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Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses

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Abstract

The rapid spread and the transmission to humans of avian influenza virus (H5N1) has induced world-wide fears of a new pandemic and raised concerns over the ability of standard influenza vaccine production methods to rapidly supply sufficient amounts of an effective vaccine. We report here on a robust and flexible strategy which uses wild-type virus grown in a continuous cell culture (Vero) system to produce an inactivated whole virus vaccine. Candidate vaccines based on clade 1 and clade 2 influenza H5N1 strains were developed and demonstrated to be highly immunogenic in animal models. The vaccines induce cross-neutralising antibodies, highly cross-reactive T-cell responses and are protective in a mouse challenge model not only against the homologous virus but against other H5N1 strains, including those from another clade. These data indicate that cell culture-grown, whole virus vaccines, based on the wild-type virus, allow the rapid high yield production of a candidate pandemic vaccine.

Keywords

H5N1 whole virus vaccine; Vero cells

1. Introduction

It is widely believed that the emergence of a new influenza pandemic caused by avian strains is only a matter of time and that a safe, effective and easily manufactured vaccine is required [1–3]. Pandemic H5N1 vaccine candidates tested to date were manufactured using attenuated reassortant viruses. These reassortants are generated using the hemagglutinin (HA) and the neuraminidase (NA) genes of the circulating wild-type (wt) virus and the six remaining genes of the H1N1 influenza strain A/PR/8/34 (6:2 reassortants) which usually confer high growth properties in embryonated hens' eggs. This reassortant virus is also attenuated by removal of the polybasic cleavage site of the HA which is associated with high pathogenicity [4–6]. These reverse genetics(RG)-derived reassortants [7,8] are then subjected to extensive safety testing

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before distribution to the influenza vaccine manufacturers. This procedure is essential to allow use of the virus under the biosafety level 2 enhanced, which is the highest safety level available in egg-based manufacturing facilities, and to generate the potential high growth phenotype required for adequate vaccine antigen yield. However, this derivation of new reassortants requires several weeks resulting in significant delay in the delivery of a new pandemic vaccine. In addition, the vaccine may provide an optimal antigenic fit with the wt circulating virus only with respect to the HA and NA genes and not with respect to the rest of the genes including the nucleoprotein and the matrix genes which are derived from the A/PR/8/34 virus. This report now describes the production and preclinical testing in animals of whole virus candidate vaccines based on wt H5N1 strains.

2. Materials and methods

2.1 Viruses and cell lines

Influenza strains used were (abbreviations and source in parentheses): A/Vietnam/1203/2004 (H5N1) (VN1203, CDC# 2004706280); A/Indonesia/05/2005(H5N1) (IN5/05, CDC #2005740199); A/HongKong/156/97(H5N1) (HK156, CDC #97013490); A/Vietnam/1194/2004(H5N1) (VN1194, CDC #2004706279); A/HongKong/213/2003(H5N1), (HK213, CDC #2/27/03); A/SP83/2004/Thai(H5N1), (Thai83, CDC #2004707254); A/FPV/Rostock/34(H7N1), (FPV/Ros, Univ. Giessen); B/Jiangsu/10/2003, (B/JS, NIBSC); A/NewCaledonia/20/99(H1N1), (NC20/99, NIBSC); A/NewYork/55/2004(H3N2), (NY55/04, NIBSC); A/Duck/Singapore-Q/F119-3/97(H5N3), (Dk/Sing, NIBSC #97/722). Viruses (as indicated) were provided by the Center for Disease Control (CDC, Atlanta, USA), the University of Giessen (Germany) or the National Institute for Biological Standards and Control (NIBSC, UK) and grown under BSL-3+ conditions. To generate the virus seed bank system, the CDC primary seed viruses IN5/05 and VN1203 were expanded resulting in three further Vero passages. The virus used for vaccine production is at Vero passage 4. The Vero cell line used is derived from ATCC CCL81 and was grown in serum protein-free medium as described previously [9].

