Induction of protective immunity against influenza A/Jiangxi-Donghu/346/2013 (H10N8) in mice

Li-Fang Kuah, Lay-Hoon Tang, Troy Sutton, Jie-Hui Lim, Wan-Ling Sin, Elaine Lamirande, Kanta Subbarao and Yuk-Fai Lau

Abstract

Human infections with A/Jiangxi-Donghu/346/2013 (H10N8) virus have raised concerns about its pandemic potential. In order to develop a vaccine against this virus, the immunogenicity of its haemagglutinin protein was evaluated in mice. Using both whole-virion and recombinant subunit protein vaccines, we showed that two doses of either vaccine elicited neutralizing antibody responses. The protective efficacy of the vaccine-induced responses was assessed using a reverse-genetics-derived H10 reassortant virus on the A/Puerto Rico/8/34 (H1N1) backbone. The reassortant virus replicated efficiently in the respiratory tract of unvaccinated mice whereas vaccinated mice were completely protected from challenge, with no detectable viral load in the lower respiratory tract. Finally, the serum neutralizing antibody responses elicited by the H10 vaccines also exhibited cross-neutralizing activity against three heterologous wild-type H10 viruses. Collectively, these findings demonstrate that different vaccine platforms presenting the H10 haemagglutinin protein induce protective immunity.

INTRODUCTION

In December 2013, the first human case of avian influenza A H10N8 virus infection was reported in China [1, 2]. The victim was a 73-year-old woman whose illness started 4 days after visiting a live poultry market. One week after the visit to the market, she was admitted to hospital and transferred to the intensive care unit 2 days later. Four days after hospitalization (11 days after visiting the market), an influenza virus was cultured from a tracheal aspirate specimen, and the viral isolate was subsequently designated A/Jiangxi-Donghu/346/2013 (A/JX346, H10N8). The patient succumbed to the infection 9 days after the illness onset (13 days after visiting the market). The second human H10N8 infection was identified in Jiangxi province in January 2014. A 55-year-old woman was hospitalized with severe pneumonia but remained in a stable condition [3]. The third H10N8 patient was a 75-year-old man from Nanchang who presented with fever and fatigue on 4 February 2014. He deteriorated with severe pneumonia and died on 8 February 2014. In addition, subclinical H10N8 infections in humans may have occurred as three individuals had antibodies against the H10N8 virus as shown by both haemagglutination-inhibition and micro-neutralization (MN) assays [4]. Consistent with the notion that the virus transmitted to humans from live poultry, genetic analysis revealed that A/JX346 is closely related (>99 % homology) to two H10N8 viruses isolated from healthy chickens in a live poultry market [5]. In addition, the internal gene segments of A/JX346 are also closely related to H9N2 virus isolates collected from local poultry, suggesting that genetic reassortment between different influenza strains has occurred within poultry [5]. Serological evidence indicates that some feral dogs in live poultry markets in Guangdong Province have also been exposed to H10 virus [6].

In terms of receptor binding specificity, Vachieri et al. showed that the haemagglutinin (HA) protein of the avian H10N8 virus has sufficient binding avidity to human receptors to account for its ability to infect humans [7]. Other studies also demonstrated that the H10N8 virus retains a strong avian receptor binding preference [8–11] and the arginine at position 137 is important for this binding preference [8]. As the H10N8 virus is a novel influenza strain for humans, the majority of the human population lacks immunity to the virus. Thus, if the virus acquired mutations required for sustained person-to-person transmission, it would have the potential to initiate a pandemic. Since eliciting neutralizing antibodies against the HA protein is the
basis of licensed influenza vaccines, we evaluated the immunogenicity of the H10 HA protein.

In this study, a recombinant H10 virus (rH10N1 virus) was rescued with the A/JX346 HA gene on the backbone of A/Puerto Rico/8/34 (H1N1; PR8). Although the recombinant virus grew to lower titres compared to PR8 in vitro, it replicated readily in mice, especially in the lungs. To evaluate the immunogenicity of the H10 HA, our first approach was to immunize mice with the rH10N1 virus via the intraperitoneal (i.p.) route; our results show that two doses of the vaccine were required for optimal protection. As an alternative strategy, the A/JX346 HA protein was expressed in HEK 293 cells as a subunit vaccine. When the recombinant protein was administered intramuscularly (i.m.) or intranasally (i.n.), with an adjuvant (PIKA), the immune responses induced following two doses provided complete pulmonary protection from challenge with the rH10N1 virus. Finally, the antibody responses induced by these vaccines cross-neutralized wild-type avian-origin H10 viruses, including A/mallard/Portugal/79906/09 (H10N7), A/mallard/Sweden/770/2002 (H10N6) and A/quail/Italy/1117/65 (H10N8), but showed reduced activity against A/northern shoveler/Washington/44249-675/2006 (H10N2). These findings provide important information on the immunogenicity of the HA protein of the novel H10N8 virus with implications for its potential as a vaccine candidate.

