Rapid Communication

Enhanced growth of seed viruses for H5N1 influenza vaccines

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Abstract

Seed viruses used to produce inactivated H5N1 influenza vaccines are recombinant viruses with modified avirulent-type hemagglutinin (HA) and intact neuraminidase (NA) genes, both derived from an H5N1 isolate, and all remaining genes from the PR8 strain, which grows well in eggs. However, some reassortants grow suboptimally in eggs, imposing obstacles to timely, cost-efficient vaccine production. Here, we demonstrate that our PR8 strain supports better *in ovo* growth than the PR8 strain used for the WHO-recommended seed virus, NIBRG-14. Moreover, inclusion of an alternative NA protein further enhanced viral growth in eggs. These findings suggest that our H5N1 vaccine candidates would increase the availability of H5N1 vaccine doses at the onset of a new pandemic.
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Introduction

The spread of H5N1 influenza A viruses from Asia to Europe and Africa, and the growing fatality toll continues to raise concerns over a possible influenza pandemic (Horimoto and Kawaoka, 2005), thus accelerating efforts to develop effective H5N1 vaccines (Wood and Robertson, 2004). Because pathogenic H5N1 viruses grow poorly in embryonated chicken eggs (the approved vessel for influenza vaccine production) and pose serious biosafety hazards, a number of candidate vaccine viruses produced by reverse genetics have been generated, including nonpathogenic recombinant viruses that possess modified avirulent-type hemagglutinin (mHA) and intact neuraminidase (NA) genes, both derived from an H5N1 strain, with all 6 remaining genes derived from a donor virus that grows well in eggs (Horimoto et al., 2006; Nicolson et al., 2005; Subbarao et al., 2003; Webby et al., 2004; Wood and Robertson, 2004). The World Health Organization (WHO) recommends A/Puerto Rico/8/34 (H1N1; PR8) as a donor strain because of its safety in humans (Wood and Robertson, 2004), but recombinant viruses do not grow as well in eggs as the original donor PR8 strain, even though they possess the same "internal" genes (i.e., those other than HA and NA) (Horimoto et al., 2006). This restriction compromises timely, cost-efficient vaccine production. Furthermore, the limited immunogenicity of H5N1 human vaccines requires higher amounts of H5 antigen than that used for annual vaccination against non-H5N1 influenza virus subtypes (Treanor et al., 2006). Therefore, a seed virus with more robust growth in eggs is needed to ensure an adequate supply of H5N1 influenza vaccine, should a pandemic occur. To address this need, we assessed the molecular basis of the growth of vaccine seed virus in eggs, and propose a construct for the vaccine seed virus with enhanced growth.

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Results

The NIBRG-14 carrying the A/Vietnam/1194/2004 (H5N1; VN1194) mHA and VN1194 NA genes and its remaining genes from PR8(Cambridge) strain (George Brownlee, personal communication) is a WHO-recommended seed virus for H5N1 inactivated vaccine now being tested in human clinical trials (Dennis, 2006). The PR8 strain used to provide the genes other than the HA and NA of the H5N1 vaccine seed strains was isolated in 1934. Since then, it has been maintained in different laboratories and differs in its growth properties depending on how it has been maintained. Consequently, the growth of seed viruses is likely affected by the PR8 strain used. Therefore, we generated a reassortant (designated PR8UW/1194-H5N1), possessing the mHA and NA identical to those of the NIBRG-14 (i.e., VN1194 mHA and VN1194 NA) and the remaining genes from the PR8(UW) strain maintained in our laboratory and compared its *in ovo* growth with that of NIBRG-14. Interestingly, we found that the growth of PR8UW/1194-H5N1 was significantly better than that of the NIBRG-14 (Fig. 1A), suggesting that our PR8(UW) strain would be superior to PR8 (Cambridge) for vaccine production in eggs. Moreover, replacing the HA and NA of PR8UW/1194-H5N1 with those of another WHO-recommended strain (A/Vietnam/1203/2004; VN1203) further enhanced *in ovo* viral growth (Fig. 1A), indicating the contribution of HA and NA to this property.

