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Genetic and phenotypic stability of cold-adapted influenza viruses in a trivalent vaccine administered to children in a day care setting

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Abstract

The genetic and phenotypic stability of viruses isolated from young children following intranasal administration of the trivalent live-attenuated influenza virus vaccine (LAIV, marketed in the United States as FluMist) was evaluated by determination of genomic sequence and assessment of the cold-adapted (*ca*), temperature-sensitive (*ts*) and attenuated (*att*) phenotypes. The complete genomic sequence was determined for 56 independent isolates obtained from children following vaccination (21 type A/H1N1, 12 A/H3N2, 1 A/H3N1 and 22 type B viruses), 20% of which had no nucleotide misincorporations compared with administered vaccine. The remaining isolates had from one to seven changes per genome. None of the observed misincorporations resulted in predicted amino acid codon substitutions at sites previously shown to contribute to the *ca*, *ts* or *att* phenotypes, and all vaccine-derived isolates retained *ca* and *ts* phenotypes consistent with the observation that none of the vaccine recipients displayed distinctive symptoms. The results indicate that LAIV strains undergo very limited genetic change following replication in vaccine recipients and that those changes did not affect vaccine attenuation.
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Introduction

Influenza virus is a major cause of respiratory illness in children and adults (Harper et al., 2004). There are currently three antigenic types circulating in humans, influenza A/H1N1, A/H3N2 and B viruses, against which immunization is recommended (Harper et al., 2004). The cold-adapted (*ca*), temperature-sensitive (*ts*) and attenuated (*att*) vaccine virus strains in the intranasal live-attenuated influenza virus vaccine (LAIV; marketed in the United States as FluMist) provide a convenient and effective approach for influenza vaccination of healthy individuals 5–49 years of age (Harper et al., 2003, 2004).

The two Type A and the Type B vaccine strains comprising LAIV are derived from two corresponding *ca* and *att* master donor viruses (MDV): A/Ann Arbor/6/60 [MDV-A] and B/Ann Arbor/1/66 [MDV-B] (Maassab and Bryant, 1999; Maassab et al., 1969; Murphy and Coelingh, 2002). Frequent antigenic updating of the vaccine formulation to include contemporary HA and NA antigens, recommended annually by the WHO and US Public Health Service, is required to compensate for continuous antigenic drift in the viral hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins mainly responsible for induction of protective immunity against influenza virus

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(Wright and Webster, 2001). New HA and NA proteins are incorporated into vaccine seed viruses by taking advantage of the segmented nature of the viral RNA genome that permits reassortment, replacing the gene segments encoding HA and NA of the MDV with those from the recommended wild-type influenza strains. Thus, vaccine viruses are designated 6:2 reassortants because they derive six RNA segments from the MDV (i.e., PB2, PB1, PA, NP, M and NS) and the remaining two segments (HA, NA) from the wild-type virus. Attenuated vaccinal viruses are *ca*, replicating efficiently in tissue culture at 25 °C, and *ts* which limits replication at higher temperatures (37 °C for Type B and 39 °C for Type A) such as are found in the lower respiratory tract. These critical *ca*, *ts* and attenuating phenotypes are conferred on new 6:2 reassortant vaccine viruses by genetic loci contained on the six RNA segments donated by the parental MDV (Hoffmann et al., 2005; Jin et al., 2003, 2004; Murphy and Coelingh, 2002).

Since LAIV is composed of replication-competent viruses, genetic changes could occur during replication of a virus within the vaccinee that could theoretically affect vaccinal virus phenotype. Polymerase errors, such as nucleotide substitutions, deletions or insertions during viral genome replication, could be incorporated into the vaccine viruses because virus-encoded RNA-dependent RNA polymerase lacks proofreading function. Based on estimated error rates for the influenza A virus RNA polymerase of 10^{-4} to 10^{-5} misincorporations per nucleotide position per replication cycle (Smith and Inglis, 1987), a typical 14 kilobase genome would be expected to accumulate an average of one base substitution per single round of replication. Therefore, it would not be unexpected that LAIV viruses isolated from the nasal secretions of a vaccinated individual would have accrued a certain degree of genetic change.

