Panblok-H1+advax H1N1/2009pdm vaccine: Insights into rapid development of a delta inulin adjuvanted recombinant pandemic influenza vaccine

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**ABSTRACT**
Timely vaccine supply is critical during influenza pandemics but is impeded by current virus-based manufacturing methods. The 2009 H1N1/2009pdm ‘swine flu’ pandemic reinforced the need for innovation in pandemic vaccine design. We report on insights gained during rapid development of a pandemic vaccine based on recombinant haemagglutinin (rHA) formulated with Advax\(^{TM}\) delta inulin adjuvant (Panblok-H1/Advax). Panblok-H1/Advax was designed and manufactured within 1 month of the pandemic declaration by WHO and successfully entered human clinical testing in under 3 months from first isolation and sequencing of the novel pandemic virus, requiring several major challenges to be overcome. Panblok-H1/Advax successfully induced neutralising antibodies against the pandemic strain, but also induced cross-neutralising antibodies in a subset of subjects against an H1N1 strain (A/Puerto Rico/8/34) derived from the 1918 Spanish flu, highlighting the possibility to use Advax to induce more broadly cross-protective antibody responses. Interestingly, the rHA from H1N1/2009pdm exhibited unusual behavior of the novel hemagglutinin, thereby demonstrating the power of computational modeling approaches for rapid characterization of new pandemic viruses. While challenges remain in ensuring ultrafast vaccine access for the entire population in response to future pandemics, the adjuvanted recombinant Panblok-H1/Advax vaccine proved its utility during a real-life pandemic situation.

**Introduction**

Seasonal and pandemic influenza vaccines have traditionally been made from inactivated virus grown in embryonated eggs. However, this approach has several limitations including manufacturing delays while vaccine seed viruses are egg-adapted and insecurity of egg supply with potential of supply disruption if pandemic virus also infects chicken flocks. Egg-based vaccines may also not be suitable for individuals with severe egg allergies.\(^1\) Despite more recent development of large-scale facilities for mammalian cell culture of influenza virus,\(^2\) this remains susceptible to delays while seed viruses suitable for cell culture are generated and screened. The predictability and speed of recombinant protein manufacture are major advantages for vaccine production. A potentially more reliable approach to pandemic vaccine production, therefore, is the use of recombinant haemagglutinin (rHA). Protective neutralising antibodies to HA are seen after influenza infection or immunization.\(^3,4\) The US government awarded contracts to a range of vaccine companies including Protein Sciences Corporation (PSC), Medicago, Novovax, Fraunhofer Institute, and Vaxinnate to produce recombinant influenza vaccines using various techniques.\(^5\) PSC pioneered the use of the baculovirus expression system to produce influenza vaccine based on rHA (Flublok\(^{\text{R}}\)), which was licensed by the FDA in 2013 for seasonal influenza protection in adults.\(^6\) The pandemic variant of this rHA vaccine is known as Panblok\(^{\text{R}}\). While Panblok protected birds against lethal H5 or H7 infection,\(^7\) only low levels of sero-protection were obtained in human subjects indicating the need for an adjuvant.\(^8\)

While many adjuvants have been described, very few have progressed as formulations for licensed human vaccines.\(^9\) Advax\(^{TM}\) is a novel polysaccharide adjuvant based on particles of semi-crystalline delta inulin, development of which was supported through the US. National Institutes of Health’s Adjuvant Development Program.\(^10\) In animal models, Advax\(^{TM}\) adjuvant has been shown to enhance the immunogenicity of vaccines against a wide range of diseases including influenza,\(^11-13\) hepatitis B,\(^14\) SARS coronavirus,\(^15\) Japanese encephalitis,\(^16\) West Nile virus,\(^17\) RSV,\(^18\) anthrax,\(^19\) Listeria,\(^20\) HIV\(^{21}\) and Peste de petit ruminants.\(^22\) Although its mechanism of action has yet to be
fully determined, Advax particles bind directly to human mono-
cytes and enhance their co-stimulatory function.\textsuperscript{23} In a pan-
demic influenza study in the ferret model, Advax significantly
enhanced protection afforded by an inactivated H5N1 vaccine
providing 100% survival versus only 66% survival seen with
the standard H5N1 vaccine alone.\textsuperscript{24} The vaccine with Advax adju-
vant provided over 3-fold antigen dose-sparing while signifi-
cantly reducing neurologic disease and viral shedding. Notably,
Advax has been shown to be safe and immune-enhancing in
humans when formulated in hepatitis B,\textsuperscript{25} seasonal influenza\textsuperscript{26}
and allergy,\textsuperscript{27} vaccines.

