A single immunization with CoVaccine HT™-adjuvanted H5N1 influenza vaccine induces protective cellular and humoral immune responses in ferrets

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ABSTRACT

Highly pathogenic avian influenza A viruses of the H5N1 subtype continue to circulate in poultry and zoonotic transmissions are reported frequently. Since a pandemic caused by these highly pathogenic viruses is still feared, there is interest in the development of influenza A/H5N1 virus vaccines that can protect humans against infection, preferably after a single vaccination with a low dose of antigen. Here we describe the induction of humoral and cellular immune responses in ferrets after vaccination with a cell-culture derived whole inactivated influenza A virus vaccine in combination with the novel adjuvant CoVaccine HT™. The addition of CoVaccine HT™ to the influenza A virus vaccine increased antibody responses to homologous and heterologous influenza A/H5N1 viruses and increased virus-specific cell-mediated immune responses. Ferrets vaccinated once with a whole-virus equivalent of 3.8 µg HA and CoVaccine HT™ were protected against homologous challenge infection with influenza virus A/VN/1194/04. Furthermore, ferrets vaccinated once with the same vaccine/adjuvant combination were partially protected against infection with a heterologous virus derived from clade 2.1 of H5N1 influenza viruses. Thus, the use of the novel adjuvant CoVaccine HT™ with cell culture derived inactivated influenza A/H5N1 antigen is a promising and dose sparing vaccine approach warranting further clinical evaluation.
INTRODUCTION

Since the first human case of infection with a highly pathogenic avian influenza A virus of the H5N1 subtype in 1997 (9-10, 37) hundreds of zoonotic transmissions have been reported with a high case-fatality rate (10, 44). Since these viruses continue to circulate among domestic birds and human cases are regularly reported, it is feared that they will adapt to their new host or exchange gene segments with other influenza A viruses, become transmissible from human-to-human and cause a new pandemic. Recently, a novel influenza A virus of the H1N1 subtype emerged. This virus, which originated from pigs, was transmitted between humans efficiently, resulting in the first influenza pandemic of the 21st century (8, 45). Although millions of people have been inoculated with the (H1N1) 2009 virus, compared to infections with the H5N1 viruses, the case-fatality rate was relatively low (11, 31). However, the unexpected pandemic caused by influenza A/H1N1(2009) viruses, has further highlighted the importance of rapid availability of safe and effective pandemic influenza vaccines. Other key issues for the development of pandemic influenza A virus vaccines include optimal use of the existing (limited) production capacity of viral antigen and effectiveness against viruses that are antigenically distinct. Ideally, a single administration of a low dose of antigen would be sufficient to induce protective immunity against the homologous strain and heterologous antigenic variant strains. However, since the population at large will be immunologically naïve to a newly introduced virus, high doses of antigen are required to induce protective immunity in un-primed subjects (23, 36). The use of safe and effective adjuvants in pandemic influenza vaccines is considered as a dose-sparing strategy. Clinical trials evaluating candidate inactivated influenza A/H5N1 vaccines showed that the use of adjuvants can increase their immunogenicity and broaden the specificity of the induced antibody responses (2, 7, 19, 23, 27, 36, 41). These research efforts have resulted in the licensing of adjuvanted vaccines against seasonal and pandemic influenza (17). The protective efficacy of immune responses induced with candidate influenza A/H5N1 vaccines was demonstrated in ferrets after two immunizations (1, 22, 24-25) or after a single immunization. The latter was achieved with a low dose of antigen in combination with the adjuvant ISCOMATRIX (26).

