Evaluation of vaccines for H5N1 influenza virus in ferrets reveals the potential for protective single-shot immunisation.

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Running title: Protective vaccines for pandemic influenza

Word count abstract = 250; word count text = 4521
ABSTRACT

As part of influenza pandemic preparedness, policy decisions need to be made about how best to utilise vaccines once they are manufactured. As H5N1 avian influenza virus has the potential to initiate the next human pandemic, isolates of this subtype have been used for the production and testing of pre-pandemic vaccines. Clinical trials of such vaccines indicate that two injections of preparations containing adjuvant will be required to induce protective immunity. However, this is a working assumption based on classical serological measures only. Examined here are the dose of viral hemagglutinin (HA) and the number of inoculations required for two different H5N1 vaccines to achieve protection in ferrets following lethal H5N1 challenge. Ferrets inoculated twice with 30µg A/Vietnam/1194/2004 HA vaccine with AlPO₄, or doses as low as 3.8µg of HA with ISCOMATRIX™ adjuvant, were completely protected against death and disease following H5N1 challenge and protection lasted at least 15 mths. Cross-clade protection was also observed with both vaccines. Significantly, complete protection against death could be achieved with only a single inoculation of H5N1 vaccine containing as little as 15µg HA with AlPO₄ or 3.8µg HA with ISCOMATRIX™ adjuvant. Ferrets vaccinated with the single-injection ISCOMATRIX™ vaccines showed fewer clinical manifestations of infection than those given AlPO₄ vaccines and remained highly active. Our data provide the first indication that in the event of a future influenza pandemic, effective mass vaccination may be achievable with a low dose “single-shot” vaccine and provide not only increased survival but also significant reduction in disease severity.
INTRODUCTION

The emergence in 2004 and continued persistence of highly pathogenic H5N1 influenza A virus in bird populations is justifiably considered a potential pandemic threat (19). The virus has become endemic in many areas of the world and has demonstrated an ability to infect humans through transmission from poultry, thus far with limited human-to-human spread (26). Of great concern is that the case fatality rate for H5N1 infection of humans is reported to be >60%, compared to 0.1% for the 1957 and 1968 pandemics and 2-3% for the 1918 pandemic, which together resulted in at least 50 million deaths (14, 20). For these reasons, the development of strategies to minimise the impact if the virus mutates to acquire efficient human-to-human spread is essential.

Vaccination is considered the best method to ultimately control an influenza pandemic and should be implemented as soon as the pandemic strain is identified and vaccines produced (9, 23). To maximise coverage, pandemic vaccines will need to be available rapidly and will have to include the minimal dose of antigen to achieve solid immunity. This poses several major problems. One is that the human population is predominantly immunologically naïve to the emerging subtype of virus and so very large numbers of people will need to be protected as quickly as possible which will place a huge demand on vaccine supply. The use of an adjuvant to lower the dose of antigen required (8) may ameliorate this problem to some degree but there are few adjuvants that are suitable for human use, particularly those in ready supply in the event of a pandemic. In addition, we have little understanding of what levels and what type of immunity will provide protection from death or severe disease due to H5N1 infection (19).

Clinical trials with candidate H5N1 vaccines have been initiated with traditional virus preparations (egg-grown whole or detergent-disrupted “split” virions) and alternative vaccine strategies (recombinant protein, live-attenuated and adjuvant-containing vaccines) (24). Using split virus alone, high amounts of antigen, containing 90 µg hemagglutinin (HA), given twice, were required to elicit what is considered to be a protective antibody response in approximately 50% of subjects (25). Adjuvants, such as those based on aluminium salts (3) or the oil-in-water adjuvants
MF59 (2, 17, 22) and ASO3 (13, 21), have provided considerable antigen dose reduction but in all clinical trials and pre-clinical animal evaluation to date, two doses of vaccine have been required to achieve what is considered to be adequate anti-HA antibody levels or protection respectively (8, 24).

One aim of the present study was to determine how suitable the ferret model is for making assumptions about human responsiveness to influenza vaccination. To do this we evaluated in ferrets the same H5N1 pandemic vaccines, formulated with or without AlPO₄₄ adjuvant, that had been examined in Phase 1 and II randomized trials in healthy adults (18). We then sought to compare whether the responses to these vaccines were protective against lethal H5N1 challenge and whether the protective effects could be achieved with less antigen by using the more potent saponin-based ISCOMATRIX® adjuvant. The ISCOMATRIX® adjuvant has been shown to be safe and well tolerated in humans and to induce both strong and long-lived antibody and cytotoxic T cell responses in both humans and animal studies (7). Finally, the encouraging results with these adjuvants led us to examine whether protection from severe disease and death could be achieved after only a single injection of the H5N1 vaccines.

MATERIALS AND METHODS

Ferrets. Healthy juvenile female or male ferrets, less than 12 months old and typically 4-6 months of age and 700-1500g in weight, were supplied by the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia. Ferrets were tested by hemagglutination inhibition (HI) tests and found to be seronegative to currently circulating influenza type A (H1N1, H3N2) and B viruses prior to use. Approximately ten days prior to virus challenge, temperature transponders were surgically implanted beneath the skin of the flank. All experiments were conducted with the approval of the CSIRO AAHL Animal Ethics Committee.