In addition to nucleotide substitutions accrued during replication, intratypic (i.e., between the Type A/H1N1 and A/H3N2 vaccine strains) reassortment provides a second mechanism by which the vaccinal virus genome may be modified. The reassortment of influenza virus gene segments during a mixed virus infection is a well-documented natural occurrence leading to the emergence of novel influenza viruses (Wright and Webster, 2001). One would expect that co-administration of A/H1N1 and A/H3N2 vaccinal viruses in the LAIV vaccine dose would provide conditions sufficient for the generation of intratypic vaccinal virus reassortants in vaccine recipients.

Notwithstanding the inherent mutability of influenza viruses, 6:2 reassortant vaccine strains produced from the MDVs have been shown to be phenotypically and genotypically stable when tested in monovalent, bivalent or trivalent formulations. This is thought to be due in large part to the fact that vaccinal virus attenuation results from the contribution of multiple mutations. Previous studies conducted on vaccinal viruses shed from immunized adults or children have not detected virus that was reverted for the *ts*, *ca* or *att* phenotypes (reviewed in Maassab and Bryant, 1999; Murphy and Coelingh, 2002). However, in these studies, genetic stability has largely been inferred from phenotypic stability, although a single study used limited RNA sequence analysis to demonstrate that recovered virus retained the expected 6:2 vaccinal virus genotype (Cha et al., 2000).

The present study was initiated to provide a comprehensive assessment of the genetic and phenotypic stability of LAIV vaccinal viruses following their replication in the upper respiratory tract of young vaccine recipients. Young vaccine recipients were chosen for this study because their limited prior exposure to influenza would theoretically allow vaccine viruses to replicate for a longer time period and thereby increase the chances of mutations to occur in the viral genomes. Consensus RNA sequence analysis was employed to determine nucleotide sequences of entire genomes of over 50 influenza viruses recovered from nasal secretions of children who were administered LAIV as part of a randomized, placebo-controlled, clinical trial conducted in a day care setting. The phenotypes of these clinical isolates were assessed in vitro as well, and several were further tested for attenuation in ferrets.

Results

Characterization of influenza viruses shed by clinical study participants

A total of 197 healthy children between 8 and 36 months old were inoculated intranasally with LAIV ($n=99$) or placebo ($n=98$) (Vesikari et al., submitted for publication). Twenty percent of the nasal specimens (197 of 1006) collected from LAIV recipients during the 21-day study were influenza-virus-positive. At least one vaccine strain was recovered from nasal swabs of 80% of LAIV recipients. LAIV A/H1N1, A/H3N2 and B viruses were recovered from 31, 12 and 72 vaccinees, respectively (Table 1). All viruses isolated from vaccine recipients retained the vaccinal *ca* and *ts* phenotypes in tissue culture cells (data not shown), and no wild-type viruses were isolated from vaccinated individuals.

Only 1% of the nasal specimens (10 of 1020) obtained from placebo recipients during the study were influenza-virus-positive. Influenza virus was isolated from the nasal swabs of 7 placebo recipients (Table 1). Two children shed wild-type influenza A/H3N2 virus that was closely related to A/Panama/

Table 1
Influenza virus type/subtype analysis of virus shed by study participants

Total LAIV recipients	98	(100.0%)
Influenza virus negative	20	(20.4%)
ca A/H1N1	4	(4.1%)
ca A/H3N2	2	(2.0%)
ca B	35	(35.7%)
ca B+*ca* A/H1N1	21	(21.4%)
ca B+*ca* A/H3N2	4	(4.1%)
ca B+*ca* A/H1N1+*ca* A/H3N2	6	(6.1%)
A (not sub-typed)+*ca* B	6	(6.1%)
Total placebo recipients	99	(100.0%)
Influenza virus negative	94	(95.0%)
wt A/H3N2[a]	2	(2.0%)
A (not sub-typed)	4	(2.0%)
ca B[b]	1	(1.0%)

[a] Wild-type influenza A/Panama-like virus.
[b] *ca* influenza B/Ann Arbor/1/94 virus.

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