RESULTS

Replication of rH10N1 virus in Madin–Darby canine kidney (MDCK) cells and mice

For immunization and challenge studies, we first rescued a recombinant H10 influenza virus (rH10N1) by reverse genetics [12]. The rH10N1 virus is a (7+1) reassortant virus that contains the HA gene of the A/JX346 isolate with the remaining genes (internal protein genes and the neuraminidase gene) derived from A/Puerto Rico/8/34 (PR8; H1N1). A recombinant PR8 (rPR8; H1N1) virus was also rescued as a control. These viruses were rescued in HEK 293 cells and further amplified in MDCK cells.

To evaluate the replication of the rH10N1 virus in cell culture, rH10N1 or rPR8 virus were inoculated onto MDCK cells in six-well tissue culture plates at an m.o.i. of 0.000625 (1/1600). Supernatant samples were collected at different time points and titrated on MDCK cells. As shown in Fig. 1(a), from 12 h after inoculation, the rH10N1 virus was significantly slower in growth than the rPR8 virus. The difference in titre between the two viruses was about 100- to 1000-fold over a 3 day period. To demonstrate that the growth attenuation is not restricted to MDCK cells, the experiment was repeated using A549 and Mv 1 Lu cells, which are commonly used for isolating influenza A and B viruses from clinical samples [13, 14]. As shown in Fig. 1(b, c), similar trends of growth restriction of the rH10N1 virus were observed in all three cell lines.

To determine whether the rH10N1 virus could replicate in mice, a group of five naive BALB/c mice were inoculated i.n. with 50 µl of the undiluted MDCK-derived virus supernatant (−2.5×10^4 TCID₅₀). Nasal turbinates (NT) and lungs were harvested 3 days later and viral titres in the tissues were determined. As shown in Fig. 1(d), the virus replicated to high titres in both the upper and lower respiratory tract. The mean lung titre was significantly higher than that in NT (P<0.002).

![Fig. 1. Growth kinetics of rH10N1 virus in vitro and in vivo. (a) Five hundred TCID₅₀ of rH10N1 (dashed line) or rPR8 viruses (solid line) was inoculated onto MDCK monolayers in triplicates. Culture supernatants were collected at the indicated time points and viral titres were determined in MDCK cells. Five hundred TCID₅₀ of the aforementioned viruses was inoculated onto A549 (b) and Mv 1 Lu (c) monolayers in triplicates, and the viral titres of the collected supernatants were determined in MDCK cells. The mean and standard deviations of the groups are presented. Asterisk (*) indicates that the difference between the two groups is statistically significant. (d) A group of five mice were inoculated with 2.5×10^4 TCID₅₀ of rH10N1 virus i.n. The viral titres in nasal turbinates (NT) and lungs were determined 3 days later. Asterisk (*) indicates that the difference between NT and lungs is statistically significant (P<0.05).]
**rH10N1 virus elicited robust neutralizing antibody responses in mice**

Since the most native form of HA recognized by the immune system is that found on the surface of the virion, we first evaluated the immunogenicity of the H10 protein by immunizing mice with live rH10N1 virus by the i.p. route. Unlike mice that received the virus i.m., mice that received the rH10N1 virus by i.p. showed no evidence of productive replication in the respiratory tract as well as weight loss and mortality (Fig. 2a–c). Groups of five mice were injected with different doses of virus by the i.p. route. Serum samples were collected on day 22 and the mice received a second dose of virus on day 28. Additional serum samples were collected on day 49. Mice were injected with rPR8 virus for comparison. The neutralizing antibody (MN) and ELISA titres in the sera were tested against the respective viruses. As shown in Fig. 2(d), for mice that received a single dose of rH10N1 virus, only the group that received $2 \times 10^5$ TCID$_{50}$ had detectable MN titres. After two doses of virus, the titres in the $2 \times 10^5$ TCID$_{50}$ group increased further and were significantly higher than the primary response. For the $2 \times 10^4$ TCID$_{50}$ group, neutralizing antibodies were detected after the second dose of virus, but the MN titres were significantly lower than the $2 \times 10^5$ TCID$_{50}$ group ($P<0.05$). Two doses at $2 \times 10^4$ TCID$_{50}$ failed to elicit a detectable MN titre in mice. The serum ELISA titres are consistent with the MN titres (Fig. 2e). In terms of the kinetics and magnitude of the neutralizing antibody responses, the mice vaccinated with rH10N1 virus and those with the rPR8 virus showed similar results (Fig. 2f, g).