To address the molecular mechanism for the superior *in ovo* growth of PR8(UW) relative to PR8(Cambridge), we constructed a series of reassortants between PR8UW/1194-H5N1 and

NIBRG-14 by reverse genetics and tested their growth (Fig. 2). Although two of six single-gene-reassortants showed significantly lower plaque titers than PR8UW/1194-H5N1, their titers were still higher than that of NIBRG-14. By contrast, simultaneous replacement of the polymerase and nucleoprotein (NP) genes of PR8UW/1194-H5N1 with those of NIBRG-14 (see multiple-gene ressortants, Fig. 2) resulted in a decrease in virus titers to a level equivalent to NIBRG-14. We, therefore, conclude that the polymerase subunit proteins and NP in concert are responsible for the improved growth of PR8(UW) relative to PR8(Cambridge).

Although the titer of a single gene reassortant possessing the PR8(Cambridge) NS gene was higher than that of PR8UW/ 1194-H5N1 when titrated with MDCK cells by a plaque assay, this was not the case in eggs (Fig. 2); this increase in virus titers with MDCK does not, therefore, appear to be useful for vaccine production in eggs.

The HA–NA functional balance has been reported to affect the growth in eggs of seed viruses for influenza seasonal vaccines (Lu et al., 2005). Therefore, we next generated a series of reassortant viruses bearing the VN1194 mHA, the remaining genes other than NA from PR8(UW), and NA from the following N1 strains: VN1203, A/Hong Kong/213/2003 (H5N1; HK213), A/Hong Kong/486/1997 (H5N1; HK486), A/WSN/33 (H1N1; WSN), A/Kanagawa/173/2001 (H1N1; Kanagawa), and PR8(UW). Among these reassortants, only the virus with PR8(UW) NA showed significantly enhanced growth compared to the PR8UW/1194-H5N1 virus (Fig. 1B). Similarly, we generated reassortant viruses bearing VN1203
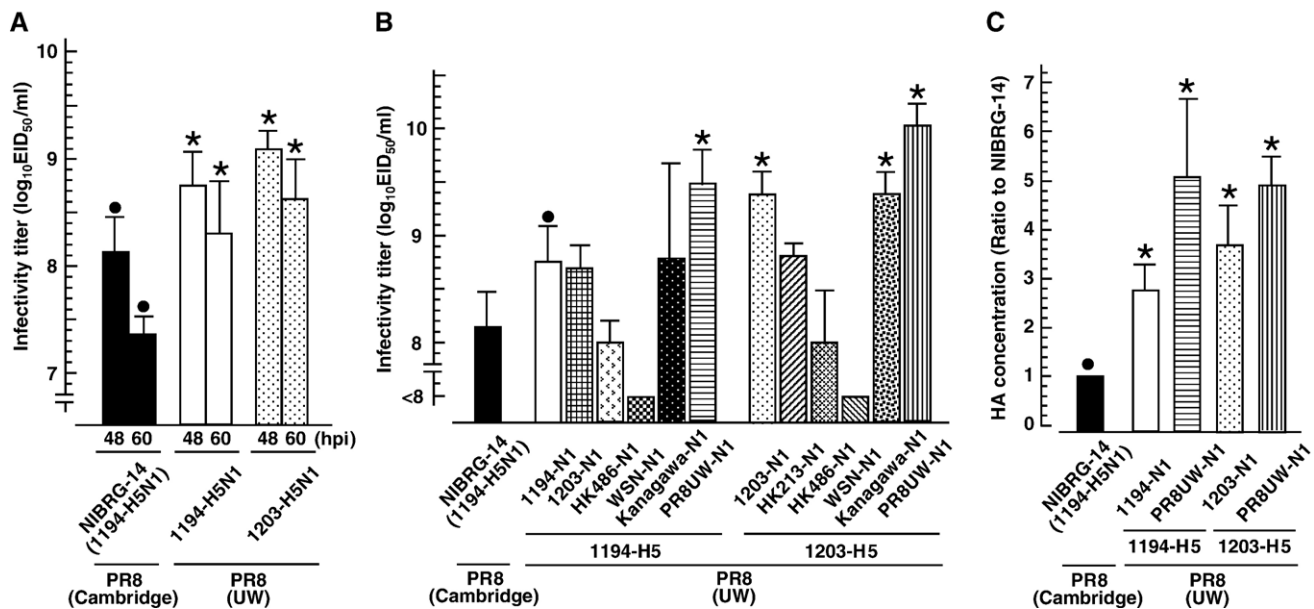