Viruses. The wildtype H5N1 human influenza virus, A/Vietnam/1203/04 (which differs in the HA from A/Vietnam/1194/04 by only a single amino acid) and A/Indonesia/5/05 were obtained.
from the World Health Organisation Collaborating Centre for Influenza Reference and Research, Parkville, Victoria, Australia. Stock viruses were propagated in the allantoic cavity of 10-day embryonated chicken eggs at 35°C for 24-36 h and stored at -70°C. All experiments with these highly pathogenic viruses were conducted at the BSL3+ containment facility of the Commonwealth Scientific and Industrial Research Organisation’s Australian Animal Health Laboratory at Geelong, Victoria, Australia.

**Vaccine formulations.** The vaccine used was based on the reverse engineered strain (NIBRG-14) of the A/Vietnam/1194/04 human isolate of clade 1 H5N1 virus supplied by NIBCS, Mill Hill, UK. The purified inactivated detergent-disrupted vaccine was prepared from this virus by CSL Limited in an identical manner to seasonal influenza vaccine. In brief, virus was propagated in embryonated eggs, mixed with β-propiolactone (ICN Pharmaceuticals Inc., Costa Mesa, CA), subjected to rate zonal centrifugation on a sucrose gradient, and treated with sodium taurodeoxycholate (Sigma, St. Louis, MI) to yield a “split” virus preparation (5). The concentration of viral antigen was expressed in terms of hemagglutinin (HA) protein, which was determined by standard single radial immunodiffusion and compared to a known standard of the relevant strain. The vaccine was used either in the absence of adjuvant or formulated with aluminium phosphate (AlPO₄) or ISCOMATRIX™ adjuvant (60 µg/dose) in PBS pH 6.2 prior to inoculation. The AlPO₄-containing vaccines were equivalent to those used in phase I (ClinicalTrials.gov identifier: NCT00136331) and phase II (ClinicalTrials.gov identifier: NCT00320346) clinical trials in healthy adults.

**Vaccination and viral challenge of ferrets.** Two equivalent 0.5 ml doses of vaccine were administered to groups of 4 (unless otherwise indicated) ferrets, 21 days apart. Vaccines were delivered by the intramuscular route into the quadriceps muscle of the hind legs, using a 1 ml syringe with a 27-gauge needle. The route, antigen doses and timing of immunisation followed that of the phase I clinical trial, which examined vaccines containing 7.5 or 15 µg HA, with or with AlPO₄, and the phase II trial, which evaluated 30 (and 45) µg HA of vaccine formulated with
AlPO₄ (18). In some experiments, ferrets received only a single inoculation of vaccine. Three to four weeks after the last inoculation, the ferrets were challenged intranasally with 10⁶ 50% egg infectious doses (EID₅₀) of challenge virus (A/Vietnam/1203/2004 or A/Indonesia/5/05) in 0.5 ml as described (10) under Ketamine/Medetomidine anaesthesia (50:50 0.1 ml/kg, reversed with Atipemazone).

**Monitoring and sample collection.** Animals were visually inspected daily throughout the study and twice daily following challenge. Ferrets were euthanased when reaching a previously determined endpoint or 14 days after challenge. The humane endpoint was defined as 10% body weight loss within 7 days of challenge or exhibition of signs consistent with involvement of other organ systems (eg. tremor, abdominal discomfort). In control animals, this occurs typically within the first 7 days after viral challenge. In preliminary studies, these signs were found to correlate with the requirement to euthanase ferrets on subsequent days on humane grounds; thus they have been utilised as surrogates for lethality.

Reaction site observations (ie. erythema, oedema) were assessed at 2, 24 and 48 hrs following each vaccination. General clinical observations were documented prior to challenge and, following challenge, a detailed clinical signs sheet and an evaluation of activity based on a 5-level score were recorded daily. Animals were weighed while under sedation at the time of vaccination and challenge and at days 3, 5, 7 and 14 post-challenge. Rectal temperature was also determined at sedation using digital thermometers to augment data derived remotely from the implanted temperature transponders (16).

Blood samples were collected immediately prior to each vaccination and prior to viral challenge from either the jugular or axillary veins. A further blood sample to monitor boosting of antibody responses by the challenge virus was collected 14 days post challenge or at the time of euthanasia. In addition, nasal washes and oral and rectal swabs, each into 1 ml PBS, were taken on days 3, 5 and 7 post-challenge for virus isolation. A range of tissues were collected at autopsy and...
fixed in 10% neutral buffered formalin. Sections were prepared and stained with hematoxylin and
eosin.

**Immunological and virological evaluation.** Serum samples were assessed by micro HI
using chicken red blood cells (or horse red cells where indicated) and VN by standard methods.
The supernatants from nasal washes and swab media were assessed for infectious virus by
inoculation into the allantoic cavity of at least three 9- to 11-day embryonated fowl eggs. The eggs
were incubated at 35-37°C for up to 5 days. Allantoic fluids from eggs containing dead or dying
embryos as they arose, and all eggs remaining at the end of the incubation period were tested for
virus by hemagglutination of fowl red blood cells. All allantoic fluids with HA activity were
considered positive for the virus. **For some experiments, nasal washes were titrated for virus
growth in Vero cells and reported as TCID<sub>50</sub>**.

