MAJOR ARTICLE

Localized Mucosal Response to Intranasal Live Attenuated Influenza Vaccine in Adults

Maria Ines Barría,[1,a] Jose Luis Garrido,[1,a] Cheryl Stein,[2] Erica Scher,[2] Yongchao Ge,[3] Stephanie M. Engel,[2,6] Thomas A. Kraus,[4] David Banach,[5] and Thomas M. Moran[1]

[1]Department of Microbiology, [2]Department of Preventive Medicine, [3]Department of Neurology, [4]Department of Obstetrics, Gynecology, and Reproductive Sciences, [5]Department of Medicine, Mount Sinai School of Medicine, New York, New York, and [6]Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina–Chapel Hill

Background. Influenza virus infection is a major public health burden worldwide. Available vaccines include the inactivated intramuscular trivalent vaccine and, more recently, an intranasal live attenuated influenza vaccine (LAIV). The measure of successful vaccination with the inactivated vaccine is a systemic rise in immunoglobulin G (IgG) level, but for the LAIV no such correlate has been established.

Methods. Seventy-nine subjects were given the LAIV FluMist. Blood was collected prior to vaccination and 3 days and 30 days after vaccination. Nasal wash was collected 3 days and 30 days after vaccination. Responses were measured systemically and in mucosal secretions for cytokines, cell activation profiles, and antibody responses.

Results. Only 9% of subjects who received LAIV seroconverted, while 33% of patients developed at least a 2-fold increase in influenza virus–specific immunoglobulin A (IgA) antibodies in nasal wash. LAIV induced a localized inflammation, as suggested by increased expression of interferon-response genes in mucosal RNA and increased granulocyte colony-stimulating factor (G-CSF) and IP-10 in nasal wash. Interestingly, patients who seroconverted had significantly lower serum levels of G-CSF before vaccination.

Conclusions. Protection by LAIV is likely provided through mucosal IgA and not by increases in systemic IgG. LAIV induces local inflammation. Seroconversion is achieved in a small fraction of subjects with a lower serum G-CSF level.

Keywords. influenza; LAIV; Vaccination; IP10; G-CSF; FluMist; interferon.

Influenza is an acute viral respiratory infection that results in high morbidity and significant mortality in humans, producing significant health and economical burdens worldwide [1]. Annual vaccination has been the most effective strategy to reduce the impact of influenza virus infection [2]. As a consequence, significant effort has been made to produce effective vaccines that will reduce the incidence and severity of natural infections.

In the United States, the use of intramuscular trivalent inactivated influenza vaccine (TIV) is recommended to induce protective immunity through the induction of serum antibodies. Recently, a live attenuated influenza vaccine (LAIV) delivered by intranasal spray was licensed [3–5]. LAIV induces an immune response that more closely resembles natural immunity than the response elicited by the intramuscular vaccine [6, 7]. This vaccine provides comparable levels of protection against laboratory-documented influenza in adults (85% efficacy), compared with TIV [8, 9], but the mechanism of action might be different. Lower serum hemagglutination-inhibition (HAI) titers are seen with LAIV, and they are accompanied by a higher level of immunoglobulin A (IgA) antibodies in nasal wash [8–11], suggesting that other immunological contributors may be involved in the protection following vaccination with LAIV.

Although systems biology approaches have been used to predict the immunogenicity of the vaccine YF-17D against yellow fever [12] and, more recently, of TIV and LAIV against influenza [13], the latter study

Received 27 April 2012; accepted 31 July 2012; electronically published 19 October 2012.

[a]M. I. B. and J. L. G. contributed equally to the study.

Correspondence: Thomas M. Moran, PhD, Department of Microbiology, Mount Sinai School of Medicine, 1468 Madison Ave, New York, NY 10029 (thomas.moran@mssm.edu).

The Journal of Infectious Diseases 2013;207:115–24

DOI: 10.1093/infdis/jis641

did not examine the mucosal response to LAIV. The present study extends what is known about the systemic response and provides information about the local response to LAIV.

To identify factors associated with LAIV vaccination, we performed a study during the 2010–2011 influenza season. We developed a protocol to evaluate the immunological changes in systemic and local (upper respiratory tract) immune responses and collected blood samples and nasal secretions from 79 healthy adult subjects who were vaccinated with LAIV. Our study indicates that LAIV receipt induces a local inflammatory response, triggering nasal release of interferon (IFN) and granulocyte colony-stimulating factor (G-CSF) 2–3 days after vaccination, followed by specific IgA antibody production, with little changes in systemic immunity.

MATERIAL AND METHODS

Subjects

The study was performed at Mount Sinai Medical Center in New York City. All subjects provided informed consent on enrollment. The vaccination period was 5 October 2010 through 21 December 2010. This study was approved by the Mount Sinai School of Medicine Institutional Review Board.

Eligibility criteria were based on the Centers for Disease Control and Prevention's and manufacturer's guidance for the administration of the intranasal LAIV [2, 14]. Healthy, nonfebrile individuals aged 18–49 years were eligible. Individuals who reported recent influenza, previous receipt of influenza vaccine during the 2010–2011 seasons, asthma, concurrent pregnancy, allergy to the vaccine or its components, or chronic medical conditions were excluded.

Vaccination

All subjects were inoculated with FluMist vaccine (2010–2011 formulation; MedImmune, Gaithersburg, MD). Each 2-mL dose contained live attenuated influenza virus reassortants of each of the 3 strains for the 2010–2011 season: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008.

Study Protocol

At the initial study visit, subjects were administered a questionnaire to obtain baseline demographic information, influenza vaccination history, and risk factors for influenza infection. Phlebotomy was performed during the initial visit prior to FluMist administration (day 0). Subjects returned for a first follow-up visit 48-72 hours after administration (day 3). At this first follow-up visit, a questionnaire assessed self-reported postvaccination influenza symptoms (ie, fever, rhinorrhea, nasal congestion, sore throat, and cough). Blood pressure, temperature, and heart rate were recorded, and a 10-mL nasal wash and phlebotomy was performed. Subjects returned for a second follow-up visit at least 30 days following vaccination (day 30) for a 10-mL nasal wash and phlebotomy.