The 2009 influenza pandemic caused by the sudden emer-
gence in North America of the H1N1/2009pdm strain (ori-
ginally referred to as “swine flu”) provided a real-life opportu-
nity to test novel pandemic vaccine approaches. It was first detected
in North America in April 2009 and spread rapidly around the
world, leading to a declaration by the World Health Organiza-
tion of a global pandemic on June 11, 2009.\textsuperscript{28} The new influ-
enza virus was at least as contagious as seasonal influenza and
spread quickly, particularly among younger people. Some
degree of pre-existing immunity was seen in older adults espe-
cially those aged over 60, possibly due to previous exposure to
antigenically related in
uenza A viruses. Recent seasonal in-
uenza vaccines (2005–2009 provided minimal protection against
the novel pandemic strain.\textsuperscript{29} While the 2009 pandemic was not
as severe as initially feared, it did cause an upsurge in hospital
and intensive care unit admissions for severe respiratory illness,
hypoxemia, multi-organ failure and requirement for ventilator
support.\textsuperscript{30} Given the need for rapid development of pandemic
cases of influenza virus in eggs took 3–4 months to
establish. The influenza vaccine seed strain initially distributed
by Centers for Disease Control and Prevention (CDC) was A/
H1N1/California/04/2009. (Source: CDC ID number
2009712047; Passage 1 MDCK cells). Although this strain sub-
sequently provided unsatisfactory egg production yields delay-
ing vaccine manufacture while an alternative higher-yield
strain was identified, PSC had already cloned the HA from the
A/California/04/2009 virus and, in particular, the differen-
ces in behavior of the Pro200 vs. Ser200 rHA, we generated a
homology model of A/California/04/2009 HA using Modeler, a
homology modeling program, using a crystal structure of an
earlier HA protein, 3AL4.pdb, as a template. This was then
used to conduct receptor binding analysis using the molecular
docking program, Autodock vina, with binding energies of the
predicted binding conformation of the HA head region to avian
and human sialic acid receptors. These binding studies con

\textbf{Characterization of rHA from A/California/04/2009}

Hemagglutination activity assays were performed with fresh
chicken and swine red blood cells (RBCs). With chicken RBCs,
the undiluted Ser200 rHA monovalent bulk only had a hemag-
glutination titer of 3, equivalent to 0.07 Units/\mu g HA protein,
indicating a exceptionally low agglutination activity. By com-
parison, 1 \mu g/ml of a control rHA from A/Brisbane/59/2007
(H1N1) had a haemagglutination titer of 2048, equivalent to
20,480 units/\mu g HA protein. The Ser200 rHAsimilarly had no
agglutination activity for guinea pig RBC. Trypsinisation of the
Ser200 rHA had no effect on the lack of agglutination activity.
Interestingly, by contrast to the Ser200 rHA, the original Pro200
rHA had high agglutination activity.

\textbf{In silico assembly of a 3-D structural model of
A/California/04/2009}

At the time of the pandemic declaration, minimal information
was available on the new pandemic virus to help inform vaccine
design. This is a typical situation with any new pandemic strain,
as laboratory characterization and in particular structural
determination of key viral receptor proteins takes considerable
time. This indicates a need for better methods to more rapidly
characterize novel pandemic viruses.

