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Effect of the influenza A (H1N1) live attenuated intranasal vaccine on nitric oxide (FE\textsubscript{NO}) and other volatiles in exhaled breath

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Abstract

For the 2009 influenza A (H1N1) pandemic, vaccination and infection control were the main modes of prevention. A live attenuated H1N1 vaccine mimics natural infection and works by evoking a host immune response, but currently there are no easy methods to measure such a response. To determine if an immune response could be measured in exhaled breath, exhaled nitric oxide (FE\textsubscript{NO}) and other exhaled breath volatiles using selected ion flow tube mass spectrometry (SIFT-MS) were measured before and daily for seven days after administering the H1N1 2009 monovalent live intranasal vaccine (FluMist\textsuperscript{®}, MedImmune LLC) in nine healthy healthcare workers (age 35 ± 7 years; five females). On day 3 after H1N1 FluMist\textsuperscript{®} administration there were increases in FE\textsubscript{NO} (MEAN±SEM: day 0 15 ± 3 ppb, day 3 19 ± 3 ppb; p < 0.001) and breath isoprene (MEAN±SEM: day 0 59 ± 15 ppb, day 3 99 ± 17 ppb; p = 0.02). MS analysis identified the greatest number of changes in exhaled breath on day 3 with 137 product ion masses that changed from baseline. The exhaled breath changes on day 3 after H1N1 vaccination may reflect the underlying host immune response. However, further work to elucidate the sources of the exhaled breath changes is necessary.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The influenza A H1N1 2009 pandemic affected over 214 countries and resulted in approximately 43–89 million illnesses and 8870–18 449 deaths worldwide (Center for Disease Control, World Health Organization, 6 August 2010).

6 AM and KMP are co-first authors.
when compared to inactivated or subunit vaccines live attenuated influenza vaccines (LAIV) are found to be more effective in protecting against influenza infection on a pandemic scale [3, 4]. LAIV are successful through an immediate, innate antiviral response after vaccination. The 2009 trivalent influenza vaccine was less likely to provide protection from the newer H1N1 strain because of the virus’ differences from previous strains. The monovalent vaccine was recommended because of the induction of a more specific host immune response.

A biomolecule possibly linked to influenza infection and vaccination is nitric oxide (NO). NO is a highly reactive free radical with oxidizing properties. It is endogenously produced by an enzyme class called nitric oxide synthases (NOS) which converts the substrate L-arginine to L-citrulline with the release of NO. There are three isoforms of NOS: type I NOS (neuronal NOS (nNOS)), type II NOS (inducible NOS (iNOS)) and type III NOS (endothelial NOS (eNOS)). During an inflammatory response, iNOS is either induced by cytokines, endotoxins, or oxidants resulting in an increased NOS expression and NO synthesis [5–8]. NO is measured in exhaled breath by using the fractional exhaled nitric oxide (FENO). Past studies have identified changes in exhaled NO after influenza infection, vaccination and upper respiratory tract infections [9–13]. In particular, NO has been shown to peak after viral infection and has been speculated to play a beneficial role in viral clearance [12]. Additionally, FE\textsubscript{NO} measurement is used in the assessment of airway inflammation, particularly in asthmatics. Deviations from the normal range of FE\textsubscript{NO} have also been noted in a number of conditions such as bronchiectasis, cystic fibrosis, liver cirrhosis, pulmonary hypertension, smoking and chronic obstructive pulmonary disease [14].

Beyond FE\textsubscript{NO}, breath analysis is rapidly evolving as a new frontier in medical testing for disease states of the lung and beyond. Considerable advances have been made in the field of exhaled breath analysis during the 21st century and the utility of breath analysis in healthcare is developing quickly. Exhaled breath analysis is currently used to diagnose and monitor asthma, check for transplant organ rejection, and to detect lung cancer, alcohol intoxication and Helicobacter pylori infection, among a few of its clinical applications.

We hypothesize that the immune response to live virus vaccination can result in detectable changes in FE\textsubscript{NO} and other volatile organic compounds (VOCs) in exhaled breath. Currently, there is no non-invasive method to measure the host immune response to the H1N1 influenza vaccine or active infection.