Nasal Wash

Nasal washes were performed using a method previously described [15], by spraying sterile saline solution into the nostril followed by collecting the expelling fluid in a specimen collection cup. Both nostrils were washed with 5 mL of saline solution, resulting in the nasal wash sample. Each nasal wash sample was centrifuged at 500×*g* for 10 minutes to remove cells and debris, and cell-free supernatant was stored in aliquots at −80°C. The cell pellet of the nasal wash sample was processed for total RNA extraction using Trizol (Invitrogen).

Cell and Serum Isolation

Peripheral blood mononuclear cells (PBMCs) and serum samples were collected from fresh blood, using ethylenediaminetetraacetic acid–coated and serum Vacutainer tubes, respectively (BD). Serum samples were stored and frozen at −80° until analysis. PBMCs were isolated by Ficoll density gradient separation (Histopaque, Sigma-Aldrich). $CD14^+$ monocytes were isolated by positive selection (Miltenyi Biotec). Monocytes were lysed in Trizol (Invitrogen) and stored at −80°C.

Cytokine/Chemokine Analysis

All samples from serum and nasal wash were stored at −80°C until the end of the study. Measurements of cytokines/chemokines were performed as described before [16], using an 11-plex cytokine panel (Millipore). All samples were run in duplicate in accordance with the manufacturer's protocol, using a Luminex 200 (Luminex Corporation), and were analyzed using Milliplex Analyst software.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and Gene Expression Analysis

Monocyte RNA extraction was performed using Trizol (Invitrogen). Concentrations of total RNA were determined using a Nanodrop spectrophotometer. Reverse transcription was performed using a First Strand Reverse Transcriptase kit (Roche). qRT-PCR was performed using the Universal Probe Library and the Master Mix 480 system for LightCycler (Roche). Gene expression data were normalized to the average cycle threshold (Ct) value of the housekeeping genes *GAPDH* and *Rps13A*, and the difference in the normalized Ct value between days 3 and 0 was calculated. By use of the $\log_2$ fold-change on day 3 relative to day 0, unpaired t test analysis was done to compare subjects who were negative for seroconversion with those who were positive for seroconversion.

The cell pellet of the nasal wash sample was processed for total RNA extraction, using Trizol (Invitrogen), and RNA was amplified using the WT-Ovation RNA Amplification system (NuGEN). Gene expression was analyzed by qRT-PCR, using a LightCycler 480 II (Roche). Gene expression was performed as described above, and statistical analysis of the difference in $\log_2$ values between days 3 and 30 was performed using a t test involving a paired 2-sample analysis. In the analysis, only patients with detectable messenger RNA (mRNA) levels on days 3 and 30 after vaccination were considered. The gene expression data for *MX1, STAT1, BST2, IRF7,* and *RIG1* are representative of 25, 22, 10, 5, and 8 subjects, respectively.

HAI Assays

Titers from HAI assays were determined on the basis of standard protocol of the World Health Organization. Briefly, serum samples were treated with receptor-destroying enzyme (Sigma Aldrich) and then serially diluted with phosphate-buffered saline (PBS) in 96-well round-bottom plates (Nunc). Four HA units of influenza A virus subtype H1N1 was added to each well. HAI titers were determined as the highest dilution that displayed hemagglutination activity.

Immunohistochemical (IHC) Staining

Madin-Darby canine kidney (MDCK) cells were seeded in 96-well plates at 60% of confluence and cultured with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), L-glutamine, and penicillin-streptomycin. On the next day, cells were washed and infected at multiplicity of infection of 1.5 with the A/California/4/2009 (H1N1) influenza strain. One hour after infection, cells were cultured with FBS-containing media and stored overnight. Cells were harvested, washed with PBS, and fixed with 1% paraformaldehyde in PBS for 5 minutes, after which additional PBS washes were performed. Cells were blocked with bovine serum albumin and then incubated at different dilutions (from 1:10 to 1:20 000) of patient sera samples for 2 hours at room temperature. Cells were washed twice with PBS and then incubated with anti-total IgG-HRP for 1 hour at room temperature. Cells were washed twice and developed using AEC substrate kit (BD Pharmingen). The IHC titer was determined as the highest dilution that displayed immunodetection.

Table 1. Demographic and Clinical Characteristics of 70 Adults Who Received Intranasal Live Attenuated Influenza Vaccine

Characteristic	Value
Age, y, mean ± SD	30.3 ± 7.2
Body mass index,[a] mean ± SD	25.5 ± 6.1
Sex	
Male	51 (65)
Female	28 (35)
Race/ethnicity	
White, non-Hispanic	40 (51)
Hispanic	18 (23)
Black, non-Hispanic	6 (8)
Asian	12 (15)
Other	3 (4)
Received 2009 H1N1 vaccine	
Yes	24 (30)
No	50 (63)
Didn't know/no answer	5 (6)
Received 2009 seasonal influenza vaccine	
Yes	38 (48)
No	36 (46)
Didn't know/no answer	5 (6)
Ever received FluMist	2 in 2009, 1 in 2005
Had ILI during 2009–2010 influenza season	7 (9)

Data are no. (%) of patients, unless otherwise indicated.

Abbreviations: H1N1, 2009 pandemic influenza A virus subtype H1N1; ILI, influenza-like illness.

[a] Defined as the weight in kilograms divided by the square of the height in meters.

