Genetic stability of cold-adapted A/Leningrad/134/47/57 (H2N2) influenza virus: sequence analysis of live cold-adapted reassortant vaccine strains before and after replication in children

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We previously reported that the A/Leningrad/134/47/57 (H2N2) cold-adapted virus (A/Len/47) used in preparing reassortant live attenuated vaccines for children acquired 14 (11 coding) mutations in genes coding for proteins other than haemagglutinin and neuraminidase during cold-adaptation. Preservation of these mutations in genomes of viruses isolated from children on the second, fifth, or eighth day after vaccination was examined by sequence analysis. The sequence data demonstrated that all nine coding mutations selected for examination were conserved in the genomes of all 11 strains investigated, indicating that the mutations accompanying cold-adaptation and attenuation of the A/Len/47 master vaccine are highly stable.

Live attenuated reassortant vaccines are widely used in Russia for the control of influenza (Zhdanov, 1986). These vaccines are produced by reassortment of current epidemic viruses with attenuated cold-adapted (ca) strains selected for their ability to replicate at low (25 °C) temperature. The A/Leningrad/134/47/57 (H2N2) strain (A/Len/47) was obtained by 47 sequential passages of the A/Leningrad/134/57 wild-type virus (A/Len/wt) in fertile hens’ eggs (CE) at 25 °C. This ca and highly attenuated master strain is used to prepare live reassortant vaccines of the A(H1N1) and A(H3N2) subtypes for immunization of children (Alexandrova et al., 1984; Ghendon et al., 1984; Kendal et al., 1981). These reassortant vaccines are non-reactogenic and immunogenic for children 3–15 years old when used as monovalent (Kendal et al., 1981), bivalent (H1N1 + H3N2) (Alexandrova et al., 1984, 1986; Ghendon et al., 1984) or trivalent (H1N1 + H3N2 + type B) preparations (Rudenko et al., 1993).

Direct sequencing (Klimov et al., 1992) has revealed 14 nucleotide differences between the ‘internal’ genes (i.e. genes coding for proteins other than surface antigens, the haemagglutinin and neuraminidase) of A/Len/47 and A/Len/wt viruses. Of these, 11 encoded amino acid (aa) substitutions: three in PB1, two each in PB2 and PA, and one each in the NP, M1, M2 and NS2 proteins.

These sequence data established a basis for the evaluation of the genetic stability of these live attenuated reassortant vaccines, a critical consideration for vaccine safety. Earlier evidence of the stability of A/Len/47-based vaccines was based on the observation that ca strains are temperature sensitive (ts). All isolates from vaccinees retained their ts phenotype, an essential marker of vaccine stability. In genetic experiments (recombination tests with ts mutants of fowl plague virus; FPV), most virus isolates from vaccinated children displayed ts lesions in the same five (PB2, PB1, NP, M and NS) of six internal genes that were observed in the genome of the A/Len/47 donor strain (Ghendon et al., 1984; Medvedeva et al., 1990). These experiments demonstrated, however, that several viruses from vaccinees isolated at later times (days 5 to 9) after vaccination displayed an apparent loss of one or two ts lesions in the recombination test. This effect was observed most frequently for the PB2 and/or PB1 genes (Medvedeva et al., 1990); nonetheless, it was not clear whether these apparent changes in the ts phenotype were a result of reversion, extragenic suppression, or other events such as intracistronic complementation or leakiness of particular mutations. Unfortunately, the isolates investigated in the study of Medvedeva et al. (1990) are no longer available. More direct biochemical
Table 1. Sequence analysis of mutations' conservation in the genomes of Novgorod reisolates

<table>
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<tr>
<th>Strain, isolate</th>
<th>Subtype</th>
<th>Vaccine</th>
<th>Day of isolation</th>
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<th>PB2</th>
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experiments, using RNA–RNA hybridization followed by treatment with nuclease S1 and electrophoresis in polyacrylamide gels, indicated a high degree of stability of reasortant attenuated vaccines during replication in vaccinees (Ghendon et al., 1984); however, this technique was not sensitive enough to guarantee that all mutations in the vaccine virus genome were preserved.

In this study, we have used sequence analysis to investigate the conservation of mutations in the genomes of ca live attenuated vaccine viruses isolated during the second year (autumn 1990) of a large-scale vaccine trial in Novgorod, Russia (Rudenko et al., 1993). The ca A/Len/47 master strain (Alexandrova et al., 1984; Ghendon et al., 1984) was used to obtain live type A influenza reasortant vaccines by reassortment with the epidemic strains A/Taiwan/1/86 (H1N1) [A/Taiw] and A/Zakarpaktya/354/89 (H3N2) [A/Zak] (A/Shanghai/11/87-like). The ca B/USSR/60/69 master strain (Alexandrova, 1971) was used to obtain the type B live attenuated vaccine component by reassortment with the epidemic strain B/USSR/2/87 (B/Victoria/2/87-like).

