Mucosal antibody responses are directed by viral burden in children with acute influenza infection

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Background Influenza infection causes excess hospitalizations and deaths in younger patients, but susceptibility to severe disease is poorly understood. While mucosal antibodies can limit influenza-associated infection and disease, little is known about acute mucosal antibody responses to influenza infection.

Objectives These studies characterize mucosal antiviral antibody production in children during lower respiratory infection (LRI) with H1N1 influenza versus other viral LRI and examine the relationship between mucosal antiviral antibodies and protection against severe disease.

Methods B lymphocytes were assessed by immunohistochemistry in lung tissue from infants with fatal acute seasonal influenza infection. Nasopharyngeal secretions (NPS) were obtained at presentation from children with acute respiratory illness, including H1N1 (2009) influenza infection. Total and antiviral antibodies, and inflammatory and immune mediators, were quantified by ELISA. Neutralizing activity in NPS was detected using a pseudotyped virus assay. Viral burden was assessed by qPCR.

Results and conclusions B lymphocytes were abundant in lung tissue of infants with fatal acute influenza LRI. Among surviving children with H1N1 infection, only a small subset (11%) demonstrated H1N1 neutralizing activity in NPS. H1N1 neutralizing activity coincided with high local levels of antiviral IgM, IgG and IgA, greater detection of inflammatory mediators, and higher viral burden ($P = 0.016$). Patients with mucosal antiviral antibody responses demonstrated more severe respiratory symptoms including greater hypoxia ($P = 0.0018$) and pneumonia ($P = 0.038$). These patients also trended toward younger age, longer duration of illness and longer hospital stays. Prophylaxis strategies that heighten neutralizing antibody production in the mucosa are likely to benefit both older and younger children.

Keywords Mucosal antibody response, pandemic influenza pediatric, lower respiratory infection, neutralizing antibody response.

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Introduction

More than 100 000 hospitalizations and 40 000 deaths are attributed to influenza infections each year in the United States. In 2009, widespread infection with a swine-origin influenza A (H1N1) virus resulted in excess hospitalizations and deaths, which disproportionately affected younger patients. In some studies, hospitalizations for pediatric pandemic influenza infection were doubled as compared with seasonal influenza the previous year, with a 10-fold increased mortality rate. Nevertheless, 2009 A/H1N1 influenza infection resulted in self-limiting, mild disease in most infected children. The reasons why influenza virus infection results in severe outcomes such as pneumonia in some previously healthy children, and not in others, are incompletely understood. Improved understanding of protective antiviral responses in healthy children may help to more precisely identify individuals at risk and inform critical decision-making about therapeutic and vaccination strategies, especially during pandemic years.

Antibodies directed to influenza hemagglutinin (HA) are thought to protect against infection with influenza virus, limiting subsequent disease. The lack of an anamnestic antibody response to swine-lineage HA is proposed to increase disease susceptibility in young people as compared with older adults. However, lack of preexisting HA-directed antibodies against 2009 A/H1N1 in serum is expected in all infants and young children, and this factor by itself
cannot explain the occurrence of severe disease in a very small subset. We considered whether other mechanisms of protection, occurring in the respiratory mucosal compartment, could limit influenza pathology in some pediatric patients without preexisting HA-directed antibodies in serum.

Because influenza virus targets the respiratory tract, mucosal antiviral responses such as local antibodies are thought to contribute to immunity. In experimental models, passive local transfer of HA-directed IgA protected non-immune mice from H1N1 but not H3N2 influenza challenge.9 Data acquired from volunteers infected with wild type or attenuated influenza viruses showed that class-switched influenza-directed Ig, particularly IgA, is elicited in the respiratory tract and confers resistance to infection and illness.10–13 However, the mechanisms and timing of the local antibody response to influenza were not clarified in these studies. Mucosal antiviral antibodies might have been locally generated by recruited B lymphocytes,14 or alternatively, antibodies may have transudated into the mucosa from the peripheral blood. In addition, mucosal antibody responses detected in adults and older children were possibly facilitated by T- and B-lymphocyte responses elicited during previous natural infections and vaccinations. In infants, facilitation of mucosal antibody responses by influenza-responsive T lymphocytes is reduced, because of a limited window for previous infections and at most a single year of vaccination experience. Whether acute mucosal antiviral antibody responses could be elicited in this group, rapidly enough after influenza exposure to modify disease was not addressed in previous studies.

