An H7N1 influenza virus vaccine induces broadly reactive antibody responses against H7N9 in humans

Abstract

Emerging H7N9 influenza virus infections in Asia have once more spurred the development of effective pre-pandemic H7 vaccines. However, many vaccines based on avian influenza viruses - including H7 - are poorly immunogenic as measured by traditional correlates of protection. Here we re-evaluated sera from an H7N1 human vaccine trial performed in 2006. We examined cross-reactive antibody responses...
to divergent H7 strains including H7N9, dissected the antibody response into head versus stalk reactive antibodies, and tested the in vivo potency of these human sera in a passive transfer H7N9 challenge experiment in mice. Although only a low percentage of vaccinees induced neutralizing antibody responses against the homologous vaccine strain and also H7N9, we detected strong cross-reactivity to divergent H7 HAs in a large proportion of the cohort using a quantitative ELISA method. Furthermore, H7N1 vaccination induced antibodies to both the head and the stalk domain of the HA which is in sharp contrast to seasonal inactivated vaccines. Finally, we were able to show that both, neutralizing but also non-neutralizing antibodies improved in vivo virus clearance in a passive transfer H7N9 challenge model.

Introduction

Influenza A H7 subtype viruses have caused sporadic infections in humans in the past (1-3). These incidents have triggered the development of pre-pandemic vaccine candidates that have been tested in animal models and humans (4-10). However, H7 vaccines have proven to be of low immunogenicity in humans when traditional correlates of protection like the hemagglutination inhibition (HI) titer were used as read-out (5, 6). In the spring of 2013 human cases of infection with a novel H7N9 strain were reported to the World Health Organization (WHO) by Chinese authorities (11). Although no sustained human-to-human transmission has been detected so far for this novel H7 subtype, the outbreak triggered fears about a new pandemic since the virus causes a high case fatality rate (12), is able to transmit in mammalian animal models (13-16), shows binding to alpha-2.6 linked sialic acid (14, 17, 18) and quickly developed resistance against neuraminidase (NA) inhibitors in several cases (19). After a period with very little activity during the summer months of 2013, the virus regained momentum and...
more than 250 cases have been reported for the 2013/14 winter season (12). In order to proceed with the development of successful H7 vaccines it is necessary to understand the type of immunity that these vaccines induce. The HI titer is commonly used as correlate of protection for seasonal influenza virus vaccines. However, pre-pandemic avian H7 influenza virus vaccines are notorious for inducing no or very low HI titers. It is therefore important to investigate if, in the case of H7 vaccines, the immune response is directed against other regions of the hemagglutinin (HA) that do not induce HI-reactive antibodies, like the HA stalk domain. Stalk-reactive antibodies are known to be broadly neutralizing but even non-neutralizing HA binding antibodies could play a role in protection via mechanisms like antibody-dependent cell mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (20-23). Our study re-examined sera collected during an H7N1 vaccine trial (the vaccine containing an Eurasian H7 HA, Figure 1) conducted in 2006-07 at the University of Bergen (UIB), Norway (5). This is one of only four H7 human clinical trials conducted prior to the H7N9 outbreak. Using recombinant HA proteins from divergent H7 viruses (Figure 1A) as the substrate, we performed quantitative endpoint titer ELISA to measure the magnitude and breadth of the antibody response. Novel analytical tools allowed us to dissect the immune response into head- and stalk-reactive antibodies and we also assessed cross-reactivity to H7N9 by HI assays. Finally, we evaluated the biological relevance of our findings in an H7N9 passive transfer challenge model.

**Materials and Methods**

**Cells and viruses**

Madin Darby Canine Kidney (MDCK, ATCC# CCL-34) cells were culture in Dulbecco’s Modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, HyClone) and 100 units/ml of penicillin and 100 μg/mL of streptomycin (Pen/Strep, Gibco). Sf9 cells (ATCC# CRL-1711) were grown in
TNM-FH insect cell media (Gemini Bioproducts) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 units/ml of penicillin and 100 μg/mL streptomycin (Pen/Strep, Gibco) and 0.1% Pluronic F68. BTI-TN-5B1-4 cells (Vienna Institute of Biotechnology subclone(24)) were maintained in serum free SFX media (HyClone) containing 100 units/ml of penicillin and 100 μg/mL of streptomycin (Pen/Strep, Gibco). The 6:2 reassortant virus carrying the HA and NA segments from A/Shanghai/1/13 (H7N9) and the internal genes from A/Puerto Rico/8/34 (H1N1) was grown in 8 day old embryonated eggs as described before and the viral titer was determined using a plaque assay in MDCK cells (25, 26). H7N9 virus like particles (VLPs) based on A/Shanghai/1/13 were expressed in mammalian cells as described before (27, 28). Viruses/reagents used in this study are further described in Table 1.