Recently, a novel adjuvant has been developed that consists of a sucrose fatty acid sulphate ester (SFASE) immobilized on the oil droplets of a submicron emulsion of squalane-in-water (4). It has been demonstrated that the addition of this novel adjuvant, called CoVaccine HT™, to multiple antigens increased the immune response to these antigens in pigs and horses and was well tolerated in both-species (4, 16, 40). Furthermore, it was shown that the use of CoVaccine HT™ increased the virus-specific antibody responses in mice and ferrets after vaccination with a cell culture-derived whole inactivated influenza A/H5N1 virus vaccine (5, 13). One of the mode of actions of CoVaccine HT™ is the activation of antigen presenting cells like dendritic cells, most likely through TLR-4 signaling (5).
In the present study, we evaluated the protective potential of CoVaccine HT™-adjuvanted cell-culture derived whole inactivated influenza A/H5N1 (WIV) vaccine in the ferret model, which is considered the most suitable animal model for the evaluation of candidate influenza vaccines (6, 14-15). To this end, ferrets were vaccinated once or twice with various antigen doses with or without the adjuvant to test if dose-sparing could be achieved. The use of CoVaccine HT™ increased virus specific antibody responses and T cell responses. A single administration of 3.8µg hemagglutinin of WIV NIBRG-14 vaccine-preparation in combination with CoVaccine HT™ conferred protection against challenge infection with the homologous highly pathogenic A/H5N1 strain A/VN/1194/04 and partial protection against infection with a heterologous, antigenically distinct strain, A/IND/5/05. Therefore, it was concluded that the use of CoVaccine HT™ in inactivated influenza vaccines induced protective virus specific humoral and cell mediated immune responses and could be suitable as adjuvant in (pre)pandemic A/H5N1 vaccines. Further clinical testing of these candidate vaccines seems warranted.
MATERIALS AND METHODS

Vaccine and adjuvant formulations

The vaccine strain NIBRG-14 (H5N1), based on strain A/Vietnam/1194/04 (H5N1, clade 1) was used to prepare a whole inactivated influenza A virus (WIV NIBRG-14) vaccine. The vaccine was produced in Madin-Darby Canine Kidney (MDCK; European Collection of Cell Cultures (ECACC), London, UK) cells and inactivated using beta-propiolactone on a commercial scale by Nobilon Schering-Plough (Boxmeer, The Netherlands). The hemagglutinin content was assessed by a single radial immunodiffusion assay according to standard methods (46). CoVaccine HT™ is a proprietary adjuvant of Protherics Medicines Development Ltd., a BTG Company London, United Kingdom. The optimal dose of CoVaccine HT™ in ferrets was determined by Nobilon Schering-Plough, Boxmeer, The Netherlands before the start of this experiment (data not shown).

Influenza A viruses

Virus stocks of influenza viruses A/Vietnam/1194/2004 (H5N1, clade 1) and A/Indonesia/5/2005 (H5N1, clade 2.1) were prepared by propagation in confluent MDCK cells. After cytopathologic changes were complete, culture supernatants were cleared by low speed centrifugation and stored at −70°C. Infectious virus titers were determined in MDCK cells as described previously (32). All experiments with these viruses were performed under Bio Safety Level (BSL)-3 conditions.

Ferrets

Healthy young adult outbred female ferrets (Mustela putorius furo; between 6 and 12 months old) were purchased from a commercial breeder. Ferrets were screened for the presence of antibodies against circulating influenza A/H1N1 and A/H3N2 viruses and the influenza A/H1N1(2009) virus by hemagglutination inhibition assay. Ferrets that were tested negative for these viruses were used in this experiment. An independent animal ethics committee (DEC consult) approved the experimental protocol before the start of the experiments.

Immunizations and inoculations

Thirty-six sero-negative ferrets were divided into six groups of 6 ferrets and vaccinated with either one dose of 3.8 µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE; group S3.8+), one dose of 15µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE; group S15+), two doses of 3.8 µg HA-content of WIV NIBRG-14 in combination with CoVaccine HT™ (2 mg SFASE; group T3.8+), two doses of 3.8 µg HA-content of the WIV NIBRG-14 only (group T3.8-), two doses of phosphate buffered saline (group PBS), or two doses of CoVaccine HT™ (2 mg SFASE; Cve). Groups are listed in table 1.
Vaccinations were performed under anesthesia with ketamine in the quadriceps muscles of the left hindleg in a total volume of 0.5 ml. Ferrets that received two immunizations were vaccinated with an interval of four weeks, while ferrets that received only one immunization were vaccinated with PBS at the moment of the first vaccination of ferrets receiving two vaccinations. During vaccination experiments, ferrets were housed in groups and received food and water ad-libitum.

