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Short Communication

Induction of neutralizing antibodies to influenza A virus H7N9 by inactivated whole virus in mice and nonhuman primates

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ABSTRACT

We evaluated the immunogenicity of hemagglutinin (HA) in the context of inactivated H7N9/AH/1/13-PR8 whole-virion. At 4 weeks after immunization with 15 μg HA, mice produced hemagglutination inhibition (HI) titers of 1:192 and neutralizing antibodies of 1:317. Aluminum hydroxide (alum), or a booster immunization, or both increased HI to 1:768, 1:384, 1:896 and neutralizing antibodies to 1:1868, 1:2302, 1:10,000, respectively. Macaques generated HI of 1:190 or 1:360 and virus neutralizing titers of 1:280 or 1:658 at 3 weeks after immunization with HA alone or with alum. Sera from immunized mice and macaques protected mice from infection of A/Anhui/1/2013 (H7N9), suggesting an H7N9 vaccine is immunologically feasible.

Since the Spring of 2013, the emergence of a new avian influenza A H7N9 virus in China has caused 325 human infections and 75 deaths as of February 13, 2014, with case fatality rate at over 20% (World Health Organization, 2014). The 2013 H7N9 virus has low avian pathogenicity and thus can be spread silently in migratory and poultry birds. There is a potential risk of this virus acquiring more efficient human infection and transmission if the virus co-infects with seasonal influenza viruses in a human host. Although there is no apparent evidence to support an immediate mass H7N9 vaccine production at present, a vaccine should be considered as the most important intervention if a pandemic occurs. Some groups have stated concerns that a H7N9 vaccine may encounter the challenge of poor immunogenicity (Osterholm et al., 2013). One human trial using an inactivated subunit H7N7 vaccine showed poor antibody response even with two doses of 90 μg HA (Couch et al., 2012). An adjuvanted H7N1 subunit vaccine was also poorly immunogenic in humans (Cox et al., 2009). Based on bioinformatic analysis, it has been postulated that the emerging H7N9 may have low immunogenicity due to lack of T-cell epitopes (De Groot et al., 2013). This could become a problem if there is a need to produce large amount of vaccine within a short period of time, especially as humans have no previous exposure to this virus and have no pre-existing immunity in the population (Li et al., 2014). Therefore, we aimed to understand if the HA of H7N9/Anhui/1/2013, in the context of inactivated whole virus, can induce specific neutralizing antibody responses in mice and non-human primates.

We generated H7N9/AH/1/13-PR8 by reverse genetics (Fodor et al., 1999; Neumann et al., 1999) in the background of A/Puerto Rico/1934 (PR8) containing synthesized HA and NA coding sequences based on the sequences of A/Anhui/1/2013 in GISAID Epiflu database (www.gisaid.org). H7N9/AH/1/13-PR8 virus was purified by using a sucrose gradient and then inactivated by treating with 0.1% Formalin at 4 °C for 3 days. Evaluation of the HA protein content of purified virus was estimated to make up ~30% of the total protein of purified H7N9/AH/1/13-PR8 virus based on historical data and SDS–PAGE analysis (Oxford et al., 1981; Harvey et al., 2008). The amounts of total protein were measured by BCA protein assay kit (Pierce).