IgA Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

The HA-specific IgA antibody in nasal wash specimens was determined by ELISA as previously described [8, 17], using as antigen purified recombinant hemagglutinin (rHA) protein from influenza virus A/California/04/2009 (H1N1), obtained through the National Institutes of Health (NIH) Biodefense and Emerging Infections Research Resources Repository. Briefly, 96-well polystyrene plates were coated with rHA prior to incubation with nasal wash samples overnight. The plates were washed with PBS/0.1% Tween-20, followed by the addition of anti-human IgA-HRP (Bethyl Laboratories). ELISA titers were calculated using the positive-negative (P/N) ratio, in which the end point was the highest dilution with a P/N ratio of ≥ 2. In the calculation, the optical density (OD) of an antigen-coated well (positive) was divided by the OD of the control well lacking the antigen (negative).

Heat Map and Statistical Analysis

To avoid the problems associated with computing the log function and ratio, 1 was added to all ELISA concentrations. The ELISA-determined concentration of 4 cytokines in each patient's nasal wash at the 2 visits was then transformed by the function $\log_2(x)$, and a Manhattan distance was computed for each of 79 sample pairs, where each sample contains 8 cytokine expressions (4 at the baseline visit and 4 at the postvaccination visit). A hierarchical clustering was performed on these Manhattan distances with the agglomeration method 'ward' [18, 19], resulting in a dendrogram (clustering tree) that places samples with smaller Manhattan distances in neighboring positions. While maintaining the constraints imposed by the dendrogram, the 79 samples were further reordered according to the average of the 8 transformed cytokine expression measurements. After the samples were reordered, a heat map was generated for the 79×8 cytokine expression matrix.

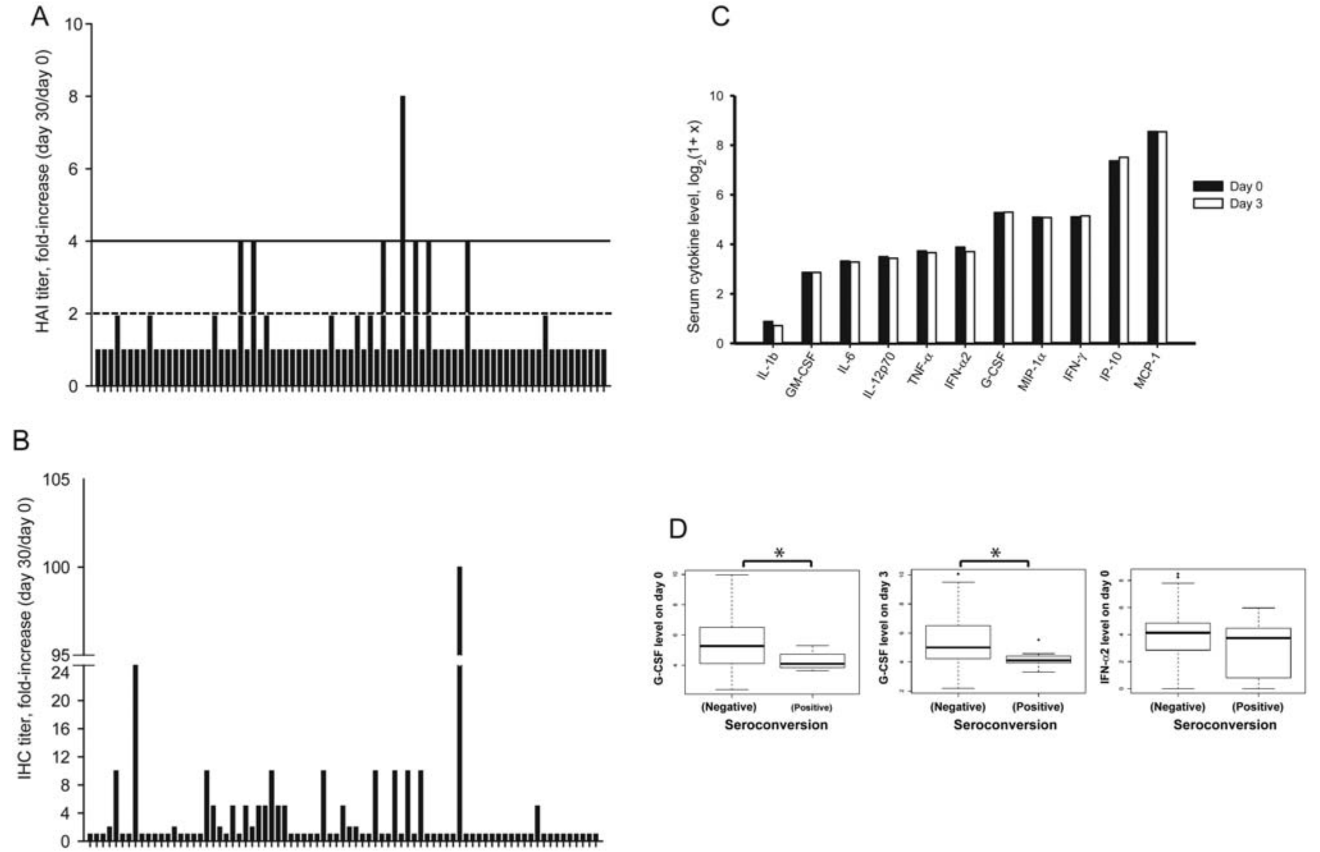

Figure 1. Systemic immunity after receipt of live attenuated influenza vaccine (LAIV). *A*, Hemagglutinin inhibition (HAI) titer in serum on day 30 after LAIV vaccination relative to day 0 (before vaccination). Data are from 1 experiment with 79 subjects assayed in duplicate, using influenza A virus subtype H1N1. Continued line represents subjects with a ≥4-fold increase above baseline (seroconversion). Dashed line represents subjects with a 2-fold increase. *B*, Immunohistochemical analysis (IHC) to recognize serum antibodies against influenza A virus subtype H1N1. Data are the fold-increase on day 30 relative to day 0. *C*, Cytokine concentrations on days 0 and 3 after vaccination were analyzed by multiplex enzyme-linked immunosorbent assay (ELISA). The *y*-axis displays the transformed concentration of each cytokine, calculated as $\log_2(1+x)$, where *x* is the ELISA-determined value. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-monocyte colony-stimulating factor; IFN-α2, interferon α2; IFN-γ, interferon γ; IL-1b, interleukin, 1b; IL-6, interleukin 6; IL-12p70, interleukin 12p70; TNF-α, tumor necrosis factor α. *D*, Box plots of serum levels of G-CSF on day 0 (left), G-CSF on day 3 after vaccination (middle), and IFN-α2 on day 0 (right) between subjects who were positive (n = 9) or negative (n = 70) for seroconversion. The top and bottom of each rectangle give the 75th and 25th percentiles, respectively, while the bold line in the middle of the rectangle gives the 50th percentile. Outliers are indicated by open circles. *$P < .05$, by the paired Wilcoxon test.

