An H7N1 Influenza Virus Vaccine Induces Broadly Reactive Antibody Responses against H7N9 in Humans

Florian Krammer, Åsne Jul-Larsen, Irina Margine, Ariana Hirsh, Haakon Sjursen, Maria Zambon, Rebecca J. Cox

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York, USA; The Influenza Centre, Department of Clinical Science, University of Bergen, Bergen, Norway; Graduate School of Biological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA; Section for Infectious Diseases, Medical Department, Haukeland University Hospital, Bergen, Norway; Public Health England, London, United Kingdom; Jebsen Centre for Influenza Vaccine Research, Department of Clinical Science, University of Bergen, Bergen, Norway; Department of Research and Development, Haukeland University Hospital, Bergen, Norway

Emerging H7N9 influenza virus infections in Asia have once more spurred the development of effective prepandemic H7 vaccines. However, many vaccines based on avian influenza viruses—including H7—are poorly immunogenic, as measured by traditional correlates of protection. Here we reevaluated 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- and stalk-reactive antibodies, and tested the in vivo potency of these human sera in a passive-transfer H7N9 challenge experiment with 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 hemagglutinins (HAs) in a large proportion of the cohort with a quantitative enzyme-linked immunosorbent assay. Furthermore, H7N1 vaccination induced antibodies to both the head and stalk domains of the HA, which is in sharp contrast to seasonal inactivated vaccines. Finally, we were able to show that both neutralizing and nonneutralizing antibodies improved in vivo virus clearance in a passive-transfer H7N9 challenge mouse model.

Influenza A H7 subtype viruses have caused sporadic infections in humans in the past (1–3). These incidents have triggered the development of prepandemic 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 the readout (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 of this novel H7 subtype has been detected so far, the outbreak triggered fears about a new pandemic since the virus causes a high case fatality rate (12), is transmitted in mammalian animal models (13–16), shows binding to alpha-2,6-linked sialic acid (14, 17, 18), and quickly developed resistance to 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 in the 2013–2014 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 a correlate of protection for seasonal influenza virus vaccines. However, prepandemic 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 nonneutralizing HA binding antibodies could play a role in protection via mechanisms like antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (20–23). Our study reexamined sera collected during an H7N1 vaccine trial (the vaccine containing an Eurasian H7 HA, Fig. 1) conducted in 2006 and 2007 at the University of Bergen (UIB), Bergen, 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 (Fig. 1A) as the substrate, we performed a quantitative endpoint titer enzyme-linked immunosorbent assay (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 using 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) cells (ATCC CCL-34) were culture in Dulbecco’s modified Eagle’s medium (Gibco) containing 10% fetal bovine serum (FBS; HyClone) and 100 U/ml of penicillin–100 µg/ml of streptomycin (Pen/Strep; Gibco). Sf9 cells (ATCC CRL-1711) were grown in TNM-FH insect cell medium (Gemini Bioproducts) supplemented with 10% FBS (HyClone), Pen/Strep, and 0.1% Pluronic F68. BTI-TN-5B1-4 cells (Vienna Institute of Biotechnology subclone [24]) were maintained in serum-free SFM medium (HyClone) containing Pen/Strep. 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 with 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). The viruses and 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/13474/99 (H7N1, chickIT99), A/Shanghai/1/13 (H7N9, SH13), 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 a hexahistidine tag (to facilitate purification) as described before (29, 30) (Table 2). The phylogenetic relationship between the HAs used in this study is shown in Fig. 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

![Phylogenetic relationships between the surface glycoproteins of the vaccine strain and relevant HAs and NAs.](http://cvi.asm.org/)

**FIG 1** Phylogenetic relationships 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 NAs. Red stars indicate the HAs and NAs used as reagents in this study, and green stars indicate the HA and NA of the vaccine strain. Scale bars represent a 5% amino acid change. (C to E) 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 nonconserved amino acids in red. Structures are based on PDB accession no. 4LN3 (52). It is of note that antigenic site A (indicated by red arrows in panels C and D) is completely conserved among avian H7 HAs. H15 has a 10-amino-acid insertion in antigenic site E that is not shown here since the structures are based on H7.