**RESULTS**

**Challenge experiments indicate not only protection from death but also from clinical
disease in ferrets immunised with split influenza vaccines formulated with AlPO<sub>4</sub>.** Ferrets were
immunised with the same formulations of 7.5 and 15 µg HA vaccines, with or without AlPO<sub>4</sub>, that
had been evaluated in humans. Table 1 shows the serum hemagglutination-inhibitory (HI) and
virus-neutralising (VN) antibody responses of these animals after the first and second of two
injections given 3 weeks apart. Ferrets did not mount any detectable responses to the vaccines
without AlPO<sub>4</sub>. However, responses were observed 21 days after the first immunisation with each
of the vaccines containing AlPO<sub>4</sub> and these increased after the second immunisation. By 4 weeks
after the second immunisation, all ferrets in both the 7.5 and 15 µg HA + AlPO<sub>4</sub> groups had HI and
VN titres of 16 or greater, representing at least a 4-fold rise for both assays. An additional group of
10 ferrets was immunised with 30 µg HA + AlPO<sub>4</sub> and all of these had HI titres between 16 and 64
and VN titres between 32 and 256 at 4 weeks post secondary immunisation (data not shown).
To address whether these responses were protective, the animals were challenged with A/Vietnam/1203/2004 virus 4 weeks after the second immunisation, and weight (Figure 1A) and activity (Figure 1B) monitored for 14 days. All four ferrets in the PBS control group showed a dramatic drop in weight following viral challenge and a decrease in activity accompanied by fever and nasal discharge from day 3 onwards. Virus was present in the nasal washings and oral swabs at all sampling times (data not shown). Two control ferrets were culled at a pre-determined humane endpoint on day 7 and a third on day 8. The remaining control ferret survived, its weight stabilised and, despite development of a cough, transient diarrhoea and conjunctival discharge, was alert throughout most of the observation period. This ferret developed very high HI (512) and VN (>4000) antibody titres post-challenge (Table 1). Histopathological evaluation of organs from the PBS control ferrets culled prior to day 14 revealed multi-organ involvement including non-suppurative meningoencephalitis, necrotising bronchiolo-alveolitis, hepatitis, pancreatitis and nephritis. No involvement of the heart, intestine, spleen or bladder was observed.

In ferrets immunised with vaccines that did not contain AlPO₄ adjuvant, there were 2 of 4 and 1 of 4 survivors in the 7.5 and 15 µg HA groups respectively (Table 1). Despite fever, nasal discharge and a slight decrease in activity, these ferrets maintained their weight and had largely recovered by day 12 (Fig. 1). The other ferrets in these two groups were culled on or before day 7. In the group of animals immunised with the vaccine containing 7.5 µg HA and AlPO₄ adjuvant two ferrets needed to be culled (Fig. 1) despite their having substantial pre-challenge (VN titres 16-32) responses to the vaccine (Table 1). Clinical signs and post-mortem histology were indicative of predominantly brain rather than respiratory tract involvement in both culled animals (data not shown). The remaining two ferrets in this group and all animals in the group that was immunised with the 15 µg HA + AlPO₄ vaccine were largely free of clinical signs, showed no temperature rise, no drop in activity and maintained their weight throughout the 14-day observation period (Fig. 1). Similarly, all ten ferrets immunised with the vaccine containing 30µg HA + AlPO₄ showed no
clinical signs of infection, decrease in activity, nor a substantial temperature rise, and either
maintained or gained weight over the 14-day period.

Protection against morbidity and mortality can also be obtained with split influenza
virus formulated with ISCOMATRIX™ adjuvant but with at least 8-fold lower antigen doses.
In an effort to further reduce the amount of antigen required for protection, the same viral challenge
model was used to evaluate vaccines containing the more potent ISCOMATRIX™ adjuvant.
Ferrets were immunised twice with a vaccine containing 15, 7.5, 3.8 or 1.9 µg HA formulated with
ISCOMATRIX™ adjuvant and subsequently challenged with A/Vietnam/1203/2004 virus.
Irrespective of the antigen dose, all ferrets seroconverted after the second immunisation by the VN
test (summarised in Table 2) and all but one animal in the 1.9 µg group by the HI test, and survived
the lethal challenge (Fig. 2). In addition, none of these ferrets showed a rapid drop in weight or any
other any clinical sign of infection, except for one that had a transient temperature rise. This was in
marked contrast to the PBS control ferrets, all of which lost weight (9.5 to 19.8%) and showed a
decrease in activity score (Fig. 2) with three culled within one week post challenge due to the
development of pneumonia and/or neurological disease. The disease-free state induced by as little
as 1.9 µg HA in the presence of ISCOMATRIX™ adjuvant was similar to that achieved with 15 µg
HA formulated with AlPO₄, indicating a dose reduction of at least 8-fold.