Four weeks after the (last) immunization, the animals were anesthetized with ketamine/medetomidine (reversed with atipamezole), weighed and subsequently inoculated intratracheally with $1 \times 10^5$ TCID$_{50}$ influenza A/VN/1194/04 (H5N1) virus in a total volume of 3 ml PBS. After inoculation, ferrets were monitored three times daily for the development of clinical signs. Before inoculation and two and four days after inoculation, throat swabs of each ferret were collected while the ferrets were anesthetized with ketamine. Four days after inoculation, animals were weighed and subsequently killed by exsanguination while under anesthesia with ketamine and medetomidine. In previous experiments, it has been demonstrated that inoculated ferrets start to lose weight as early as one day after infection and continue to lose weight as disease progresses (14, 47). It was anticipated that also during the course of our experiments the maximum weight loss was at day 4 post inoculation and therefore the difference in body weight between the day of inoculation and 4 days post inoculation was considered to be a good clinical indicator for protection against the development of disease.

Necropsies were performed according to standard procedures. After finishing this experiment, the experiment was repeated following exactly the same procedure (n=6 animals per group) except that ferrets were challenged with $1 \times 10^5$ TCID$_{50}$ of influenza A/IND/5/05 (H5N1) virus. Because no differences were observed between mock-vaccinated ferrets and ferrets vaccinated with CoVaccineHT only (group Cve) in the first experiment, the latter group was omitted in the second experiment to reduce the number of animals. One ferret of the group that was vaccinated twice with 3.8 µg HA of WIV NIBRG-14 (T3.8-) died between the second vaccination and inoculation with influenza virus A/IND/5/05 due to reasons unrelated to the experiment.

Serology

Serum samples were collected before, twenty-eight days after the first and twenty-eight days after the second vaccination. Sera were stored at −20°C until use. Sera were tested for the presence of anti-HA antibodies using a hemagglutination inhibition assay (HI-assay) with 1% turkey erythrocytes and for the presence of virus neutralizing antibodies using a micro virus neutralization assay (VN-assay) as described previously (12, 29). Sera were tested for the presence of antibodies reactive with influenza A/H5N1 viruses A/VN/1194/04 and A/IND/5/05. For this purpose, reverse genetics viruses of both viruses were produced. The titers obtained with these viruses were comparable with those against the wild-type strains (data not shown). A hyperimmune rabbit serum to influenza strain A/Tern/South Africa/1/63 and serum obtained from
a swan immunized twice with inactivated H5N1 influenza virus A/Duck/Potsdam/1402/86
(Intervet, Boxmeer, The Netherlands) (30) were used as positive control sera specific for
influenza viruses A/VN/1194/04 and A/IND/5/05, respectively.

T cell proliferation assay
Blood samples were collected from the jugular vein of ferrets before, twenty-eight days after the
first vaccination and twenty-eight days after the second vaccination in EDTA-tubes (Greiner Bio-
One, Alphen a/d Rijn, The Netherlands). Peripheral blood mononuclear cells were isolated by
density gradient centrifugation using lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and then
cryopreserved at -135°C until use. Thawed PBMC were washed twice with PBS, labeled with
0.3µM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS (Invitrogen, Breda, The
Netherlands) for five minutes at 37°C, washed twice and subsequently cells were resuspended in
RPMI 1640 (Cambrex, East Rutherford, USA) containing 10% (v/v) fetal calf serum, penicillin
(100 µg/ml), streptomycin (100 U/ml), L-glutamine (2mM). Cells were seeded (10⁵ cells per well)
in a 96-well round-bottom plate in the presence or absence of WIV NIBRG-14 (200ng HA content)
or phytohaemagglutinin (PHA) (1 µg/ml) and incubated at 37°C/5% CO₂ for six days. For each
condition duplicate samples were tested. Two days after stimulation, 100 µl supernatant of
Concanavalin A-stimulated ferret lymph node cells was added, which was prepared essentially as
described previously (34). After the remaining four days of incubation, cells were transferred to a
96-wells V-bottom plate, washed and subsequently stained with a monoclonal antibody directed
to human CD8 (OKT-8)-Pacific Blue (eBioscience, San Diego, USA). To exclude dead cells in the
analysis, cells were also stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen,
Breda, The Netherlands). Cells were subsequently fixed and permeabilized with Cytofix and
Cytoperm (BD Pharmingen, Alphen a/d Rijn, The Netherlands) and stained with a Alexa Fluor
647-labelled monoclonal antibody specific for human CD3 (PC3/188A) (Santa Cruz
Biotechnology, Santa Cruz, USA). It has been demonstrated that these CD3 and CD8-specific
monoclonal antibodies are cross-reactive with ferret CD3 and CD8 (35, 43). Data were acquired
using a FACSCanto-II and analysed with FACS Diva software (BD). The proliferation of PBMC
from at least 9 ferrets of each group was assessed in this assay, except for group Cve, since this
group only contained six ferrets. The proliferation of CD3+CD8- cells was calculated by
subtracting the mean number of CD3+CD8-CFSElow cells of the medium only controls from the
mean number of CD3+CD8-CFSElow cells stimulated with WIV NIBRG-14. The same calculation
was performed for CD3+CD8+ cells.