Fig. 1. Growth of PR8/H5N1 reassortant viruses in chicken embryonated eggs. (A) Viral titers (EID$_{50}$/ml) of the WHO-recommended NIBRG-14, PR8UW/1194-H5N1, and PR8UW/1203-H5N1 were determined at 48 or 60 h post-inoculation (hpi) for comparison. The data are reported as mean titers and standard deviations for >3 eggs inoculated with each virus. Viruses with significant growth enhancement compared with NIBRG-14 (●) ($p<0.05$, Student t-test with two-tail analysis) are shown by an asterisk (*). (B) Virus titers of reassortants containing mHA from either VN1194 (1194-H5) or VN1203 (1203-H5) and NA from a different N1 virus (strain name-N1) were determined. The data are reported as mean titers and standard deviations for >3 eggs inoculated with each virus. Viruses with significant growth enhancement compared with PR8UW/1194-H5N1 (●) ($p<0.05$) were shown by an asterisk (*). (C) HA concentrations of the selected reassortants are shown as ratio values to that of NIBRG-14. The data were determined as mean values and standard deviations in four independent experiments. Viruses with significant enhancement of the HA concentration compared with that of NIBRG-14 (●) ($p<0.05$) are shown by an asterisk (*).
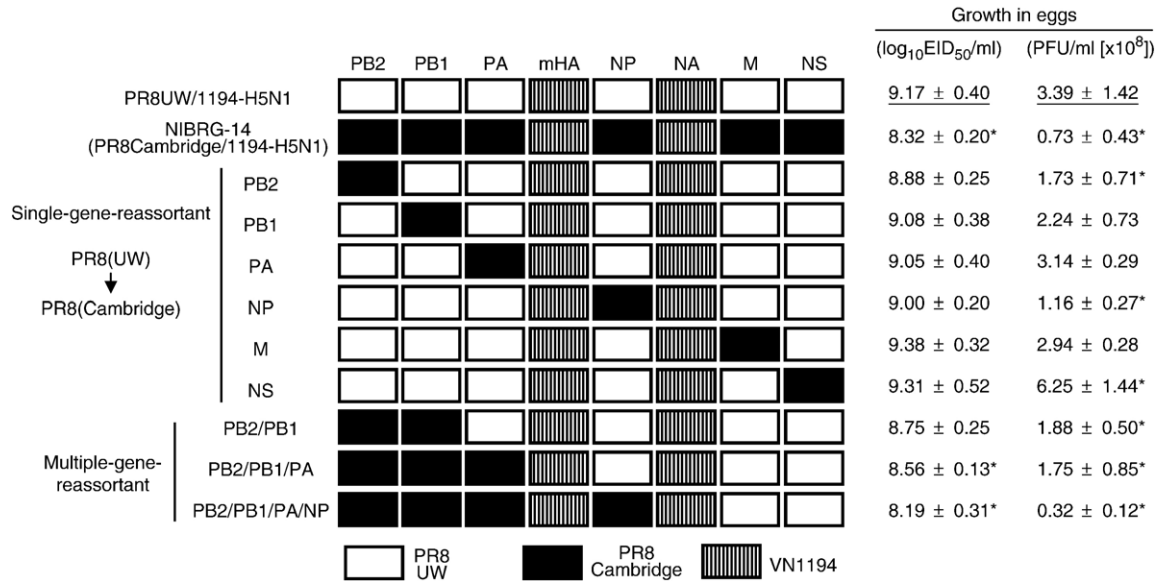
Fig. 2. Growth of PR8/H5N1 reassortant viruses containing heterologous internal genes, each derived from either PR8(UW) or PR8(Cambridge), in chicken embryonated eggs. All viruses possess mHA and NA, both derived from VN1194. Virus titers were determined in eggs (EID_{50}/ml) and a plaque assay with MDCK cells (PFU/ml) and are shown as mean titers with standard deviations. Significantly decreased or increased titers compared with that of the PR8UW/1194-H5N1 virus (underlined) ($p < 0.05$) are shown by an asterisk (*).

mHA, the different NAs, and the remaining genes from PR8 (UW). Notably, the virus with PR8(UW) NA showed the highest growth of all of the viruses tested. A greater than 20-fold enhancement of growth was observed with the virus containing VN1203 mHA and PR8(UW) NA compared to PR8UW/1194-H5N1, and a greater than 70-fold growth enhancement compared to NIBRG-14 (Fig. 1B).