2. Methods

2.1. Study population

Healthy Cleveland Clinic workers scheduled to receive the 2009 H1N1 monovalent live intranasal vaccine (FluMist\textsuperscript{®}, Medimmune LLC) were recruited to participate in the study. A total of 11 individuals (MEAN±SD: age 35 ± 7 years; females = 5) were enrolled. One exhaled breath sample and FE\textsubscript{NO} measurement were obtained prior to receiving the vaccine (day 0) and daily after vaccination for seven days (days 1–7). All subjects were non-smokers and signed an Institutional Review Board approved consent form prior to participation in the study. Subjects were tested for influenza A (nasopharyngeal swab for influenza A, B and respiratory syncytial virus (RSV) by polymerase chain reaction) on day 0 (prior to vaccination) and day 1. Subjects who tested positive for influenza or RSV prior to vaccination (n = 2) were released from the study.

2.2. H1N1 influenza vaccine

All subjects received the 2009 H1N1 monovalent live attenuated intranasal vaccine. The vaccine was administered at a dose of 0.2 ml (0.1 ml each nare) on day 0 of the study.

2.3. FE\textsubscript{NO} measurement

One FE\textsubscript{NO} sample was provided by each study participant prior to vaccination (n = 6) on day 0 and after vaccination on days 1–7 (n = 9). The FE\textsubscript{NO} measurement was completed prior to exhaled breath sample collection. FE\textsubscript{NO} was measured using a NIOX MINO\textsuperscript{®} (Aerocrine AB, Solna, Sweden).

2.4. Exhaled breath sample collection

One exhaled breath sample was provided by each study participant (n = 9) prior to vaccination on day 0 and again on days 1–7. Subjects were asked to produce one exhaled breath sample through an exhaled breath collection device (figure 1). They were asked to exhale normally to release residual air from the lungs. The subject was prompted to inhale to the capacity of the lungs through a disposable mouth filter (Aerocrine, AB, Solna, Sweden) and an attached N7500-2 Acid Gas Cartridge (North Safety LLC, USA). The subject exhaled at an approximate rate of 350 ml s\textsuperscript{–1} through the device. The exhaled breath sample was collected in the attached Mylar\textsuperscript{®} bag, capped and analyzed within 4 h. Mylar\textsuperscript{®} bags were cleaned by flushing with 5 l of nitrogen between subjects.

2.5. Analysis of exhaled breath using SIFT-MS

Exhaled breath samples were analyzed using selected ion flow tube mass spectrometry (SIFT-MS) [15]. The instrument used in this study was a VOICE200\textsuperscript{®} SIFT-MS instrument (Syft Technologies Ltd, Christchurch, New Zealand, www.syft.com) (figure 1) that has been described elsewhere [16].

Mass-selected H\textsubscript{3}O\textsuperscript{+}, NO\textsuperscript{+} and O\textsubscript{2}\textsuperscript{+} ions generated from a microwave air discharge at ~0.5 Torr are admitted into a flow tube reactor where they are carried along the flow tube in a stream of helium. Breath samples containing the analytes for analysis are admitted into the flow tube at a known rate through a heated variable leak valve at the sample inlet. All the ion products of the chemical ionization reaction and un-reacted reagent ions are monitored by a quadrupole mass filter and an
Table 1. Exhaled breath compounds, their sources and precursor ion masses used in the SIM analysis.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Possible source</th>
<th>Precursor ion</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-propanol</td>
<td>Reduction of acetone, exogenous</td>
<td>H$_3$O$^+$</td>
<td>43 [27]</td>
</tr>
<tr>
<td>Acetone</td>
<td>Ketone bodies, stress, dieting, fasting</td>
<td>NO$^+$</td>
<td>88 [28]</td>
</tr>
<tr>
<td>Benzene</td>
<td>Exogenous</td>
<td>O$_2$+</td>
<td>78 [29]</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>Gut bacteria, exogenous</td>
<td>O$_2$+</td>
<td>76 [30]</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>Oral malodor, incomplete breakdown of methionine, gut bacteria</td>
<td>O$_2$+</td>
<td>62 [30]</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Gut bacteria, exogenous</td>
<td>NO$^+$</td>
<td>45 [27]</td>
</tr>
<tr>
<td>Isobutane</td>
<td>Cholesterol biosynthesis, ageing</td>
<td>O$_2$+</td>
<td>56 [29]</td>
</tr>
<tr>
<td>Isoprene</td>
<td>Exogenous</td>
<td>O$_2$+</td>
<td>53 [29]</td>
</tr>
<tr>
<td>Methanol</td>
<td>Gut bacteria, hydrolysis of leaving methyl groups</td>
<td>H$_2$O$^+$</td>
<td>33 [27]</td>
</tr>
<tr>
<td>Pentane</td>
<td>Lipid peroxidation</td>
<td>O$_2$+</td>
<td>72 [29]</td>
</tr>
<tr>
<td>Toluene</td>
<td>Exogenous</td>
<td>O$_2$+</td>
<td>92 [29]</td>
</tr>
</tbody>
</table>