2.2 Fermentation and inactivation of H5N1 viruses

Growth of influenza virus in Vero cells was as described [9]. Briefly, a single aliquot of the fully characterized Vero production cell bank was expanded through successive passages to the required volume in serum-free DMEM/Ham's F12 medium. Either 30L or 100L-scale fermenters containing microcarrier cultures were infected with an aliquot of the H5N1 influenza virus A/Vietnam/1203/2004(H5N1) or A/Indonesia/05/2005(H5N1), respectively, at a multiplicity of infection of 0.001. Supernatant virus was harvested and double-inactivated with 0.05% formalin at 37°C for 72h, followed by UV-irradiation.

2.3 Downstream processing and vaccine preparation

Double-inactivated influenza virus was purified by sucrose gradient ultracentrifugation followed by ultra-/diafiltration and sterile filtration to produce the monovalent bulk material (MVB) used for testing. Ultra- and diafiltration steps serve to concentrate the harvest supernatant and to achieve a buffer exchange, respectively. Influenza antigen content was determined by hemagglutination and single radial immunodiffusion assays (see below).

2.4 Immunization and challenge of animals

Mice and guinea pigs were immunized by subcutaneous injection of 500 µl of vaccine as described in the text. Mice were challenged intranasally with 20 µl containing 1×10^5 TCID₅₀ of virus and monitored for death or survival over a period of 14 days. H5N1 strains used for challenge were grown and titrated via TCID₅₀ assays in Vero cells. Prior to the challenge

studies, the virus dose that kills 50% of the CD1 mice (LD_{50}) was determined for the H5N1 strains HK156, VN1203 and IN5/05 to be 2.9, 3.3 and 4.1×10^3 TCID₅₀, respectively. Despite similar LD_{50} values, at doses $\geq 10^4$ TCID₅₀/animal, the HK156 strain killed all, the VN1203 usually killed 90–100% and the IN5/05 strain killed 70–100% of the mice showing that IN5/05 was somewhat less virulent for the CD1 mouse.

2.5 ELISA (Enzyme Linked Immunosorbant Assay)

The specific IgG titer against the H5 hemagglutinin (H5 HA) was determined by an indirect ELISA. Microtiter plates were coated with 50 ng recombinant baculovirus-derived H5 hemagglutinin (derived from VN1203 strain and obtained from Protein Sciences, Meriden, CT, USA) per well or left uncoated, respectively. Subsequent to washing and blocking, serial 1:4 dilutions of the sera starting with a 1:100 dilution were applied. Following incubation with the HRP-labeled secondary anti-mouse IgG antibody the ELISA was developed using OPD/ H_2O_2 . Color development was stopped by addition of H_2SO_4 and plates were read at 490/620 nm. To determine the endpoint antibody titer, all absorbance readings equal or greater than the cut-off value (4 times the mean absorbance value of a negative control serum at a 1:100 dilution) was considered positive.

2.6 Hemagglutination assay

Influenza virus samples were diluted in two-fold steps in PBS, mixed with an equal volume of a 1% erythrocyte suspension (chicken) and incubated in a V-shaped microtiter plate for 30 min at room temperature.

2.7 Microneutralization assays

For the standard microneutralization assay (done with wt viruses which have the polybasic cleavage sites in the HA allowing trypsin-independent growth in Vero cells), heat-inactivated serum samples were serially diluted with cell culture medium in two-fold steps. The dilutions were mixed at a ratio of 1:1 with VN1203 virus (100 TCID₅₀ per well), incubated for 1 h at RT and transferred to a microtiter plate with a Vero cell monolayer. After 5–7 days incubation at 37°C, the cultures are inspected for cytopathic effect (cpe). The neutralizing titer, expressed as the reciprocal of antiserum dilution at which virus growth is 50% inhibited, was calculated by the number of virus negative wells and the serum dilution according to [10].

For the ELISA-based microneutralization assay (done with viruses lacking the polybasic cleavage sites in the HA allowing trypsin-independent growth only in MDCK cells), heat-inactivated serum samples were serially diluted with PBS in twofold steps, starting at a 1:10 dilution. 50 μ L of each dilution were transferred to a microtiter plate, mixed 1:1 with virus (adjusted to the appropriate concentration) and incubated for 1h at RT. After addition of 100 μ L/well MDCK cell suspension (5×10^5 /mL), the plates were incubated for 16h at 37°C. After removal of medium, fixation with methanol/acetone and washing, the first antibody (anti-influenza A matrix) was added and incubated 1h at 37°C. After washing, the second antibody (rabbit anti-goat IgG, HRP conjugated) was added and incubated 1h at 37°C. Freshly prepared substrate (TMB) was added and incubated for 20 min at RT in the dark. Stop solution was added and the absorbance (OD) of the wells was read at 450/620 nm. The neutralization titer of a sample is determined as the highest serum dilution with an OD lower than the 50% neutralization point of the plate, calculated from positive cell control (medium + test dilution of virus + MDCK cells) and negative cell control (medium + MDCK cells).