To determine whether the rH10N1 virus- or rPR8-immunized mice were protected from subsequent infection, the mice were lethally challenged on day 59 with $2.5 \times 10^4$ TCID$_{50}$ of the respective viruses i.n. and viral titres in the respiratory tract were determined 3 days later. A group of mice that received PBS were challenged as controls. For rH10N1 virus-immunized mice, only the $2 \times 10^5$ TCID$_{50}$ group showed a significant reduction in challenge virus titres in the NT compared with the PBS control group ($P=0.0104$, Fig. 3a). For rPR8-immunized mice, two groups achieved a similar level of reduction of challenge virus ($P=0.0109$ and 0.0432 for $2 \times 10^4$ and $2 \times 10^5$ TCID$_{50}$ groups, respectively; Fig. 3c). In the lungs, the rH10N1 virus-immunized mice and rPR8-immunized mice showed similar results – all immunized mice showed a significant reduction in challenge viral titres; however, only the $2 \times 10^5$ TCID$_{50}$ groups showed complete viral clearance in lungs (Fig. 3b, d). The viral clearance achieved in the $2 \times 10^5$ TCID$_{50}$ in both the rH10N1 and PR8 groups was more variable. Although the mean challenge virus titre in the $2 \times 10^5$ TCID$_{50}$ group was significantly lower than in the PBS group, it was $1000$-fold lower than that observed in the $2 \times 10^5$ TCID$_{50}$ group. In addition to humoral responses detected in serum, a modest influenza-specific CD8$^+$ T-cell response with effector function was detected in the lungs and spleens of rH10N1-primed mice at the time of challenge (Fig. 3e, f). To determine whether the protection observed is subtype specific, rH10N1-immunized mice were challenged with a serologically distinct virus (PR8, an H1N1 virus). As shown in Fig. 3(g), compared to the PBS control group, the rH10N1-primed mice showed 1000-fold reduction in pulmonary viral titre, which was statistically significant ($P=0.0102$). As for the NT, although the difference did not reach statistical significance ($P=0.1105$), the average viral load of the rH10N1-primed mice was $10$-fold lower than mice that received PBS on day 3 post-infection (Fig. 3h). This suggests that apart from the neutralizing humoral responses, certain immune effectors that recognize conserved regions of the two viruses may have contributed to the viral clearance observed in this platform. In summary, the dose required to induce neutralizing antibodies by rH10N1 virus was comparable to that of rPR8-immunized and challenged mice. The observed protection in the challenge experiments was mediated by a combination of neutralizing anti-HA antibody responses as well as other non-neutralizing immune effectors.

**Expression of h10 HA protein in eukaryotic cells**

In order to determine whether humoral immunity against HA protein alone can mediate protection in a challenge experiment, we proceeded to evaluate the immunogenicity of purified recombinant H10 HA protein in mice. Unlike rH10N1 virus, purified rH10 HA protein does not contain other viral proteins such as nucleoprotein, allowing the evaluation of the protective efficacy of the HA-specific responses against viral challenge. The HA gene was cloned into a commercial protein expression vector, which was transfected into HEK 293 cells to establish a stable H10 HA-expressing cell line. The H10 HA-expressing cells were cultured in serum-free medium. Two days later, the serum-free culture supernatant was collected, purified by anion exchange and concentrated for SDS-PAGE and Western blot analyses. Under reducing conditions, there was a clear band at $75$ kDa that was positive for V5 tag (Fig. 4a), representing the monomer of the HA protein. In addition, there was also a positive signal between $125$ kDa (dimer) and $250$ kDa (trimer). Silver staining of the SDS-PAGE gel also revealed that the aforementioned bands made up the majority of the proteins present in the processed culture supernatant (Fig. 4b).