The comparative experiments described above were all conducted at 37 °C, raising the possibility that differences in growth among the test viruses may be influenced by temperature. In further experiments, we found that PR8UW/1194-H5N1 grew better at 33 °C than at 37 °C by 2.2-fold ($p = 0.048$), whereas the growth of NIBRG-14 and of the reassortant containing the 1194 mHA and PR8 NA showed essentially the same growth at both temperatures (data not shown). Thus, the potential superiority of PR8(UW) relative to PR8(Cambridge) for vaccine production is independent of culture temperatures within 33 °C to 37 °C.

To evaluate whether enhanced growth in eggs correlates with an increase in HA antigens for vaccine production, we purified each reassortant from an equal amount of allantoic fluid (30 ml), following virus propagation for 48 h at 33 °C, and measured the HA concentration of each virus. Compared to the reference NIBRG-14, significantly higher (2.7- to 5.1-fold) HA concentrations were evident for our reassortants (Fig. 1C), indicating that more HA doses of H5N1 inactivated vaccines could be manufactured from a given number of eggs using our seed viruses than with NIBRG-14.

Discussion

In this study, we produced H5N1 influenza vaccine seed viruses with enhance growth in chicken embryonated eggs.

Seed viruses such as these, with more robust *in ovo* growth potential, are needed to ensure an adequate supply of H5N1 influenza vaccine, for pre-pandemic and pandemic use. We demonstrated that the PR8(UW) strain maintained in our laboratory is a superior donor virus for H5N1 vaccine production compared to the PR8(Cambridge) used to produce the reference NIBRG-14 seed virus, with respect to *in ovo* growth. PR8 strains differ in their growth properties depending on their passage histories in a laboratory; PR8(UW) may be more highly adapted in eggs than PR8(Cambridge). Since the high growth property in eggs of PR8(UW) was determined by polymerases and NP (Fig. 2), several mutations in these internal genes (as shown in Table 1) may be responsible in concert for this optimal growth adaptation.

Here, we found that HA–NA functional balance determined growth of the vaccine seed viruses in eggs. The WHO recommends that 6:2 reassortant viruses containing mHA and NA, derived from H5N1 viruses, and the remaining genes from PR8 be used as seed viruses for H5N1 inactivated vaccine production

Table 1
Amino acid differences in internal proteins between PR8 strains

Protein	Amino acid difference [Cambridge/UW(position)]
PB2	M/I(105), K/R(251), K/R(299), S/Y(360), V/I(504), R/K(702)
PB1	K/N(175), I/M(205), R/K(208), G/S(216), R/I(563)
PB1-F2	K/R(59), Q/R(60)
PA	R/K(158), L/I(550)
NP	V/L(353), V/I(425), T/N(430)
M1	None
M2	A/T(27), I/T(39)
NS1	E/K(55), E/D(101)
NS2	V/I(89)

(Wood and Robertson, 2004). However, we demonstrated that 7:1 reassortant viruses containing only mHA from H5N1 viruses (and PR8 NA) grew significantly better than 6:2 reassortant viruses. One might argue that reassortants lacking NA from an H5N1 isolate would induce a less protective immune response than recombinant viruses with the H5N1 NA, because of antigenic differences in these proteins (even though the NA of PR8 is of the N1 subtype) (Chen et al., 2000; Kilbourne et al., 1968). However, since HA is the major protective antigen in inactivated vaccines, the enhanced growth potential conferred by the PR8 NA should offset the limited antigenic mismatch in this minor protective antigen. In the event of a pandemic caused by a highly pathogenic avian influenza virus, chicken eggs will likely be in short supply. Under such conditions, a reassortant seed vaccine carrying the PR8 NA would offer an attractive option for the generation of H5N1 vaccines.