2.8 Viral infectivity (TCID₅₀) and single-radial-immunodiffusion (SRD) assays

The H5N1 virus titers of samples (TCID₅₀) were determined by titration on standard Vero cells by serial ten-fold dilutions of samples inoculated into 96 well microtiter plates as described

earlier [9]. SRD tests were performed according to [11] in duplicate gels using standard sheep antiserum to H5N1 (NIBSC, lot 04/214 raised against the VN1194 strain) and a Baxter internal working reference standard vaccine antigen calibrated against the NIBSC standard antigen. The Baxter internal working reference standard vaccine antigen (WRV, #11-01-06) is derived from the VN1203 strain that has an identical HA amino acid sequence as the VN1194 strain. The standard antiserum was used at 15 µl/ml agarose, as recommended by NIBSC.

2.9 ELISPOT assay

The frequency of interferon- γ (IFN- γ) or interleukin-4 (IL-4) secreting cells was analysed using mouse IFN- γ and IL-4 ELISPOT kits (Mabtech AB, Nacka, Sweden) following the instructions of the manufacturer. Polyvinylidene difluoride-coated 96-well plates (Millipore Corp. Bedford, MA) were coated with anti-IFN- γ or anti-IL-4 monoclonal antibodies overnight at 4°C. Serial dilutions of spleen cells were distributed in the wells ranging from 6×10^4 to 2×10^5 cells/well and stimulated with either H5N1 candidate vaccines, inter-pandemic influenza strains A/NewCaledonia/20/99(H1N1), A/NewYork/55/2004(H3N2), B/Jiangsu/10/2003 or with recombinant H5 protein at a concentration of 0.1 µgHA/ml. Wells containing no antigen or 0.1 µg/ml of pokeweed mitogen (Sigma, St. Louis, USA) were used as negative and positive control, respectively. The plates were incubated overnight at 37°C and the cells were discarded. After extensive washing with PBS, IFN- γ or IL-4 spots were detected by biotinylated IFN- γ - or IL-4-specific antibody followed by addition of streptavidin-alkaline phosphatase and development with BCIP/NBT substrate solution. Spots were counted using an automated ELISPOT reader (AID, Strassberg, Germany). The number of spots observed in wells containing no antigen was subtracted from the number of spots observed in wells containing specific antigen and the results were expressed as the number of spot forming cells (SFC)/ 10^6 spleen cells.

2.10 Sequencing of hemagglutinin genes

Viral RNA extracted from passage 10 (post production virus) was transcribed into cDNA using the primer oFLURT-2 (5'- AGC AAA AGC AGG GGT ATA ATC TGTC-3'). The primers oHA_v-10 (5'-AAC CAT GGA GAA AAT AGT GCT TC-3') and oHA_u-2 (5'-GTC GAC TTA AAT GCA AAT TCT GCA TTG TAAC-3') were used for PCR amplification. The resulting HA genes were cloned resulting in plasmids pDD4mH55TNT-VN-HA (VN1203 insert) and pPCR-Script-HA-INa#13 (IN5/05 insert) and sequenced using standard procedures. The VN1203 and IN5/05 inserts were compared with the master sequences (Genbank accession# AY818135 for VN1203 and Los Alamos Data base number ISDN125873 for IN5/05).

2.11 Statistical analysis

Data were analyzed with the Statistica software (version 7.0; Statsoft, Tulsa, USA); P values <0.05 were the criterion for statistical significance. The two sided t-test was used to assess the significance level of survival after challenge with different viruses (difference between two proportions). The nonparametric Kolmogorov-Smirnov two-sample test was used to assess the significance of difference in the ELISA and micro-neutralization titer between the mice immunized with adjuvanted or non-adjuvanted vaccine.