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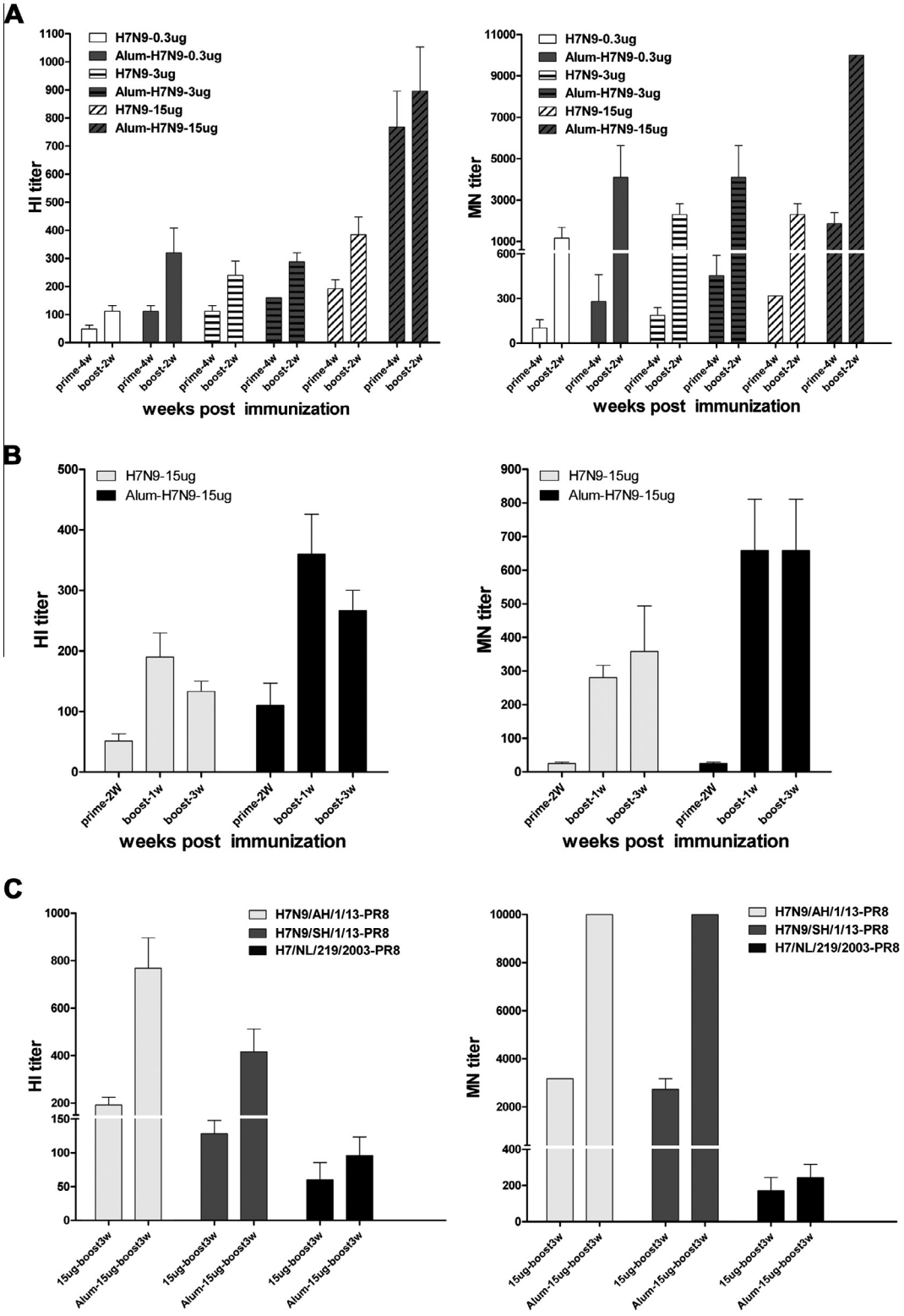

Fig. 1. Hemagglutination inhibition and virus neutralizing titers of serum samples from immunized mice and rhesus macaques. (A) HI and neutralizing antibody titers against H7N9/AH/1/13-PR8. Serum samples were collected from mice at 4 weeks after a single immunization or 2 weeks after a booster immunization (4 weeks after the first immunization) with 0.3, 3, and 15 μg of HA alone or with alum. Each bar represents mean titers from 5 mice ± SEM (standard error of means). (B) HI and neutralizing antibody titers from macaques against H7N9/AH/1/13-PR8, H7N9/SH/1/13-PR8 and H7/Netherlands/219/2003-PR8. Four rhesus macaques received the first immunization and a booster immunization at 2 weeks with 15 μg of HA without or with alum. Serum samples were collected at 2, 3, and 5 weeks after the first immunization. (C) HI titers and neutralizing antibody titers against recombinant viruses carrying HAs of A/Anhui/1/2013 (H7N9), A/Shanghai/1/2013 (H7N9), and A/Netherlands/219/2003 (H7N7). Serum samples were collected from mice at 3 weeks after a booster immunization with 15 μg of HA alone or with alum. Each bar represents mean titers from 5 mice ± SEM. The rhesus macaques and mice were housed and handled in accordance with the guidelines set by the Association for the Assessment and Accreditation of Laboratory Animal Care. The study protocol was approved by the GIBH Institutional Animal Care and Use Committee (Protocol number 2011038, 2013010).