All heat map analyses were performed using the statistical programming language R, version 2.13 (http://www.r-project.org) [20, 21]. The box plot and the *P* values of paired Wilcoxon tests were computed by the functions boxplot() and wilcox.test(), respectively, in R, version 2.13 [20]

RESULTS

Subjects Characteristics

Seventy-nine subjects completed the study protocol. Baseline demographic characteristics of the study cohort are described in Table 1. In the study cohort, 24 patients (30%) reported receipt during the previous year of 2009 pandemic influenza A virus subtype H1N1, and 38 (48%) reported receipt of the 2009 trivalent seasonal influenza vaccine. Only 3 subjects (4%) had previously received FluMist, and 7 subjects reported a history of influenza-like illness during the 2009–2010 influenza season. Symptoms present 48–72 hours after vaccination are described in Supplementary Table 1. Two subjects (3%) reported fever following administration of the vaccine. Reports of other symptoms varied, with 28% reporting rhinorrhea and nasal congestion, 18% reporting sore throat, and 6% developing a mild cough. Overall, 40 subjects (51%) reported at least 1 symptom.

Systemic Antibody Response

All serum samples were analyzed for the production of antibodies to both influenza A viruses included in the vaccine.

Table 2. Findings From Hemagglutination Inhibition (HAI) and Immunohistochemical (IHC) Analyses to Determine Serum Antibody (Ab) Response to Intranasal Live Attenuated Influenza Vaccine

	HAI Findings				IHC Findings			
Group	Log_2 Ab Titer Before Vaccination	Log_2 Ab Titer After Vaccination[a]	Response,[b] Proportion (%)	P[c]	Log_2 Ab Titer Before Vaccination	Log_2 Ab Titer After Vaccination[a]	Response,[b] Proportion (%)	P[c]
All subjects	4.56 ± 1.24	4.85 ± 1.22	7/79 (9)	.139	7.33 ± 3.18	8.15 ± 2.85	19/79 (24)	.089
Subjects with serum HAI ≤1:10	2.44 ± 0.33	3.08 ± 1.29	6/33 (18)	.008	4.56 ± 2.18	5.86 ± 2.34	13/33 (39)	.023

Data are mean ± SD, unless otherwise indicated. HAI analysis was performed using A/California/4/2009 (H1N1) influenza virus. IHC analysis was performed using Madin-Darby canine kidney cells infected with A/California/4/2009 (H1N1) influenza virus to detect immunoglobulin G Ab to the virus.

[a] Measured on day 30 after vaccine receipt.

[b] Defined as a ≥4-fold increase in titer between samples obtained before and after vaccination. Data are no. of seroconverted vaccine recipients/total no. of vaccine recipients (%).

[c] Comparison of vaccine Ab levels before and after vaccination, calculated by a 2-tailed unpaired Student *t* test.

Most subjects had high prevaccine titers to the H3N2 component (data not shown); as a result, we focused this study on the response to the H1N1 component of the vaccine.

We determined HAI titers for influenza A virus subtype H1N1 in serum samples at days 0 and 30 after vaccination. Only 7 subjects (9%) were positive for seroconversion, with an increase of at least 4-fold in the antibody response (Figure 1*A*), a finding consistent with many published reports showing that LAIV induces less serum antibody than TIV [8, 13, 22]. Serum HAI titers are the most commonly measured correlates of protection, and a protective serum antibody response (defined as an HAI titer of ≥1:40) [23] was detected in 34 subjects (43%) prior to vaccination, indicating prior exposure to the antigen. The majority of individuals who seroconverted in this study (6 subjects) showed a prevaccination HAI titer of 1:10 or less (Table 2). None of the subjects who seroconverted reported receiving the previous years' influenza vaccine, and only 1 subject reported having influenza-like illness in the year previous to LAIV administration.

We further evaluated the serum antibody response by performing IHC using MDCK cell monolayers infected with influenza A virus subtype H1N1 and incubated with dilutions of serum collected at the time of vaccine administration or 30 days later. Nineteen subjects (24%) showed at least a 4-fold increase in staining after vaccination (Table 2). By use of this assay, 39% of subjects without preformed antibody