3 Results

3.1 Vaccine manufacturing based on H5N1 wild-type virus in Vero cells

A novel strategy was developed to avoid the delay associated with vaccine production using reverse genetics-derived reassortant virus. This involves use of wild-type virus to produce vaccine antigen in Vero cell culture, one of the most promising cell culture systems for production of influenza viruses [9,12]. Large scale vaccine manufacture is carried out at BSL-3

+, the level required by WHO for wild-type H5N1 [13], using state-of-the-art serum protein-free cell culture fermentation technology. The studies described here used vaccine manufactured at 100 liter pilot scale but this process has been successfully upscaled to 6,000 liter and multiple batches have already been manufactured at this scale. Candidate vaccine was manufactured using the clade 1 strain A/Vietnam/1203/2004 [14] (VN1203) and the clade 2 strain A/Indonesia/05/2005 (IN5/05) [15]. Consistent high virus titers were obtained for eight pilot fermentation runs with the VN1203 strain (range 0.4 to 1×10^9 TCID₅₀/ml), which was up to 10-fold higher than yields obtained for standard seasonal vaccine strains grown in Vero cells (6:2 reassortants optimized for growth in eggs). Excellent and consistent yields were also obtained for HA antigen as measured by standard HA determination (range 512–1024 hemagglutination units). Similar titers were obtained for other clade 1 H5N1 isolates tested (strains A/HongKong/213/2003, A/Vietnam/1194/2004, and A/SP83/2004(Thai)) and also for the IN5/05 clade 2 isolate. For vaccine production, the virus harvest was inactivated using a highly stringent procedure involving two separate steps, formalin and UV treatment. Formalin alone was sufficient to achieve total inactivation as confirmed by safety (passage) assays of the bulk vaccine in two highly susceptible cell systems, i.e. Vero and chicken embryo cells. Double inactivation was chosen to enhance the safety margin. The inactivated virus was then purified by continuous sucrose gradient centrifugation followed by ultra-/dialfiltration steps prior to formulation. Re-sequencing of the HA gene of both strains at the post production level at passage 10 confirmed that virus grown in Vero cells did not result in the selection of antigenic variants, the sequences derived from post-production virus RNA were identical to the corresponding gene bank entries (see materials and methods).

3.2 Immunogenicity and cross-neutralization in guinea pigs

In addition to vaccine supply, the other critical issue is vaccine efficacy. Ideally the H5N1 vaccine should protect not only against the virus strain used for vaccine manufacture but also against viruses that have undergone antigenic drift. Initial immunogenicity studies in guinea pigs and mice determined the ability of the whole virus vaccine to induce broadly reactive humoral and cellular immune responses. Guinea pigs were immunized twice subcutaneously with either 5µg alum-adjuvanted, inactivated VN1203 vaccine (corresponding to 1.7µg HA antigen as measured by SRD assay) or 5µg recombinant baculovirus-derived alum-adjuvanted HA protein (rH5-HA, derived from the VN1203 strain). Since the baculovirus-derived HA did not react in the SRD assay, standardization according to SRD values was not possible. Antisera to the whole virus candidate vaccine neutralized the infectivity of all H5N1 strains (see Materials and Methods) tested (Table 1), ranging from the original Hongkong/1997 to the recent clade 2 Indonesia/2005 isolate. Moreover, substantial cross-neutralization was also seen with a H5N3 strain (A/Duck/Singapore-Q/F119-3/97) but, as expected, no activity was measured against a control H7N1 strain. Despite being administered in the guinea pigs at a higher concentration, the recombinant antigen induced lower neutralizing antibody titers to the homologous strain (VN1203) and was ineffective against the clade 2 IN5/05 strain, consistent with reports that very high antigen doses of rHA vaccine were required to induce seroconversion in human trials [16]. These data indicate that the candidate whole virus vaccine has the potential to be effective against a broad range of H5N1 viruses as antisera generated by immunization with the VN1203 vaccine not only neutralized a number of human pathogenic H5N1 clade 1 strains ranging from 1997 to 2004 but also the clade 2 Indonesia strain.