Two additional groups of 5 ferrets were inoculated twice with 3.8 µg HA +
ISCOMATRIX™ adjuvant or 30 µg HA + AlPO₄ as above but were not challenged until 15 months
after the second inoculation. Serum samples taken at 2, 3, 6, 9 and 12 months after immunisation
showed very low levels of HI antibody but using the more sensitive horse RBC rather than chicken
RBC in the assay, responses could be detected at all time points and ranged from a maximum of
160 at 2 months to 40 at 12 months; mean responses to the two vaccines never varied more than 2-
fold throughout (data not shown). After challenge, all ferrets survived and had subclinical
infections only.
Protection of the upper respiratory tract is greatly enhanced in ferrets immunised with split influenza virus formulated with ISCOMATRIX™ adjuvant. Despite being free of clinical disease, many of the ferrets throughout the study continued to have virus present in nasal washings and oral swabs for up to 10 days after viral challenge. Figure 3 shows the fraction of ferrets that had virus isolated from nasal washings or throat swabs taken on days 3, 5 and 7 post-challenge. Unexpectedly, all of the vaccines appeared to have the ability to reduce the duration of viral shedding. While only 1 of 9 (11%) control ferrets that remained alive on day 7 post challenge had stopped shedding virus, the proportion of all immunised ferrets that had cleared virus from their nose and throat by day 7 was between 50 and 100% depending on the vaccine. At day 5 post challenge, none of the animals immunised with vaccines containing 7.5 or 15 µg HA with or without AlPO₄ adjuvant had cleared virus from the upper respiratory tract but 70% of ferrets immunised with 30 µg HA + AlPO₄ had cleared the virus. Significant differences in virus shedding compared to the control group were observed on all sampling days in this highest dose AlPO₄-containing vaccine group.

Overall, the rate of viral clearance in ferrets immunised with vaccines containing ISCOMATRIX™ adjuvant was much greater than those containing the equivalent antigen dose formulated in AlPO₄. When compared to control animals, ferrets immunised with vaccines containing 3.8, 7.5 or 15 µg HA and ISCOMATRIX™ adjuvant showed a significant decrease in the proportion that shed virus even at day 3 after challenge, and this virus-positive proportion decreased from as early as day 5 in the 3.8 and 7.5 µg HA dose groups. Only 3 out of the 28 ferrets given vaccines containing ISCOMATRIX™ adjuvant were still shedding virus on day 7 post challenge. The 3.8 µg HA vaccine formulated with ISCOMATRIX™ adjuvant induced a particularly potent viral-clearing response with 8 of 12 (66%) ferrets having no detectable virus in their noses and throats on day 3 and highly significant viral clearance at each time point. In terms of viral shedding, this vaccine was not significantly different (Fisher’s exact test) from the vaccine containing AlPO₄ and eight-fold more (30 µg) HA.
Protective cross-clade responses can be achieved with vaccines formulated with AlPO$_4$ and ISCOMATRIX™ adjuvant. To test whether the vaccines induced cross-reactive immunity capable of protecting against related H5N1 isolates, ferrets were immunised twice with vaccines prepared from the A/Vietnam/1194/04 clade 1 virus, containing either 15 µg HA + AlPO$_4$ or 15 or 3.8 µg HA + ISCOMATRIX™ adjuvant, and subsequently challenged with the clade 2.1.3 virus A/Indonesia/5/05. In general, HI and VN titres were slightly lower against the clade 2 virus than the homologous clade 1 virus prior to challenge (Table 3). Nevertheless, all immunised ferrets survived the clade 2 viral challenge, which proved to be lethal to all four of the non-immunised control animals. The immunised ferrets had subclinical infections (data not shown), reduced viral shedding compared to the controls, and managed to maintain and, in most cases, increase their weight over the 7 days post challenge (Table 3). Consistent with the data from homologous challenge experiments (Fig. 3), there were less cases of viral shedding in the ISCOMATRIX™ vaccine groups compared with the AlPO$_4$ group (Table 3).