The Novgorod vaccine study design and results obtained are described elsewhere (Rudenko et al., 1993).

Virus isolates for our analyses were obtained from nasal swabs collected from 7- to 10-year-old children on days 2, 5 and 8 after vaccination. The viruses were passaged once or twice in embryonated eggs (CE) (Table 1). The children from whom isolates were taken showed no symptoms of illness. Isolates 8, 10, 11 and 18 were obtained from children vaccinated with monovalent A/Taiw (H1N1) vaccine, while isolates 2/2, 2/8, 6, 7 and 2015 were obtained from children vaccinated with monovalent A/Zak (H3N2) vaccine. Isolates 2/2 and 2/8 were obtained from the same child on days 2 and 8 after vaccination, respectively. Isolates 15 and 2035 were collected from children vaccinated with trivalent [A/Zak (H3N2) + A/Taiw (H1N1) + B/USSR] live attenuated ca vaccine; antigenic analysis has shown that both of the latter isolates belong to the A(H3N2) subtype. The fact that only A(H3N2) subtype viruses were available from children receiving trivalent vaccine is most likely explained by the low numbers of isolates, since antibody responses to the A(H1N1) vaccine component were observed among at least 50% of vaccinees (Rudenko et al., 1993). The A/Len/wt virus was used as a reference strain in sequencing experiments.

All isolates recovered from children in Novgorod retained the ts phenotype of the vaccines administered; none of the type A isolates was able to grow in CE at 40 °C (not shown). It has been shown previously, by using the recombination test with FPV ts mutants, that the A/Len/47 ca master strain displays ts mutations in genes PB2, PB1, NP, M and NS (Ghendon et al., 1984). We used this test to examine whether the same ts lesions were detected in the genomes of the Novgorod reasortant vaccine strains both before and after replication in vaccinated children. Both type A vaccines [A/Zak (H3N2) and A/Taiw (H1N1)] and all virus isolates retained the ts phenotype in all the genes in which they were detected in the attenuated master strain. The titres determined at non-permissive temperature after double infection of CEF cells with the vaccines/isolates and FPV ts mutants having mutations in the genes PB2, PB1, NP, M and NS genes were at least 10^4 times less than the corresponding titres at the permissive (36 °C) temperature (data not shown).

For sequence analysis, virion RNAs were extracted from allantoic fluids by using phenol–chloroform. The
indicate a high degree of stability of mutations were sequenced for each virus isolate: PB2 1374-1614, PB1-740 (5'd GAGAATTGACATACGA), PA-5 (5'd AAAGCACGTCTGATCCG), PA-900 (5'd TGAGACACCAAGTCACGA), NP-886 (5'd TATGGACCTGCTGACGCC), M-785 (5'd CCTCTTGGTTTGCGC) and NS-673 (5'd CAAAAAGAAACCGAAAA), and the corresponding reverse complementary primers PB2rc-1614 (5'd TATTGTCAGTTTCTCTGT), PB1rc-1044 (5'd TGGATGCTCAGGACGTT), PAre-233 (5'd GCATTTGGATCATCAAGC), PAre-1511 (5'd GAGCAATTGACATACGA), Mcrc-1001 (5'd CAGCTCTATGGCTGACAA), and NSrc-865 (5'd ATAAATTGATTGCTGAAG). The same primers were used for sequencing the asymmetrically amplified PCR products in a standard Sequenase (US Biochemical) sequencing reaction (Klimov et al., 1992). In most cases sequencing in both directions was carried out.

In this study, for all isolates we investigated the stability of nine of the 14 mutations present in the ca master strain (see Table 1). We did not examine, for the majority of strains, the stability of the non-coding changes (in genes PB2, PB1 and PA), the mutation at position 1795 of the PB1 gene coding for the conservative aa substitution Val$_{1795}$ → Ile, or the mutation at position 68 of the M gene that codes for the Ile$_{68}$ → Val substitution in the M1 protein [the last mutation seems to be irrelevant to the attenuation phenotype of the A/Len/47 virus (Klimov et al., 1992)]. However, for two isolates (2/2 and 2/8) the entire PB1 and PB2 genes were sequenced and conservation of two silent mutations in these genes as well as the coding mutation at position 1795 of the PB1 gene were examined (see below).

To determine whether the nine nucleotide changes were retained in genomes of viruses isolated from vaccinated children, seven PCR-amplified fragments were sequenced for each virus isolate: PB2 1374-1614, PB1 740-1044, PA 5-233, PA 900-1218, NP 886-1200, M 785-1001 and NS 673-865. In control experiments, the sequences of similar RNA fragments of the A/Len/wt, A/Len/47 and A/Zak (H3N2) NP genes were determined. The data in Table 1 show that all nine coding changes (in genes PB2, PB1 and PA), the mutation at position 920 and two bases (A and G) at position 923 (A is the darker band in both cases). The heterogeneity observed for nucleotides 919-924 of the NP gene of virus isolates is therefore likely to be a result of the selection of different sequence variants from a mixed vaccine virus population during replication of the vaccine strains in vaccinees.