For vaccination in mice, 6–8 weeks old female Balb/c mice were intramuscularly injected with 100 μl of inactivated whole-virus containing 0.3 μg, 3 μg or 15 μg HA, either alone or with aluminum hydroxide (alum) as an adjuvant. We used two assays to assess the antibody response: the hemagglutination inhibition (HI) assay using chicken red blood cells, which measures the antibodies that act against the globular head of HA and thus inhibit the interaction between virus and sialylated host receptor; and the microneutralization assay using MDCK cells and anti-NP based ELISA, which directly measures the neutralizing activity (Hirst, 1942; Rowe et al., 1999). At three weeks after immunization, the mice immunized with 15 μg of HA alone or with alum elicited HI titers of 1 to 48 or 152. At 4 weeks after immunization, mice immunized with 0.3, 3, or 15 μg HA alone had the HI titers reached 48, 112, and 192, whereas the virus neutralizing antibodies reached 102, 186, and 317, respectively (Fig. 1A). With alum, the same amount of HA produced HI titers of 112, 160, and 768, whereas the virus neutralizing antibodies reached 280, 453, and 1868 respectively (Fig. 1A). Therefore, the use of alum could enhance the virus neutralizing antibodies. We also boosted a group of mice with the second injection 2 weeks later. These mice had HI and neutralizing titers significantly higher than the mice that received one injection (Fig. 1A). The booster effect is more drastic for the lower HA group. HI titers increased from 48 to 112, whereas the virus neutralizing titers increased from 102 to 1160 for the 0.3 μg HA alone. For the 0.3 μg HA with alum, a booster immunization increased HI titers from 112 to 320 and increased the virus neutralizing titers from 280 to 4102. This result indicated that a booster immunization, especially with alum, could effectively elicit HI and neutralizing antibodies. The enhancement at the lower antigen dosage indicates that it is possible to spare the use of antigen if there is a need to maximize the number of people for immunization within a short period of time. On the other hand, a single immunization with higher-dose HA, especially with alum, is able to rapidly induce higher HI and neutralizing titers. We used alum, the most commonly used and the only adjuvant that has license in China, in this study. Other adjuvants, such as MF59, and AS03, which have been shown to enhance antibody response with influenza vaccines (Langley et al., 2010; Khurana et al., 2010), could be tested in countries where there is regulatory approval. Indeed, clinical trials using MF59 and AS03 are being tested in the United States (http://clinicaltrials.gov/ct2/results?term=h7n9&Search=Search).

To understand if the HA of H7N9/AH/1/13 in the context of inactivated whole-virus could induce HI and neutralizing antibodies to H7N9 virus in animal species that may be more relevant to humans, we immunized four Chinese rhesus macaques via intramuscular injection. At 2 weeks after immunization with 15 μg HA alone or with alum, HI titers were 51 or 110 (Fig. 1B). At 1 week after a booster immunization (3 weeks after the first immunization), HI titers increased to 190 or 360, and the virus neutralizing titers reached 280 or 658 in macaques immunized with 15 μg HA alone or with alum (Fig. 1B). The HI titers and the virus neutralizing titers maintained at the similar levels at 3 weeks after a booster immunization.