Robust local antibody production has been reported in infants and young children after first exposure to some respiratory infections, despite immaturity of the immune system in these patients. For example, many studies have demonstrated antiviral IgG, IgM and IgA in respiratory secretions of infants within days after primary respiratory syncytial virus (RSV) infection [reviewed in Ref. 15]. We recently showed that detection of RSV-directed antibodies in respiratory secretions appeared to be clinically relevant, as high levels of antiviral IgM and IgA in secretions were inversely correlated with disease severity.16 Also, detection of RSV-directed antibodies in respiratory secretions positively correlated with local production of proinflammatory cytokines, BAFF and APRIL, suggestive of T-lymphocyte-independent antibody responses.15 To our knowledge, a similar study has not yet been performed in infants and young children with influenza infection. However, several studies have found a substantial mucosal proinflammatory cytokine burst occurs with seasonal influenza 17 and particularly with H1N1 2009 H1N1 infection. These robust inflammatory responses might be sufficient to support antibody responses in the mucosa, similar to those described in RSV and in experimental models of influenza infection.21–23

Based on these observations, we hypothesized that rapid local antibody responses generated during acute exposure to influenza could be disease modifying in some patients.

In this study, we explored mucosal B-lymphocyte responses in postmortem lung specimens from infants with acute influenza infection and the mucosal immune responses in NPS from infants and children with viral lower respiratory infection (LRI) including 2009 A/H1N1 influenza. In lung tissue from infants with fatal acute seasonal influenza infection, prompt B-lymphocyte recruitment was confirmed by immunohistochemistry (IHC), similar to findings in infants with RSV infection.16 We demonstrated for the first time H1N1-directed neutralizing antibodies in respiratory secretions using a pseudovirus-based neutralization assay, which offers improved sensitivity over other methods and obviates the need for high-level biocontainment.24,25 Surprisingly, we found that exposure to 2009 A/H1N1 influenza in most cases did not elicit strong cytokine or mucosal antibody responses in children, even in the presence of significant acute LRI symptoms. Instead, local antibody responses were detected only in younger children who had the most severe respiratory symptoms, greatest local cytokine response, and highest viral burden.

Methods

Study populations

Postmortem lung tissues were obtained as previously described 17 from infants with fatal acute seasonal influenza infection (n = 11) autopsied at Hospital Roberto del Rio, Santiago, Chile. Dying infants had not been subjected to prolonged mechanical ventilation or to the use of anti-inflammatory agents or antiviral agents. Lung tissues from three age-matched infants, one who died in a car accident and two with asphyxia, were provided by the National Institute of Child Health and Development (Baltimore, MD, USA).

Nasopharyngeal secretions (NPS) were collected in two cohorts. In the first cohort, NPS were collected on presentation from 63 children with confirmed H1N1 influenza infection and 25 children with confirmed RSV infection at Women and Children’s Hospital in Buffalo, New York, during the 2009–2010 season. Nasopharyngeal secretions were obtained by direct aspiration and centrifuged to collect epithelial cells for rapid diagnosis. Supernatants were reserved for cytokine and antibody analyses and determination of viral burden by quantitative PCR (qPCR). Values of oxygen saturation (O2sat) were determined by pulse oximetry at the time of NPS collection.

In the second cohort, 55 NPS were collected from children with influenza-like illness presenting to the Emergency Department at Dell Children’s Medical Center of Central
Texas. These samples were collected for influenza surveillance testing during the winter of 2007–2008 and from April to June 2009 during the early H1N1 pandemic season. These samples were analyzed specifically for cytokine content.