3.3 T helper cell responses and cross-stimulation in mice

Subsequently, homologous T helper cell responses and potential heterologous cross-stimulation responses were investigated using inbred Balb/c mice, widely used for influenza virus protection and virulence studies [17]. Animals were immunized twice (days 0 and 21) with either VN1203 or IN5/05 whole virus vaccines, a B/Jiangsu (B/JS) strain whole virus vaccine, rH5-HA antigen and saline. All preparations were injected at a dose of 1.5µg HA with

0.2% alum as adjuvant, a dose expected to induce robust immunity. Splenocytes were prepared on days 8 and 28 and stimulated in-vitro with the non-adjuvanted whole viral and recombinant antigens. The number of INF- γ and IL-4 producing cells were then determined by ELISPOT assays. TH-1 type responses (INF- γ) were found to be predominant at day 8 (not shown), while a mixed TH-1 and TH-2 response was observed on day 28 (Fig. 1). All three H5 vaccines induced substantial TH-1 responses to stimulation with H5-containing antigens, but little or no response to H1, H3 or B-strain antigen stimulation (Fig. 1a). Immunization with either VN1203 or IN5/05 resulted in high cross-reactive responses to either strain. Immunization with the B-strain vaccine did not result in cross-reactive responses, only a highly specific response to stimulation to B-strain antigen. The TH-2 responses showed a different picture, however, in that immunization with whole virus VN1203 or IN5/05 vaccines resulted in cross-reactive responses after stimulation with either H5-, H1- or H3-containing antigen (Fig. 1b). No cross-reactive TH-2 responses were obtained to the B-strain and, as expected, the whole virus B-strain vaccine did not induce cross-reactions. Interestingly, immunization with recombinant subunit H5 did not induce cross-reactive TH-2 responses to H1 or H3, suggesting that the cross-reactivity induced by the whole virus vaccine is due to responses to antigens other than HA, for instance, nucleoprotein and matrix proteins.

3.4 Protection studies in mice

As guinea pigs, initially chosen for serology studies, could not be infected with H5N1 virus, mice were used for subsequent challenge and protection studies. Outbred mice (CD1 strain) were used to reflect more the human genetic situation. The animals were immunized twice, subcutaneously, after a three weeks interval with doses ranging from 0.001 to 3.75 μ g HA antigen using non-adjuvanted material and vaccine adjuvanted with 0.2% aluminum hydroxide. Control groups received buffer or adjuvanted buffer. Sera, drawn 21 days after the immunizations, were assayed for H5 HA-specific ELISA- and VN1203-specific neutralizing antibodies (see materials and methods). Three weeks after the first immunization, dose-dependent ELISA titers were detectable (not shown); however, neutralizing antibodies were below the detection limit. Three weeks after the booster immunization, ELISA antibodies increased strongly and virus-neutralizing antibodies could also be detected. There were no statistically significant differences in ELISA titers between the mice immunized with adjuvanted and nonadjuvanted vaccine. The only statistical significant difference was seen for the microneutralization assay at the highest dose of 3.75 micrograms ($P=0.0134$).

At the three week time point, all animals were challenged intranasally with 10^5 TCID₅₀ of the VN1203 virus strain and survivors were counted 14 days post challenge. The non-adjuvanted vaccine protected 100% of immunized mice with a dosage as low as 30ng HA antigen, whereas 750ng of the alum-adjuvanted vaccine were required to provide full protection against lethal infection (Table 2). The statistical comparison of survival rates showed no significant difference at the higher dose levels, only at the lowest dose was the difference significant ($P=0.0192$). Thus, the whole virus vaccine was highly effective in inducing protective immunity in CD1 mice and complete protection correlated with positive neutralization results. The non-adjuvanted vaccine formulation appeared more potent than the alum-formulated material, an effect not seen in challenge studies in Balb/c mice (not shown). Subsequent CD1 mouse challenge studies were carried out with non-adjuvanted preparations.