**TABLE 1** Reagents and viruses used for MN and HI assays and in vivo studies

| Reagent or virus | Strain (HA) | Assay(s) | Comment |
|------------------|-------------|----------|---------|
| RD3 virus        | A/chicken/Italy/13474/99 (H7N1) | HI<sup>a</sup>, MN<sup>b</sup> | Homologous vaccine strain, 6:2 reassortant on A/Puerto Rico/8/34 backbone |
| SH13 VLPs        | A/Shanghai/1/13 (H7N9) | HI<sup>d</sup> | Expressed in 293T cells |
| SH13 virus       | A/Shanghai/1/13 (H7N9) | Passive-transfer exp<sup>a</sup> | 6:2 reassortant on A/Puerto Rico/8/34 backbone |

<sup>a</sup> See Fig. 2B.<br><sup>b</sup> See Fig. 2B.<br><sup>c</sup> See Fig. 2F.<br><sup>d</sup> See Fig. 6.
TABLE 2 Recombinant proteins used for ELISAs

| Protein          | Strain (HA)                                      | Comment                                      |
|------------------|-------------------------------------------------|----------------------------------------------|
| chickIT99 H7 HA  | A/chicken/Italy/13474/99 (H7N1)                  | Homologous to vaccine strain, Eurasian lineage |
| mallIN100 H7 HA  | A/mallard/Netherlands/12/00 (H7N3)               | Eurasian lineage                             |
| SH13 H7 HA       | A/Shanghai/1/13 (H7N9)                          | Eurasian lineage                             |
| chickkJ12 H7 HA  | A/chicken/lalisco/12283/12                       | North American lineage                       |
| H15 HA           | A/shearwater/Western Australia/2576/79 (H15N9)  |                                              |
| NC93 F1 HA       | A/New Caledonia/20/99 (H1N1)                    |                                              |
| chH47 HA         | Consists of H4 head domain of A/duck/Czech/56 and H7 stalk domain of A/Shanghai/1/13 | Used to measure stalk-reactive antibodies |
| H4 head          | Amino acids 52–277 of H4 HA of A/duck/Czech/56  | Used as a control for chH47                  |
| H7 head          | Amino acids 52–277 of H7 HA of A/Shanghai/1/13  | Used to measure cross-reactive antibodies against H7 head domain |
| Avian-origin N1 NA | A/California/4/09                               | N1 of 2009 pandemic H1N1 strain, member of avian N1 clade<sup>a</sup> |

<sup>a</sup> H3 numbering.
<sup>b</sup> See Fig. 1B.

were assigned a value of 5 for calculation purposes. Assays were performed in duplicate or triplicate for each sample.

**MN assay.** Pre- and postvaccination microneutralization (MN) assay titers were determined as previously described (32). Serum samples were tested from an initial dilution of 1:20 against the Per.C6-grown RD 3 virus. Sera with titers of ≥20 were considered positive, and antibody titers of <20 were assigned a value of 5 for calculation purposes. Assays were performed in duplicate or triplicate 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 μl/well). Plates were then blocked at room temperature for 1 h with phosphate-buffered saline (PBS)–0.1% Tween 20 (TPBS) containing 3% New Zealand goat serum (Gibco) and 0.5% nonfat dry milk powder (GM-TPBS). Serum was prediluted 1:100 and then serially diluted in GM-TPBS in 1:2 steps. After 2 h 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 1:3,000 in GM-TPBS (50 μl/well). After 2 h of incubation, plates were washed again as described above and developed with o-phenylenediamine dihydrochloride (SigmaFast OPD; Sigma) as the substrate. Reactions were stopped after exactly 10 min with 3 M HCl (50 μl/well), and the optical density at 490 nm (OD<sub>490</sub>) was read. 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 (OD<sub>490</sub>) was above a cutoff of the average plus 3 standard deviations of negative-control wells (secondary antibody only). The samples examined by ELISA were taken on day 0 (n = 60 for all analyses), on day 21 (performed only for the homologous HA, n = 60), on day 42 (52 available serum samples, except for H15, in which case 52 samples were analyzed), and at 6 months postinfection (26 available samples).