We also tested whether the antibodies elicited in mice immunized with H7N9/AH/1/2013-PR8 have cross-reactivity against other H7 viruses. We detected a comparable reactivity in both HI and virus neutralizing activities against H7/SH/1/13-PR8, a recombinant virus carrying HA from A/Shanghai/1/2013 (H7N9) which has 9 amino acid differences in its HA versus A/Anhui/1/2013 (H7N9) (Gao et al., 2013). Interestingly, there is a significant cross-reactivity against H7/NL/219/03-PR8, a recombinant virus carrying HA from A/Netherlands/219/2003 (H7N7). The HI titer is 60 and 96 without or with alum, while the virus neutralizing titer is 172 and 245 without or with alum respectively (Fig. 1C). This H7N7 virus caused 89 human infections and one death in Netherlands in 2003 (Fouchier et al., 2004; Koopmans et al., 2004). The HA of A/Netherlands/219/2003 (H7N7) has 24 amino acid differences in its HA versus A/Anhui/1/2013 (H7N9), which is 95.9% identity in amino acid sequence with the HA of A/Anhui/1/2013 (H7N9). This result suggested that a vaccine based on HA from A/Anhui/1/2013 is likely to elicit highly reactive neutralizing antibodies against variants of H7N9 virus and have certain cross-protectivity against other H7 subtypes. These cross-reactive antibodies are likely bind to the conserved antigenic sites, since the antigenic sites especially site A are highly conserved among H7 viruses (Goff et al., 2013). Furthermore, we reconfirmed the HI activities against two wild type H7N9 viruses isolated from infected people,

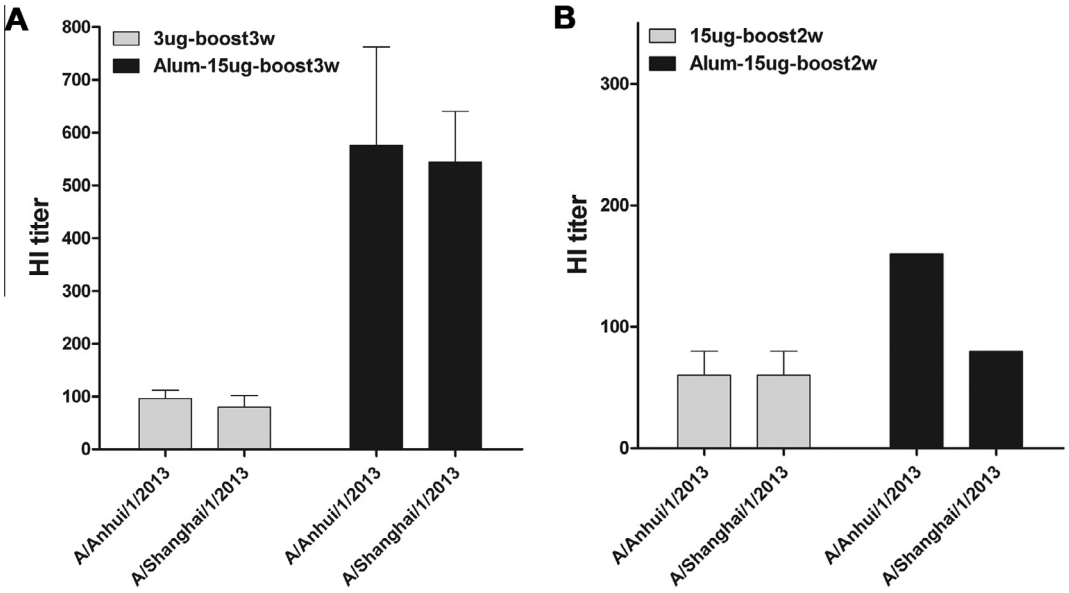

Fig. 2. Hemagglutination inhibition and virus neutralizing activities against wild type viruses A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9). (A) Serum samples were collected from mice at 3 weeks after a booster immunization (5 weeks after the first immunization) with 3 μg of HA alone or 15 μg of HA with alum. Each bar represents mean titers ± SEM from 5 mice. (B) Serum samples were collected from 4 macaques at 2 weeks after a booster immunization (2 weeks after the first immunization) with 15 μg of HA alone or with alum.