3.5 Cross-protection of the VN1203 and IN5/05/05 candidate vaccines in mice

An important question for potential pre-pandemic vaccines is their cross-protective potential against antigenically different strains of the same subtype. Therefore, CD1 mice were immunized as described above with VN1203 vaccine in a dose range of 0.006 to 0.750 micrograms with the non-adjuvanted material. Three weeks after the second immunization, the animals were challenged with either the homologous clade 1 VN1203 strain, the

heterologous clade 1 HK156 strain or the heterologous clade 2 IN5/05 strain. Challenge with VN1203 resulted in full protection against lethal infection with doses >30 ng (Table 3, upper panel). Challenges with IN5/05 and HK156 showed surprisingly good dose-dependent cross-protection, which reached 95–100% at a dose of 750ng. Next, the reciprocal study was carried out with mice being immunized with the clade 2 Indonesian strain vaccine and challenged with the homologous IN5/05 or the heterologous VN1203 clade 1 strain. The study confirmed the high level of cross-protection obtainable by immunization with the whole virus vaccine (Table 3, lower panel). At the higher antigen doses of 3.75 and 0.75µg, 100% protection was conferred against challenge with clade 2 or clade 1 virus and close to 100% protection against heterologous challenge was obtained with as little as 0.15µg. The statistical comparison of survival rates showed no significant difference after challenge with the different H5N1 viruses (not shown). The high degree of cross-protection of VN1203 or IN5/05 vaccinated mice from challenge with divergent H5N1 strains suggests that the virus strain used for vaccination need not fully match the challenge virus to achieve high levels of protection against lethal infection.

4. DISCUSSION

The pandemic potential of the highly pathogenic avian influenza H5N1 virus highlights the urgent need for an effective vaccine manufactured using a robust, secure production system capable of producing large amounts of vaccine in a short time-frame. Ideally, this vaccine should afford protection against the different H5N1 strains which may arise by antigenic drift. Standard technologies using 6:2 reassortant viruses and embryonated eggs to produce split or subunit vaccines may not fulfill all those criteria. We describe here the advantages of using an inactivated wild-type whole virus H5N1 vaccine manufactured using a novel Vero-cell based cell culture system.

Vero cells have been widely used for human vaccine production over the past 30 years [18] and are the only continuous cell line fully accepted by regulatory authorities for production of whole virus vaccine. This extremely robust system has already been scaled up to 6,000 liter scale without loss of cell viability or virus productivity compared to the pilot scale process. Importantly, this process is completely independent of the supply of hens' eggs which may be endangered during a pandemic. Further, this technology can be utilized at BSL-3+, the standard biohazard requirements for working with wild-type pathogenic avian influenza viruses. Use of wild-type virus instead of the 6:2 reassortant strain for H5N1 vaccine production not only reduced the manufacturing time but also lead to 2 to 4-fold higher HA antigen yields than obtained using reassortant virus in eggs (data not shown). This confirms data that the original viruses attenuated by reverse genetics deliver only 30% to 40% of HA yields obtained for normal seasonal vaccine strains [19]. However, new or improved reassortant viruses generated by reverse genetics may result in higher yields.

Use of an inactivated whole virus vaccine, instead of split or subunit vaccine, may also improve the vaccine supply. It has been reported that only 10µg of antigen per dose was required to generate acceptable seroconversion with a reassortant-derived whole virus vaccine produced in eggs [20]. In contrast, results from clinical trials with split/subunit vaccines were disappointing as 30–90µg of antigen per dose were required to generate acceptable antibody responses [21,22]. Potential superiority of whole virus wild-type based H5N1 vaccines over 6:2 reassortant split vaccines, however, can only be evaluated in direct comparisons in humans.

In addition to vaccine supply, the other critical issue is vaccine efficacy. Ideally the H5N1 vaccine should protect not only against the virus strain used for vaccine manufacture but also against viruses that have undergone antigenic drift. Our data indicate that the candidate whole virus vaccine has the potential to be effective against a broad range of H5N1 viruses. Antisera generated by immunization with the VN1203 vaccine, prepared using the clade 1 strain

Vietnam/1203/2002, not only neutralized a number of H5N1 clade 1 strains ranging from 1997 to 2004 isolates (differing in HA antigenicity [23–25]) but also neutralized the first clade 2 strain to emerge (Indonesia/05/2005) [15]. The baculovirus-derived recombinant HA antigen was less effective in inducing neutralizing antibodies against the clade 1 strains, and it did not induce neutralizing responses to the clade 2 strain, in agreement with reports that very high antigen doses of rHA were required to induce seroconversion in humans [16].