Protection against lethal H5N1 challenge can be achieved with only a single inoculation of vaccine. Another means of significantly reducing the amount of vaccine antigen required and/or the time to achieve protective immunity within a population is by use of a vaccine that is sufficiently potent to induce the required responses with only a single inoculation. Govorkova et al. (11) had previously observed protection against death due to A/Vietnam/1203/04 infection with a single dose of 7 and 15 µg HA in the form of whole inactivated virus vaccine and AlOH adjuvant. However these experiments were performed in ferrets that had previously been infected with influenza virus of a heterologous subtype and so protection may have been due to recently induced cytotoxic T cell responses. In this study we tested vaccines in the naïve ferrets that contained 30 µg HA either alone or formulated with AlPO$_4$ or ISCOMATRIX™ adjuvant given as a single inoculation and challenged the animals with A/Vietnam/1203/04 four weeks later. As a positive control, a group of ferrets was immunised twice with the vaccine containing 3.8 µg HA + ISCOMATRIX™ adjuvant, a schedule that we knew to be totally protective against clinical disease.
and death. Table 4A shows the serology data, weight change, temperature status and virus isolation from nose and throat and Figure 4A shows the activity scores post challenge. All vaccinated ferrets survived the challenge, which was fatal for all four of the control animals. However, the course of infection differed between vaccine groups. Ferrets immunised with two inoculations of 3.8 µg HA + ISCOMATRIX™ adjuvant had very mild and largely subclinical infections as expected, with additional clinical signs restricted to mucus in the nasal wash of ferrets 149 and 150 on day 5 only and a transient temperature rise in ferret 137 on day 7. This, plus the fact that half the animals showed no virus in the nose or throat samples from day 3 onwards, was in accordance with the observations for similarly immunised animals elsewhere in this study. Importantly, ferrets immunised with just a single inoculation of 30 µg HA + ISCOMATRIX™ adjuvant showed no clinical signs of infection whatsoever and 3 of the 4 animals had no virus in their nose or throat samples from day 3 onwards. Ferrets immunised with a single inoculation of 30 µg HA + AlPO₄ remained healthy except for the presence of mucus in the nasal washes from days 3 to 5 post challenge in all animals and conjunctive discharge in ferrets 153 and 154, which was preceded by a drop in their activity score. In contrast, some ferrets immunised with 30 µg HA in the absence of adjuvant had a spike in temperature on day 3 post challenge, a transient drop in activity score and had virus in their noses and throats on days 3 and 5 post challenge. Individual animals showed signs of depression, transient conjunctivitis and mucus in the nasal wash but, nevertheless, showed better activity scores (Fig. 4A) and reduced weight loss (Table 4A) compared to animals vaccinated with two doses of 15 µg HA (Fig. 1). These ferrets vaccinated once with 30 µg HA alone fully recovered despite the fact that they had not seroconverted to the vaccine. Antigen dose titration of the vaccines containing adjuvant revealed that complete protection against death could be achieved with a single dose as low as 3.8 µg HA when formulated with ISCOMATRIX™ adjuvant or 15 µg HA with AlPO₄ (Table 4B and Fig. 4B). Overall, clinical signs of infection were fewer and limited to transient conjunctival and nasal discharge (data not shown), weight loss was somewhat less severe (Table 4B) and activity less compromised (Fig. 4B) with vaccines containing.
ISCOMATRIX™ adjuvant compared to AlPO₄ at these lower doses of antigen. Virus titres in nasal washes (Fig. 4C) were significantly reduced in all vaccinated groups compared to the control group (ANOVA with Tukey’s Multiple Comparison test performed for each sampling day) and no virus was detected in the nasal wash of any of the vaccinated ferrets on day 7 post-infection. A single inoculation of 15 or 7.5 µg HA + ISCOMATRIX™ adjuvant also yielded virus-free samples on day 5 post infection, indicating a faster rate of viral clearance compared to AlPO₄ vaccines. However, at the lowest dose of 3.8 µg HA there was no significant difference in viral clearance between the two adjuvant groups.

DISCUSSION

Successful pandemic vaccines that can be registered and implemented relatively quickly build on existing technology that has proven to be safe and effective for seasonal influenza vaccines and for which the manufacturing process is already in place and well validated (6, 24). For this reason we have used inactivated, detergent-split, purified egg-grown influenza virus as a starting point for these pandemic vaccines and added components as adjuvants that have already been registered, in the case of AlPO₄, or are in development and clinically proven, as is the case for ISCOMATRIX™ adjuvant (7). Our studies have provided data in the ferret model that supports the conclusion that the recently licensed H5N1 vaccine consisting of split virus in the form of 30 µg HA + AlPO₄ adjuvant delivered as two inoculations 21 days apart, is highly immunogenic and further show that the responses induced are completely protective against death and disease following lethal H5N1 challenge. In the ferret model we also show, for the first time, that protection against death from lethal challenge can be achieved with only a single inoculation of this vaccine and although some clinical signs of disease were apparent, these were relatively minor. This is very encouraging data that may inform process during mass vaccination, and imply that provision of a single inoculation to a larger number of people may have a more beneficial outcome than trying to achieve better seroconversion with two inoculations in a smaller number of people.
That said, the precision with which these findings translate from the ferret to humans remains unknown. The phase I and II clinical trial results reported by Nolan et al. 2008 (18) showed some degree of responsiveness to 7 and 15 μg HA vaccines in humans whereas ferrets showed no response in the absence of adjuvant. Addition of AlPO₄ adjuvant to these vaccines enabled a very modest rise in the frequency of significant responses in humans, most notably for VN antibodies, to give approx. 40% responders to the 15 μg HA + AlPO₄ vaccine, but allowed HI and VN responses to become detectable in ferrets. Increasing the antigen dose to 30 μg in the vaccine formulated with AlPO₄ resulted in a further increase in the frequency of human responders (>50%) and robust immunity in ferrets. It therefore appears that the ability to mount a seemingly successful response in both species requires roughly similar antigen doses. While protection data must be extrapolated with caution, these data would give hope to the idea that similar fully protective responses can be generated in man using this type of vaccine. This notion is further strengthened by the fact that the disease pathogenesis appears similar in ferrets to that observed in humans (4, 12), as previously noted in other studies (1, 10, 11).

When making assumptions about the success of vaccines in humans solely on the basis of seroconversion some caution should be exercised. Here, as in other studies (11) (15), there are several instances where the classical serological measures of immunity normally associated with protection did not provide a good correlation with disease outcome. One such example is the group of ferrets that received a single inoculation of 30 μg HA alone (Table 4A) where all survived viral challenge without prior seroconversion to the vaccine. Additional studies of the mechanisms underpinning vaccine-induced protection are warranted.