Our group has previously used in silico structural modeling
approaches to rapidly build 3-D models of molecules of interest
including HA from novel influenza strains.\textsuperscript{31,32} This permits
rapid analysis of virus behavior in advance of wet-laboratory
characterization. To better understand the unusual behavior of
the A/California/04/2009 virus and, in particular, the differen-
ces in behavior of the Pro200 vs. Ser200 rHA, we generated a
homology model of A/California/04/2009 HA using Modeler, a
homology modeling program, using a crystal structure of an
earlier HA protein, 3AL4.pdb, as a template. This was then
used to conduct receptor binding analysis using the molecular
docking program, Autodock vina, with binding energies of the
Pro200 vs. Ser200 rHA calculated for both avian and human
sialic acid receptors. These binding studies confirmed that the
Pro200 \rightarrow Ser200 substitution had a significant impact on the
predicted binding conformation of the HA head region to avian
and human sialic acid receptors (Fig. 1, top panels). The pre-
dicted binding energy of the Ser200 HA variant for both the
human and avian sialic acid receptors was lower than the binding energy of Pro^{200} HA (Table 1), which could help explain the lack of RBC agglutination activity of the Ser^{200} rHA variant. The model further showed the Pro^{200} \rightarrow\text{Ser}^{200} HA mutation altered the binding mode of the HA head to the sialic acid receptors. Having Ser^{200} allowed formation of H-bonds between Ser^{200} and sialic acid causing the receptor molecule to move toward the \(\alpha\) helix and lose contact with Y_{104} (Fig. 1, bottom panels). Since Ser^{200} is located above the H_{192} that forms the base of the pocket, H-bonding between Ser^{200} and sialic acid prevents the receptor contacting the base of the binding pocket and interacting with H_{192}. This may cause the HA to bind its receptor with a reduced affinity. Furthermore, since the receptor is not entering deep into the binding pocket, entry of water will hydrate potential contact sites and further reduce the stability of the interaction. In contrast, Pro^{200} loses the H-bonding ability of this position with sialic acid and as a result the receptor can now form H-bonds with the base of the pocket.

**Figure 1.** Predicted receptor binding of A/California/04/2009 P^{200} or S^{200}. Top 4 panels depict predicted binding of \(\alpha-2,3\) sialyllactose or \(\alpha-2,6\) sialyllactose to receptor binding pocket of P^{200} or S^{200} A/California/04/2009 HA. Bottom 4 panels depict residues P^{200} or S^{200} colored in green and with other receptor contacting residues highlighted in yellow. Predicted hydrogen bonds between sialic acid and hemagglutinin are shown by black lines.
alone or with Advax delta inulin adjuvant, then bled 2 weeks after the second immunization for measurement of anti-influenza IgG1 and IgG2a levels by ELISA (left figure). HI assays (right figure) were performed using the Pro200 Cal04 rHA variant that possessed high agglutination activity. *p < 0.05.

**Preclinical assessment of Panblok-H1**

Given its inability to induce agglutination activity in vitro, in vivo studies were considered necessary to identify whether or not the Ser200 Panblok-H1 antigen would induce neutralising antibodies that could protect against H1N1/2009pdm infection. Ser200 Panblok-H1 vaccine was tested for immunogenicity in mice alone or with Advax adjuvant. Despite the Ser200 rHA antigen having no agglutination activity in vitro, when used to immunise mice it still induced antibodies (Fig. 2) able to neutralise the agglutination activity of the Pro200 rHA variant which retained agglutination activity as well as the agglutination activity of a local pandemic virus isolate named SF3 strain that was characterized as an A/California/07/2009-like virus. SF3 was isolated from a patient admitted to Flinders Medical Center in South Australia with acute influenza infection. The isolate was propagated in MDCK cells and the HA gene sequenced and confirmed to match the published H1N1/2009pdm sequence. The immune sera from Ser200 rHA immunized mice was also able to neutralise the HA activity of a commercial inactivated A/California/07/2009-like vaccine (Panvax™, CSL Australia). Notably, addition of Advax adjuvant to the Ser200 rHA resulted in a significant increase in both anti-influenza serum IgG1 and IgG2a levels in immunised mice. This suggested that although it did not have agglutination activity itself, the Ser200 rHA could still induce antibodies able to neutralise the agglutination activity of wildtype H1N1/2009pdm viruses.