Recombinant proteins

Recombinant HA proteins from A/chicken/Jalisco/12283/12 (H7N3, chickJal12), A/mallard/Netherlands/12/00 (H7N3, mallNL00), A/chicken/Italy/5093/99 (H7N1, chickIT99), A/Shanghai/1/13 (H7N9, SH13) and A/shearwater/West Australia/2576/1979 (H15N9, H15) and A/New Caledonia/20/99 (H1N1, NC99) were expressed in the baculovirus system as soluble trimers with a C-terminal trimerization domain and hexahistidine tag (to facilitate purification) as described before (29, 30) (see Table 2). The phylogenetic relation between the HAs used in this study is shown in Figure 1A. Expression constructs for the chimeric H4/7 protein (H4 head domain from A/duck/Czech/1956 (H4N6) on top of an H7 stalk domain from A/Shanghai/1/13 (H7N9), cH4/7) were designed as described before with the disulfide bond between cysteins 52 and 277 (H3 numbering) as demarcation line between head and stalk domain (31). The protein was expressed in the same way as wild type HAs. Globular head-only constructs from A/Shanghai/1/13 (H7N9, H7 head-only) and A/duck/Czech/1956 (H4N6, H4 head-only) were expressed by N-terminal fusion of the globular head domain (amino acid 52 to 277, H3 numbering) to a signal peptide from the HA of A/Puerto Rico/8/34. On the C-terminus a GCN4pII trimerization domain (32).
domain and a hexahistidine tag were added. The head domain was connected to the signal peptide and the trimerization domain via glycine linkers. Recombinant NA proteins from A/California/04/09 (H1N1, avian-like NA) and A/Shanghai/1/13 (H7N9) were expressed in the baculovirus expression system featuring an N-terminal hexahistidine tag and tetramerization domain as described before (30). The phylogenetic relationship between NAs used in this study is shown in Figure 1B. All proteins (except the H4 head construct) were secreted into the cell supernatant, purified via Ni-NTA (Qiagen), buffer exchanged to PBS pH7.4 and concentrated as described before (29, 30).

Human Serum samples

Sixty healthy young volunteers (aged 19–39 years) were enrolled in an open label phase I dose escalating clinical trial in 2006-7 (Figure 2A). The trial was approved by the regional ethics committee (REK Vest) and the Norwegian Medicines Agency. The trial is registered in the WHO Initiative for Vaccine Research (http://www.who.int/immunization/diseases/influenza/clinical_evaluation_tables/en/) and the EudraCT Database (EudraCT #. 2006-001582-42). All subjects provided written informed consent before inclusion in the study and to subsequent use of their sera in analysis of the influenza specific immune response. Volunteers were randomly assigned to one of four vaccine groups and were intramuscularly vaccinated with two doses of split cell culture derived H7N1 (RD3) virus vaccine (21 days part) containing 12μg or 24μg hemagglutinin (HA) alone or with aluminum hydroxide adjuvant (300ug or 600ug respectively). The RD3 strains was generated by reverse genetics using the genetically modified HA (multibasic cleavage site deleted) and the NA from the A/chicken/Italy/13474/99 (H7N1, chickIT99) virus on a A/Puerto Rico/8/34 (H1N1, PR8) backbone. Serum samples were collected pre (0), 21, 42 (2 patients were sampled on d41, 13 on day 43 and 1 on day 45) and 180 (6 months) days after vaccination. All samples were coded with a unique identification number, aliquoted and stored at –80 °C until used in this study.
Hemagglutination inhibition (HI) assay

Pre- and post-vaccination receptor-destroying enzyme (RDE) treated serum samples from each subject were serially diluted and incubated with 4 hemagglutination units of the homologous vaccine virus (PER.C6® cell grown RD3 virus) or SH13 (H7N9) VLPs (UIB) and subsequently with 1% (v/v) horse erythrocytes. For HI assays with SH13 VLPs pre- and post-vaccination RDE treated sera were pretreated with packed horse erythrocytes prior to analysis in the HI assay as described above. The individual HI titers are expressed as the reciprocal of the highest dilution at which hemagglutination was inhibited and titers <8 were assigned a value of 5 for calculation purposes. Assays were performed in duplicates or triplicates for each sample.

Microneutralization (MN) assay

Pre- and post-vaccination microneutralization titres were determined by a MN assay as previously described (32). Serum samples were tested from an initial dilution of 1:20 against the Per.C6 grown RD3 virus. Sera with titres of ≥20 were considered positive and antibody titres <20 were assigned a value of 5 for calculation purposes. Assays were performed in duplicates or triplicates for each sample.