**IgG avidity ELISA.** Sera were evaluated for relative avidity of IgG antibodies against the SH13 HA. Ninety-six-well plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 0.3 μg/ml HA in PBS and incubated at 2 to 8°C overnight. Plates were blocked with 5% milk–0.1% Tween 20–1% BSA solution in PBS prior to the addition of appropriate serum dilutions giving an OD<sub>450</sub> of 0.7 ± 0.3 and incubation for 1 h. Subsequently, sera were treated with 1.5 M sodium thiocyanate (NaSCN) for 1 h. Bound antibodies were detected with mouse anti-human IgG horseradish peroxidase (BD) at a final dilution of 1:4,000 and 3,3′,5,5′-tetramethylbenzidine substrate (Europa Ltd.). After the reaction was stopped with 0.5 N HCl, the absorbance at 450 nm was read by subtracting a background reference of 620 nm. The antibody binding resistance to 1.5 M NaSCN was calculated as follows: (OD<sub>450</sub> treated serum/OD<sub>450</sub> untreated serum) × 100%.

**Passive-transfer challenge experiment.** We chose patients (10 per group) with similar increases in reactivity who had HI-active antibodies (H<sup>+</sup>) on day 42 postvaccination or did not induce HI-active antibodies (H<sup>−</sup>). Samples for both pools came predominantly from the adjuvant-
treated groups; the HI− group had a mean induction (by ELSA endpoint titer) of 4.93 compared to 6.06 in the HI+/H11001 group. The geometric mean endpoint titer on day 42 was 1:5,701.8 for the HI+/H11001 group and 1:4,354.5 for the HI+/H11002 group. Four pools of sera (equal volumes from all of the patients) in these two cohorts were generated, i.e., day 0 HI+/H11002, day 42 HI+/H11002, day 0 HI−, and day 42 HI−. All day 0 samples were HI+/H11002 in both groups. Pooled sera (250 μL/mouse) were then injected intraperitoneally into 6- to 8-week-old female BALB/c mice (six per group). Two hours later, the mice were anesthetized by the intraperitoneal injection of 0.1 ml of a ketamine-xylazine mixture (0.15 and 0.03 mg/kg). Mice were then infected with 300 PFU of SH13 H7N9 virus. On days 3 and 6, three mice per group were sacrificed and their lungs were harvested and homogenized in 600 μL of pH 7.4 PBS with a BeadBlaster 24 (Benchmark) homogenizer. To determine the lung virus titers, the homogenates were allowed to form plaques on MDCK cells. Plaques were counted after 3 days of incubation at 37°C and immunostaining with convalescent-phase serum from H7-infected mice. Mice were handled according to the Mount Sinai Institutional Animal Care and Use Committee.

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 with a parametric unpaired t test in Prism 6 (GraphPad). Correlation analysis was performed with the standard linear regression model in Prism 6. Trees and sequence alignments were computed with ClustalW. Trees were visualized in FigTree; molecular structures were visualized in Protein Workshop.

RESULTS

Induction of homologous and heterologous neutralizing antibodies. In 2006, 60 healthy volunteers were divided into four groups and vaccinated with an H7N1 vaccine containing 12 or 24 μg 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, Fig. 2Band D) of the vaccinees reached an HI titer of 1:32, the criterion that shows protection (5). A total of 39% of the vaccinated individuals tested positive for HI-active or neutralizing antibodies (Fig. 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 reevaluated against H7N9 with SH13 H7N9 VLPs (27). HI activity was detectable in 9 out of 10 sera preadsorbed with horse red blood cells (Fig. 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 the substrate, we analyzed day 0, day 21, day 42, and 6-month serum samples for reactivity to the vaccine strain. Day 0 endpoint titers were low, with a geometric mean of 1:673. The low titers indicate low levels of cross-reactive antibodies against group 2 HAs, confirming previous findings (33) (Fig. 3A). Upon vaccination, the geometric mean titer increased to 1:1,270 on day 21 and to 1:3,200 on day 42.
(both highly statistically significant, \( P < 0.0001 \)), which are inductions of 1.89- and 4.74-fold, respectively (Fig. 3A and C, geometric means of induction). On day 42, 69.8% of the vaccinees had experienced a titer increase of ≥4-fold relative to day 0, 24.5% had experienced 2-fold induction, and only 5.7% were nonresponders (Fig. 3B). This is in sharp contrast to 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 toward the end of the observation period, with a geometric mean endpoint of 1:3,755 (highly significant, \( P < 0.0001 \)) and a mean induction over day 0 of 6.13-fold (Fig. 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, Fig. 1A). However, titers of antibodies against the NC99 H1 HA did not increase significantly (Fig. 3C and D).

