa was lowered by about 35%–50%. These results suggest augmented IgE and IgG antibody responses to influenza A H1N1 with respect to the vaccinated and infection states. Although there may exist antigenic mimicry to certain components of the virus which are shared in the immune response to vaccination as well as infection, the demonstration of increased virus-specific responses following vaccination in the presence of infection compared with vaccination alone, in the presence of our fractionated protein assay, suggests unique responses to each of these viral strains in their own right. Because the laboratory diagnosis of clinical H1N1 infection differs

**Figure 2** Immunoblot analysis of IgG and IgE anti-H1N1 virus antibodies.

**Notes:** Serum from subjects with a past history of influenza virus vaccination and H1N1 infection, influenza virus vaccine and no H1N1 infection, or unvaccinated and uninfected controls was incubated with nitrocellulose strips containing H1N1 virus vaccine antigen (see Materials and methods section). IgG (left panel) representative blot of subject vaccinated with influenza virus vaccine, and infected with H1N1 virus; development time, 10 seconds. IgE (right panel), representative blot of subject vaccinated with influenza virus vaccine and infected with H1N1 virus; development time, 7 seconds. Data represent one of two patients with similar results.

**Abbreviation:** Ig, immunoglobulin.



**Figure 3** Comparison of IgG and IgE anti-H1N1 virus antibodies after vaccination with subsequent infection.

**Notes:** Serum from subjects vaccinated with influenza vaccine with a past history of H1N1 infection (V + I) compared with vaccine alone (V) were assessed for IgG (Figure 3A) and IgE (Figure 3B) anti-H1N1 antibody production. Data represent comparative scoring of intensity of Western blot (from Figure 2) of two patients, as determined by averaging mean assessments by four blinded referees (score range 1–10).

**Abbreviation:** Ig, immunoglobulin.

between laboratories, the uniqueness of epitopes among influenza strains and preparations remains unclear. Future studies utilizing manufacturer's preparations are warranted to elucidate precisely which antigens are more versus less immunogenic with respect to isotype as well as idio type.

However, of notable interest, is the fact that both H1N1-infected patients had low serum IgE levels. Taken together, these results suggest that the total serum IgE level is not as important as the specific fraction of IgE anti-H1N1 influenza antibodies representing a percentage of the total IgE responsible for mediating IgE anti-viral immune responses, and levels of serum IgE do not necessarily correlate with virus-specific IgE. The active role of IgE in viral disease is unknown but in earlier preliminary observations<sup>14</sup> and other studies<sup>1–5</sup> we have suggested that the IgE molecule has evolved to have other beneficial functions, including those of an antiviral nature.

Other studies in our laboratory have investigated the role of IgE in other disease states, ie, anticancer antibodies, which were found in patients with normal and high serum IgE levels and had the ability to mediate antibody-dependent cell-mediated cytotoxicity against cancer cells in vitro.<sup>15</sup> Similarly, in the present study, these specific immune responses did not correlate with total serum IgE levels.<sup>15</sup>

Some limitations in the design of this study should be borne in mind, including its small sample size and ethnic homogeneity, and any generalizations should be made cautiously. However, the strengths of this research include demonstration of the selectivity and specificity of antiviral IgE responses to viruses which maintain some degree of shared antigenicity (influenza versus H1N1) and further supports the uniqueness of antiviral IgE responses. To this end, the distinct and independent regulation of IgE in viral pathogenesis (ie, HIV and varicella zoster virus) as distinct from other immunoglobulin isotypes (IgM, IgG, and IgA) has also been reported.<sup>5,16</sup> Studies reported by Ferrazzi et al<sup>16</sup> have shown that although adult HIV-1 infected individuals often exhibit hypergammaglobulinemia, the relative increase in serum IgE levels is greater than that of other serum immunoglobulins, with elevation especially apparent in end-stage disease (ie, acquired immune deficiency syndrome) in patients with decreased numbers of blood CD4+ T cells (<200/mm<sup>3</sup>).<sup>16</sup> However, it is well established that T cell responses play a role during the early stage of viral infection.<sup>17</sup> Thus, future experiments are warranted to quantify the H1N1 influenza A virus-specific T cell response(s) in combination with humoral responses.

To our knowledge, this study is the first to describe the presence and persistence of IgE anti-H1N1 influenza virus antibodies in serum from human subjects previously infected with H1N1 influenza virus during the 2009 pandemic. Although additional studies are required for further elucidation of the possible molecular mechanisms involved, our results raise the possibility that IgE could be used as a novel biomarker for human viral disease and that IgE may have a possible functional role in virus memory responses and the pathogenesis of viral disease.

## Disclosure

The authors report no conflicts of interest in this work.

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