**Mice vaccinated with h10 HA protein i.m. or i.n. in the presence of an adjuvant elicited robust neutralizing antibody responses**

To test whether rH10 HA protein could elicit neutralizing antibody responses, groups of five BALB/c mice were vaccinated i.m. with $300$ ng of rH10 HA protein and subsequently boosted twice, 2 weeks apart. Because recombinant proteins are poorly immunogenic, one group received the rH10 HA with PIKA, which acts as an adjuvant. PIKA has been shown to be an effective adjuvant [15]. Sera were collected on days 13 and 27 for detection of humoral responses against rH10N1 virus by MN assay. As shown in Fig. 5(a), only the mice that received two doses of the adjuvanted vaccine had detectable MN titres. In terms of protective efficacy
Fig. 2. Inoculation of rH10N1 or PR8 virus by i.p. route led to induction of MN and ELISA antibody responses without disease or productive viral replication in mice. (a, b) Groups of five BALB/c mice were inoculated with $2 \times 10^5$ TCID$_{50}$ of rH10N1 or rPR8 viruses by i.p. route, and viral titres in NT (a) and lungs (b) were determined 3 days later. The dashed lines represent the lower detection limit of the assay. (c) Groups of five BALB/c mice were inoculated with $2 \times 10^5$ TCID$_{50}$ of rH10N1 virus by i.p. or i.n. route, and body weight was monitored for 2 weeks. All mice that received the virus by i.p. route survived on day 14, whereas only one in five mice that received the virus by i.n. route survived the infection, with a massive weight loss. (d–g) Groups of five mice were primed with rH10N1 or PR8 virus at the indicated doses i.p. Mice in the $10^5$ group received $2.5 \times 10^5$ TCID$_{50}$ of virus. The $10^4$ and $10^3$ groups received 10- and 100-fold less virus as inoculant. The mice received a boost 28 days later. Serum samples were collected on day 22 and day 49. MN (d, f) and ELISA (e, g) titres against the homologous viruses (d, e for rH10N1; f, g for PR8) were determined and expressed on a log$_2$ and log$_{10}$ scale, respectively. Each point represents the titre in an individual mouse and the short solid line is the mean of the group. Asterisk (*) indicates that there is a significant difference between the two groups. Undetectable serum neutralizing and ELISA antibody titres are assigned a value of 3.82 and 2.5, respectively.
against rH10N1 virus challenge, the groups that received the adjuvanted vaccine showed complete protection in the lungs (Fig. 5b). For the NT samples, mice that received the adjuvanted vaccine had ~10-fold reduction in viral titres compared with the PBS control mice ($P=0.0135$, Fig. 5c).

Given the importance of the upper respiratory tract in the establishment of influenza infection and previous observations that mice receiving vaccines by i.n. route had better protection in the NT, we proceeded to evaluate whether the protective efficacy in the NT could be improved through i.n. administration of the vaccine formulation. Groups of five BALB/c mice were vaccinated i.n. with 300 ng of rH10 HA protein (with or without PIKA) and subsequently boosted twice, 2 weeks apart. Sera were collected on days 13, 27 and 46 for detection of humoral responses against rH10N1 virus by MN and ELISA assays. As shown in Fig. 6(a, b), even after the third dose of the unadjuvanted vaccine, the mice did not mount significant ELISA or MN titres against the rH10N1 virus. Significant ELISA and MN titres were detected for day 27 samples from mice that received the adjuvanted rH10HA. An additional boost with adjuvanted rH10HA on day 28 led to a further increase in ELISA and MN titres on day 46. In particular, the increment in the ELISA titre reached statistical significance ($P=0.0079$), though not the MN titre ($P=0.3991$). In addition, we found that increasing the dose of the rH10 HA protein to 1100 ng did not accelerate the kinetics or increase the magnitude of the MN responses (data not shown). Furthermore, our data also showed that two doses of adjuvanted rPR8 HA protein were required to develop significant ELISA titre with some MN activity against the wt PR8 virus (Fig. 6a, b). An additional boost on day 27 further enhanced the humoral responses. To determine the protective efficacy of the induced MN responses, groups of five mice were vaccinated with two doses of rH10 protein with or without adjuvant