**Effect of Al(OH)\(_3\) adjuvant.** Vaccinees in the original clinical trial were divided into four different groups that received vaccine doses with 12 or 24 \( \mu \)g of HA with or without Al(OH)\(_3\) as an adjuvant. The low vaccine dose induced HI or neutralizing titers in 21.4% of the non-adjuvant-treated cohort and 50% of the adjuvant-treated group (Fig. 4A). Similarly, the high vaccine dose induced HI titers in 23.1% of the individuals in the non-adjuvant-treated group and resulted in 61.5% positives in the adjuvant-treated group (Fig. 4A) (5). Endpoint titers at day 42 in the non-adjuvant-treated low-vaccine-dose group were induced 3.1-fold, on average, while adjuvant addition resulted in a 7.3-fold induction (Fig. 4B). Likewise, induction in the non-adjuvant-treated high-dose group was, on average, 3.6-fold compared to 6.9-fold in the comparable adjuvant-treated group (statistically significant, \( P = 0.0374 \)) (Fig. 4B). These 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 ELISAs 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

---

**FIG 3** ELISA endpoint titers of antibodies against the homologous H7 HA. (A) ELISA endpoint titers of antibodies against chickIT99 HA at 0, 21, and 42 days and 6 months postvaccination. (B) Percentages of vaccinees that reacted with a ≥4-fold, a 2-fold, or no response to the vaccine. (C) Fold induction of endpoint titers of antibodies against the chickIT99 HA and the NC99 H1 HA. (D) ELISA endpoint titers of antibodies against the NC99 H1 HA that was used as a control. The red bars in panels A and D indicate the geometric mean titers, and the columns in panel C indicate the geometric mean levels of induction. n.s., not significant.
ELISA reactivity and HI activity of sera, we performed a correlation analysis of ELISA endpoint titers and HI titers (Fig. 4C). Analysis of HI titers versus ELISA endpoint titers showed no correlation ($r^2$, 0.02571) (Fig. 4C), indicating that the antibody response might be directed against noncanonical epitopes (non-HI) on the HA.

**Reactivity to heterologous and heterosubtypic HAs and avian-origin NA.** Next, we examined whether vaccination with H7N1 vaccine is also able to induce antibodies against HA from the novel H7N9 strain and against other heterologous H7 strains from the Eurasian and North American H7 lineages (Fig. 1). Endpoint titers of antibodies against H7 from H7N9 strain SH13 (Eurasian lineage) increased significantly ($P < 0.0001$) from 1:1,361 on day 0 to 1:3,600 on day 42, which represents an average increase (geometric mean) of 2.63-fold (Fig. 5A and M) with 43.4% of the vaccinees having 4-fold induction, 32.1% having 2-fold induction, and 24.5% being nonresponders (Fig. 5E). Similarly, titers of antibodies against another Eurasian lineage strain, mallNL00, rose from an average endpoint of 1:1,838 to 1:4,738 (Fig. 5B and M). This represents a mean induction of 2.56-fold between days 0 and 42, with 47.2% of the individuals reacting with 4-fold induction, 22.6% reacting with 2-fold induction, and 30.2% not responding (Fig. 5F and M). The average endpoint titers of antibodies against the North American lineage H7 HA from chickJal12 started with 1:867 on day 0 and increased, on average, 2.85-fold to 1:2,432 on day 42 (Fig. 5C and M), with 49.1% of the vaccinees having 4-fold induction, 32.1% having 2-fold induction, and 18.9% not responding (Fig. 5G).