In conclusion, we propose that, in addition to the 6:2 reassortant viruses recommended by the WHO, 7:1 reassortant viruses (containing only a modified H5 derived from circulating strains) in the background of the PR8(UW) strain may be considered as vaccine seeds for H5N1 inactivated vaccine production. This approach would allow the production of more doses of pre-pandemic or pandemic H5N1 vaccines in a timely, cost-efficient manner.

Materials and methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (MEM) with 5% newborn calf serum. African green monkey Vero WCB cells, approved for use in human vaccine production (Sugawara et al., 2002), were maintained in serum-free VP-SFM medium (GIBCO-BRL) with antibiotics. Cells were maintained at 37 °C in 5% CO_2. The VN1194 and VN1203 H5N1 strains were propagated in 10-day-old embryonated chicken eggs for 2 days at 37 °C, after which time the allantoic fluids containing virus were harvested. All experiments with infectious H5N1 viruses were carried out in a Biosafety Level 3 containment laboratory. The WHO-recommended vaccine seed virus, NIBRG-14 (PR8/VN1194 6:2 reassortant) (Dennis, 2006), a kind gift from Drs. John Wood and James Robertson, National Institute for Biological Standards and Control, UK, was propagated once in eggs for further experiments.

Generation of vaccine seed viruses

To generate reassortant viruses, we used plasmid-based reverse genetics (Neumann et al., 1999). Viral RNA from VN1194 or VN1203 was extracted from allantoic fluid by using a commercial kit (ISOGEN LS, Nippon Gene) and was converted to cDNA by using reverse transcriptase (SuperScript III; GIBCO-BRL) and primers based on the consensus sequences of the 3-prime ends of the RNA segments for the H5 viruses. The full-length cDNAs were then PCR-amplified with ProofStart polymerase (QIAGEN) and H5 subtype-specific primer pairs, and cloned into a plasmid under the control of the human/mouse polymerase I promoter/terminator (referred to as PolI plasmids), generating a PolI-VN1194/HA or a -VN1203/HA construct containing each HA gene. By using an inverse PCR method, we altered the HA cleavage site sequence (RERRRKKR) of the wild-type virus to create the avirulent-type sequence (RETR), as described previously (Horimoto et al., 2006). We similarly constructed PolI-VN1194NA and VN1203NA, containing each NA gene, by the same procedure with N1-specific primers. Six pPolI plasmids each containing an internal gene of PR8 (Cambridge) were constructed from NIBRG-14. Primer sequences are available upon request. We also used our previously produced PolI plasmids, derived from WSN, PR8(UW), HK213, HK486, and Kanagawa strains for reverse genetics (Hatta et al., 2001; Horimoto et al., 2006; Kobasa et al., 2004; Neumann et al., 1999). Using these PolI plasmids, we conducted reverse genetics (12-plasmid) systems (Neumann et al., 1999). Briefly, a total of 12 plasmids containing 8 PolI and 4 plasmids expressing PR8(UW) NP, PA, PB1, or PB2 under control of the chicken β-actin promoter were transfected to Vero cells with an electroporator (Amaxa) according to the manufacturer's instructions. Sixteen hours after transfection, freshly prepared Vero cells were added to the transfected cells and, 6 h later, TPCK-trypsin (1 µg/ml) was added to the culture, which was then incubated for 4 days in serum-free medium. Supernatants containing infectious viruses were harvested, biologically cloned once by limiting dilution in embryonated eggs, and used in further experiments.

HA concentration of vaccine seed viruses

Viruses were purified through 25% sucrose from the allantoic fluid of eggs by ultracentrifugation. Pellets were resuspended in phosphate-buffered saline. For standardization of the HA content, the protein concentration of the purified viruses was measured by using a Micro BCA Protein assay kit (PIERCE). Viral proteins were then separated by SDS-polyacrylamide gel electrophoresis on a 10–20% gel and stained with Coomassie brilliant blue. The gel image was captured and analyzed by using a CS analyzer (ATTO, Tokyo), and the ratio of HA protein to total protein was determined.

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