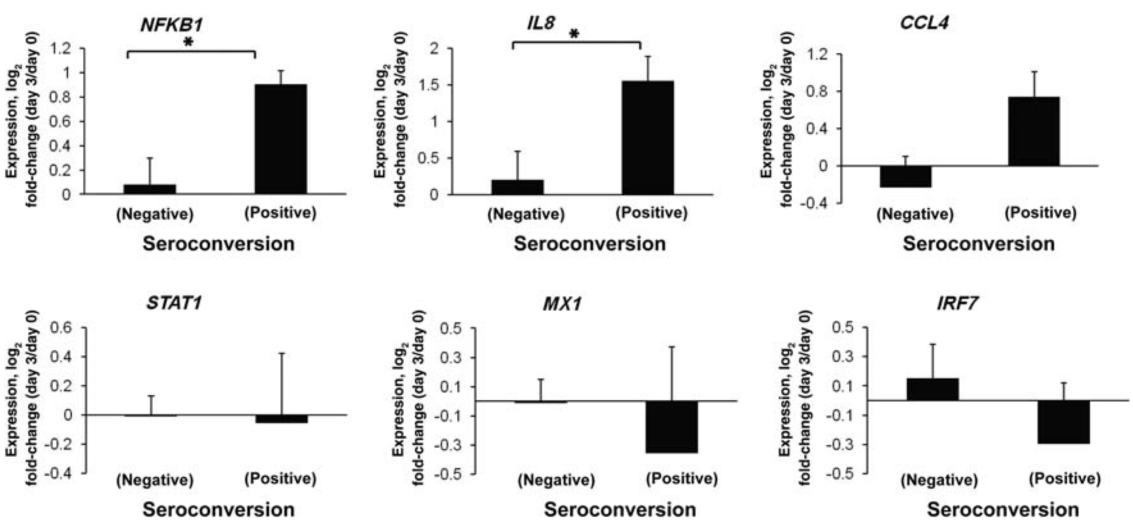

Figure 2. Analysis of gene expression induced by live attenuated influenza vaccine receipt in monocytes of peripheral blood, using quantitative reverse transcription polymerase chain reaction analysis of CD14[+] monocytes on day 0 and 3 after vaccination. Fold-changes in expression are shown as the mean of the log_2 value between days 3 and 0 for 7 subjects who were negative for seroconversion (–) and 5 subjects who were positive for seroconversion (+). Error bars represent the standard error of the mean. *$P < .05$.

Table 3. Findings From Enzyme-Linked Immunosorbent Assay (ELISA) to Determine the Hemagglutinin-Specific Immunoglobulin A (IgA) Antibody Response to A/California/04/2009 (H1N1) Influenza Virus in Nasal Wash Specimens

	ELISA Findings			
Group	Log_2 IgA Titer 3 d After Vaccination	Log_2 IgA Titer 30 d After Vaccination	Response,[a] Proportion (%)	P[b]
All subjects	3.36 ± 1.58	3.57 ± 1.99	26/79 (33)	.4723
Subjects with ≥2-fold increase in IgA titer	3.18 ± 1.75	5.03 ± 2.52	26/26 (100)	.0033

Data are mean ± SD, unless otherwise indicated.

[a] Defined as a ≥2-fold increase in titer between samples obtained on day 30 and day 3 after vaccination. Data are no. of seroconverted vaccine recipients/total no. of vaccine recipients (%).

[b] Comparison of vaccine antibody levels 30 d and 3 d after vaccination, calculated by a 2-tailed unpaired Student *t* test.

seroconverted (Figure 1*B*), suggesting that a systemic response to nasally administered LAIV can be seen with highly sensitive assays in subjects with low preformed antibody titers.

Serum Cytokine Levels

During virus infection, a rise in levels of several cytokines occurs in serum 48–72 hours after infection [24]. To determine whether LAIV causes a systemic change in serum levels of cytokines, samples collected at the time of vaccination and 3 days later were analyzed by multiplex ELISA. No predictable patterns could be detected in the measured cytokines (Figure 1*C* and Supplementary Table 2). In contrast to virus infection, LAIV does not appear to trigger a change in serum cytokine profiles.

Analysis of baseline serum cytokine expression was performed to determine the influence of serum cytokines on the systemic antibody response to LAIV. While subjects who seroconverted had slightly lower levels of IFN-α2 in blood than those who did not, the difference did not reach statistical significance ($P = .30$). Interestingly, subjects who seroconverted had significantly less G-CSF in prevaccine sera than subjects who did not seroconvert ($P = .047$ on day 0 and $P = .025$ on day 3; Figure 1*D*).

Monocyte Gene Expression Following Administration of LAIV

Monocytes are an important element of the response to virus infection and have been shown to be in an activated state in the blood and bone marrow of influenza virus–infected mice [25]. To determine whether monocytes become activated following LAIV administration, cells were isolated from the blood of subjects at the time of vaccination and 3 days later to measure gene expression associated with antiviral immunity.

Subjects who seroconverted, as determined by both HAI and IHC assays, were compared to randomly selected subjects who did not seroconvert (all were vaccinated but had no detectable systemic antibody response). Among the genes analyzed, only *NF-kB1* and *IL8* demonstrated a statistically significant rise in the seroconverted group relative to the group that did not seroconvert (Figure 2). A modest increase (1.67-fold) was observed in *CCL4* in the group positive for seroconversion, although this did not reach statistical significance ($P = .06$). Other genes associated with the IFN response, such as *MX1, STAT1*, and *IRF7,* did not seem to be affected. These data confirm that subjects who seroconverted had a detectable immune response to the vaccine in blood cells.

Nasal Antibody Responses and Nasal Cytokine Secretion

To evaluate the local response to virus replication, a nasal wash specimen was collected from subjects 3 days after administration of the vaccine and was compared to a nasal wash sample collected 30 days after vaccination. An ELISA was performed to quantify the HA-specific IgA antibodies, using the rHA from influenza A virus subtype H1N1 as an immunoadsorbent. In contrast to results obtained by HAI in serum, 26 subjects (33%) showed a significant, ≥2-fold increase in IgA at day 30, which has been proposed by others to be a positive nasal antibody response [8] (Table 3). Moreover, the magnitude of the HA-specific IgA antibody response between the subjects showed large variation, from 2- to 22-fold (Figure 3*A*).

The nasal wash samples were analyzed in a multiplex ELISA assay to determine cytokine expression. IP-10 and MCP-1 were detected in 100% and G-CSF in 84% of subjects on day 3 after vaccination, while only 34% of the subjects had detectable IFN-α2 on day 3 after vaccination. The cytokine levels in the nasal wash samples on day 30 and the levels observed 3 days after vaccination for all 79 subjects are displayed as a heat map (Figure 3*B*). The cytokine levels on day 30 after vaccination were considered to be baseline values because nasal wash was not performed prior to vaccine, owing to concern that this procedure might interfere with the administration and absorption of the intranasal vaccination. Moreover, nasal cytokine levels are reported to return to baseline levels by day 9 after LAIV receipt [26] and by day 8 after experimental influenza virus infection [27].