ELISA

Immulon 4 HBX plates were coated overnight at 4°C with 2 μg/ml recombinant protein in a pH 9.4 carbonate/bicarbonate coating buffer (50 ul/well). Plates were then blocked at room temperature for 1 hour with PBS containing 0.1% Tween 20 (TPBS) containing 3% New Zealand goat serum (Gibco) and 0.5% non-fat dry milk powder (GM-TPBS). Serum was pre-diluted 1:100, and then serially diluted in GM-TPBS in 1:2 steps. After 2 hours of incubation at room temperature plates were washed three times with...
100 μl of TPBS/well and incubated with an anti-human IgG horseradish peroxidase labeled secondary antibody (Sigma) diluted 1:3000 in GM-TPBS (50 μl/well). After 2 hours of incubation plates were washed again as described above and developed using o-phenylenediamine dihydrochloride (SigmaFast OPD, Sigma) as substrate. Reactions were stopped after exactly 10 minutes with 3M HCl (50 μl/well) and plates were read at an optical density of 490 nm. To be able to quantitatively compare ELISA results we performed an endpoint titer analysis. The endpoint titer was defined as the last serum dilution at which the reactivity (signal at OD490nm) was above a cutoff of the average plus three standard deviations of negative control wells (secondary antibody only). Samples examined by ELISA included day 0 (n=60 for all analysis), day 21 (performed only for the homologous HA, n=60), day 42 (n=53 available serum samples except for H15 were n=52 samples were analyzed) and 6 month post infection (n=26 available samples).

IgG avidity ELISA

Sera were evaluated for relative avidity of IgG antibodies against the SH13 HA. Ninety-six well plates (Nunc maxisorp, Roskilde, Denmark) were coated with 0.3 μg/ml HA in PBS and incubated at 2-8 °C overnight. Plates were blocked with 5% milk, 0.1% Tween-20, 1% BSA solution in PBS prior to addition of appropriate serum dilutions giving an OD450 of 0.7 ± 0.3 and incubation for 1 hour. Subsequently, sera were treated with 1.5M NaSCN for 1 hour. Bound antibodies were detected using mouse anti-human IgG HRP (BD, USA) at a final dilution of 1:4000 and TMB substrate (Europa Ltd). After stopping the reaction with 0.5N HCl, the absorbance was read at 450nm subtracting a background reference of 620nm. The antibody binding resistance to 1.5M NaSCN was calculated as: (OD450 treated serum/OD450 untreated serum) × 100%.
Passive transfer challenge experiment

We chose patients (n=10 per group) with similar increase in reactivity who had HI active antibodies (HI+) on day 42 post vaccination or did not induce HI active antibodies (HI-). Samples for both pools came predominantly from the adjuvanted groups; the HI+ group had a mean induction (by ELSA endpoint titer) of 4.93 as compared to 6.06 in the HI- group. The geometric mean endpoint titer on day 42 was 1:5701.8 for the HI+ group and 1:4354.5 for the HI- group. Four pools of sera (equal volumes from each patient) from these two cohorts were generated: 'day 0 HI-', 'day 42 HI-', 'day 0 HI+' and 'day 42 HI+'. All day 0 samples were HI negative in both groups. Pooled sera (250 ul/mouse) were then injected intraperitoneally into 6-8 week old female BALB/c mice (n=6 per group). Two hours later the mice were anesthetized by injecting 0.1 ml of a ketamine/xylazine mixture (0.15 mg/kg and 0.03 mg/kg) intraperitoneally. Mice were then infected with 300 plaque forming units of SH13 H7N9 virus. On day 3 and day 6 three mice per group were sacrificed and lungs were harvested and homogenized in 600 ul of pH 7.4 PBS using a BeadBlaster 24 (Benchmark) homogenizer. To determine the lung virus titers the homogenates were plaqued on MDCK cells. Plaques were counted after three days of incubation at 37°C and immunostaining with convalescent serum from H7 infected mice. Mice were handled according to the Mount Sinai Institutional Animal Care and Use Committee (IACUC).