No subject was vaccinated for H1N1 or seasonal influenza during the 2009–2010 season, and none received therapeutic antibody preparations, corticosteroids, or antiviral agents. All studies performed on NPS specimens were reviewed and approved in advance by the Research in Human Subjects Committee at the Food and Drug Administration/Center for Biologics Evaluation and Research.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded lung tissues were sectioned and processed through xylene to remove paraffin. Heat-induced epitope retrieval was performed prior to IHC analysis. Primary antibodies reactive with CD20 (Thermo Fisher Scientific, Kalamazoo, MI, USA), CD4, IgM, IgA, IgD, and IgG (Millipore, Billerica, MA, USA) were used according to manufacturers’ recommendations. Bound primary antibodies were detected with biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) and streptavidin-horseradish peroxidase conjugate (GE Healthcare, Piscataway, NJ, USA) followed by peroxidase substrate (Sigma Aldrich, St. Louis, MO, USA).

**Analysis of NPS**

**H1N1 neutralizing activity**

H1N1-specific influenza HA-pseudotype viral particles were generated in 293T cells cotransfected with plasmids expressing HA (H1N1:A/Mexico/4108/2009), HIV gag/pol, and luciferase reporter, as previously described.24 Pseudovirus infection was measured with a luciferase assay kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. For each control and NPS sample, luciferase activity was assessed in duplicate wells, and neutralization was defined as mean luciferase activity decreased by three STD compared with control infected wells.

**Immune and inflammatory mediators**

TNF-α, IL-1β, IL-6, IL-8, IFN-γ, IL-2, IL-4, IL-21 BAFF and APRIL (R&D Systems; eBioscience, Minneapolis, MN, USA) and total IgG, IgM, and IgA levels (Bethyl Laboratories Inc., San Diego, CA, USA) were measured using capture-detect ELISA systems (Montgomery, TX, USA) according to manufacturer’s recommendations. For H1N1 antibody detection flat-well ELISA plates (Maxisorp, Nalge Nunc International, Rochester, NY, USA) were coated with H1N1 monovalent vaccine (FLULAVAL, GSK, Philadelphia, PA, USA) at a nominal concentration of 1 μg/ml HA antigen. Non-specific protein interactions were blocked by incubation in saline containing 3% BSA (Sigma, St. Louis, MO, USA). Nasopharyngeal secretions were added to wells at a 1:10 dilution in blocking buffer. Bound antibodies were detected with isotype-specific, HRP-labeled polyclonal antisera (Jackson Immunoresearch) followed by HRP substrate (SureBlue, KPL, Gaithersburg, MD, USA). OD was quantified at 450 nm using a 96-well plate reader (Bio-Tek Instruments, Winooski, VT, USA).

**H1N1 influenza and RSV viral burden measurements**

Viral RNA was isolated from nasopharyngeal aspirates of children with confirmed either H1N1 or RSV infections using the QIAamp Viral RNA extraction kit (Qiagen Inc., Valencia, CA, USA). RT-PCR-based detection of pandemic influenza A/H1N1 2009 including primers and probes was performed according to the CDC protocol. Primers for influenza A (universal) were the following: forward 5’ GAC CRA TCC TGT CAC CTC TGA C 3’; reverse 5’ AGG GCA TTY TGG ACA AAK CGT CTA 3’; probe FAM-TGC AGT CCT CGC TCA CTG GGC ACG MGB. Pandemic influenza 2009 specific primers were the following: forward 5’ GTG CTA TAA ACA CCA GCC TYC CA3’; reverse 5’ CGG GAT ATT CCT TAA TCC TGT RGC 3’; probe FAM-CA GAA TAT ACA TCC RGT CAC AAT TGGAR A-MGB. Influenza virus DNA oligos for assay qualification were prepared commercially (IDT, Coralville, IA, USA). Primers and probes for RSV detection were prepared as previously described.25 A DNA oligo encoding nucleotides 428–535 from human RSV fusion gene (RSV-F; accession number M22643) was used for qualification. Amplification of target DNA and detection of PCR products were performed with an ABI® 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to manufacturer’s recommendation. Virus copy number in NPS samples, expressed as mean copies per ml obtained from duplicate assessments, were based on a standard curve generated using virus-specific oligos.