The heat map shows an increase in IP-10 and G-CSF levels after LAIV receipt in the majority of the patients. A total of 62 (78%), 42 (53%), 27 (34%), and 17 patients (21%) showed an increase in IP-10, G-CSF, MCP-1, and IFN-α2 levels, respectively, on day 3 after vaccination. Among those cytokines, we observed a statistically significant increase in IP-10 ($P < .0001$) and G-CSF ($P = .0005$) levels after LAIV administration. In contrast, no statistically significant differences were observed for MCP-1 and IFN-α2 levels (Figure 3*C*).

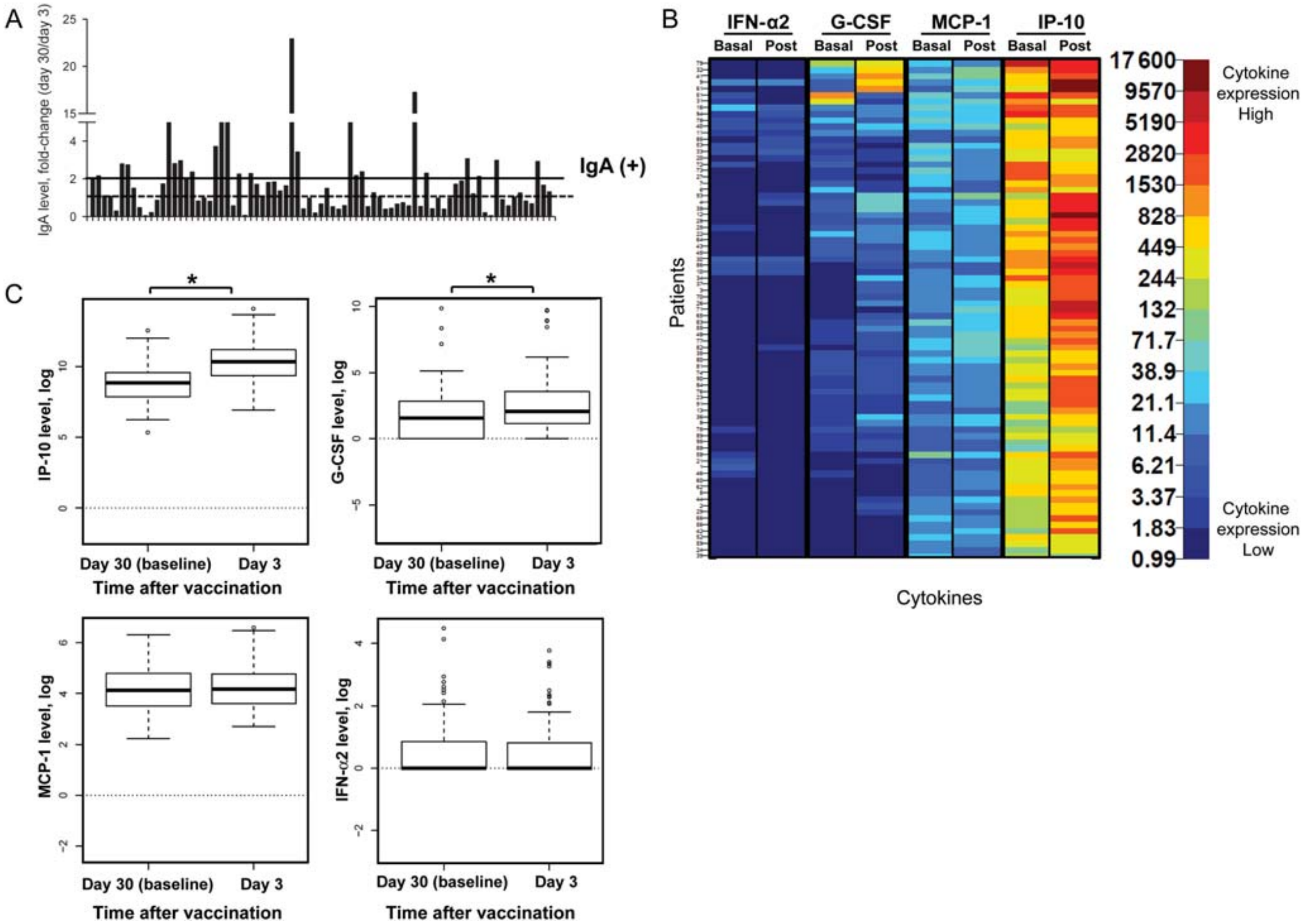

Figure 3. Analysis of mucosal immunity induced by live attenuated influenza vaccine (LAIV) receipt. *A*, Nasal hemagglutinin-specific immunoglobulin A (IgA) antibody to influenza A virus subtype H1N1 are shown as fold-changes on day 30 after LAIV vaccination relative to day 3. Continued line represents the subjects with a ≥2-fold increase above the baseline value. Dashed line denotes a fold-change equal to 1 (ie, no change). *B*, Nasal cytokine concentrations on day 3 and 30 after vaccination were analyzed by multiplex enzyme-linked immunosorbent assay and are represented as a heat map. Each column represents 1 cytokine. The Basal column represents the concentration on day 30, and the Post column represents the concentration on day 3 after vaccination. *C*, Box plots of cytokine levels from nasal wash specimens on day 30 after vaccination (baseline) and day 3 after vaccination. The top and bottom of each rectangle give the 75th and 25th percentiles, respectively, while the bold line in the middle of the rectangle gives the 50th percentile. Outliers are indicated by open circles. *$P < .0005$, by the paired Wilcoxon test.

Nasal Mucosa Gene Signature Profile After LAIV Receipt

Gene expression in nasal mucosa was measured to determine whether an IFN signature existed in the mucosa of the IgA converters. We analyzed different IFN-stimulated genes, including *MX1*, *STAT-1*, *IRF7*, *RIG1*, and BST2. All IFN-stimulated genes were statistically significantly upregulated on day 3 after vaccination (Figure 4). These results indicated that LAIV induces a local inflammatory response that may influence LAIV efficacy.