Fig. 3. Mice primed with rH10N1 or PR8 viruses i.p. showed reduced viral titres in the respiratory tract in a challenge experiment and the presence of influenza-specific CD8$^+$ T cells. Groups of five mice were primed with rH10N1 (a, b) or PR8 (c, d) viruses i.p. at the indicated doses ($2.5\times10^5$, $2.5\times10^6$ and $2.5\times10^7$, respectively). The mice received an additional dose of virus on day 28. On day 58, mice were challenged i.n. with $2.5\times10^4$ TCID$_{50}$ of the respective viruses. Viral titres in the NT (a, c) and lungs (b, d) were determined 3 days later. (e, f) NP-specific CD8$^+$ T cells were detected on day 58 in lungs (e) and spleen (f) of mice primed and boosted i.p. with $2\times10^5$ TCID$_{50}$ of rH10N1 virus on day 0 and day 28. The corresponding non-specific staining in Q2 for lungs and spleen, as determined by incubating the samples without the peptide stimulant, were $0.012$ and $8.52\times10^{-3}$ %, respectively. The figures are representative of three individual mice. (g, h) Five $2\times10^5$ TCID$_{50}$ rH10N1-primed and boosted mice were challenged with $2.5\times10^7$ TCID$_{50}$ of PR8 virus on day 58. Viral titres in the lungs and NT of the mice 3 days later are shown. Each point represents the titre in an individual mouse and the short solid line is the mean of the group. The long dotted line represents the lower limit of detection. Asterisk (*) indicates that there is a significant difference between the two groups.
and challenged on day 30 with $2.5 \times 10^4$ TCID$_{50}$ of rH10N1 virus i.n. Viral titres in NT and lungs were examined 3 days later. As shown in Fig. 6(c, d), the mice that received the adjuvanted rH10 HA vaccine showed complete protection from viral challenge, with no detectable virus in the upper or lower respiratory tract ($P<0.05$). In mice that received the unadjuvanted rH10 HA vaccine, consistent with the lack of MN antibodies in serum (Fig. 6a), the challenge viral titres in the respiratory tracts were similar to the PBS control group, with no significant protection observed. To ascertain whether the observed protection is subtype specific, mice that received two doses of the adjuvanted rH10 HA vaccine i.n. were challenged with PR8 virus on day 31. As shown in Fig. 6(e, f), no significant viral reduction was observed in these mice, confirming that the protection was specific against the rH10 HA protein.

**Humoral responses induced by either rH10N1 virus or rH10 HA protein neutralize other wild-type H10 viruses in vitro**

To determine whether the antibody responses induced by the rH10N1 virus or rH10 HA could cross-neutralize heterologous wt H10 viruses, sera from five mice vaccinated with either strategy were tested against four different wt H10 viruses. The HA genes of these viruses share $\sim 90$–96% sequence homology with the HA protein of A/JX346 and are from the H10N2, H10N6, H10N7 and H10N8 subtypes (Fig. 7a). The antibody response induced by i.p. immunization with rH10N1 virus neutralized the H10N7, H10N6 and H10N8 viruses (Fig. 7b). However, only two out of five mice had MN titres of 80 ($=2^{6.3}$) against the H10N2 virus. In the case of antibody response induced by immunization with adjuvanted rH10 HA protein (Fig. 7c), we detected stronger neutralizing titres against the H10N7 and H10N6 viruses, whose HA proteins are more closely related to the A/JX346 HA (Fig. 7a). Comparing the MN titres achieved by the two strategies (rH10N1 virus and adjuvanted rH10 HA protein), the MN titres against the H10N7 and H10N6 viruses were similar ($P$ values of 0.2222 and 0.0952, respectively). Most of the mice that received the rH10 HA vaccine did not have significant MN titres against the H10N8 and H10N2 viruses. Notably one particular mouse had MN titres against these two viruses ($2^9$ and $2^8$, respectively; Fig. 7c).

![Fig. 4. Expression of rH10 HA in HEK 293 cells. HEK 293 cells stably expressing rH10 HA protein were established and the culture supernatant was tested for the presence of V5-tagged rH10 HA protein by Western blot (a). The Western blot was done in reducing conditions. After separating the proteins in the processed culture supernatant by electrophoresis in reducing conditions, the protein bands were visualized by silver staining (b).](image)

![Fig. 5. Induction of humoral responses and protective efficacy of rH10 HA administered by the i.m. route with or without PIKA. Groups of five mice were primed i.m. with 300 ng rH10 HA, with or without PIKA. The mice received additional booster dose on day 14. Serum samples collected on days 13 and 27 were tested for neutralizing activity against rH10N1 virus. (a) MN titres against the homologous viruses were determined and expressed on a log$_2$ scale. Undetectable serum neutralizing antibody titre is assigned a value of 3.82. (b, c) The vaccinated mice were challenged with $2.5 \times 10^4$ TCID$_{50}$ rH10N1 virus on day 31 and the viral titres in lungs (b) and NT (c) were determined 3 days later. Each dot represents the titre in an individual mouse and the short solid line is the mean of the group. The long dashed line represents the lower limit of detection. Asterisk (*) indicates that there is a significant difference between the two groups.](image)
DISCUSSION