**Table 1.** Calculated binding energies for hemagglutinin variants.

| Binding energy u−2,3 Sialyllactose | Binding energy u−2,6 Sialyllactose |
|-----------------------------------|-----------------------------------|
| A/California/04 [P200]            | −7.4 kCal/mol                     |
| A/California/04 [S200]            | −6.9 kCal/mol                     |

**Assessment of vaccine efficacy**

Given the speed of the pandemic and the need for rapid vaccine manufacture, a major challenge for the clinical trial was to identify a suitable HA antigen for use in HI assay design. While it would have been preferable to use the rHA vaccine antigen itself for the assays, the reference Ser200 rHA antigen, as described above, had no agglutination activity against either chicken or guinea pig RBC. Surprisingly, the 2 variant rHA sequences produced during the initial baculovirus cloning that contained Pro200 in the receptor-binding site did have potent RBC agglutination activity. To assess its suitability for use as an antigen in HI assays, we tested the Pro200 rHA for recognition by a reference ferret hyperimmune sera to A/California/07/2009 (BEI No. NR-15429, NIH). Pro200 rHA gave the correct HA titer (1:5000), indicating that it contained an equivalent agglutination epitope to wild type H1N1/2009pdm virus. When a monovalent inactivated A/California/07/2009 vaccine (Panvax, CSL Australia) became available this was also used to conduct HI assays in parallel with the SF3 and Pro200 rHA antigens. HI titres were measured by HI assay using guinea pig RBC, as described previously.

At study baseline, when using the SF3 antigen for the HI assays, only 9.1% of study subjects had HI titres ≥ 40, suggesting a low level of pre-existing immunity to H1N1/2009pdm in the South Australian population at the time of study commencement. As previously reported, HI assay results are highly dependent on both the actual HA antigen as well as the species of RBC used, making international standardisation of such assays problematic. High inter-assay variability may help explain the markedly different baseline estimates of H1N1/2009pdm seroprotection reported by various laboratories around the world. Indeed, we obtained different estimates of baseline seroprotection rates in our study population depending on which HA antigen we used for the HI assays. The lowest baseline seroprotection estimate for the HI assays was obtained when using the SF3 antigen, then inactivated A/California/07/2009 grown in eggs, and with the highest estimate obtained using the Pro200 rHA (data not shown). Importantly, while the different HA antigens provided different estimates of baseline seroprotection, the fold-change in pre-
post-immunisation HI titers in study subjects was highly correlated between the 3 different HA antigens (data not shown). Given the differences in HI results obtained using the 3 different H1N1/2009pdm HA antigens, we reported the study using the SF3 virus antigen.34

Three weeks after a single rHA immunization, the highest responders were younger subjects who received the highest rHA dose together with Advax adjuvant who had a seroprotection rate of 80% with SF3 as antigen for the HI assay. Given the difficulties of comparing HI assay results between studies, we compared results from the FLU005 study to convalescent sera from hospital patients recovering from H1N1/2009 infection.35 This confirmed that subjects that received Panblok-H1 with Advax adjuvant had comparable or higher HI titres than patients recovering from H1N1/2009pdm infection.34

Microneutralization (MNT) assays test the ability of immune sera to prevent live influenza virus from infecting cell lines, in vitro. They thereby measure a broader array of neutralising antibodies than HI assays, which just measure the ability to block binding of HA to sialic acid. In addition to HI activity, MNT assays detect antibodies that block other vital virus functions including fusion and nucleic acid unwrapping.36 HI and MNT results were compared at baseline and post the second immunisation in the groups that received 45 μg Panblok-H1 with or without Advax adjuvant. At baseline, most samples had low or undetectable HI titres, although many were positive by the MNT assay, indicating the HI assay had a lower sensitivity than the MNT assay. There was consequently only a very weak correlation between baseline HI and MNT results in each individual (Fig. 3A), with many subjects with undetectable HI titres having positive MNT titres against H1N1/2009pdm. This suggests that MNT can identify subjects who despite being negative by HI nevertheless have seroprotective levels of neutralising non-HI antibodies. Interestingly, post-immunization there was a much stronger positive correlation between HI and MNT levels in each subject (Fig. 3B), suggesting the vaccine induced similar proportions of both HI and MNT antibodies. Advax adjuvant had equally favorable effects on both HI and MNT responses with the mean fold increase in HI and MNT being ~2–3-fold higher when compared with Panblok-H1 alone.34