2.6. SIFT-MS normalization

The MS data were normalized to account for variability in the precursor ion intensity by dividing each mass signal by the sum of the corresponding reagent ion signals. The inbuilt LabSYFT software and accompanying libraries (Syft Technologies Ltd) calculated absolute concentrations of SIM analytes from the precursor ion to product ion count ratio, so normalization was not required.

2.7. Data analysis

The paired t-test was used to analyze changes in breath FENO and to identify significant changes in VOCs and other product ion masses. The Pearson’s correlation test was used to identify correlations between the relative change in FENO and other VOCs and product ion masses.

Additionally, MS data were analyzed using linear discriminant analysis. All product ion masses were entered into the discriminant analysis and the ten best masses that discriminate between day 0 and day 3 were chosen during a stepwise variable selection.

3. Results

3.1. FENO results

FENO levels were normal at baseline and peaked on day 3 (MEAN±SEM: day 0 14.7 ± 2.7 ppb, day 3 19.2 ± 2.8 ppb; p < 0.001). There was no difference in FENO on any other study days compared to baseline (figure 2).

3.2. SIFT-MS results (SIM and MS data)

Analysis of the SIM data revealed that of the 11 compounds specifically monitored, only breath isoprene changed significantly during the study. Breath isoprene levels were elevated on day 3 compared to baseline (MEAN±SEM: day 3 98.8 ± 16.8 ppb, day 0 59.2 ± 14.9 ppb; p = 0.02).

The MS data (15–200 amu for each reagent ion) provided evidence that there were many masses where the intensities
changed during the study. These masses are the product ions of dozens of compounds which were not specifically identified prior to analysis. The biggest difference in exhaled breath was on day 3 after H1N1 vaccination (figure 3). When the intensities of the product ion masses on each sample day were compared to baseline there were 38 product ion masses that differed on day 1, 94 product ion masses that differed on day 2, 137 product ion masses that differed on day 3, 78 product ion masses that differed on day 4, 25 product ion masses that differed on day 6 and 80 product ion masses that differed on day 7. Interestingly, nearly all of the 137 significant product ion masses on day 3 were decreased compared to baseline.

3.3. Pearson’s correlation test

The relative change in FENO was negatively correlated to the relative change (baseline and day 3) in intensities of 37 of the product ion masses (table 2).

Table 2. Correlations between the relative change in product ion masses from the MS data and the relative change in FENO. Relative change is a comparison of the change between day 3 and baseline (day 0). The relative change in the intensity of 37 product ion masses was correlated to the relative change of FENO.

<table>
<thead>
<tr>
<th>Number of correlations</th>
<th>Pearson’s correlation (r)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>−0.95</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>23</td>
<td>−0.86</td>
<td>p = 0.01–0.05</td>
</tr>
</tbody>
</table>

3.4. Linear discriminant analysis

Ten discriminant masses were chosen during a stepwise variable selection as part of the discriminant analysis. We scanned between 15–200 amu for each reagent ion (H₃O⁺, NO⁺, O₂⁺) in the MS analysis resulting in 558 product ion masses scanned for. All 558 product ion masses were entered into the discriminant analysis and the 10 best masses that discriminate between day 0 and day 3 were chosen. These 10 masses are derived from any of the three reagent ions and they identify with at least a couple different VOCs. However, we are unsure of which specific VOCs they represent. The three most significant masses (H₃O⁺143⁺, NO⁺108⁺, O₂⁺89⁺) are displayed in figure 4.