There have been limited studies on the virulence of H10 influenza viruses in experimental animals. Recently, Deng et al. have shown that wt H10N8 influenza viruses replicated well in the respiratory tract of mice [16]. The study also demonstrated that although most of the H10 wt viruses replicated throughout the respiratory tract, the viral load in the lungs was consistently higher than in NT [16]. In this study, the rescued (7+1) reassortant virus carrying the HA of A/JX346 caused a productive infection in the upper and lower respiratory tract upon i.n. inoculation in mice. Furthermore, the reassortant virus replicated to higher titre in lungs than the NT, analogous to what was observed with wt H10N8 viruses such as CK/JX/S3755/13 [16]. Therefore, we concluded that the (7+1) reassortant rH10N1 virus was appropriate as a surrogate challenge strain in place of the wt A/JX346 strain, to which we did not have access.

Fig. 6. Induction of humoral responses and protective efficacy of rH10 HA administered by the i.n. route with or without PIKA. Groups of five mice were primed i.n. with rH10 HA, with or without the adjuvant PIKA, or PR8 HA with PIKA. The mice received additional booster doses on days 15 and 28. Serum samples collected on days 13, 27 and 46 were tested for neutralizing activity against the respective viruses. MN (a) and ELISA (b) titres against homologous viruses were determined and expressed on a log2 and log10 scale, respectively. Each point represents the titre in an individual mouse and the short solid line is the mean of the group. Undetectable serum neutralizing and ELISA antibody titres are assigned a value of 3.82 and 2.5, respectively. (c–f) Groups of five mice were primed and received a boost with rH10 HA on day 14 as previously described. The mice were challenged with 2.5×104 TCID50 of rH10N1 virus (c, d) or PR8 (e, f) i.n. on day 30. Viral titres in the lungs (c, e) and NT (d, f) were determined 3 days later. Each dashed represents the titre in an individual mouse and the short solid line is the mean of the group. The long dashed line represents the lower limit of detection. Asterisk (*) indicates that there is a significant difference between the two groups. Results are representative of two independent experiments. The MN titre and viral load data of the second experiment can be found in Fig. S1 (available in the online Supplementary Material).
We previously demonstrated that priming mice twice by the i.p. route with a reassortant H7 virus provided complete protection against challenge infection with wt A/AnHui/1/2013 (H7N9) virus [17]. In this study, we extended this approach by examining the viral dose required for eliciting robust immune responses and protection from viral challenge. Based on the serum MN titres (Fig. 2) and viral titres 3 days after challenge (Fig. 3a, b), our results showed that $2 \times 10^5$ TCID$_{50}$ of virus was required to induce a neutralizing antibody response, especially for the first dose because only mice that received $2 \times 10^5$ TCID$_{50}$ had significant MN titres on day 22 (Fig. 2). In addition, the significant increase in MN titres between day 22 and day 49 suggested that the additional dose was essential to achieve optimal protection. Since the genomes of rH10N1 and PR8 viruses differ only in the HA gene, the i.p. inoculation could also have elicited immune responses against other structural proteins, such as neuraminidase. These 'non-neutralizing' immune effectors have been shown to be capable of providing protection from homologous and heterologous challenge in vivo [18, 19] potentially accounting for the subtype-independent protection observed in rH10N1-primed mice. On the other hand, our rH10 HA protein data demonstrated that a robust HA-specific humoral immunity alone could provide complete protection from viral challenge (Fig. 6c, d). The inclusion of an adjuvant, such as PIKA, in the vaccine formulation was essential for the induction. The kinetics of the antibody responses increased significantly [15, 17, 20]. In terms of immunogenicity, a certain recombinant HA, such as rH7 HA, has been reported to be less immunogenic than seasonal HA in mice [21]. H5 HA is also less immunogenic.