Although functional antibody responses are an accepted correlate for vaccine induced protection for vaccine licensure purposes [26], there are increasing reports that T-cell responses are important [27] and may be better correlates of vaccine protection especially in the elderly [28]. Whole virus vaccine may induce a more efficient cellular response than split or sub-unit vaccines [29,30] and this may be critical in preventing severe disease and death if not critical for prevention of infection. The candidate VN1203 and IN5/05 vaccines were both effective in inducing TH-1 cell responses (induced by CD4+ T helper cells). Both vaccines and the rH5-HA antigen induced cross-reactive T-cell responses with responses to stimulation with any H5 antigen but not to H1, H3 or B strain antigen. However, the TH-2 response was more broadly cross-reactive. Immunization with whole virus VN1203 or IN5/05, but not with rH5-HA, induced cross-reactive responses following stimulation with H5, H3 or H1 containing antigens. This cross-reactivity may be due to responses to other antigens in the whole virus vaccine and not in the rH5-HA formulation, for instance, matrix and nucleoproteins which are highly effective in inducing T-cell responses [31]. Thus a whole virus vaccine may have the potential to induce a stronger and more broadly reactive cellular and humoral response due to the presence of the full set of virion proteins (including their helper cell epitopes) in the vaccine. It should further be mentioned that an essential arm of T cell immunity, cytotoxic T cell (CTL) response by CD8+ T cells, is primarily induced by live virus or live vaccines.

The cross-protective potential was further analyzed with protection studies in mice. The whole virus vaccine was highly effective in inducing protective immune responses in CD1 mice and this protection appeared to be correlated with induction of neutralizing antibodies. The non-adjuvanted vaccine formulation appeared more potent than the alum formulated material. The reason for this is unknown but the effect was reproducible in repeat studies in CD-1 mice, but has not been seen in challenge studies carried out in Balb/c mice (data not shown) and may finally not be relevant for the human situation.

In a previous report, an inactivated H5N3 alum-adjuvanted whole virus vaccine at high dosages (10µg) generated 100% protection of mice against a clade 1 H5N1 challenge [17]. Further, a single dose of a clade 1 H5N1 whole virus vaccine adjuvanted with incomplete Freund's adjuvant also fully protected mice against challenge with clade 1 virus at dosages of 7µg HA [32]. Recent studies with ferrets demonstrated that a 7 or 15µg dose of whole virus egg-derived vaccine (based on the clade 1 HK213 strain) protected against challenge with heterologous strains (clade 1 strains HK156 and VN1203) [33]. Using live, attenuated influenza H5N1 candidate vaccines broad cross-protection in mice and ferrets could be demonstrated. Mice were protected following challenge with H5N1 viruses from clades 1, 2 and 3 [34]. Our studies demonstrate full protection against the homologous strain challenge at doses as low as 30 ng HA with the VN1203 clade 1 vaccine using a two dose regimen in CD1 mice. We further confirm and extend cross clade immunity by showing protection against the clade 2 virus IN5/05 using the VN1203 vaccine in mice. In addition, we show that the IN5/05 vaccine cross-protects against clade 1 VN1203 and HK156 virus challenge. Altogether, these observations support the use of a stock-piled vaccine until a strain-specific pandemic vaccine is available. As it may require up to 6 months to produce the first batches of H5N1 vaccine after a pandemic strain is identified, the availability of vaccine to prevent substantial morbidity and mortality would be greatly facilitated by the stockpiling of pre-pandemic strain vaccine.

However, the advantages associated with use of wild-type virus must be balanced against the potential environmental risks associated with use of an highly pathogenic virus, when attenuated reassortant strains can be generated, although with some considerable delay. Vaccine manufacture with wild-type viruses is carried out in facilities specifically designed for virus containment. For example, virus growth is carried out in closed systems with double physical containment which ensures no virus can be released into the environment. Double inactivation with an extremely large safety margin and stringent quality control is carried out to ensure there is no risk of residual infectious virus after virus inactivation. However, other strategies which would allow use of attenuated viruses must also be considered. These include removal of the polybasic cleavage site but retention of all other wild-type H5N1 genes. This strategy could be considered if the yield obtained with wild-type virus could be retained, although it would involve more delay than immediate use of the wt virus for vaccine production.

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Fig. 1.