4. Discussion

The main finding from our study is that FE NO levels peaked at day 3 after live attenuated H1N1 vaccination, which was associated with major changes in the breath profile of other VOCs in the breath. These findings provide preliminary evidence that the immune response to vaccination (and possibly a natural infection) can be detected by analyzing the exhaled breath for FE NO and possibly other volatile compounds.

Using FE NO to determine which timeframe to target for further analysis of other VOCs in breath is a novel concept.
that we introduced in this study. While analyzing all breath scans from the seven study days could be a daunting task and may have little yield, we expected that focusing on the study day with the largest change in $\text{FE}_{\text{NO}}$ would be the most effective way to find corresponding changes in a large number of breath VOCs. Our working hypothesis was that if the increase in $\text{FE}_{\text{NO}}$ on day 3 is the reflection of the induced immune response, then we expect to identify other related breath compounds in the MS data on the same day. Our approach was validated when we found that of all the days studied day 3 had the largest magnitude of change from baseline in both $\text{FE}_{\text{NO}}$ and exhaled breath compounds (figure 3). Additionally, previous findings suggest that day 3 after H1N1 influenza and human rhinovirus infection has the highest number and severity of symptoms related to infection validating why a host immune response would be prominent on day 3 after viral exposure [10, 12]. Interestingly, many previous studies identified a slight increase in $\text{FE}_{\text{NO}}$ 1 week after infection or vaccine, whereas we identified the increase on day 3 after vaccination. This may be due to a faster host immune response to the monovalent live vaccine as opposed to actual infection or use of the trivalent vaccine. Additionally, in previous studies the $\text{FE}_{\text{NO}}$ and symptom score (number and severity of symptoms) are negatively correlated, suggesting that a $\text{FE}_{\text{NO}}$ increase helps with viral clearance and protection from exacerbations of asthma [12, 18].

On day 3 we identified an increase in isoprene, a major constituent of exhaled breath produced endogenously. As reported in several studies, increased levels of breath isoprene can reflect airway oxidative stress, offering a possible explanation for the increase in isoprene on day 3 along with $\text{FE}_{\text{NO}}$ [19]. However, isoprene is mainly produced endogenously but can be influenced by variables such as exercise, sleep, age, cholesterol biosynthesis, medications and certain diseases [20–26]. Our sampling protocol accounted for many of these variables by enrolling healthy subjects who were sampled in the morning at work and not during exercise.

Thus, it may be plausible to assume that the increase in breath isoprene on day 3 was also associated with the host response to the vaccine.

Interestingly, the relative change of $\text{FE}_{\text{NO}}$ was negatively correlated to the relative change of the 37 product ion masses we identified in the MS analysis between baseline and day 3. Thus, as $\text{FE}_{\text{NO}}$ increased many breath VOCs decreased in a related way. While this is not what we expected, it is certainly possible that airway inflammation may cause a decrease in the rate of diffusion of VOCs into the lungs explaining why we found a reduction in exhaled breath compounds on day 3.

Our group and others have studied the signature of the Mylar bags. We recognize that Mylar, much like any other collection reservoir, contributes VOCs to the sample. Our rationale for using this material was that all subjects had an off-line breath collected into Mylar bags and the same Mylar bags were used throughout the study. Since we looked at the change in exhaled breath volatiles we would not expect a Mylar bag VOC contribution to change overtime (duration of this study) and we believe it was not a contributing effect.

The mechanism(s) underlying this change in $\text{FE}_{\text{NO}}$ and other breath volatiles in response to the live vaccine remain to be elucidated and require further study. Further work is also needed to identify the specific compounds in the MS data and link those compounds to the corresponding metabolic and inflammatory pathways. While the compounds identified in the SIM and MS data may be explained by metabolic end products that differ because of the underlying disease process or immune response, it is also possible that some differences can be attributed to exogenous sources such as exposure to VOCs, ingestion of certain foods, or gut bacterial flora. In either case, our findings have several important practical implications given the fact that there are currently no easy non-invasive methods to measure the immune response to a vaccine or actual infection.
5. Acknowledgment

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