**Results**

**B lymphocytes in lung tissue of infants with fatal acute LRI**

To investigate mucosal B-lymphocyte responses to acute influenza infection, we first evaluated lung tissue acquired at autopsy from infants with fatal acute seasonal influenza LRI. CD20+ B lymphocytes were prevalent particularly in perivascular spaces in infants with influenza LRI (Figure 1). In contrast, minimal CD20+ staining was observed in lung tissue of uninfected infants. IgA and IgM positive cells (Figure 1) along with IgD and IgG positive cells (Figure S1) with lymphocyte morphology were similarly prevalent in lung tissues from infants with fatal acute LRI, but not in tissue of uninfected infants. CD4+ cells were sparse...
in lung tissue of infants with fatal acute LRI, but were greater in number than in control tissues (Figure S1).

**H1N1 antibody responses in NPS**

We hypothesized that acute mucosal antibody responses to influenza infection may contribute to protection against severe disease in children. To assess whether respiratory secretions from pediatric patients contain neutralizing antibody responses against pandemic influenza, a sensitive *in vitro* assay was used, based on a pseudotyped reporter virus. Briefly, pseudovirions were cotransfected with three individual plasmids, encoding H1N1 HA, HIV gag-pol, and the luciferase reporter gene (Figure S2a). Recovered pseudoviral particles were collected and incubated with 293 cell substrates, to generate a luciferase signal detectable at 48 hours post infection. Luciferase activity was not inhibited by control antiserum, but pre-incubation of pseudovirions with convalescent serum from a patient with H1N1 influenza infection significantly reduced luciferase signal at high dilutions (Figure S2b). Our initial data set demonstrated that the pseudovirus-based neutralization assay was able to detect neutralizing antibody responses in NPS acquired from children in Buffalo, NY, seeking medical attention for H1N1 infection. A small subset of NPS samples from the Buffalo cohort (7/63, 11%) significantly reduced luciferase activity in duplicate at low dilutions (Figure 2A). Nasopharyngeal secretions samples that neutralized H1N1 pseudoviruses demonstrated no neutralizing activity against seasonal influenza-based pseudovirions (Figure 2B), demonstrating that antiviral activity detected was specific to HA presented by H1N1 2009. Also, no H1N1 2009 neutralizing activity was observed in aspirates acquired from patients with RSV LRI (Figure S3). Specificity of neutralizing activity was further demonstrated in antigen-down ELISA using monovalent H1N1 vaccine antigen (Figure 3). All NPS samples with neutralizing activity...
demonstrated H1N1-directed IgG, IgM, and IgA, while non-neutralizing NPS samples demonstrated very low or undetectable anti-H1N1 reactivity.

**H1N1 antibody responses correlation with disease severity, inflammatory mediators, and viral burden**

To determine whether local antiviral antibody responses might be clinically relevant, the demographic features and clinical measures for patients in the Buffalo, NY cohort with and without mucosal H1N1-directed antibody responses were compared (Table 1). Nasopharyngeal secretions had been collected from H1N1 patients at a wide range of ages, from 0.5 to 19 years. Surprisingly, neutralizing antiviral antibody responses tended to occur in younger patients, although the relationship was not statistically significant (\( P = 0.0516 \); Table 1). Patients with mucosal antiviral antibodies demonstrated more severe respiratory symptoms including greater hypoxia and pneumonia (Table 1) and trended toward longer duration of illness and longer hospital stays. There was no evidence of less severe disease in patients with greater viral neutralizing activity, although the cohort was too small for definitive analysis.

To determine whether local antibodies might be effective in reducing viral load, we evaluated H1N1 viral burden in NPS from the Buffalo, NY cohort using qPCR. The antiviral antibody level in NPS was associated with a significantly greater detection of virus by qPCR (Figure 4). To put the H1N1 viral burden results in context, we examined viral load in NPS obtained from infants with primary RSV infection during the same season (\( n = 25 \); Figure 5). Mean viral burden in NPS was 100-fold higher in the RSV cohort compared with the H1N1 influenza cohort. Antibodies reactive with RSV antigen in ELISA were found in approximately half of NPS samples obtained from RSV patients (Figure S4). For comparison, antibodies reactive with H1N1 were observed in only 11% of NPS obtained from H1N1 patients.