Statistical analysis, phylogenetic analysis and structure visualization

For statistical analysis of ELISA endpoint titers we used a parametric paired t-test, statistical analysis of lung titers in the passive transfer experiment was performed using a parametric unpaired t-test in Prism 6 (GraphPad). Correlation analysis was performed using the standard linear regression model in Prism 6. Trees and sequence alignments were computed using ClustalW. Trees were visualized in FigTree, molecular structures were visualized in Protein Workshop.
Results

Induction of homologous and heterologous neutralizing antibodies

In 2006, sixty healthy volunteers were divided into four groups and vaccinated with an H7N1 vaccine containing 12 or 24µg HA alone or with aluminum hydroxide adjuvant. Fifty-four volunteers received a second dose of vaccine at day 21. Upon initial analysis of day 42 sera only a very small proportion (1.9% or 1 out of 54, Figure 2B and D) of the vaccinees reached an HI titer of 1:32, the criteria to show protection (5). A total of 39% of the vaccinated individuals tested positive for HI active or neutralizing antibodies (Figure 2B, C and E), although all but one had HI titers lower than 1:32 (5). A selection of the strongest 10 responders was chosen to be re-evaluated against H7N9 with SH13 H7N9 VLPs (27). HI activity was detectible in nine out of ten sera pre-adsorbed with horse red blood cells (Figure 2F) showing good HI cross-reactivity between the vaccine strain and H7N9.

Induction of long-lasting immunity as measured by ELISA

Using an ELISA with homologous chickIT99 HA as substrate we analyzed day 0, day 21, day 42 and 6 months serum samples for reactivity to the vaccine strain. Day 0 endpoint titers were low with a geometric mean titer of 1:673. The low titers indicate low levels of cross-reactive antibodies against group 2 HAs, confirming previous findings (33) (Figure 3A). Upon vaccination the geometric mean titer increased to 1:1270 on day 21 and to 1:3200 on day 42 (both highly statistically significant, p<0.0001) which are inductions of 1.89- and 4.74-fold respectively (Figure 3A and C, geometric means of induction). On day 42 69.8% of the vaccinees had experienced an increase in titer of 4-fold or more relative to day 0, 24.5% had experienced a 2-fold induction and only 5.7% were non-responders (Figure 3B). This is in sharp contrast with the results from the classical serology (HI/MN) data where only 39% of the vaccinees showed any reactivity. Interestingly, the titers did not wane over a period of 6 months and even increased towards the end of the observation period with a geometric mean endpoint titer of...
1:3755 (highly significant, p<0.0001) and a mean induction over day 0 of 6.13 (Figure 3A and C). As a negative control, an H1 HA from the human seasonal NC99 strain was used since we did not expect an increase in antibodies against this HA after vaccination because it belongs to a different HA group (group 1, Figure 1A). However, titers against the NC99 H1 HA did not significantly increase (Figure 3C and D).

**Effect of the Al(OH)₃ adjuvant**

Vaccinees in the original clinical trial were divided into four different groups that received vaccine doses with 12 or 24 ug of HA with or without Al(OH)₃ as adjuvant. The low vaccine dose induced HI/neutralizing titers in 21.4% in the non-adjuvanted cohort and 50% in the adjuvanted group (Figure 4A). Similarly the high vaccine dose induced HI titers in 23.1% of the individuals in the non-adjuvanted group while resulting in 61.5% positives in the adjuvanted group (Figure 4A) (5). Endpoint titers at day 42 in the non-adjuvanted low vaccine dose group were induced 3.1-fold on average while addition of adjuvant resulted in a 7.3-fold induction (Figure 4B). Likewise, induction in the non-adjuvanted high dose group was on average 3.6-fold compared to 6.9-fold in the comparable adjuvanted group (statistically significant, p=0.0374) (Figure 4B). This data strongly suggest that the adjuvant, but not the dose, had an impact on vaccine immunogenicity.

**No correlation between ELISA endpoint titers and HI reactivity**

ELISA reactivity and HI activity both require binding of antibodies to the HA. While ELISA assays detect binding without restriction to a specific location on the HA molecule, HI active antibodies have to bind in close proximity or directly at the receptor binding site of the HA and are considered a surrogate correlate for neutralization (34). The receptor binding site is located in the membrane distal, genetically highly flexible globular head domain of the HA. To assess if there is a correlation between the ELISA reactivity and the HI activity of sera we performed a correlation analysis between the ELISA endpoint
titers and the HI titers (Figure 4C). Analysis of HI titers versus ELISA endpoint titers showed no correlation ($r^2 = 0.02571$) (Figure 4C) indicating that the antibody response might be directed against non-canonical epitopes (non-HI) on the HA.