Vaccines formulated with ISCOMATRIX™ adjuvant were even more potent in terms of their ability to achieve protection of ferrets against clinical disease with very low antigen doses. Those containing as little as 1.9 μg HA were effective in preventing disease in all animals tested although the vaccine containing 3.8 μg HA and ISCOMATRIX™ adjuvant as two inoculations gave more complete seroconversion. This latter vaccine also demonstrated cross-clade protection and
was still completely protective against death and disease when animals were challenged 15 months
after the last immunisation. Most importantly, a single immunisation with any of 3.8, 7.5, 15 or 30
µg HA + ISCOMATRIX™ adjuvant provided complete protection against death, an outcome only
achieved at the higher antigen levels with AlPO₄. This implies the possibility of an effective single
immunisation approach to H5N1 pandemic vaccination that has the advantage of reducing both the
time to achieve protective immune status as well as the amount of antigen required.

While all vaccines reduced the period of viral shedding from the upper respiratory tract,
vaccines containing ISCOMATRIX™ adjuvant were most effective in this regard when given in a
two injection regime. The majority of animals vaccinated twice with greater than 3.8 µg HA and
ISCOMATRIX™ adjuvant had cleared detectable virus from the site of inoculation by day 3
whereas those inoculated with comparable antigen doses of the other vaccines were still shedding
virus 2 days later. Single dose vaccines also showed significant benefit in reducing the viral load in
the upper respiratory tract compared to PBS control animals. Titration of nasal washings in Vero
cells from these animals confirmed this; the GMT of infectious virus for each vaccinated group was
at least 2 logs lower compared to the control ferrets at the equivalent time point. These data indicate
that the vaccines might have a role in decreasing the rate of person-to-person spread of the virus
within a community as well as having benefits for the vaccinee.

These studies confirm that the recently licensed human pandemic vaccine containing 30 µg
HA + AlPO₄ adjuvant has the potential to be completely protective against lethal H5N1 challenge
and may even prevent death and significantly reduce disease after a single administration. The data
also highlight the further dose reduction effect of ISCOMATRIX™ adjuvant, even after a single
inoculation, to provide disease-free protective immunity. This study provides the incentive for
further evaluation of vaccines containing ISCOMATRIX™ adjuvant in humans.

ACKNOWLEDGMENTS
The authors wish to thank Tim Hancock for participation in the ferret experiments, Eleanor Cummins for formulation of vaccines and Alan Hampson for his advice in the initial stages. This work was supported by the National Health and Medical Research Council of Australia’s Urgent Research into a Potential Avian Influenza-Induced Pandemic Granting scheme. The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health and Ageing.


26. World Health Organisation. posting date 28 January 2008. H5N1 avian influenza timeline of major events. [Online.] Available at:
FIGURE LEGENDS

FIG. 1. Changes in weight and activity score of ferrets immunised with vaccines formulated with or without AlPO₄. Ferrets were immunised with vaccines based on the reverse engineered A/Vietnam/1194/04 NIBRG-14 (H5N1) virus containing 7.5 µg HA (ferrets 37-56), 15 µg HA (ferrets 41-44), 7.5 µg HA formulated in AlPO₄ (ferrets 45-48), 15 µg HA formulated in AlPO₄ (ferrets 49-52) or PBS (ferrets 53-56). Ferrets received 2 inoculations of vaccine on days 1 and 21 and were challenged with wild-type A/Vietnam/1203/04 virus 4 weeks after the last immunization. A: Weights of individual ferrets on days 0, 3, 5, 7, 10 and 14 after challenge. B: Activity scores for each day after challenge out to day 12. Scores on days 13 and 14 were the same as day 12. Scores are depicted for each ferret by a strip of colored squares corresponding to the activity of the animal on the different days as indicated in the legend. Squares that are half black indicate the activity score on the day of culling at the humane end point.

FIG. 2. Changes in weight and activity score of ferrets immunised with vaccines formulated with ISCOMATRIX™ adjuvant. Ferrets were immunised with vaccines based on the reverse engineered A/Vietnam/1194/04 NIBRG-14 (H5N1) virus containing 1.9, 3.8, 7.5 or 15 µg HA + 60 µg ISCOMATRIX™ adjuvant (IMX) or with PBS. Ferrets received 2 inoculations of vaccine on days 1 and 21 and were challenged with wild-type A/Vietnam/1203/04 virus 3 weeks after the last immunization. A: Weights of individual ferrets on days 0, 3, 5, 7 and 14 after challenge. B: Activity scores for each of the indicated days after challenge. Scores are depicted as in Figure 1.

FIG. 3. The influence of vaccine type on the presence or absence of virus in nose and throat samples taken on days 3, 5 and 7 post challenge with homologous clade A/Vietnam/1203/04 virus. The data are combined from the different experiments described in this study that included ferrets immunised with the indicated vaccines or PBS control animals. Nasal washings and also oral swabs...
taken into PBS were sampled on the indicated days post challenge and tested for the presence of infectious virus by amplification in eggs. Data indicate the fraction of ferrets: red square, virus present in nasal and/or throat samples; white square, no virus detected in either nasal or throat samples; black square, animal previously culled. Pair-wise statistical analyses of the vaccine groups compared to the control group for the relevant day post-infection were performed using Fisher’s exact test with 95% confidence levels. The p values for those groups that were significantly different from the control group are shown as stars below the group, the number of stars indicative of the p value as shown by the range in the legend. NS: not significantly different. No indicator: statistics unable to be performed as all shedding virus (therefore not different).

