Chimeric neuraminidase and mutant PB1 gene constellation improves growth and yield of H5N1 vaccine candidate virus

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We previously showed that a mutated PB1 gene improved the growth kinetics of a H3N2 influenza reassortant. Here, we showed that the same mutations improved the growth kinetics of a virus containing the A/Vietnam/1203/2004 (H5N1) haemagglutinin and neuraminidase (NA).

The object of this work was to improve the genomic backbone segments used in vaccine candidate viruses whilst avoiding detrimental gene constellation effects. It is not known what attributes from each segment affect the gene constellation or reassortment, but it is desirable that vaccine seed strains containing contemporary haemagglutinin (HA) and neuraminidase (NA) segments in the context of known backbone segments can be recovered. It is also desirable that those strains replicate well and that satisfactory protein yields can be harvested. We have previously shown that five mutations to the PB1 gene of the high-growth backbone virus PR/8 improved the growth kinetics of a reassortant containing the HA and NA segments from an H3N2 virus (Plant et al., 2009). Co-cultured 293T and Madin–Darby canine kidney (MDCK) cells were transfected using the method of Bourret et al. (2012). Culture supernatant was used to infect eggs and viruses containing either the WT (PB1wt) or mutant (PB1m5) PB1, in conjunction with either the WT (NAwt) or chimeric (NAC3) NA from A/VN/1203 was recovered. The HA, NA and PB1 genes were sequenced, and did not contain any additional mutations.

The stalk region of the H5N1 NA has been shown to contribute to pathogenicity (Zhou et al., 2009). Here, we confirmed that removing this region reduced pathogenicity, with or without the PB1m5 allele, by determining mean death time (MDT). Virus particles (106) were inoculated into the allantoic fluid of eight eggs, which were then incubated at 35°C and candled at 12 h intervals. Eggs were scored as alive or dead and the time of death recorded. MDT was obtained by multiplying the number of embryos dead at each interval post-inoculation by the hours post-inoculation and dividing by the total number of embryos inoculated. The MDT range was similar for all viruses (Table 1).

Optimal polymerase activity is required for efficient replication (Giesendorf et al., 2013). PB1 from a seasonal strain or with targeted mutations has been shown to improve growth or HA yield of H3 reassortants (Cobbin et al., 2013; Plant et al., 2012); however, mixed results have been observed for H5 reassortants (Abt et al., 2011; Rudneva et al., 2007; Wanitchang et al., 2010). All three polymerase segments reassort together more frequently in experimental settings than natural reassortment, suggesting gene constellation effects (Macken et al., 2006; Varich et al., 2008; Wille et al., 2013). Here, we examined the growth of H5 reassortants with the PB1m5 allele with or without a chimeric NA.

Virus was generated using the eight-plasmid reverse genetics system (Hoffmann et al., 2000). The polybasic cleavage site of the A/VN/1203 HA had previously been removed (Adamo et al., 2009). Co-cultured 293T and PR/8 improved the growth kinetics of a H3N2 influenza reassortant. Here, we showed that the same mutations improved the growth kinetics of a virus containing the A/Vietnam/1203/2004 (H5N1) haemagglutinin and neuraminidase (NA).

Total protein yield and NA activity were increased when a chimeric NA was included. These increases indicated that the synergistic effect was due to the gene constellation containing both the altered PB1 gene and the chimeric NA gene.
Improving influenza vaccine virus yield

Virus names and corresponding gene segments are indicated together with the HA titre of the virus stock and MTD (with the range).

<table>
<thead>
<tr>
<th>Virus name</th>
<th>PB1</th>
<th>NA</th>
<th>HA titre</th>
<th>MDT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV1</td>
<td>PB1M5</td>
<td>PR8/PR8/VN1203</td>
<td>1024</td>
<td>81 (72–96)</td>
</tr>
<tr>
<td>PV2</td>
<td>PB1M5</td>
<td>PR8/PR8/VN1203</td>
<td>1024</td>
<td>81 (72–96)</td>
</tr>
<tr>
<td>PV3</td>
<td>PB1M5</td>
<td>PR8/PR8/VN1203</td>
<td>1024</td>
<td>81 (72–96)</td>
</tr>
<tr>
<td>PV4</td>
<td>PB1M5</td>
<td>PR8/PR8/VN1203</td>
<td>1024</td>
<td>81 (72–96)</td>
</tr>
</tbody>
</table>

*a hemagglutinating unit.

plaque assay (Fig. 1b). Plaque assays were performed on MDCK cells incubated for 72 h at 33 °C with an Eagle’s minimum essential medium/0.8% agarose overlay and 1 μg TPCK trypsin ml−1. The Pm5Nwt and Pm5NCh viruses reached higher peak titres than analogous viruses containing the PB1wt allele. The presence of the chimeric NA and the PB1m5 allele together resulted in the highest titres among the four viruses (Fig. 1b).