Antiviral mucosal antibodies may be transported from serum to the lung or may be produced locally by B lymphocytes present in tissue.\(^{14}\) To understand whether heightened mucosal cytokine responses to H1N1 infection \(^{18–20}\) might facilitate local antibody production, we initially examined a few immune and inflammatory mediators present in NPS from the Buffalo, NY cohort using commercially available ELISA kits (Table 2). Similar to recent findings in RSV LRI,\(^{17}\) inflammatory mediators TNF-\( \alpha \) and IL-1\( \beta \) were detected in NPS of H1N1 infected children, while T-lymphocyte-associated cytokines IL-2 and IL-4 were marginally detected. Inflammatory mediators TNF-\( \alpha \), IL-1\( \beta \), and the B-cell-tropic cytokine APRIL were significantly increased in NPS of patients with antiviral antibody responses. BAFF, another B-cell stimulatory
cytokine, was also elevated in patients with mucosal anti-viral antibodies but the relationship was not significant \( (P = 0.0912; \text{Table 2}) \). We expanded these observations by exploring cytokine levels in a second cohort of 55 NPS samples, collected as part of routine influenza surveillance activities at the Dell Children’s Medical Center of Central Texas, Austin, Texas from the winter season of 2007–2008 through June 2009. In patients with either confirmed seasonal influenza infection or pandemic influenza infection, IL-6, IL-8, and IL-1β were consistently detected and positively correlated with TNF-α levels (Figures S5 and S6). In both seasonal influenza and pandemic influenza groups, IFN-γ was detected in a smaller subset and was not correlated with TNF-α or other measured cytokines (Figures S5 and S6 and data not shown). Follicular T-lymphocyte cytokine IL-21 was rarely detected in NPS from patients with either seasonal influenza or pandemic H1N1 infection (Figure S5).

**Discussion**

Mucosal antibodies have been previously implicated in increasing resistance to severe influenza disease.\(^\text{11}\) Nevertheless, the precise relationship between local antibody responses and disease susceptibility has been difficult to
assess. One problem is the limited availability of respiratory tissue samples. The current study benefitted from access to a unique panel of lung tissues from infants with fatal acute influenza LRI. In these tissues, we observed robust lung recruitment of CD20+ and antibody-secreting B lymphocytes. In contrast, essentially no CD20+ or antibody-secreting B lymphocytes were detected in control lung tissue of uninfected infants. Thus, rapid local B-cell responses to influenza infection are possible in infants with immature immunity. Unfortunately, the tissue study was limited to a single time point analysis and was not sufficient to determine whether B-cell responses were early enough to potentially modify disease.

To partially address this deficiency, we assessed anti-influenza antibody responses in two separate panels of respiratory secretions from surviving children. Typically, high dilution of antibodies in respiratory secretions hampers the measurement of neutralizing activity. To address this issue, we used a novel, pseudovirion-based assay that allowed for rapid, highly sensitive detection of neutralizing activity in dilute respiratory secretions, without need for working with infectious H1N1 influenza. Data from our analysis of NPS confirm that rapid development of influenza-neutralizing antibodies can occur in the respiratory tract of children at the peak of acute symptoms. Low-titer H1N1-directed antibodies were detected in NPS of younger children with more serious respiratory symptoms and higher viral burden. These observations are reminiscent of the substantial local antibody induction reported in young children after infection with ubiquitous respiratory pathogens such as RSV, Haemophilus influenza, and Streptococcus pneumoniae.\textsuperscript{15,26–30} In these previous studies, mucosal pathogen-specific antibodies were observed in children with more serious or prolonged illness, or longer duration of carriage, pointing to a possible role for pathogen burden in triggering or amplifying mucosal antibody responses. In these reports, pathogen-specific antibodies were still detected up to 2 years after primary exposure. The duration of influenza-specific mucosal antibody detection after natural influenza infection in children is the subject of ongoing work.