ChickIT99, mallNL00, and SH13 are all closely related members of the Eurasian H7 lineage, and this cross-reactivity is not unexpected because they have very similar amino acid sequences and structures (Fig. 1A and C). Interestingly, strong cross-reactivity toward chickJal12, which is a member of the more distantly related North American lineage, also exists (Fig. 1A). This cross-reactivity is probably based on the completely conserved antigenic site A (Fig. 1D) and on shared epitopes in the conserved stalk domain. In addition to cross-reactivity to divergent H7 HAs, we also wanted to see if the H7N1 vaccine induced antibodies against heterologous members of the group 2 HAs. Interestingly, the endpoint titers of antibodies against H15 also rose from a mean of 1:641 to 1:1,428, which represents an induction (geometric mean) of 2.25-fold (Fig. 5D and M). However, only 30.8% of the vaccinees reacted with a 4-fold increase and the majority of the individuals showed an only 2-fold (46.2%) increase or no (23.1%) increase (Fig. 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 1:1,318 on day 42 ($P = 0.0038$), this represents an only 1.24-fold increase, on average (Fig. 5L and M). It has been noted that both antibody titers and antibody affinity or avidity play an important role in virus neutralization. We therefore also examined the long-term avidity of sera at day 42 and 6 months postvaccination in a sodium thiocyanate elution ELISA with SH13.
FIG 5. H7N1 vaccination induces broad reactivity to divergent HAs which is directed against the HA head and stalk domains. (A to D) Day 0 and 42 endpoint titers of H7N1 vaccinees (n = 52/53) against HA from the novel H7N9 strain SH13 (A, Eurasian lineage), malNL00 (B, Eurasian lineage), chickJal12 (C, North American lineage), and H15 (D). The percentages of vaccinees that reacted with a ≥4-fold, a 2-fold, or no response to SH13 (E), malNL00 (F), chickJal12 (G), or H15 (H) are shown. ELISA endpoint titers of antibodies against the stalk domain of the 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 a control. (L) ELISA endpoint titers on days 0 and 42 against the avian-origin N1 NA. (M) Fold induction of reactivity to proteins shown in panels A to D and I to L upon H7N1 vaccination. (N) Antibody avidity for the SH13 HA in an NaSCN avidity assay at 42 days and 6 months postvaccination. The red bars in panels A to D, I to L, and N indicate the geometric mean titers. The columns in panel M indicate the geometric mean levels of induction (see Fig. 1 for the phylogenetic distances between the HAs tested).
HA as the 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 months postvaccination (Fig. 5N).

Reactivity to the divergent head and conserved stalk domains 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 directed mainly 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 the mean endpoint titer from 1:1,008 on day 0 to 1:2,107 on day 42 for cH4/7, a 2.17-fold increase, on average (Fig. 5I and M). The mean endpoint titer for the H7 head-only construct was 1:888 and increased to 1:1,686 on day 42, which is a 1.95-fold increase, on average ($P < 0.0001$) (Fig. 5J and M). To control for reactivity toward the H4 head domain, we also analyzed the response against an H4 head-only construct. The mean endpoint titer for the H4 head-only control increased minimally (although statistically significantly, $P = 0.029$) from a mean of 1:673 on day 0 to 1:721 on day 42, a marginal increase of 1.1-fold (Fig. 5K and M). These results suggest that H7N1 vaccination induced antibodies against the head and stalk domains.

In vivo relevance and potency of neutralizing and nonneutralizing antibodies. Finally, we assessed the biological impact that these antibodies have on viral replication in vivo. For this purpose, we used a passive-transfer H7N9 challenge mouse model (Fig. 6A). H7N9 was chosen as the challenge virus because of its relevance to global health and because it has an NA subtype to which humans are naive and to 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 or neutralizing activity. Samples were pooled, resulting in day 0 HI$^+$, day 42 HI$^+$, day 0 HI$^-$, and day 42 HI$^-$ serum pools. Groups of mice corresponding to the pools were then injected intraperitoneally with 250 μl of serum and infected 2 h later with SH13 H7N9 virus. Lungs were harvested on days 3 and 6 postinfection (Fig. 6A). Mice treated with postvaccination HI$^+$ sera had a significant reduction ($P = 0.0186$) of about 1 log on day 3 compared to mice that received prevaccination sera from the same individuals. The difference increased on day 6 postinfection, with an almost 2-log reduction in the lung virus titers of mice treated with HI$^+$ postvaccination sera compared to those of mice treated with prevaccination sera (Fig. 6A). HI$^-$ postvaccination sera were still able to reduce the viral titers on day 3 about 4-fold compared to prevaccination sera from the same individuals (not significant.
**DISCUSSION**