We also sequenced the entire PB1 and PB2 genes of two isolates from the same child, early isolate 2/2 and late isolate 2/8. This was done to further evaluate the possibility of genomic changes in areas other than those containing mutations that are likely to be associated with attenuation and to determine if there was a conservation of silent mutations and the coding mutation at position 1795 of the PB1 gene. The data obtained have shown that the coding mutation G-1795-A in the PB1 gene (Val$_{1795}$ → Ile substitution in PB1 protein) as well as silent mutations G-1170-A in the PB2 gene and G-360-A in the PB1 gene were conserved in the genomes of both isolates investigated. However, an additional substitution, C-1964-A in the PB1 gene of the late 2/8 isolate (Pro$_{441}$ → Gln substitution in PB1 protein), was detected. No differences between isolates 2/2, 2/8 and the A/Len/47 donor strain were found in their PB2 genes.

Our data concerning nucleotide heterogeneity in the region 919–924 of the NP gene and the existence of one additional mutation at position 1964 of the PB1 gene of a late isolate (2/8) indicate that mutations at positions other than those accompanying cold-adaptation may be selected during their replication in vaccinees. To determine the influence of such possible hidden mutations on virus attenuation, we studied the pathogenicity of the 2/2 and 2/8 isolates in ferrets. A/Shanghai/11/87 (H3N2) wt virus, which is antigenically similar to the A/Zak (H3N2) vaccine strain, was used as a control.
Ferrets (9- to 10-month-old females; three per virus) were infected intranasally with approximately $10^6$ EID$_{50}$ infectious doses. On days 2 and 4 the ferrets' nasal expectorations were taken and diluted in 100 μl of PBS. Animals were euthanized on day 4 and their lungs were taken for analysis. We were unable to isolate viruses from lung preparations in any animals investigated; however, viruses were isolated from the nasal specimens of infected ferrets. All animals infected with the wt A/Shanghai/11/87 (H3N2) virus had shown about a 1000-fold increase in virus titres in nasal expectorations between days 2 and 4 after infection. The average virus titres were about $10^2$ EID$_{50}$/0.1 ml on day 2 and $10^6$ EID$_{50}$/0.1 ml on day 4. A less than 10-fold (if any) increase in virus titres was observed during this period in nasal specimens of ferrets infected with isolates 2/2 or 2/8. Increases in temperature (of 1.0–1.5 °C above normal during the first day after infection) were observed only in ferrets infected with the wt A/Shanghai/11/87 virus but not in animals inoculated with isolates 2/2 or 2/8 (data not shown). Thus the late isolate 2/8, which had at least one extra coding mutation in the PB1 gene, was as attenuated for ferrets as its progenitor strain 2/2.

In summary, the results obtained in this study provide biological and molecular evidence for the stability of the A/Len/47 live ca master strain. Study of important biological characteristics such as the ts phenotype and virulence for ferrets indirectly demonstrated stability of mutations related to attenuation of vaccine reassortants. In terms of molecular data, our most important observation was that mutations which occurred as a result of cold-adaptation of A/Len/47 were stable during live vaccine replication in children. Although we observed the appearance of an additional mutation in the PB1 gene of one vaccine reisolate (2/8) at a position different from those encoding mutations that occurred during cold-adaptation, this mutation did not appear to diminish vaccine attenuation for the vaccinated child or for unprimed ferrets. In addition, although we observed genetic heterogeneity in the master donor and reassortant vaccine strains at two additional nucleotide positions in the NP gene, there was no detectable accompanying change in attenuation. Our observations of sequence heterogeneity were not unexpected since it is known that RNA viral populations are heterogeneous and consist of a distribution of related nonidentical genomes rather than a single defined species (Domingo & Holland, 1988). Hence, although occasional mutations at positions that seem to be irrelevant to attenuation may appear during replication in vaccinees, they do not appear to be an impediment to widespread use of live ca attenuated vaccines. The same conclusion was stated by Treanor et al. (1991) for avian–human reassortant live vaccines; however, additional studies demonstrated that reassortants of the A(H1N1) subtype sustained residual virulence for seronegative children (Steinhoff et al., 1991).

To our knowledge, the present study is the first attempt to evaluate at the molecular level the genetic stability of live attenuated influenza vaccines used during mass vaccination. Only a limited number of strains (especially late isolates) were investigated here and more isolates should be examined in order to prove the genetic stability of ca live attenuated vaccines. However, our results confirm and extend previous studies that demonstrated the genetic stability and safety of reassortant live attenuated vaccines made with the A/Len/47 donor strain for control of influenza among children.

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References


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