Congruent with previous observations in RSV LRI,\textsuperscript{16} our initial studies of NPS demonstrated local H1N1-specific antibodies were positively correlated with both increased viral burden and higher levels of inflammatory mediators elaborated from infected cells, such as TNF-α, IL-6, IL-9 and IL-1β, and the B-cell-directed cytokine APRIL, with less detection of IFN-γ and almost no detection of T-cell-derived cytokines IL-2, IL-4, and the follicular T-cell-associated cytokine IL-21. Together, these data may implicate low levels of T-cell help in inefficient development of mucosal virus-directed antibodies in infants with LRI. Development of mucosal antibody responses in these patients may require strong, proinflammatory cytokine production by infected cells to recruit B lymphocytes to the mucosa. If true, B-cell recruitment might be happening too late after virus infection and spread to limit disease. The data may also suggest that induction of antiviral antibodies in the respiratory tract possibly occurs when other, more efficient antiviral responses are defective, contributing to unchecked viral spread and progressive pneumonia.\textsuperscript{17} This could explain the concurrent observation of high levels of local antibodies in patients with more severe respiratory symptoms. We did not observe more rapid or robust local antibody production in older children during peak H1N1 infection. There was no evidence of either heightened mucosal antibody responses or facilitation of antibody responses with T-lymphocyte help. Thus, rapid induction of mucosal antibodies does not appear to be a protective mechanism against acute influenza infection in older children. Additionally, there was no evidence that skewed T-lymphocyte responses produced more severe disease during H1N1 infection.

Our current observations show that acute mucosal antibody responses to H1N1 are inefficient in children, independent of age. Based on this data set, the lack of mucosal antibodies does not appear to be a major risk factor for severe outcomes after H1N1 infection. Nevertheless, vaccination strategies that heighten local antibody responses are clearly not redundant in the pediatric population and are likely to provide additional protective benefit in a prophylaxis setting. Mucosal vaccination with attenuated influenza virus (LAIV) has been suggested as the method of choice in young children,\textsuperscript{31} but mechanisms of LAIV-mediated protection in children are still under investigation. Systemic immunologic responses to influenza elicited by LAIV do

### Table 2. Inflammatory and immune mediators in Buffalo, NY H1N1 nasopharyngeal secretions (NPS) samples*

| Mediator   | Non-neutralizing | Neutralizing | P value |
|------------|------------------|--------------|---------|
| TNFα (pg/ml) | 1.925 (0.06) | 2.497 (0.79) | 0.0005  |
| IL-1β (pg/ml) | 1.774 (0.18) | 2.807 (0.82) | 0.0008  |
| BAFF (pg/ml) | 1.602 (0.01) | 1.674 (0.13) | 0.0912  |
| APRIL (ng/ml) | 0.5445 (0.65) | 1.338 (0.66) | 0.0073  |
| IL-2 (pg/ml)  | n.d.            | n.d.         | –       |
| IL-4 (pg/ml)  | n.d.            | n.d.         | –       |

n.d., none detected.

*Mediators were quantified in NPS using commercially available ELISA kits. NPS with virus neutralizing activity were compared with samples lacking virus neutralizing activity. Geometric mean values are shown with standard deviation in parentheses. Statistical significance was determined by unpaired t-test.
not correlate with degree of protection against infection, and at least one study has shown that children receiving LAIV rapidly developed HA-directed IgA and were 4.5 times more likely to develop a mucosal antibody response than an HAI response in serum. LAIV vaccination as currently performed requires high doses of attenuated virus (approximately 10^7 focus assay units per strain) and, in one study, was associated with respiratory illness in very young patients. An interpretation is that in young children, proinflammatory cytokines elaborated by high doses of LAIV mediate B-lymphocyte recruitment to tissue and local antibody production, similar appears to be occurring during severe LRI. Based on data collected in this study, the contribution of B-cell tropic cytokines such as BAFF and APRIL, secreted by infected cells, to antibody production after LAIV may be critical. Providing these B-cell-directed mediators as adjuvants may improve the safety profile of LAIV by facilitating local antibody production at lower doses of virus.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Detection of lymphocytes in lung tissues from infants with influenza LRI.

Figure S2. Pseudotyped virus assay for H1N1 neutralization.

Figure S3. Aspirates of RSV patients fail to neutralize H1N1 pseudoviruses.

Figure S4. Detection of antiviral antibodies in aspirates from patients with primary RSV infection.

Figure S5. Detection of cytokines in NPS from a second influenza cohort.

Figure S6. Correlation analysis of cytokines in NPS from the Austin, Texas cohort.

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