FIG. 4. Changes in activity score of ferrets immunised with a single inoculation of vaccine. A. Ferrets were immunised with vaccines containing 30 μg HA and either ISCOMATRIX™ adjuvant (IMX), AlPO₄ or no adjuvant. Ferrets were challenged with the homologous clade virus 4 weeks later and activity scores up to day 7 are shown. As a positive control, a group of ferrets was immunized with 2 inoculations of vaccine containing 3.8 μg HA + IMX three weeks apart and challenged 4 weeks later. B: Ferrets were inoculated once with 3.8, 7.5 or 15 μg doses of vaccine with either ISCOMATRIX™ (IMX) or AlPO₄ adjuvant. Activity was recorded and presented as in panel A. C: Nasal washes from the ferrets described in panel B, sampled on days 3, 5 and 7 post challenge, were titrated in Vero cells. The limit of detection for this assay was 10⁻⁰.⁵.
TABLE 1. Serological responses to human H5N1 candidate vaccines in ferrets before and after viral challenge

<table>
<thead>
<tr>
<th>Ferret</th>
<th>Vaccine</th>
<th>Primary</th>
<th>Secondary</th>
<th>Terminal bleed</th>
<th>F&lt;sup&gt;b&lt;/sup&gt;</th>
<th>D&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HI</td>
<td>VN</td>
<td>HI</td>
<td>VN</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>7.5 µg HA</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>+ 7</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>64</td>
<td>128 +</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>+ 4</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>64</td>
<td>256 +</td>
</tr>
<tr>
<td>41</td>
<td>15 µg HA</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4 + 7</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4 + 6</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4 + 3</td>
</tr>
<tr>
<td>44</td>
<td></td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>128</td>
<td>512 +</td>
</tr>
<tr>
<td>45</td>
<td>7.5 µg HA + AlPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>&lt;4</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>32 + 7</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>128 12</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>64 128</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>&lt;4</td>
<td>4</td>
<td>32</td>
<td>64</td>
<td>32 128</td>
</tr>
<tr>
<td>49</td>
<td>15 µg HA + AlPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>&lt;4</td>
<td>4</td>
<td>32</td>
<td>16</td>
<td>64 128</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>128</td>
<td>32 128</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>&lt;4</td>
<td>4</td>
<td>64</td>
<td>32</td>
<td>64 256</td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>32 128</td>
</tr>
<tr>
<td>53</td>
<td>PBS</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4 + 7</td>
</tr>
</tbody>
</table>
Vaccines were administered on days 0 and 21.

Hemagglutination inhibition (HI) and virus neutralisation (VN) tests were performed on A/Vietnam/1203/04 virus on sera taken 3 wk after the primary immunisation and 4 wk after the secondary immunisation immediately prior to homologous viral challenge.

Sera was tested again on day 14 post challenge or earlier at the time of culling.

Rectal temperature >40°C on at least one sampling day (d3, d5, d7) post challenge.

Day post challenge of euthanasia at the humane endpoint; blanks indicate ferrets surviving to day 14 when the experiment was terminated.
### TABLE 2. Serological responses to vaccines formulated with ISCOMATRIX™ adjuvant before and after viral challenge

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Primary</th>
<th>Secondary</th>
<th>Terminal bleed</th>
<th>F&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI</td>
<td>VN</td>
<td>HI</td>
<td>VN</td>
</tr>
<tr>
<td>1.9 µg HA + IMX</td>
<td>3/4</td>
<td>1/4</td>
<td>23</td>
<td>3/4</td>
</tr>
<tr>
<td>3.8 µg HA + IMX</td>
<td>7/2</td>
<td>1/4</td>
<td>45</td>
<td>4/4</td>
</tr>
<tr>
<td>7.5 µg HA + IMX</td>
<td>11/3</td>
<td>32/4</td>
<td>76</td>
<td>4/4</td>
</tr>
<tr>
<td>15 µg HA + IMX</td>
<td>8/2</td>
<td>16/4</td>
<td>108</td>
<td>4/4</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;4/0</td>
<td>&lt;4/0</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

**Vaccines** were inoculated on days 0 and 21; IMX = ISCOMATRIX™ adjuvant.

**HI and VN tests** were performed against A/Vietnam/1203/04 virus on sera taken 3 wk after the primary immunisation and 3 wk after the secondary immunisation immediately prior to homologous viral challenge.

Sera was tested again on day 14 post challenge or earlier at the time of culling for the PBS group.