The level of IFN- γ (a) and IL-4 (b) spot-forming cells (SFC)/10⁶ in spleens of Balb/c mice as determined by ELISPOT assay. Mice were immunized as described in the text either with 1.5 μ g hemagglutinin of inactivated whole virus vaccines (VN1203, IN5/05 or B/JS) or recombinant H5 vaccine (rH5-HA, derived from VN1203). Splenocytes were stimulated in-vitro with 0.1 μ g/mL hemagglutinin of inactivated whole influenza viruses or rH5-HA (stim. antigens) as shown in the insert of the upper panel. Bars represent means $\pm$ SD of spot counts in quadruplicate wells.

Cross-neutralization titers (microneutralization assay) of anti-H5N1 guinea pig sera raised against whole virus candidate vaccine VN1203 or recombinant rH5-HA and tested against H5N1, H5N3 and H7N1 strains.

Table 1

vaccine Antigen	HA dose (µg)	VN1203 (H5N1) 2004 ^b	VN1194 (H5N1) 2004	Thai83 (H5N1) 2004	HK156 (H5N1) 1997	Dk/ Sing (H5N3) 1997	IN5/05 (H5N1) 2005	FPV/ Ros (H7N1) 1934 ^d
VN 1203	1.7	2,092 ^c	1,660 ^d	1,810 ^d	4,695 ^c	1,280 ^{de}	1,174 ^c	≤28 ^d
rH5 HA	5.0	830 ^c	538 ^d	538 ^d	1,522 ^c	640 ^d	≤28 ^c	≤28 ^d
Control ^f	-	≤28 ^c	≤28 ^d	≤28 ^d	≤28 ^c	10 ^d	≤28 ^c	≤28 ^d

^a for full name of strains see methods;

^b year of strain isolation;

^c GMT of 3 independent determinations;

^d single determination;

^e ELISA-based microneutralization assay;

^f nonimmunized.

Table 2
Humoral immune response and protection induced in mice by the H5N1 VN1203 candidate vaccine with and without alum adjuvant

dose (ug HA)	ELISA ^a - alum	+ alum	μ-NT ^b - alum	+ alum	survival ^c -alum	+alum
3.75	45,614	28,735	739	143	12/12	12/12
0.75	28,735	45,614	570	370	12/12	12/12
0.15	3,408	9,051	120	≤57	12/12	11/12
0.03	3,200	2,263	74	≤57	12/12	9/12
0.006	252	317	≤57	≤57	4/12	4/12
0.0012	317	141	≤57	≤57	5/12	0/12
TBS	<100	n.a.	≤57	n.a.	0/24	n.a.
TBS+Alum	n.a.	<100	n.a.	≤57	n.a.	1/24

^a geometric mean titer (GMT) of ten animals;
^b microneutralization assay, the VN1203 strain was used for neutralization, GMT of 3 pools of 4 animals;
^c challenge with 1×10^{4.5} TCID₅₀/ml of VN1203; n. a., not applicable.

Table 3
Protection induced in mice by the VN1203 and the IN5/05 candidate vaccines three weeks after boosting immunizations.

Dose (ug HA)	Survivors (n/n _{total} (%))		Challenge strain
	VN1203	IN5/05	HK156
<i>Vietnam VN1203 vaccine</i>			
0.75	10/10 (100%)	19/20 (95%)	10/10 (100%)
0.15	10/10 (100%)	17/20 (85%)	8/10 (80%)
0.03	8/10 (80%)	12/20 (60%)	6/10 (60%)
0.006	5/10 (50%)	10/20 (50%)	6/10 (60%)
PBS Contr.	1/10 (10%)	3/20 (15%)	1/10 (10%)
<i>Indonesia IN5/05 vaccine</i>			
3.75	9/9 (100%)	9/9 (100%)	n.d.
0.75	9/9 (100%)	9/9 (100%)	n.d.
0.15	8/9 (89%)	9/9 (100%)	n.d.
0.03	7/9 (78%)	8/9 (89%)	n.d.
0.006	4/9 (44%)	6/9 (67%)	n.d.
0.0012	1/9 (11%)	5/9 (56%)	n.d.
PBS Control	1/9 (11%)	3/9 (33%)	n.d.

n. d., not determined. legends