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Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist®)

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Abstract

Cold adapted (*ca*) B/Ann Arbor/1/66 is the master donor virus for the influenza B (MDV-B) vaccine component of the live attenuated influenza vaccine (FluMist®). The six internal genes contributed by MDV-B confer the characteristic cold-adapted (*ca*), temperature-sensitive (*ts*) and attenuated (*att*) phenotypes to the vaccine strains. Previously, it has been determined that the PA and NP segments of MDV-B control the *ts* phenotype while the *att* phenotype requires the M segment in addition to PA and NP. Here, we show that the PA, NP and PB2 segments are responsible for the *ca* phenotype of MDV-B when examined in chicken cell lines. Five loci in three RNA segments, R630 in PB2, M431 in PA and A114, H410 and T509 in NP, are sufficient to allow efficient virus growth at 25 °C. Substitution of these five amino acids with wt (wild type) residues completely reverted the MDV-B *ca* phenotype. Conversely, introduction of these five *ca* amino acids into B/Yamanashi/166/98 imparted the *ca* phenotype to this heterologous wt virus. In addition, we also found that the MDV-B M1 gene affected virus replication in chicken cells at 33 and 37 °C. Recombinant viruses containing the two MDV-B M1 residues (Q159, V183) replicated less efficiently than those containing wt M1 residues (H159, M183) at 33 and 37 °C, implicating the role of the MDV-B M segment to the *att* phenotype. The complexity of the multigenic signatures controlling the *ca*, *ts* and *att* phenotypes of MDV-B provides the molecular basis for the observed genetic stability of the FluMist® vaccines.
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Keywords: FluMist®; Influenza B virus; B/Ann Arbor/1/66 strain; Cold-adaptation; Reverse genetics

Introduction

In the U.S., annual influenza epidemics are responsible for approximately 36,000 deaths and over 100,000 hospitalizations per year. Occasional worldwide pandemics can escalate the number of deaths into the millions (Simonsen et al., 2000; Thompson et al., 2003). Human influenza epidemics are usually caused by type A and type B influenza viruses. The host range of influenza B viruses is very restricted and these viruses have only been isolated from humans and seals. The emergence of new influenza B strains is mainly driven by antigenic drift due to amino acid changes in the hemagglutinin (HA) and neuraminidase (NA) proteins and occasionally by reassortment among different strains of influenza B virus (McCullers et al., 1999).

Vaccination is the most effective means to prevent illness and complications caused by influenza infection. The FluMist® vaccine licensed in 2003 in the U.S. is a live, attenuated, trivalent influenza vaccine that effectively protects people from influenza illness (Belshe and Gruber, 2001; Belshe et al., 2004; Murphy and Coelingh, 2002). FluMist® contains two influenza A virus strains (H1N1 and H3N2) and one influenza B strain that are frequently updated based on the epidemiological studies and recommendations of the CDC and FDA. Each vaccine strain of FluMist is a 6:2 genetic reassortant that contains the six internal gene segments (PB2, PB1, PA, NP, M and NS) derived from the master donor virus for type A influenza viruses, MDV-A (*ca* A/Ann Arbor/6/60), or the master donor virus for type B influenza viruses, MDV-B (*ca* B/Ann Arbor/1/66), and the HA and NA gene segments from a currently circulating wild type (wt) strain. The six internal gene segments from MDV reproducibly confer the attenuation and safety features to the vaccine strains. Similar to MDV-A, MDV-B was originally derived by serial passage of

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the parental wt B/Ann Arbor/1/66 isolate at successively reduced temperatures in primary chicken kidney (PCK) cells (Maassab, 1967; Maassab et al., 1986). The resulting *ca* B/AA/1/66 grows efficiently at 25 °C (cold-adapted, *ca*), but its growth is restricted at 37 °C (temperature-sensitive, *ts*). In addition, MDV-B replicates in the upper respiratory tract of ferrets but inefficiently in their lungs (attenuated, *att*) (Belshe and Gruber, 2001; Murphy and Coelingh, 2002).

The *ca* and *ts* phenotypes of MDV-B are believed to contribute to the biological activity of the vaccine by facilitating efficient replication in the upper airways without producing clinical disease. The genetic basis of the *ts* and *att* phenotypes has only recently been established. Earlier studies using reassortment techniques implicated PA in the *ts* and *att* phenotypes of B/AA/1/66 (Donabedian et al., 1987). Using a plasmid-based reverse genetics system, the *ts* loci of MDV-B have since been mapped to three residues, one in PA (M431) and two in NP (A114 and H410). These three *ts* loci in combination with two amino acids in M1 (Q159 and V183) contribute to the expression of the *att* phenotype (Hoffmann et al., 2005).