To test the ability of the Panblok-H1/Advax to induce broadly neutralising antibodies against other influenza strains, HI and MNT assays were also performed against the historic H1N1 strain, A/Puerto Rico/8/34 (A/PR8) and the H3N1 strain, A/Hong Kong/1/68 x A/Puerto Rico/8/34 (A/X31). The MNT assay detects neutralising antibodies targeting the HA stem that thereby neutralise both homologous and heterologous strains,36 whereas HI assays only detect neutralising antibodies directed against the HA head and hence are specific to homologous virus. As expected from the differences in the sequences of the HA head region, FLU005 study subjects showed no increase in HI against heterologous A/PR8 or A/X31 viruses, post-immunization (Fig. 4, left side). However, the more sensitive MNT assay revealed seroconversion to A/PR8 in ~2/5th of subjects in the Panblok-H1/Advax group vs. ~1/6th of subjects in the Panblok-H1 alone group (Fig. 4, right side). Given the lack of detectable HI to A/PR8 in these subjects, this suggests the MNT assay was detecting other types of neutralising antibodies.

**Figure 3.** Panblok-H1/Advax vaccine responses in adult human subjects. Shown is HI and MNT results against A/SF3 after 2 immunizations in FLU005 study subjects that received 45 μg rHA (Cal04 Ser200) alone (white bars) or with Advax adjuvant (black bars). Graphs on the left of the panel show responses in subjects as measured by HI, whereas those on the right show corresponding results for MNT assays. Both assays were performed against the homologous A/California/04/2009-like strain (SF3) plus heterologous influenza strains, H1N1 A/Puerto Rico/8/34 (PR8) and H3N1 A/Hong Kong/1/68 x A/Puerto Rico/8/34 (X31) \( p < 0.05. \)
such as those that target the HA stem region. HA stem antibodies are difficult to induce so it will be interesting in future to confirm whether Advax-adjuvanted rHA is able to induce broadly neutralising stem antibodies.

We next assessed the effect of subject age on antibody levels. Subject age had an effect on baseline antibody responses, with subjects older than 40 y having higher baseline titres against A/PR8 and A/X31 virus (Fig. 3C), suggesting older subjects were more likely to have encountered these viruses in the past. However, for the homologous SF3 virus there was no difference in baseline titres between those over and under 40 y of age (Fig. 3C), consistent with this being a new virus to which humans had not been previously exposed. Interestingly, there was no correlation between age at the time of immunization and the fold-change in MNT to H1N1/2009pdm (Fig. 3D), which contrasts with previous data showing a negative correlation between age and seasonal influenza HI responses.

**Discussion**

The 2009 pandemic highlighted the need for new technologies to allow more rapid design and manufacture of pandemic influenza vaccines. A seasonal vaccine (Flublok®) based on insect-cell expressed rHA was at the time of the 2009 pandemic still
multimerize we speculate that another potential reason for the as the ability of HA to agglutinate RBC relies on its ability to have the ability to in of binding properties and with a broad range of HA residues viruses. Indeed, development of Panblok-H1/Advax was per-
show any agglutination activity. This is not a unique occurrence surprisingly, when rHA was expressed from the initial CDC recom-
the fastest vaccine development programs in history. Surpris-
produce Panblok-H1/Advax was extremely fast as it avoided the need for creation of egg- or cell culture adapted influenza viruses. While the H1I/2009pdm MNT assay provided a much higher estimate of baseline immunity to H1N1/2009pdm across the study population, as many subjects that had a nega-
tive HI were seropositive by MNT assay. Advax adjuvant induced a ~3-fold increase in MNT titters vs. ~2-fold higher titters by HI assay. While the HI assay did not detect any vac-
cine effects on heterologous antibodies against the A/PR8 or A/X31 virus strains, the more sensitive MNT assay showed that Panblok-H1/Advax induced sroconversion against A/PR8 in ~2/5th of the trial subjects. This raises the possibility that Panblok-H1/Advax induced HA stem antibodies in some sub-
jects. As stem antibodies confer broad cross-protection against heterologous influenza viruses, this will be an important area for future study. The ability of Advax adjuvant to induce broader cross-protective responses has been seen in animal studies where formulation of an inactivated Japanese encephali-
tis virus antigen with Advax resulted in robust cross-protection against heterologous flaviiviruses including Murray Valley Encephalitis and West Nile virus. This enhanced protection was transferrable from immunised to naïve mice using memory B cells, suggesting Advax enhances production of broadly cross-protective memory B cells. Recent human influenza stud-
ies showed that subjects that received Advax-adjuvanted inacti-
ated influenza vaccine had a higher day 7 post-immunisation plasmablast responses and enhanced expression of activation-
induced cytidine deaminase translating into greater antibody avidity and CDR3 complexity.