Reactivity to heterologous and heterosubtypic HAs and avian-origin NA

Next, we wanted to examine whether vaccination with H7N1 vaccine was also able to induce antibodies against HA from the novel H7N9 strain and against other heterologous H7 strains from the Eurasian and the North American H7 lineage (Figure 1). Endpoint titers against H7 from H7N9 strain SH13 (Eurasian lineage) increased significantly ($p<0.0001$) from 1:1361 on day 0 to 1:3600 on day 42 which represents an average increase (geometric mean) of 2.63-fold (Figure 5A and M) with 43.4% of the vaccinees having a 4-fold or higher induction, 32.1% having a 2-fold induction and 24.5% being non-responders (Figure 5E). Similarly, titers against another Eurasian lineage strain, mallNL00, rose from an average endpoint titer of 1:1838 to 1:4738 (Figure 5B and M). This represents a mean induction of 2.56 between day 0 and day 42 with 47.2% of the individuals reacting with a 4-fold or higher induction, 22.6% with a 2-fold induction and 30.2% who did not respond (Figure 5F and M). Average endpoint titers against the North American lineage H7 HA from chickJal12 started with 1:867 on day 0 and increased on average 2.85-fold to 1:2432 on day 42 (Figure 5C and M) with 49.1% of the vaccinees having a 4-fold or higher induction, 32.1% having a 2-fold induction and 18.9% non-responding (Figure 5G). ChickIt99, mallNL00 and SH13 are all closely related members of the Eurasian H7 lineage and this cross-reactivity is not unexpected due to very similar amino acid sequences and structures (Figure 1A and C). Interestingly, strong cross-reactivity also exists towards chickJal12, which is a member of the more distantly related North American lineage (Figure 1A). This cross-reactivity is probably based on the completely conserved antigenic site A (Figure 1D) and on shared epitopes in the conserved stalk domain. In addition to cross-reactivity towards divergent H7 HAs we also wanted to see if the H7N1 vaccine induced antibodies...
against heterologous members of the group 2 HAs. Interestingly endpoint titers against H15 also rose from a mean of 1:641 to 1:1428 which represents an induction (geometric mean) of 2.25 fold (Figure 5D and M). However, only 30.8% of the vaccinees reacted with a 4-fold or higher increase, the majority of individuals showed only a 2-fold (46.2%) or no (23.1%) increase (Figure 5H). Finally, we also wanted to see if the vaccine induced an increase in reactivity against avian-type N1 NAs. Although the average endpoint titer increased from 1:981 on day 0 to 1:1318 on day 42 (p=0.0038) this represents only a 1.24-fold increase on average (Figure 5L and M). It has been noted that both antibody titers but also antibody affinity/avidity play an important role in virus neutralization. We therefore also examined the long term avidity of day 42 and 6 month post vaccination sera in a sodium thiocyanate elution ELISA with SH13 HA as substrate. Interestingly, avidity increased significantly (p=0.004) from a mean of 12.9% on day 42 to a geometric mean of 18.9% 6 month post vaccination (Figure 5N).

**Reactivity to the divergent head and conserved stalk domain of the HA**

The lack of correlation between HI and ELISA titers and the good reactivity against heterosubtypic H15 HA suggested that a proportion of the antibody response induced by the H7N1 vaccine is directed against epitopes outside the classical antigenic sites. In order to assess whether this response is mainly directed against the head domain (where classical antigenic sites are located (34)) or the conserved stalk domain of the HA we expressed a chimeric H4/7 HA that consists of the head domain from H4 and the stalk domain from SH13 H7. In addition we also expressed trimeric globular head only-domains from H7 SH13 and from H4. These recombinant proteins were then used in endpoint titer ELISAs. We measured a highly significant (p<0.0001) increase in mean endpoint titers from 1:1008 on day 0 to 1:2107 on day 42 for cH4/7, a 2.17 fold increase on average (Figure 5I and M). Mean endpoint titers for the H7 head-only construct were 1:888 and increased to 1:1686 on day 42 which is 1.95-fold increase on average (p<0.0001) (Figure 5J and M). To control for reactivity towards the H4 head domain we also analyzed the...
response against an H4 head-only construct. Mean endpoint titers for the H4 head-only control increased minimally (although statistically significant, p=0.029) from mean endpoint titers of 1:673 on day 0 to 1:721 on day 42, a marginal increase of 1.1-fold (Figure 5K and M). These results suggest that H7N1 vaccination induced antibodies against the head and the stalk domain.