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Accession number</th>
<th>Differences in amino acid residues compared to A/JX346</th>
</tr>
</thead>
</table>

**Fig. 7.** Neutralization of heterologous wt H10 viruses with serum samples generated in this study. (a) The amino acid sequences of the indicated viruses were aligned with the A/JX346 sequence and their differences are listed. The numbering is in reference to the methionine at the start of the ORF of the HA protein. The common differences shared by all the wt H10 viruses are in bold. (b, c) The rH10N1 virus- (a, day 49 samples from Fig. 2) and rH10 HA-induced (b, day 46 samples from Fig. 6) serum samples generated in this study were tested for their ability to neutralize other wt H10 viruses. Each point represents the titre in an individual mouse and the short solid line is the mean of the group. The long dotted line represents the lower limit of detection. Hash symbol (#) indicates that there is a significant difference between MN titre against the rH10N1 virus and the tested heterologous H10 virus.
in humans, requiring two doses of 90 μg HA to achieve neutralizing antibody responses typically associated with protection [22, 23]. The strong MN titres achieved in both the rH10 HA (Fig. 6) and rH10N1-prime mice (Fig. 2d), as compared to those induced by rPR8 HA, demonstrated the good antigenicity of the H10 HA in mice.

In this study, mice that received the adjuvanted vaccine by the i.n. route were better protected than the i.m. group in the upper respiratory tract (Figs 5c and 6d), likely because the i.n. route is more effective than parenteral routes in inducing IgA responses [15]. Using baculovirus-derived recombinant HA, Wohlbold et al. showed that mice that were immunized twice i.m. with 5 μg HA with poly I: C were protected from weight loss during challenge [24]. Using 300 ng of HEK cell-derived HA, our results are consistent with their findings and show significant reduction of virus in lungs and NT of vaccinated mice 3 days post-challenge (Figs 5b and 6c, d). Although PIKA’s stimulatory effect on the innate immune system induces a transient protection against the replication of a broad spectrum of influenza virus in mice [25], the subtype-specific protection observed in the rH10 HA-prime mice (Fig. 6e, f), together with the protection observed in the i.m. model (Fig. 5), contradicts the likelihood that the protection observed in this study was mediated through anti-viral effects of PIKA on the innate immune system, such as type I interferon production.

Using the serum samples generated by the two platforms, we found that the A/JX346-induced antibodies exhibited neutralizing activity against three of the four wt H10 viruses tested. The fact that the HA gene of A/JX346 belongs to the Eurasian avian lineage might explain why the A/JX346 HA-induced responses showed reduced MN activity against the North American lineage H10N2 virus isolated from a northern shoveller in the USA in 2006. A separate vaccine might be required to elicit robust responses against H10 viruses in the North American lineage.

In summary, we have generated a reassortant rH10N1 influenza virus and demonstrated that it replicates efficiently in the respiratory tract of mice. The immunogenicity of the H10 HA protein was evaluated in mice using two different vaccination platforms. Similar to other HA proteins studied previously, two doses of vaccine are required for induction of an immune response that provides complete protection from viral challenge. In the event that an H10N8 influenza virus gains sustained human-to-human transmissibility and poses a pandemic threat, these findings will assist in the design of a vaccine strategy to mitigate the burden of this virus.

METHODS

Viruses

rH10N1 virus is a (7+1) reassortant virus on a A/PR/8/34 (PR8) background, containing the HA gene of A/Jiangxi-Donghu/346/2013 (H10N8, GISAID accession no. EPI497477), generated using reverse-genetics techniques previously described [12]. The DNA fragment of the HA gene was chemically synthesized by Life Technologies and inserted between the pol I and pol II promoters of pATE plasmid. The original pATE plasmid was provided by Dr Brendon Hanson, DSO National Laboratories, Singapore [20]. The virus stock was propagated in Madin–Darby canine kidney (MDCK) cells and subsequently aliquoted for storage at −80°C. The wild-type H10 isolates A/mallard/Portugal/79906/09 (H10N7) and A/mallard/Sweden/770/2002 (H10N6) were kindly provided by Dr J. Waldenstrom, Linnaeus University, Sweden. A/quail/Italy/1117/65 (H10N8) was obtained through BEI Resources, NIAID, NIH and A/northern shoveler/Washington/44249-675/2006 (H10N2) was provided by Dr H. Ip, US Geological Survey, National Wildlife Health Center, WI, USA. All wt H10 isolates were grown in 10-day-old embryonated chicken eggs.