The amount of HA produced from vaccine strains is important because it is the main antigenic component of inactivated vaccines. The protein profiles of each of the strains were assessed. Virus stocks were quantified using a Virus Counter (ViroCyt) and eggs were inoculated with 10⁴ virus particles. After 48 h, 36 ml allantoic fluid was harvested and virus was pelleted at 30 000 r.p.m. for 90 min in a Beckman SW32Ti rotor. The washed pellets were then purified through a sucrose cushion, pelleted and resuspended in 0.5 ml PBS. The purified virus was subjected to deglycosylation, and the resultant HA1 and HA2 fragments separated by SDS-PAGE under reducing conditions (Fig. 2a). Deglycosylation has previously been shown to improve the quantification of HA (Harvey et al., 2012). The protein bands were stained and the density of the bands was used to determine the percentage of total protein. The viral bands were quantified and the proportion of HA calculated. The viruses containing the chimeric NA (PwtNCh and Pm5NCh) had a slightly higher proportion of HA than the viruses with the WT NA (Fig. 2b).

Total protein content for each of the reassortants was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Measurements from three independent virus amplifications resulted in similar yields of ~200 μg ml⁻¹ for the virus with the A/VN/1203 surface proteins in a PR/8 background (PwtNwt) and the viruses with either the chimeric NA (Pm5NCh) or the PB1m5 allele (Pm5Nwt). The yield of total proteins for the virus containing both the chimeric NA and PB1m5 alleles (Pm5NCh) was significantly higher at 1200 μg ml⁻¹ (Fig. 2c).

Modulation of HA and NA activities has been shown to be important for viral growth (Mitnaul et al., 2000; Wagner et al., 2000) and protein yield (Adamo et al., 2009; Jing et al., 2012). We previously showed that viruses with WT or chimeric A/VN/1203 NA (JV20 and JV24, respectively) had similar NA activity (Adamo et al., 2009). We assessed the analogous viruses produced in this work (PwtNwt and PwtNCh) along with the viruses containing the PB1m5 allele (Pm5Nwt and Pm5NCh). Three independent sucrose-purified preparations for each virus were serially diluted and assayed for NA activity as described previously (Sultana et al., 2011). Vibrio cholerae NA (Sigma) was used to generate a standard curve for each assay from which activity was calculated. NA activity for the PwtNwt and PwtNCh viruses was similar (Fig. 2d), as previously observed for the analogous JV20 and JV24 viruses (Adamo et al., 2009). The mean NA activity was higher for the Pm5NCh virus containing both the chimeric NA segment and the PB1m5. It has been shown that replacement of the non-coding region of the NA segment of another H5N1 strain with a PR/8 backbone also improved growth and HA content through increased packaging of the NA segment (Pan et al., 2012).

Thus, we increased protein yield in eggs by changing the PB1 and NA genes of a reassortant H5N1 vaccine candidate strain. This work builds on previous research improving the backbone segments of donor viruses used for vaccine candidate strain production. A small number of donor viruses with high protein yield and good growth characteristics are used to produce vaccine candidate strains. However, introduction of HA and NA segments from
contemporary strains sometimes results in reduced growth and protein yield. Amino acids common to PB1 segments from circulating strains that had reassorted with the HA and NA into vaccine candidate strains have previously been shown to improve the growth of an H3N2 reassortant when they were introduced to the backbone PB1 segment from PR/8 (Plant et al., 2012). Here, we show that these mutations also enhance growth of a reassortant containing HA and NA segments from an H5N1 strain. We also recapitulate the introduction of an altered stalk region in the NA. This by itself resulted in a slight increase in the proportion of HA produced from each virus. However when the chimeric HA was present in combination with the PB1 mutations, an increase in both NA activity and total protein content was observed. As neither NA activity nor protein content increased when only one of the mutant segments was present, we suggest that this result is due to a beneficial gene constellation effect. It will be of interest to examine the mechanism behind this effect and determine whether it is at the nucleotide or protein level. Regardless of the mechanism, this particular gene constellation effect can be harnessed to improve virus growth and protein yield in vaccine manufacture.

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