In vivo relevance and potency of neutralizing and non-neutralizing antibodies

Finally, we wanted to assess the biological impact that these antibodies have on viral replication in vivo. For this purpose we used a passive transfer - H7N9 challenge mouse model (Figure 6A). H7N9 was chosen as challenge virus because of its relevance for global health and because it has an NA subtype to which humans are naive and for which no cross-reactivity is induced by H7N1 vaccination (data not shown). We chose 10 vaccinees with measurable HI and/or neutralizing activity and 10 vaccinees with comparable induction by ELISA but no measurable HI and neutralizing activity. Samples were pooled resulting in day 0/HI+, day 42/HI+, day0/HI- and day42/HI- serum pools. Groups of mice corresponding to the pools were then injected intraperitoneally with 250 ul of serum and infected two hours later with SH13 H7N9 virus. Lungs were harvested on day 3 and day 6 post infection (Figure 6A). Mice treated with post-vaccination HI+ sera had a significant reduction (p=0.0186) of about 1 log on day 3 as compared to mice that received pre-vaccination sera from the same individuals. The difference increased on day 6 post infection with an almost 2 log reduction in lung titers of mice treated with HI+ post-vaccination sera as compared to the mice treated with pre-vaccination sera (Figure 6B). HI- post-vaccination sera was still able to reduced the viral titers on day 3 about 4-fold as compared to pre-vaccination sera from the same individuals (not significant, p=0.0525) (Figure 6C). A similar trend was seen on day 6. This data suggests that even sera that only exhibit binding but no neutralizing activity in vitro could impact on virus replication in vivo.
Pre-pandemic avian influenza virus vaccines - and H7 vaccines specifically - are of low immunogenicity in humans if the classical HI titer is used as correlate of protection (5, 6, 9, 35). Emerging viruses like H7N9 but also H6N1 (36) and H10N8 (37) warrant research to better understand the human immune response to vaccines based on avian influenza virus strains. Here, we re-evaluated sera from an H7N1 clinical trial performed in 2006/07 in Norway (5). Only one individual in this trial reached an HI titer of 1:32 and none of the four vaccine formulations tested fulfilled the licensing criteria of the European Committee for Medicinal Products for Human Use (CHMP) (5). A similar outcome was reported for a trial in the US (6).

There are two possibilities to explain why these vaccines failed. The first possibility is that they are not immunogenic. The second, and more likely explanation is that the immune response is not directed against the classical antigenic sites which would induce strong HI active antibodies as it is the case for seasonal influenza virus vaccines (38, 39). In the present study we set out to test this hypothesis. Only 1.9% (1 out of 54) of the vaccinees met the required 1:32 HI titer and only 39% (21 out of 54) of the vaccinees had any detectible HI or MN titer in the initial trial. A re-evaluation of a selection of 10 initially HI and/or MN positive serum samples showed that H7N1 vaccination is also able to induce cross-reactive HI titers against the novel H7N9 virus, an important finding which suggests that currently available H7 vaccines could be deployed as first line of defense in case of an H7N9 pandemic (26, 40).