DISCUSSION

We performed a prospective cohort study to identify factors associated with immunological responses to LAIV systemically and in the local respiratory tract during the 2010–2011 influenza season in a generally healthy adult population. Two major questions asked in this study were whether LAIV triggers a systemic immune response, including production of HA-specific antibodies, production of cytokines, and innate cell activation, and whether similar changes occurring in the upper respiratory cavity can be measured.

By use of HAI, we found that only 9% of subjects seroconverted, which is less than previously reported [3]. However, a more sensitive method (IHC) revealed that 24% showed an enhancement in serum antibody response. IHC is probably more sensitive because it identifies antibodies binding to different antigens, including HA and NA epitopes present on the cell surface. No changes in serum cytokine levels were found on day 3 after vaccination either in the full group or in the seroconverters only. However, monocytes collected from

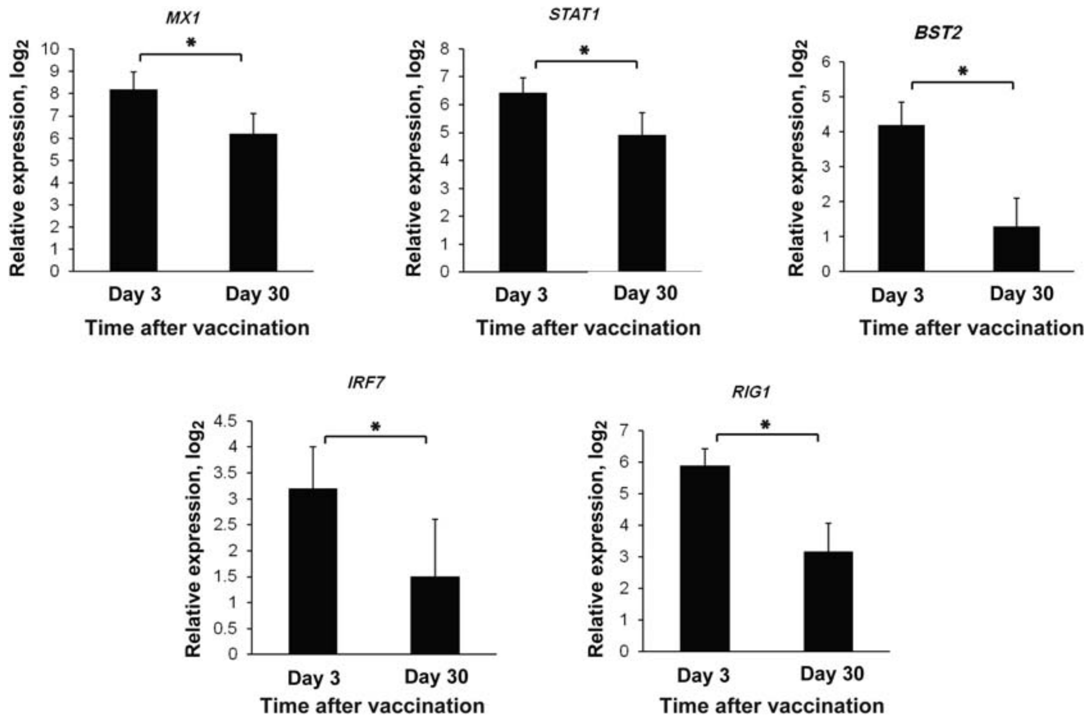

Figure 4. Nasal mucosal gene signature profiles after live attenuated influenza vaccine receipt. RNA amplification and quantitative reverse transcription polymerase chain reaction of cell pellets of nasal wash specimens obtained on days 3 and 30 after vaccination were performed to analyze the expression of the interferon-regulated genes *MX1, STAT1, BST2, IRF7*, and *RIG1*. Error bars represent the standard error of the mean. *$P < .05$.

subjects with seroconversion showed a mildly activated phenotype, as manifested by a rise in NF-kB and interleukin 8 mRNAs. These results are in agreement with the findings by Nakaya et al [13], showing upregulation of different genes in monocytes 7 days after vaccination. Our data suggest that monitoring the status of blood monocytes might be an extremely sensitive assay for detecting systemic immune activation. Moreover, the demonstration that this vaccine is protective reinforces the division between mucosal and systemic immunity and mandates that measures of efficacy should be chosen appropriately.

LAIV depends on some level of virus replication to generate an adaptive immune response. Two immune elements that might interfere with that are preformed neutralizing antibodies and very efficient innate immune activation. Thus, we evaluated seroconversion with respect to these host factors. Our data suggests that subjects lacking preformed antibody to the virus were more likely to generate a systemic response to LAIV. Of the 7 subjects who seroconverted by HAI, 6 had no preformed antibodies.

Our studies in mouse models of virus infection emphasized the systemic rise in levels of serum cytokines that function in successful viral clearance [28]. In contrast to findings for mice, we documented great variation in human serum cytokine profiles [16], and indeed, similar variation in cytokine levels was observed in prevaccine serum from patients in this study. Therefore, we asked whether cytokine profile variation might influence vaccine efficacy. Type I IFN levels vary greatly, and subjects who seroconverted had slightly lower levels of IFN-α2 at vaccination. Because of the low percentage of seroconverters, a statistically significant difference was not observed. In contrast, the observation that subjects who seroconverted had lower serum concentrations of G-CSF at vaccination was quite striking.

As previously reported, LAIV receipt resulted in an increase of IgA antibodies in nasal wash samples [5]. To further characterize the response elicited by the vaccine, we also determined the cytokine expression and gene profile in the nasal mucosa.