Growth kinetics

MDCK (CCL-34), A549 cells (CCL-185) and Mv 1 Lu (CCL-64) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Eagle’s minimal essential medium (EMEM; Lonza) supplemented with 10% FCS, L-glutamine and antibiotics. The cells were seeded in six-well plates in serum-containing medium. After overnight incubation, the cells were washed twice with plain EMEM. Five hundred TCID₅₀ of viruses was inoculated into the plates in 3 ml of EMEM supplemented with l-glutamine, antibiotic and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Culture supernatants were collected at 8, 12, 24, 48 and 72 h after inoculation. The viral titres of the collected supernatants were determined in a standard TCID₅₀ assay using MDCK cells.

Cloning of recombinant H10 HA protein expression plasmid

A DNA fragment encoding the amino acid sequence HA₁₆₅₋₅₂₃ was amplified from a synthetic DNA plasmid using Pfu DNA polymerase (Stratagene). A human interleukin-2 secretary signal peptide sequence was introduced upstream of the HA sequence. A spacer sequence, V5 epitope tag and the trimeric GCN4pII heptad repeat [26, 27] were introduced at the C terminus and cloned into the pcDNA5 plasmid using InFusion HD Cloning kit (Clontech). The sequence of the resultant recombinant plasmid was confirmed by sequencing.

Recombinant H10 HA protein

HEK 293 cells stably expressing the rH10 HA were created by transfecting Flp-In 293 cells (Life Technologies) with the confirmed H10 HA plasmid and pOG44 using the manufacturer’s protocol. HEK 293 cells were seeded in 100 mm tissue culture dishes (Greiner Bio-One) at 1.5×10⁵ cells in 10 ml of EXCELL 293 Serum-Free Medium for HEK 293 cells (Sigma-Aldrich) supplemented with l-glutamine and Dulbecco’s modified Eagle’s medium. The supernatant was collected 2 days later and clarified by low-speed centrifugation. The HA protein was adsorbed onto a Sartobind Q15 membrane adsorber (Sartorius) and eluted using a Tris-base buffer with 1 M sodium chloride. The eluted protein was further
Serum samples were serially diluted in half-log dilutions in 96-well plates pre-coated with 50 µl of rH10N1 virus (10^{5.7} TCID_{50} ml^{-1}) or rH10 protein (20 µg ml^{-1}). After overnight incubation at 4°C, bound antibodies were detected using polyclonal goat anti-mouse immunoglobulin conjugated with HRP (Dako). ABTS [2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); Sigma-Aldrich] was used as a substrate; the reaction was stopped with 1% SDS solution after 20 min, and the color intensity was measured with a microplate reader (VersaMax; Molecular Devices) at a wavelength of 405 nm and reference wavelength of 450 nm. An optical density of >0.2 was considered to be positive.

Micro-neutralization assay

The assay was performed as previously described [15]. Serially, twofold-diluted serum samples were tested against 100 TCID_{50} rH10N1 virus or wt H10 influenza viruses. After incubation at room temperature for an hour, the serum/virus mixture was inoculated onto MDCK cells in four replicates. The plates were scored for cytopathic effect 4 days later, and the neutralizing antibody titre was defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of the inoculated virus and expressed on a log_{2} scale.

Intracellular cytokine staining

The assay was performed as previously described [28]. In brief, single cell suspensions from various organs were stimulated with GolgiPlug (BD BioSciences) in the presence or absence of 1 μM CTL epitope (TYQRTRALV) for 5 h. Epitope-specific gamma interferon-secreting CD8+ cells were enumerated using a BD FACS Canto II flow cytometer. Data were analysed using FlowJo.

Statistical analysis

Experimental groups were compared using the Mann–Whitney test in Prism 5 (GraphPad Software). P values of <0.05 were considered significantly different.

Funding information

This work is supported by funds from DSO National Laboratories and Future Systems and Technology Directorate (FSTD), Ministry of Defence, Republic of Singapore and in part from the Division of Intramural Research, NIAID, NIH. The funders had no role in study design, data collection and interpretation of the data.

Acknowledgements

We thank Ms Glennis Ong Yu Xuan and Tan Li Si from Ngee Ann Polytechnic for their technical assistance. The authors also gratefully acknowledge Yang Lei of WHO Chinese National Influenza Center, Virology Institute, Chinese CDC for depositing the sequence of the HA gene of A/JX346 in the GISAID Epiflu database.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The animal study protocols were approved by the Institutional Animal Care and Use Committee of DSO National Laboratories.

References


Lau YF, Tang LH, Ooi EE. A TLR3 ligand that exhibits potent inhibition of influenza virus replication and has strong adjuvant activity has the potential for dual applications in an influenza pandemic. *Vaccine* 2009;27:1354–1364.