Advax has been shown in animal models to be a potent inducer of memory CD4+ and CD8+ T cells. One important aspect that was not able to be addressed in our study was whether Panblok-H1/Advax also enhanced human T-cell immunity against influenza. CD4+ T cells have recently been shown to make an important contribution to human influenza protection. Future studies will be needed to identify whether better immune correlates of influenza protection can be found to supplement or replace the information provided by HI assays.

While the merits of adding adjuvants to seasonal influenza vaccines has been widely debated, studies of H5N1 vaccines have demonstrated the vital importance of adjuvants for high levels of seroprotection. The reason why seasonal vaccines generally do not require adjuvants is that most people have already been primed by past seasonal influenza infections with related strains. Furthermore, inactivated virus vaccines contain contaminants including viral RNA and neuraminidase and nuclear protein that act as inbuilt adjuvants. Inactivated influenza vaccines lose their immunogenicity when administered to toll-like receptor (TLR)-7 knockout mice, thereby demonstrat-
ing the importance of viral RNA to innate immune activation and vaccine immunogenicity. Panblok-H1 alone or together with Advax adjuvant was very well tolerated. The most common local reaction was injection site discomfort with other
local reactions such as bruising, redness, and swelling with most of local reactions being low grade. Surprisingly, post-immunisation headaches were significantly lower in subjects that received Advax adjuvant (2.9%) compared with Panblok-H1 alone (10.9%), suggesting the Advax adjuvant suppressed immunisation headaches though a currently unidentified mechanism. While adjuvants were ultimately not essential for immunisation headaches though a currently unidentifi- ed H1 alone (10.9%), suggesting the Advax adjuvant suppressed

Very little time is available for vaccine design when the world is faced with a real-life pandemic given the exceptional rates of virus spread. In particular, wet-laboratory methods for study of new viruses require that the virus first be successfully cultured, and even then there is a risk that the virus will mutate in culture and not be fully representative of the wild-type viruses causing the pandemic. This was highlighted by the problems of the A/California/04/2009 reference sequence, with the rHA from this sequence having no agglutination activity. While this problem was ultimately solved by the more representative A/California/07/2009 strain, this caused time delays due to the problems with vaccine manufacture. As we show here, rapid analysis of new mutant influenza strains based in silico structural modeling can provide important early insights into key virus properties, including its ability to bind human and avian sialic acid receptors. In the future such in silico approaches are likely to become more routine in the analysis of new pandemic viruses.

Limitations of the current study included the inability during study follow up to assess actual protection against the pandemic virus using viral cultures in individuals developing symptoms of influenza-like illness. However, as detailed in the initial study report, exposure to the pandemic virus was indirectly assessed by looking for serological evidence of a boost in antibody titres, in the period between 4 weeks post the second immunisation and the end of the study with the expectation that infection with the pandemic virus should result in a boost to these titres. In fact, there was an extremely low 3.9% rate of late H1N1/2009pdm seroconversion between weeks 6 and 26, suggesting most of subjects were protected during the peak period of the pandemic when the pandemic virus was widely circulating in the community. It would also have been useful to do a direct comparison within the study between the immunogenicity of the Advax-adjuvanted recombinant hemagglutinin vaccine and a commercial inactivated virus vaccine, but this was not possible as at the time that the FLU005 study commenced, no pandemic vaccine based on inactivated virus was yet available. While it would be useful to have crystal structures of Pro\\textsuperscript{200} and Ser\\textsuperscript{200} HA bound to the human and avian sialic acid receptors to confirm the predictions of our in silico structural models, we have previously used a similar modeling approach to predict the structure and behavior of HA from the novel H7N9 influenza strain and were able to confirm the accuracy of the model against the published crystal structure.