The genetic signature controlling the *ca* phenotype of MDV-B has not yet been established. A previous study using classical reassortment technology showed that the PA segment from *ca* B/AA/1/66 could transfer the *ts*, but not the *ca*, phenotype to a heterologous wt strain, implying that a genetic difference exists between the *ts* and *ca* phenotypes (Donabedian et al., 1987). In this report, a series of recombinant viruses derived from MDV-B were constructed and their *ca* phenotypes analyzed. It was determined that five loci in three RNA segments, including the three *ts* loci in PA and NP plus two additional loci found in PB2 (R630) and NP (T509), were required for controlling the *ca* phenotype. In addition, the contribution of the M1 gene to virus growth in vitro was also determined.

Results

Recombinant MDV-B exhibits the ca phenotype

Plasmid rescue systems have allowed the mapping of the *ts* and *att* phenotypes of MDV-B by analyzing the biological properties of MDV-B and isogenic recombinants with defined mutations (Hoffmann et al., 2005). To characterize the *ca* phenotype, the growth of recombinant MDV-B and wt B/AA/1/66 (wt B/AA) in chicken embryo kidney (CEK) cells at 25 °C was compared to their growth at 33 °C. The CEK cells were infected with MDV-B or wt B/AA at an MOI of 0.001 and incubated at 33 or 25 °C. Virus supernatants were collected daily for 4 days and the quantity of virus was measured by plaque assay (Fig. 1). Both MDV-B and wt B/AA grew well at 33 °C, reaching titers of approximately 7.0 $\log_{10}$PFU/ml after 2–3 days of infection. In contrast, the kinetics and extent of replication of wt B/AA at 25 °C were significantly decreased compared to MDV-B. At 3 days postinfection, the titer of MDV-B at 25 °C was similar to its titer at 33 °C, while the titer of wt B/AA/1/66 was approximately 3.5 $\log_{10}$PFU/ml lower than its titer at 33 °C. Thus, MDV-B exhibited the *ca* phenotype in CEK cells while wt B/AA/1/66 did not. This difference was used to further evaluate the *ca* phenotype of MDV-B in this study.

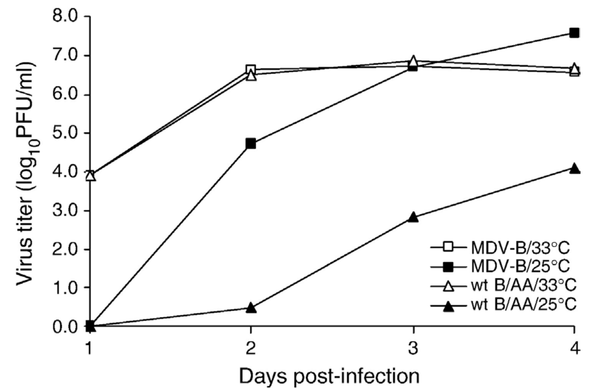

Fig. 1. Virus growth comparison of MDV-B and wt A/AA/1/66 in CEK cells. CEK cells were infected with MDV-B and wt B/AA/1/66 at an MOI of 0.001 and incubated at 33 or 25 °C. At the indicated times, the infected cell culture supernatants were harvested, and infectious virus particles were determined by plaque assay. The data represent the means of three independent experiments with standard deviations less than 10%.

Altering nine amino acids in MDV-B completely reverts the ca phenotype

MDV-B contains eight amino acids that are not present in any other wt influenza B strains and one that was only identified in one wt strain (Hoffmann et al., 2005). These residues are located in the PB2 (R630), PA (M431, H497), NP (A55, A114, H410 and T509) and M1 (Q159 and V183) gene segments. To characterize the effect of these amino acids on the *ca* phenotype, sequences encoding these nine wt amino acids were introduced into MDV-B by site-directed mutagenesis of the appropriate MDV-B gene segment plasmids. The resulting recombinant virus, rWt, was produced and examined in CEK cells for its growth at 25 °C. As shown in Table 1, MDV-B grew well at both 33 and 25 °C; the titer difference between 33 and 25 °C was only 0.3 $\log_{10}$PFU/ml. The nine amino acid substitutions incorporated in rWt thus completely reverted the *ca* phenotype. Similar to the biologically derived wt B/AA (Fig. 1), the titer of rWt at 25 °C was only 3.0 $\log_{10}$, which was 4.4 $\log_{10}$ lower than its titer at 33 °C. A recombinant virus containing seven of the nine amino acid changes (wt PB2/PA/NP) also replicated poorly at 25 °C, demonstrating that these seven amino acids were sufficient to revert the *ca* phenotype of MDV-B.

The PB2, PA and NP segments of MDV-B synergistically control the ca phenotype

A series of recombinants were constructed to identify whether the *ca* phenotype required loci in only one or multiple gene segments. Replication of these recombinant viruses at 33 and 25 °C in CEK cells was analyzed. As shown in Table 2, all these recombinant viruses grew similarly at 33 °C. The viruses containing a single wt gene segment (wt PB2, PA or NP) grew

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