Prepandemic avian influenza virus vaccines—and H7 vaccines specifically—are of low immunogenicity in humans if the classical HI titer is used as a 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 reevaluated sera from an H7N1 clinical trial performed in 2006 and 2007 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 (5). A similar outcome was reported for a trial in the United States (6). There are two possible explanations for these vaccines failures. 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 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 detectable HI or MN titer in the initial trial. A reevaluation 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 of antibodies against the novel H7N9 virus, an important finding which suggests that currently available H7 vaccines could be deployed as the first line of defense against an H7N9 pandemic (26, 40). Although we obtained comparable results with 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 pretreated with red blood cells—a protocol that also increases sensitivity. Using a different readout, namely, an endpoint titer ELISA that measures all of the antibodies binding to HA independently of their epitope locations, we found that a large proportion (69.8%) had a 4-fold increase in reactivity. If the individuals that had a 2-fold 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 postvaccination. 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 in antibody avidity over time, indicating affinity maturation. Interestingly, we also found that alum [Al(OH)₃] had a relatively strong adjuvant effect. No correlation between ELISA endpoint titers and HI was found, suggesting that the immune response against H7 HA is directed mostly against noncanonical 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 HAs. These strong cross-reactive responses can be explained by conserved epitopes in both the head and stalk domains of HA. To further investigate this, we measured reactivity against the head and stalk domains of the heterologous H7N9 HA separately and found that the immune response was almost equally directed against the head and stalk domains. 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) with humans and in quantitative studies with 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, induced only a primary response. On the basis of these 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 who exhibited low but detectable HI activity showed a very strong effect in vivo, resulting in a reduction in virus lung titers of almost 2 logs. Serum from H1⁺ individuals who showed ELISA binding activity was also able to reduce lung titers in mice, but to a lesser extent. However, these results might be skewed since binding nonneutralizing antibodies likely protect through mechanisms like ADCC and CDC (20–23) and the human antibodies might have lost potency in the mouse model because of decreased interactions of the human Fc fragment with mouse Fc receptors and mouse complement. Ultimately, tests with Fc receptor- or complement-humanized mice will answer the question of how much potency was lost because of interspecies incompatibility. Our results from the mouse model suggest that both neutralizing antibody HI titers below 1:32 and nonneutralizing 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. On the basis of data obtained from studies with pigs, it has been speculated that nonneutralizing antibodies against epitopes in the stalk domain might, in the absence of neutralizing antibodies, correlate with enhanced pathogenicity upon virus infection (50). Importantly, the data from our mouse passive-transfer studies do not support this hypothesis and show 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 a correlate of protection against pandemic influenza virus infections.
ACKNOWLEDGMENTS

Florian Kramer was supported by an Erwin Schrödinger fellowship (J3232) from the Austrian Science Fund (FWF). This work was partially supported by a Centers for Excellence for Influenza Research and Surveillance (CEIRS) grant (HHSN26620070010C), NIH grant U19 AI109946, and NIH adjudvant development contract HHSN22720090032C. The work in Bergen was supported by the Ministry of Health and Care Services Helge Vesth Influenza A (H7) studies, the K. G. Jebsen Centre for Influenza Vaccine Research, EU FP7 UniVax (601738), and RCN GloVac (220670). The work in London was supported by the Health Protection Agency.

REFERENCES

1. Fouchier RA, Schneerberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD. 2004. Avian influenza virus A (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc. Natl. Acad. Sci. U. S. A. 101:1356–1361. http://dx.doi.org/10.1073/pnas.0308352100.