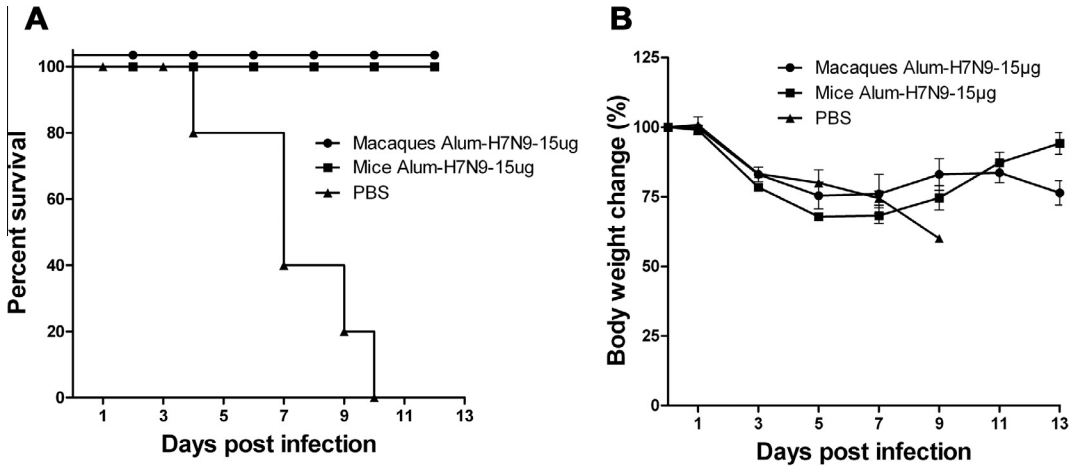

Fig. 3. Passive transfer of sera from immunized mice and macaques protected mice from infection with A/Anhui/1/2013 (H7N9). Female 6 week old Balb/c mice were divided into 3 groups ($n = 5$) and received a single intraperitoneal injection of sera from immunized mice or macaques at 2 h before intranasal infection with 10 LD_{50} A/Anhui/1/2013 (H7N9) virus. One group of mice received 150 μl of sera collected from mice immunized with 15 μg HA with alum, which had HI and virus neutralizing titers at 1 to 768 and 1868. Another group of mice received 200 μl of sera from rhesus macaques at 4 weeks after a booster immunization with 15 μg of HA, which had HI and virus neutralizing antibody titers at 220 and 620. (A) The survival curve. (B) The change of body weight.

including A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9). Sera collected from immunized mice and rhesus macaques after a booster immunization showed comparable HI titers to both A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) (Fig. 2).

Since the animal facility at GIBH is not qualified to perform direct challenge of immunized mice with H7N9 virus, we sent the sera collected from immunized animals to a Bio-safety Level 3 animal facility at ILS to test if mice received immunized sera can be protected from lethal infection of A/Anhui/1/2013 (H7N9). After receiving a single injection of sera collected from immunized mice or macaques, mice were challenged with 10 LD_{50} of A/Anhui/1/2013 (H7N9). Body weights were measured every 2 days. Mice that lost over 35% of original body weight were euthanized and counted as dead (Hu et al., 2012). All mice that received sera from immunized mice or macaques did survive, whereas all mice that received PBS instead of serum died by 9 days (Fig. 3A, B). It is important to note that the transfer of only 150 μl of sera, which accounts for only 1/4–1/3 of total blood volume in a mouse, could confer 100% protection from lethal infection of H7N9. This result demonstrated the antibody generated is highly effective in protecting mice against H7N9 infection. These data also suggest that the sera from H7N9 vaccine-immunized or convalescent individuals may have prophylactic or therapeutic uses.

In summary, we demonstrate here that immunization with HA in the context of inactivated whole virus H7N9/AH/1/13-PR8 can induce HI and neutralizing antibodies to H7N9 viruses in mice and non-human primates. The use of alum or a booster immunization increased the antibody titers. The sera from immunized mice or rhesus macaques could confer protection to recipient mice challenged with lethal dose of H7N9 virus. We conclude it is immunologically feasible to make an effective H7N9 vaccine. During the process of this work, several other labs reported positive studies in evaluating the immunogenicity of H7N9 vaccines in the context of inactivated whole virus, or split vaccine, or surface antigen as VLP, and have even started clinical trials (Krammer et al., 2014; Smith et al., 2013; Fries et al., 2013, ClinicalTrials.gov. Identifier NCT01934127). Therefore, a H7N9 vaccine is reachable if there is ever a need.

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