**Fraction of ferrets with rectal temperatures >40°C at one or more sampling points 3, 5, 7 after challenge**

**Geometric mean titre (GMT) calculated using a value of 2 when the titre was below detection limits (<4).**

**Fraction of responder animals determined by a greater than four-fold rise in titre (16 or more).**
TABLE 3. Responses to vaccines formulated with AlPO₄ or ISCOMATRIX™ adjuvant after infection with the heterologous clade 2 virus.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Pre-challenge</th>
<th>Terminal bleed</th>
<th>Virus in nose</th>
<th>% weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI clade 1</td>
<td>HI clade 2</td>
<td>VN clade 1</td>
<td>VN clade 2</td>
</tr>
<tr>
<td>15 µg HA + AlPO₄</td>
<td>45 (4/4)</td>
<td>27 (4/4)</td>
<td>108 (4/4)</td>
<td>23 (3/4)</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>1218</td>
<td>724</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d3 4/4</td>
<td>d5 2/4</td>
<td>d7 1/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+4 (+3 to +10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 µg HA + IMX</td>
<td>51 (3/3)</td>
<td>64 (3/3)</td>
<td>161 (3/3)</td>
<td>81 (3/3)</td>
</tr>
<tr>
<td></td>
<td>815</td>
<td>323</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>3/3</td>
<td>1/3</td>
<td>0/3</td>
<td>+5 (+5 to +14)</td>
</tr>
<tr>
<td>3.8 µg HA + IMX</td>
<td>32 (4/4)</td>
<td>19 (4/4)</td>
<td>76 (4/4)</td>
<td>27 (3/4)</td>
</tr>
<tr>
<td></td>
<td>1218</td>
<td>609</td>
<td>2435</td>
<td>2896</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;4</td>
<td>4</td>
<td>&lt;4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>&lt;4</td>
<td>3</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>-15 (+13 to -17)</td>
</tr>
</tbody>
</table>

- **a**: Vaccines were administered on days 0 and 21; IMX = ISCOMATRIX™ adjuvant
- **b**: HI and VN tests were performed against the homologous clade virus A/Vietnam/1203/04 (clade 1) and A/Indonesia/5/05 (clade 2.1.3) virus on sera taken 4 wk after the secondary immunisation immediately prior to challenge with the heterologous A/Indonesia/5/05 clade 2 virus. Shown is the GMT of the group and fraction of vaccine responders (titres of 16 or above) in brackets.
- **c**: Sera was tested again on day 14 post challenge or earlier at the time of culling and GMT shown
- **d**: Nasal washings and oral swabs were taken on day 3, 5 and 7 post challenge and inoculated into eggs; fraction of animals with virus detected
- **e**: Median and range of percentage change in weight from the day of viral challenge to 7 days post challenge
- **f**: Only three animals completed the study in this group for reasons unrelated to influenza infection.
TABLE 4. Responses to single inoculation vaccines

<table>
<thead>
<tr>
<th>Expt Vaccine</th>
<th>Pre-challenge</th>
<th>Terminal bleed</th>
<th>Virus in nose and throat</th>
<th>% weight change</th>
<th>Fever</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI</td>
<td>VN</td>
<td>HI</td>
<td>VN</td>
<td>d3</td>
<td>d5</td>
</tr>
<tr>
<td>A 2 x 3.8 µg + IMX</td>
<td>16 (2/4)</td>
<td>128 (4/4)</td>
<td>76</td>
<td>1448</td>
<td>2/4</td>
<td>1/4</td>
</tr>
<tr>
<td>30 µg HA + IMX</td>
<td>8 (1/4)</td>
<td>45 (3/4)</td>
<td>54</td>
<td>1024</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>30 µg HA + AlPO4</td>
<td>19 (3/4)</td>
<td>108 (4/4)</td>
<td>45</td>
<td>609</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td>30 µg HA</td>
<td>&lt;4 (0/4)</td>
<td>&lt;4 (0/4)</td>
<td>91</td>
<td>861</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;4 (0/4)</td>
<td>&lt;4 (0/4)</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>4/4</td>
<td>2/2</td>
</tr>
<tr>
<td>B 15 µg HA + IMX</td>
<td>19 (3/4)</td>
<td>27 (3/4)</td>
<td>152</td>
<td>512</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>7.5 µg HA + IMX</td>
<td>14 (2/4)</td>
<td>54 (4/4)</td>
<td>91</td>
<td>215</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>3.8 µg HA + IMX</td>
<td>10 (1/4)</td>
<td>8 (1/4)</td>
<td>215</td>
<td>304</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>15 µg HA + AlPO4</td>
<td>16 (3/4)</td>
<td>23 (3/4)</td>
<td>152</td>
<td>362</td>
<td>0/4</td>
<td>2/4</td>
</tr>
<tr>
<td>7.5 µg HA + AlPO4</td>
<td>7 (1/4)</td>
<td>7 (2/4)</td>
<td>152</td>
<td>512</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td>3.8 µg HA + AlPO4</td>
<td>11 (2/4)</td>
<td>10 (2/4)</td>
<td>256</td>
<td>512</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;4 (0/4)</td>
<td>&lt;4 (0/4)</td>
<td>10</td>
<td>4</td>
<td>4/4</td>
<td>2/2</td>
</tr>
</tbody>
</table>
a Vaccines were administered as a single inoculation, except 2 x 3.8 µg HA + IMX which was two inoculations on day 0 and 21.

b HI and VN tests were performed against A/Vietnam/1203/04 virus on sera taken 4 wk after the single or last immunisation, immediately prior to challenge with the homologous virus. Shown is the GMT of the group and fraction of vaccine responders (titres of 16 or above) in brackets.

c Sera was tested again on day 14 post challenge or earlier at the time of culling and GMT shown.

d Nasal washings and oral swabs were taken on day 3, 5 and 7 post challenge and inoculated into eggs; + = virus detected in eggs inoculated from either the nose or throat sample, - = no virus detected.

e Percentage change in weight from the day of viral challenge to 7 days post challenge or earlier in culled animals.

f Fraction of ferrets with rectal temperatures >40°C at on at least one sampling point (d3, d5, d7) after challenge.

F Fraction of ferrets surviving to day 14 when the experiment was terminated.