Overall, many lessons were learned from the 2009 pandemic which demonstrated the need for greater speed and scalability of pandemic vaccine manufacturing. This real-life pandemic scenario proved a recombinant hemagglutinin vaccine could be

designed and manufactured extremely quickly, making it an ideal pandemic platform.

Materials and methods

Manufacture of panblok-H1

Recombinant baculovirus stock was added to a bioreactor containing SF+ cells and incubated for 48–72 hours. Infected cells were then removed from the bioreactor(s), separated from the culture media by centrifugation, solubi- lized using non-ionic detergent and rHA purified by depth filtration, ion-exchange exchange chromatography, hydrophobic interaction chromatography, Q-membrane and finally ultrafiltration, as described previously. Because the reagents used in the SRID assay for HA antigen content were not available at the time of Panblok-H1 for- mulation, initial lots were formulated based on the protein content determined by the bicinchoninic acid (BCA) assay. The extractable antigen of the filled vials was 3, 11 and 45 μg respectively. Although cGMP manufacture was completed by mid-June 2009 release of the final product for the clinical trial was delayed by 30 d by the need to undertake a 30-day mycoplasma culture test. As soon as the myco- plasma result was available the Panblok-H1 antigen was released and the clinical trial commenced on 18 July 2009, making this the first 2009 pandemic vaccine to enter clinical testing worldwide.

Clinical trial design

The clinical trial of Panblok-H1 was conducted as a random- ized, subject- and observer-blinded, parallel group trial, with 6 groups receiving one of 3 rHA doses (3, 11 or 45 μg) alone or formulated with Advax delta inulin adjuvant. The trial included adults over 18 y and exclusions included pregnancy, immuno-suppressive therapy, oral corticosteroids, HIV infec- tion, or a history of drug or alcohol abuse. However, unlike many other studies no exclusions were made based on older age or presence of chronic disease. Two immunisations were administered intramuscularly 3 weeks apart and antibody responses were measured 3–4 weeks after each immunisation. As this was a first in man study, assessment of vaccine safety was a major priority and solicited local and systemic reactions were collected with a 7-day memory aid and serious adverse events collected throughout the study period. As previously reported, 281 subjects were enrolled, randomized, had baseline serology and received the first vaccine dose, with subjects being predominantly Caucasian (96.4%), with a median age of 52 y. Seventy 3 percent of subjects were overweight or obese and 54.7% were on medication for one or more chronic disease. Many trial subjects belonged to high-risk groups recommended for seasonal influenza vaccination and 71.2% had already received the 2009 season trivalent influenza vaccine.
**HI assays**

Antibody titres were determined by HI assay, with all paired serum run simultaneously in the same assay. Briefly, serum was pre-treated with 3 volumes of receptor destroying enzyme (Denka Seiken, Tokyo, Japan). The enzyme was inactivated at 56°C for 30 min and and samples were mixed with 6 parts of 1% guinea pig red blood cells (gRBCs) to remove non-specific agglutinins and inhibitors of haemagglutinin. The gRBCs were pelleted by brief centrifugation and sera mixed with 6 parts PBS to obtain a 1:10 v/v dilution of the samples. HI assays were performed with gRBC following the WHO Manual on Animal Influenza Diagnosis and Surveillance. Pre-treated sera was incubated with 4 HA units of the relevant HA antigens for 1 hour at room temperature before the addition of a 1% gRBC cell suspension. Plates were incubated for a further 1 hour at room temperature before reading. HI titres were expressed as the reciprocal of the final serum dilution completely inhibiting haemagglutination. Endpoints were seroprotection as defined by an HAI titer of ≥1:40, seroconversion (a 4-fold increase in titer and an HAI titer of ≥1:40), and fold increase in geometric mean titer (GMT). The geometric mean, 95% confidence intervals and p-values were determined for pre- and post- patient serum HA data sets using Graphpad Prism 5.0 (GraphPad Software, Inc., San Diego, California, USA).