Although we found comparable results between H7N9 VLPs and infectious virions in HI assays in a previous study (27) it needs to be kept in mind that the VLPs used in this study for HI assays could have a lower surface HA density than wild type virions. Lower copy numbers of HA on the surface of the cross-linking particle could lower the limit of detection, making them a more sensitive reagent for HI assays. Additionally, sera for the HI assays were pre-treated with red blood cells - a protocol that also increases sensitivity. Using a different readout, namely endpoint titer ELISA which measures all antibodies binding to HA independent of their epitope location, we found that a large proportion
(69.8%) had a fourfold or higher increase in reactivity. If the individuals that had a twofold increase are included, more than 94% of the vaccinees responded to the vaccine. Importantly - and in stark contrast to seasonal influenza virus vaccines – the antibody titer continued to rise throughout the observation period with the highest titers at 6 months post vaccination. This phenomenon has been reported in animal models for live attenuated H7 vaccines (7, 41) before but has never been shown in humans with inactivated H7 vaccines. It is of note that there was also an increase of antibody avidity over time indicating affinity maturation. Interestingly, we also found that alum (Al(OH)₃) had a relatively strong adjuvanting effect. No correlation was found between ELISA endpoint titers and HI, suggesting that the immune response against H7 HA is directed mostly against non-canonical epitopes. The vaccinees also exhibited broad cross-reactivity covering H7N9 and other Eurasian and North American lineage H7 HAs when ELISA measurements were considered. Surprisingly, a good proportion of the individuals mounted a heterosubtypic response against H15, which is also a member of group 2 HAs and closely related to H7. These strong cross-reactive responses can be explained by conserved epitopes in both the head and the stalk domain of HA. To further investigate this we measured reactivity against the head and the stalk domain of the heterologous H7N9 HA separately and found that the immune response was almost equally directed against head and stalk. This is highly unusual and in contrast to seasonal influenza virus vaccines, where a response almost exclusively directed against the globular head domain was reported in quantitative (42) and qualitative studies (38, 39) in humans and in quantitative studies in mice (42, 43). We hypothesize that - analogous to pH1N1 infection and vaccination (44-49) - shared epitopes exist within the group 2 HAs and that B cells with specificities for cross-reactive epitopes in the H3 stalk domain from earlier exposure have been boosted by the H7N1 vaccine. The novel H7 head domain which is highly divergent from the H3 head domain only induced a primary response. Based on this data we would expect that a proportion of the antibodies induced by the vaccine would also react with the stalk domain of other group 2 HAs like H4, H3, H10 and H14. Exploring the breadth of this cross-
reactivity will be the subject of future studies. Finally, we also wanted to assess the in vivo relevance of
the immune response detected in an H7N9 passive transfer challenge model. Serum from individuals
that exhibited low but detectible HI activity showed a very strong effect in vivo resulting in a reduction in
virus lung titers of almost two logs. Serum from HI negative individuals who showed ELISA binding
activity was also able to reduce lung titers in mice, but to a lower extent. However, these results might
be skewed since binding non-neutralizing antibodies are likely protecting through mechanisms like ADCC
and CDC (20-23) and the human antibodies might have lost potency in the mouse model due to
decreased interactions of the human Fc fragment with mouse Fc receptors and mouse complement.
Ultimately, tests in FcR- or complement- humanized mice will answer the question how much potency
was lost due to interspecies incompatibility. Our results from the mouse model suggest that both
neutralizing antibodies below HI titers of 1:32 and non-neutralizing but binding antibodies are
biologically relevant and could be protective in humans. This implies that avian influenza vaccines that
seem to have low efficacy in humans might nevertheless be protective. Based on data obtained from
studies in pigs it has been speculated that non-neutralizing antibodies against epitopes in the stalk
domain might - in the absence of neutralizing antibodies - correlate with enhanced pathogenicity upon
virus infection (50). Importantly, our data from the mouse passive transfer studies does not support this
hypothesis and shows a negative impact of cross-reactive antibodies on lung virus titer even in the
absence of neutralizing activity. We think that our findings warrant further investigation of the
multifaceted immune response to influenza virus vaccines (51). As shown in this study novel reagents
and tools for extending the analysis of the antibody repertoire can expose previously undocumented
immune responses and give new insights into how influenza virus vaccines work. Specifically, detailed
reactivity to the HA surface glycoprotein should be further evaluated as correlate of protection against
pandemic influenza virus infections.
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References


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### Tables

**Table 1: Reagents/viruses used for MN and HI assays and *in vivo* studies**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Strain name (HA)</th>
<th>Assay</th>
<th>Comments</th>
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<td>RD3 virus</td>
<td>A/chicken/Italy/5093/99 (H7N1)</td>
<td>HI (Fig. 2B) and MN (Fig. 2C)</td>
<td>homologous vaccine strain, 6:2 re-assortant on A/Puerto Rico/8/34 backbone</td>
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<td>SH13 VLPs</td>
<td>A/Shanghai/1/13 (H7N9)</td>
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**Table 2: Recombinant proteins used for ELISA assays**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Strain name</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>chickIT99 H7 HA</td>
<td>A/chicken/Italy/5093/99 (H7N1)</td>
<td>homologous to vaccine strain, Eurasian lineage</td>
</tr>
<tr>
<td>mallNL00 H7 HA</td>
<td>A/mallard/Netherlands/12/00 (H7N3)</td>
<td>Eurasian lineage</td>
</tr>
<tr>
<td>SH13 H7 HA</td>
<td>A/Shanghai/1/13 (H7N9)</td>
<td>Eurasian lineage</td>
</tr>
<tr>
<td>chickJal12 H7 HA</td>
<td>A/chicken/Jalisco/12283/12</td>
<td>North American lineage</td>
</tr>
<tr>
<td>H15 HA</td>
<td>A/shearwater/Western Australia/2576/79 (H15N9)</td>
<td></td>
</tr>
<tr>
<td>NC99 H1 HA</td>
<td>A/New Caledonia/20/99 (H1N1)</td>
<td></td>
</tr>
</tbody>
</table>
cH4/7 HA consists of the H4 head domain of A/duck/Czech/56 and the H7 stalk domain of A/Shanghai/1/13 used to measure stalk-reactive antibodies

H4 head amino acids 52-277a of the H4 HA of A/duck/Czech/56 used as control for cH4/7

H7 head amino acids 52-277a of the H7 HA of A/Shanghai/1/13 used to measure crossreactive antibodies against the H7 head domain

avian-origin N1 NA A/California/4/09 the N1 of the 2009 pandemic H1N1 strains is a member of the avian N1 clade (see Fig. 1B)

