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Mutational pattern of influenza B viruses adapted to high growth replication in embryonated eggs

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Abstract

Improved replication of influenza viruses in embryonated chicken eggs (CE) permits increased vaccine production and availability. We investigated the growth properties of influenza B viruses in relation to specific mutations occurring after serial passage in CE. In serial passage experiments yielding high growth variants of B/Victoria/504/2000, mutations predicted to alter amino acid (AA) composition occurred only near the receptor-binding pocket of the hemagglutinins (HA) and in no other genes. Two B/Victoria/504/2000 high growth variants had the same AA substitutions in HA (R162M and D196Y), but the higher yield variant had a third substitution (G141E), which also altered antigenic characteristics. In a serial passage experiment yielding a high growth variant of B/Hong Kong/330/2001, mutations predicted to alter AA composition occurred only in PB2 and NP in domains predicted to relate to RNP formation and function. Our results indicate that adaptation of influenza B viruses to high-yield replication by serial passage in CE requires few mutations either in internal or external genes. Specific modifications of genes or a combination of genes could be used to optimize or create influenza B viruses for specific growth substrates.

Keywords: Influenza B virus; Adaptation; Growth characteristics; Genotype

1. Introduction

Influenza A and B viruses cause epidemics associated with morbidity, mortality and economic loss annually. Unlike influenza A viruses which circulate in many animal species (avian and mammalian), influenza B viruses have been isolated almost exclusively from humans (Lamb and Krug, 2001; Osterhaus et al., 2000). Although influenza B viruses are not divided into subtypes as influenza A viruses are, two independently evolving lineages of influenza B viruses have been categorized by antigenic and genetic differences of their hemagglutinins (HA), and are represented by the prototype strains, B/Victoria/2/87 and B/Yamagata/16/88. Although one or the other may predominate, descendents of these two lineages coexist and reassort (Hiromoto et al., 2000; Lindstrom et al., 1999; Luo et al., 1999; Nerome et al., 1998; Rota et al., 1990, 1992; Shaw et al., 2002; Yamashita et al., 1988).

The complex evolution of influenza B viruses requires frequent alteration of influenza vaccine composition, which in turn means that new influenza B viruses must be adapted to high growth in the primary substrate for vaccine production, embryonated chicken eggs (CE). Although use of high growth influenza A virus reassortants incorporating the HA and neuraminidase of a wild-type virus with the internal genes of a CE-adapted donor has been a standard practice for many years (Kilbourne, 1969), similar methodology for influenza B virus has been neglected, partly because suitable CE-adapted influenza B virus donor strains have not been fully developed (Goodeve et al., 1985). In the absence of high growth reassortants, influenza B viruses are adapted to CE using serial passage as the primary means of obtaining high growth influenza B viruses suitable for producing inactivated vaccines. The unpredictability of adaptation by serial passage, however, sometimes leads to use of alternate strains

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for manufacturing purposes (HHS-CDC News, 2004; WHO, 2000).

Progress in molecular biology provides new options for preparation of high growth influenza viruses that could be used for vaccine preparation. Direct control over influenza viruses by transfection of susceptible cells with influenza virus genes inserted into plasmid vectors (reverse genetics) suggests opportunities to design viruses with required characteristics (Hoffmann et al., 2002; Jackson et al., 2002; Neumann and Kawaoka, 2001). To support the use of reverse genetics, however, better fundamental knowledge of influenza B virus replication and the role of specific genes is crucial. One way to gather information on the genetics of growth properties is to examine influenza B viruses that have been successfully adapted by serial passage to high growth in CE. Therefore, we have determined the nucleic acid sequences of the entire genomes of two specific reference influenza B viruses (B/Victoria/504/2000 and B/Hong Kong/330/2001) and descendents of them adapted to high growth in CE by serial passage. The strains chosen for examination are strains actually used in vaccine production and have passage histories that mimic typical experiences in the development of manufacturers' seed viruses.

2. Materials and methods

2.1. Viruses and cells

Reference influenza viruses B/Victoria/504/2000 and B/Hong Kong/330/2001 were received from the Centers for Disease Control and Prevention, Atlanta, GA (CDC) at CE passage levels 13 and 4, respectively. High growth virus variants (titer of hemagglutination $\geq$1:512 and infectivity $\geq$8.5 $\log_{10}$ EID_{50}/ml, Table 1) were selected by consecutive passaging of reference strains in CE with propagation of the viruses recovered from the best yielding eggs.

For virus propagation and titration, 9–10-day-old CE and Madin Darby canine kidney (MDCK) cells were used. MDCK cells were obtained from the American Type Culture Collection and maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) at 37 °C in an atmosphere with 5% CO_2.

2.2. Sera

Antisera against the reference strain B/Victoria/504/2000 and its CE-adapted variants were obtained from ferrets 3 weeks after intranasal inoculation with 10^5 EID_{50} of the reference viruses or one of the variants. Before use in hemagglutination inhibition (HI) test, each serum sample was treated with receptor-destroying enzyme from *Vibrio cholerae* (RDE, Denka Seiken Co. Ltd., Japan) to inactivate non-specific serum inhibitors. For the virus neutralization test, sera were sterilized by filtration (Millipore nylon membrane filter, Ø 0.22 μm) and inactivated by heating at 56 °C 30 min.

2.3. Titration of viral infectivity in CE and MDCK cells

Virus infectivity (EID_{50}) was determined by inoculating CE with 0.2 ml of 10-fold dilutions of allantoic fluid (3–5 eggs per dilution) and incubating at 33 °C for 72 h. The presence of virus was determined by hemagglutination. The hemagglutination titer was measured by standard methods using 0.5% chicken red blood cells (CRBC) in phosphate buffered saline (PBS) pH 7.2.

$TCID_{50}$ titers were determined in MDCK cells. MDCK cells grown to confluence in 12-well plates were washed with PBS and inoculated with serial 10-fold dilutions of virus. After 1 h at 37 °C, MDCK cells were washed and medium with 5% FCS without exogenous trypsin was added. The endpoint for calculation of $TCID_{50}$ was hemagglutination in the supernatant media. $TCID_{50}$ titers were calculated by the method of Reed and Muench (1938).

2.4. Estimation of the growth kinetic

Replicative abilities of the reference strains over time were compared to their CE-adapted variants. Ten-day-old CE were inoculated with 10^3 EID_{50} of virus. At specified times, allantoic fluids were collected for analysis of hemagglutination titer and infectivity as described above.

2.5. Determination of plaque phenotype

Plaque phenotype was determined using MDCK cells. MDCK cells grown to confluence in six or 12-well plates

Table 1
Passage history and phenotypic characteristics of B/Victoria/504/2000, B/Hong Kong/330/2001 and CE-adapted variants

Virus strain (variant)	Passages in CE	HA/titer	EID_{50}/ml, $\log_{10}$	Maximal plaque size (mm)[a]	Amino acids in HA at position[b]		
					141	162	196
B/Victoria/504/2000	13	128	6.25	3.0	G	R	D
Vict-CE-1	30	512	8.50	1.0	G	M	Y
Vict-CE-2	30	1024	9.00	3.0	E	M	Y
B/Hong Kong/330/2001	4	128	7.25	1.0	G	K	S
HK-CE	18	512	8.50	1.0	G	K	S

[a] Plaque size was measured 96 h post infection.
[b] The numbering of AA in HA molecules is aligned to that of prototype strain B/Lee/40.