Nasal wash could not be administered prior to vaccination because it is not part of standard clinical care and might influence the vaccine efficacy. Therefore, we used the nasal wash sample obtained 30 days after vaccination as a baseline to analyze the immediate (day 3) cytokine response to LAIV, and we used the day 3 nasal wash specimen as baseline for the IgA response on day 30. Although IFN was not detected in the nasal washes, the rise in the IP-10 level in 78% of the patients, as well as the induction of IFN-stimulated genes in mucosal cells, likely resulted from release of IFN at a level below the lower limit of detection. G-CSF expression was also increased in the majority of patients. This IFN signature observed in the nasal mucosa may be the most sensitive measure of successful LAIV vaccination.

One reason this study was undertaken was to determine whether the study of LAIV could provide information useful for understanding influenza virus infection in humans. The observation that subjects with lower levels of G-CSF were more likely to seroconvert is novel and may have implications for future studies of vaccination and infection. G-CSF is involved in the generation and recruitment of neutrophils, among other functions [29, 30]. It is possible that phagocytosis is an important control mechanism in the upper respiratory tract and that, if phagocytosis is reduced, more virus replication and possibly dissemination might occur, leading to systemic responses. Examination of nasal wash specimens revealed that the G-CSF level clearly rose in response to the vaccine, suggesting that G-CSF has an important antiviral function, which probably involves recruiting cells to and/or activating cells in the nasal cavity. We speculate that these cells may function during influenza virus infection to restrict virus to the upper airway, preventing dissemination to the lungs. Many of the less severe influenza infections are restricted to the upper airway. In response to LAIV, a lower G-CSF level may indicate a weaker innate response, allowing sufficient viral replication for an optimum adaptive response. Thus, mucosal cytokines may serve as useful indicators of sensitivity to more-severe infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: H1 HA protein from influenza virus A/California/04/09 (H1N1), recombinant, from baculovirus NR-15258. We thank Dr Gene Tan and Dr Matthew Miller for experimental reagents and technical support.

Disclaimer. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of Center for Investigating Viral Immunity and Antagonism.

Financial support. This work was supported by the Center for Investigating Viral Immunity and Antagonism (grant U19AI062623 to T. M. M.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Molinari NA, Ortega-Sanchez IR, Messonnier ML, et al. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine **2007**; 25:5086–96.
2. Fiore AE, Uyeki TM, Broder K, et al. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep **2010**; 59:1–62.
3. Belshe R, Lee MS, Walker RE, Stoddard J, Mendelman PM. Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. Expert Rev Vaccines **2004**; 3:643–54.
4. Esposito S, Montinaro V, Groppali E, Tenconi R, Semino M, Principi N. Live attenuated intranasal influenza vaccine. Hum Vaccin Immunother **2012**; 8:1–5.
5. Carter NJ, Curran MP. Live attenuated influenza vaccine (FluMist(R); Fluenz): a review of its use in the prevention of seasonal influenza in children and adults. Drugs **2011**; 71:1591–622.
6. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med **2005**; 11:S45–53.
7. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol **2004**; 59:1–15.
8. Treanor JJ, Kotloff K, Betts RF, et al. Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. Vaccine **1999**; 18:899–906.
9. Ambrose CS, Levin MJ, Belshe RB. The relative efficacy of trivalent live attenuated and inactivated influenza vaccines in children and adults. Influenza Other Respi Viruses **2011**; 5:67–75.
10. Moldoveanu Z, Clements ML, Prince SJ, Murphy BR, Mestecky J. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine **1995**; 13:1006–12.
11. Boyce TG, Gruber WC, Coleman-Dockery SD, et al. Mucosal immune response to trivalent live attenuated intranasal influenza vaccine in children. Vaccine **1999**; 18:82–8.
12. Querec TD, Akondy RS, Lee EK, et al. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol **2009**; 10:116–25.
13. Nakaya HI, Wrammert J, Lee EK, et al. Systems biology of vaccination for seasonal influenza in humans. Nat Immunol **2011**; 12:786–95.
14. FluMist [package insert] **2010**. Gaithersburg, MD: MedImmune.
15. Noah TL, Becker S. Chemokines in nasal secretions of normal adults experimentally infected with respiratory syncytial virus. Clin Immunol **2000**; 97:43–9.
16. Kraus TA, Sperling RS, Engel SM, et al. Peripheral blood cytokine profiling during pregnancy and post-partum periods. Am J Reprod Immunol **2010**; 64:411–26.
17. Murphy BR, Phelan MA, Nelson DL, et al. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. J Clin Microbiol **1981**; 13:554–60.
18. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A **1998**; 95:14863–8.
19. Everitt B, Landau S, Leese M. Cluster analysis. 4th ed. New York: Oxford University Press Inc, **2001**.
20. Ihaka R, Gentleman R. R: a language for data analysis and graphics. J Comput Graph Stat **1996**; 5:299–314.
21. Team RDC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, **2003**.
22. Sasaki S, Jaimes MC, Holmes TH, et al. Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. J Virol **2007**; 81:215–28.
23. Cox NJ, Subbarao K. Influenza. Lancet **1999**; 354:1277–82.
24. Moltedo B, Lopez CB, Pazos M, Becker MI, Hermesh T, Moran TM. Cutting edge: stealth influenza virus replication precedes the initiation of adaptive immunity. J Immunol **2009**; 183:3569–73.
25. Cotter CR, Kim WK, Nguyen ML, et al. The virion host shutoff protein of herpes simplex virus 1 blocks the replication-independent activation of NF-kappaB in dendritic cells in the absence of type i interferon signaling. J Virol **2011**; 85:12662–72.

26. Noah TL, Zhou H, Monaco J, Horvath K, Herbst M, Jaspers I. Tobacco smoke exposure and altered nasal responses to live attenuated influenza virus. Environ Health Perspect **2011**; 119:78–83.
27. Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. J Clin Invest **1998**; 101:643–9.
28. Hermesh T, Moltedo B, Moran TM, López CB. Antiviral instruction of bone marrow leukocytes during respiratory viral infections. Cell Host Microbe **2010**; 7:343–353.
29. Metcalf D. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature **1989**; 339:27–30.
30. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood **1991**; 78:2791–2808.