Figure legends

**Figure 1:** Phylogenetic relationship between the surface glycoproteins of the vaccine strain and relevant HAs and NAs. A Phylogenetic tree of group 2 HAs including the Eurasian and North American H7 lineages. B Phylogenetic tree of avian and human N1 neuraminidases. Red stars indicate HAs and NAs used as reagents in this study, green stars indicate the HA and NA of the vaccine strain. Scale bars represent a 5% amino acid change. C-E show comparisons of chickIT99 with SH13 (C) (both Eurasian lineage), chickIT99 (Eurasian lineage) with chickJal12 (North American lineage)|D and chickIT99 with H15 HA (E) with conserved amino acids in green and non-conserved amino acids in red. Structures are based on PDB# 4LN3 (52). It is of note that the antigenic site A (indicated by a red arrow in C and D) is completely conserved among avian H7 HAs. H15 has a 10 amino acid insertion in antigenic site E which was not visualized here since the structures were based on H7.

**Figure 2:** Hemagglutination inhibition and microneutralization titers. A Schematic of the clinical trial. B HI titers of the vaccinees were measured against homologous chickIT99 virus on day 0 (n=60) and post vaccination (n=54). The dotted line indicates the titer of 1:32 which is considered protective and needs to be reached by more than 70% of the vaccinees in order to meet licensing criteria. C MN titers as
measured against homologous chickIT99 virus on day 0 (n=60) and post vaccination (n=54). D-E Pie diagrams with percent of vaccinees that reached an HI titer of 1:32 (D) or had any detectible HI or MN titers post vaccination (E). F HI titers against H7N9 VLPs (based on HA from SH13) were evaluated for the ten strongest responders from B and C. Red bars in B, C and F indicate the geometric mean titers. Values in B, C and F are shown as geometric mean titers of duplicate or triplicate measurements.

Figure 3: ELISA endpoint titers against the homologous H7 HA. A ELISA endpoint titers against chickIT99 HA on day 0, 21, 42 and 6 months post vaccination. B Percentage of vaccinees that reacted with a 4-fold or higher, two-fold or no response to the vaccine. C Fold induction of endpoint titers against chickIT99 HA and the NC99 H1 HA. D ELISA endpoint titers against the NC99 H1 HA that was used as control. Red bars in A and D indicate the geometric mean titer. Red bars in A and D and columns in C indicate the geometric mean of the induction.

Figure 4: The effect of the Al(OH)₃ adjuvant. A shows percentage of seroconversion as measured by HI in the four non-adjuvanted and adjuvanted vaccine groups. B shows fold-induction of ELISA endpoint titers of the four vaccine groups. C Correlation analysis of HI versus ELISA endpoint titer. Columns in B indicate the geometric mean of the induction.

Figure 5: H7N1 vaccination induces broad reactivity to divergent HAs which is directed against the head and the stalk domain of the HA. A-D shows day 0 and day 42 endpoint titer of H7N1 vaccinees (n=54) against HA from the novel H7N9 strain SH13 (A, Eurasian lineage), mallNL00 (B, Eurasian lineage), chickJal12 (C, North American lineage) and H15 (D). Percentage of vaccinees that reacted with a 4-fold or higher, two-fold or no response to E SH13, F mallNL00, G chickJal12 or H H15. ELISA endpoint titers against the stalk domain of H7 HA as measured with a ch4/7 protein (I) (based on SH13 HA) and the head domain only (J) (based on SH13 HA). K An H4 head-only construct was used as control. L ELISA
endpoint titers on day 0 and day 42 against avian-origin N1 NA. **M** shows the fold-induction of reactivity to proteins shown in **A-D** and **I-L** upon H7N1 vaccination. **N** shows antibody avidity to SH13 HA in an NaSCN avidity assay on day 42 and 6 month post vaccination. Red bars **A-D**, **I-L** and **N** indicate the geometric mean titer. Columns in **M** indicate the geometric mean of the induction (see Figure 1 for phylogenetic distances between tested HAs).

**Figure 6: In vivo potency of HI+ and HI- day 42 sera from vaccinees in a mouse passive transfer H7N9 challenge experiment.** **A** Schematic of the passive transfer challenge experiment. **B** Mouse lung titers on day 3 and day 6 upon passive transfer with day 0 or day 42 pooled sera (n=10) from vaccinees that were HI positive on day 42 and subsequent challenge with H7N9. **C** Mouse lung titers on day 3 and day 6 upon passive transfer with day 0 or day 42 pooled sera from vaccinees that were HI negative on day 42 and subsequent challenge with H7N9.