2. Tweed SA, Skowronski DM, David ST, Larder A, Petric M, Lees W, Li Y, Katz J, Krajden M, Tellier R, Halpert C, Hirst M, Astell C, Lawrence D, Brouse A. 2004. Human illness from avian influenza H7N3 British Columbia. Emerg. Infect. Dis. 10:2196–2199. http://dx.doi.org/10.3201/eid1012.040961.

3. Centers for Disease Control and Prevention. 2012. Notes from the field: highly pathogenic avian influenza (A H7N3) virus infection in two poultry workers—Jahso, Mexico, July 2012. MMWR Morb. Mortal. Wkly. Rep. 61:726–727. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm613604.htm.

4. Cox RJ, Major D, Hauge S, Madhun AS, Brokstad KA, Kuhne M, Smith J, Vogel FR, Zambon M, Haahr L, Wood J. 2009. A cell-based H7N1 split influenza virion vaccine confers protection in mouse and ferret challenge models. Influenza Other Respir. Viruses 3:368–374. http://dx.doi.org/10.1016/j.infe.2009.01.116.

5. Cox RJ, Madhun AS, Hauge S, Sjursen H, Major D, Kuhne M, Hochschild K, Saville M, Vogel FR, Barclay W, Donatelli I, Zambon M, Wood J, Haahr L. 2009. Phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. Vaccine 27:1889–1897. http://dx.doi.org/10.1016/j.vaccine.2009.01.116.

6. Couch RB, Patel SM, Wade-Bowers CL, Niño D. 2012. A randomized clinical trial of an inactivated avian influenza A (H7N7) vaccine. PLoS One 7(12):e94704. http://dx.doi.org/10.1371/journal.pone.0094704.

7. Joseph T, McAuliffe J, Lu B, Vogel I, Swanye D, Jin H, Kemble G, Subbarao K. 2008. A live attenuated cold-adapted influenza A H7N3 virus vaccine provides protection against homologous and heterologous H7 viruses in mice and ferrets. Virology 378:123–132. http://dx.doi.org/10.1016/j.virol.2008.05.021.

8. Min JY, Vogel L, Matsuoka Y, Lu B, Swanye D, Jin H, Kemble G, Subbarao K. 2010. A live attenuated H7N7 candidate vaccine virus induces neutralizing antibody that confers protection from challenge in mice, ferrets, and monkeys. J. Virol. 84:11950–11960. http://dx.doi.org/10.1128/JVI.01305-10.

9. Talata KR, Karron RA, Callahan KA, Luke CJ, Di Lorenzo SC, Chen GL, Lamirande EW, Jin H, Coelingh KL, Murphy BR, Kemble G, Subbarao K. 2009. A live attenuated H7N3 influenza virus vaccine is well tolerated and immunogenic in a phase I trial in healthy adults. Vaccine 27:3744–3753. http://dx.doi.org/10.1016/j.vaccine.2009.03.082.

10. Rudenko I, Kiseleva I, Naykhin AN, Erofeeva M, Stukova D, Donina S, Khokhlova D, Arzumanyan V, Grishkin M, Buzktsaya Z, Isakova-Sivak I, Kuznetsova S, Larionova N, Desheva J, Dubrovina I, Nikiforova A, Victor JC, Neuzil K, Flores J, Tsvetnitsky V, Kiselev O. 2014. Assessment of human immune responses to H7 avian influenza virus of pandemic potential: results from a placebo-controlled, randomized double-blind phase I study of live attenuated H7N3 influenza virus vaccine. PLoS One 9(2):e87692. http://dx.doi.org/10.1371/journal.pone.0087692.

11. Gao R, Cao B, Hasegawa H, Swayne D, Wang D, Hu Y, Chen J, Jia Z, Wilde M, Palmberger D, Hai R, Albrecht RA, Margine I, Hirsh A, Garcia-Sastre A, Grabherr R, Kramer F. 2014. One-shot vaccination with an insect cell-derived low-dose influenza A H7 virus-like...
antibodies than influenza vaccination. PLoS One 6(10):e25797. http://dx.doi.org/10.1371/journal.pone.0025797.