**MNT assays**

The MNT assay was performed in 2 steps: a virus-antibody reaction step, where the virus is mixed with the antibodies present in the samples, and an inoculation step, where the Madin-Darby Canine Kidney (MDCK) cells were added in 96-well culture plates and incubated overnight at 37°C with 5% CO₂. Every 3 plates, we included a standard antibody, corresponding to the test virus, to check if the assay has been done properly. The medium used for the dilutions was DMEM supplemented with 3% FBS and TPCK-trypsin. The working dilution of the virus had been determined by virus titration before use on MDCK cells. In all experiments, only fresh aliquots of virus were used. Firstly, 50μl of the serially diluted samples and 50μl of virus were mixed in 96-well plates and incubated 1 hour at 37°C with 5% CO₂. Half-logarithmic dilution steps were chosen to have a broad range of serum dilution. Standard antibodies with a mid-range titer were included as a positive control. Then 100μl of 1.5×10⁵ MDCK cells were added to each well. Incubation was done overnight at 37°C with 5% CO₂. After removing media, plates were washed once with PBS then fixed with 100μl of fixative (50% MeOH/Aceton) per well for 10 minutes, and followed by an immunostaining step to detect the amount of Influenza viral nucleoprotein. Hundred μl per well of mouse anti-Influenza A virus nucleoprotein antibody (BEI, NR-4544), 1:5,000 dilution in 1% BSA/PBS was added and incubated for 1 hour at room temperature. After washing, 100μl per well of detection biotin-anti-mouse IgG1 1:5,000 in 1% BSA/PBS antibody plus Streptavidin-HRP 1:2,000 were added and incubated for 1 hour. After washing, 50μl per well of DAB substrate was added and incubated for about 10 minutes to allow color development. Plates were washed thoroughly with tap water, dried inverted overnight and scanned and counted using ImmunoSpot® Image analyzer (version 5.0.41, Cellular Technology Ltd, Cleveland, OH) adapted to microneutralisation and virus titration. The microneutralisation gives results as end point titer which is expressed as the reciprocal of the highest dilution of serum with infected cells number less than X. X is half of the difference between the average number of infected cells of positive control wells and the average number of infected cells of negative control wells. All wells with several infected cells below this X value are considered as positive for neutralisation. This absence of infectivity indicates the presence of virus-specific antibodies in the samples in enough amount to prevent the Influenza virus from infecting the cells. To decrease the variability of the results, we did the microneutralisation in duplicates for each sample. Replicate assays were combined as geometric means to give a single titer. For calculation, a negative MNT titer was assigned a value of 5, which was half of the minimum detectable; and titres greater than the final dilution were assigned a value of 1280, which was twice the maximum detectable.

**Bioinformatic methods**

For HA modeling and global alignments, the UCSF Chimera software version 1.7.47 was used. The Align and CLUSTALO programs of Uniprot website (http://www.uniprot.org/) were also used for more precise and accurate alignments. Molecular docking of sialic acid receptors were performed using the molecular docking program Autodock vina version 1.1.2.48 Preparation of ligand and the receptor for the docking calculation was conducted using the program Autodock Tools (ADT). Firstly, polar hydrogen was added to the receptor and it was followed by the assignment of Kollman united atom partial charges. A search space of 26×25×27 Angstrom was defined within the receptor binding pocket of HA molecule. Autodock 4 atom types were assigned to each ligand molecule followed by the assignment of Kollman united atom partial charges. All the torsion angles in the ligand were set free to allow flexible docking. Exhaustiveness used for the search was 8. Finally, 9 possible binding modes were calculated.

To compare the structures and sequences of different hemagglutinins (HA), reference structures from the RCSB Protein Data Bank (PDB; http://www.rcsb.org/pdb/) were used:
- 1RU7 for the HA of PR8 virus
- 2VIU for the HA of X31 virus
- 3LZG of the HA of A/California/4/2009 (H1N1), which has a very close relationship with the HA of SF3 virus (A/California/04/2009 (H1N1)

**Disclosure of potential conflicts of interest**

NP, HR and YH are affiliated with Vaxine Pty Ltd. MC is affiliated with Protein Sciences Corporation. DG and DS declared no conflicts.

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