40. Rudenko I, Isaakova-Sivak I, Rekstin A. 2014. H7N9: can H7N3 live-attenuated influenza vaccine be used at the early stage of the pandemic? Expert Rev. Vaccines 13:1–4. http://dx.doi.org/10.1586/14760854.2014.864564.

41. Joseph T, McAugille J, Lu B, Jin H, Kemble G, Subbarao K. 2007. Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. J. Virol. 81:10558–10566. http://dx.doi.org/10.1128/JVI.00970-07.

42. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, Garcia-Sastre A, Palese P, Treanor JJ, Krammer F. 2013. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J. Virol. 87:4728–4737. http://dx.doi.org/10.1128/JVI.01309-12.

43. Krammer F, Pica N, Hai R, Tan GS, Palese P. 2012. Hemagglutinin stalk-reactive antibodies are boosted following sequential infection with seasonal and pandemic H1N1 influenza virus in mice. J. Virol. 86:10302–10307. http://dx.doi.org/10.1128/JVI.01336-12.

44. Li GM, Chiu C, Wrammert J, McCartney M, Andrews SF, Zheng NY, Lee JH, Huang M, Qu X, Edupuganti S, Mulligan M, Das SR, Yewdell JW, Mehta AK, Wilson PC. 2012. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. Proc. Natl. Acad. Sci. U. S. A. 109:9047–9052. http://dx.doi.org/10.1073/pnas.1118979109.

45. Wrammert J, Koutsonanos D, Li GM, Edupuganti S, Sui J, Morrisey M, McCartney M, Skountzou I, Hornig M, Lipkin WI, Mehta A, Razavi B, Del Rio C, Zheng NY, Lee JH, Huang M, Ali Z, Kaur K, Andrews S, Amara RR, Wang Y, Das SR, O’Donnell CD, Yewdell JW, Subbarao K, Marasco WA, Mulligan MJ, Compans R, Ahmed R, Wilson PC. 2011. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med. 208:181–193. http://dx.doi.org/10.1084/jem.20101352.

46. Thomson CA, Wang Y, Jackson LM, Olson M, Wang W, Liavonchanka A, Keleta I, Silva V, Didierich S, Jones RB, Gubayj P, Pasick J, Petric M, Jean F, Allen VG, Brown EG, Rini JM, Schrader JW. 2012. Pandemic H1N1 influenza infection and vaccination in humans induces cross-protective antibodies that target the hemagglutinin stem. Front. Immunol. 3:87. http://dx.doi.org/10.3389/fimmu.2012.00087.

47. Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, Tan GS, Krause JC, Moran T, Stein CR, Banach D, Wrammert J, Belshe RB, Garcia-Sastre A, Palese P. 2012. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. Proc. Natl. Acad. Sci. U. S. A. 109:2573–2578. http://dx.doi.org/10.1073/pnas.120039190.

48. Sangarter MY, Baer J, Santiago PW, Fitzgerald T, Ilyushina NA, Sundararajan A, Henk AD, Krammer F, Yang H, Luke CJ, Zand MS, Wright PF, Treanor JJ, Topham DJ, Subbarao K. 2013. B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza virus vaccination. Clin. Vaccine Immunol. 20:868–876. http://dx.doi.org/10.1128/CVI.00735-12.

49. Krammer F, Palese P. 2013. Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr. Opin. Virol. 3:52–530. http://dx.doi.org/10.1016/j.coivd.2013.07.007.

50. Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Hennington J, Vincent AL, Golden H. 2013. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci. Transl. Med. 5(200):200ra114. http://dx.doi.org/10.1126/scitranslmed.3006366.

51. Krammer F, Cox RJ. 2013. The emergence of H7N9 viruses: a chance to redefine correlates of protection for influenza virus vaccines. Expert Rev. Vaccines 12:1369–1372. http://dx.doi.org/10.1586/14760854.2013.850036.

52. Yang H, Carney PJ, Chang JC, Villanueva JM, Stevens J. 2013. Structural analysis of the hemagglutinin from the recent 2013 H7N9 influenza virus. J. Virol. 87:12433–12446. http://dx.doi.org/10.1128/JVI.01854-13.