Virus titers of lungs and throat swabs
Samples of all lobes of the right lung and the accessory lobe of inoculated ferrets were collected
and snap frozen on dry ice with ethanol and stored at –70°C until further processing. Lung

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samples were weighed and subsequently homogenized with a FastPrep-24 (MP Biomedicals, Eindhoven, The Netherlands) in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, The Netherlands) and centrifuged briefly. After collection of throat swabs, swabs were stored at -70°C in the same medium as used to homogenize lung samples. Quadruplicate 10-fold serial dilutions of both throat and lung samples were used to infect MDCK cells as described previously (32). HA activity of the culture supernatants collected 5 days post infection was used as indicator of infection. The titers were calculated according to the Spearman-Karber method and expressed as log TCID$_{50}$ per gram for lung tissue or per milliliter (ml) for swabs (18).

Histopathology and immunohistochemistry.
Four days after inoculation with influenza A/VN/1194/04 and A/IND/5/05 virus, ferrets were euthanized and lungs were observed macroscopically and weighed before samples from the right lungs were collected to determine the virus titers. Subsequently left lung lobes were inflated with 10% neutral buffered formalin. After fixation and embedding in paraffin, lungs were sectioned at 4 µm and tissue sections were examined by staining with hematoxylin and eosin (HE). Using an immunoperoxidase method, serial lung tissue sections were also stained with a monoclonal antibody directed against the nucleoprotein of the influenza A virus (33).

Statistical analysis
The presence of overall statistical significant differences between groups regarding weight loss, the number of CFSE$^{low}$ cells, percentage of affected areas in the lungs, viral load in the lungs and throat swab specimens was calculated using the Kruskal-Wallis test. Before individual When calculated P values were less than an $\alpha$ of 0.05, data of each group was individually compared with data from the PBS group using the Games-Howell test. Differences were considered significant when P values were less than an $\alpha$ of 0.05. The number of CFSE$^{low}$ cells were converted to 10$^{log}$ values before statistical analysis was performed.
RESULTS

Antibody responses after vaccination

To assess antibody responses after vaccination with various NIBRG-14 antigen/CoVaccine HT™ combinations, pre- and post-vaccination ferret sera were tested for the presence of antibodies against influenza viruses A/VN/1194/04 and A/IND/5/05 by HI and VN-assay. Like in mock-vaccinated ferrets (group PBS) and ferrets receiving CoVaccine HT™ only (group Cve), virus-specific antibodies were not detectable by HI or VN assay in ferrets that received two vaccinations with 3.8 µg un-adjuvanted WIV NIBRG-14 (group T3.8-) (Table 2). In contrast, a single immunization with as little as 3.8 µg HA of WIV NIBRG-14 adjuvanted with CoVaccine HT™ (group S3.8+) induced HI and VN antibodies against the homologous virus in seven and eight out of twelve animals respectively. The geometric mean antibody titers were low (24 and 9, respectively) and only in a small number of ferrets, antibodies against the heterologous virus A/IND/5/05 were detected. Increasing the antigen dose did not improve the serological outcome of vaccination. Five out of 12 ferrets receiving a single dose of 15 µg WIV NIBRG-14 with CoVaccine HT™ (group S15+), developed antibodies against influenza virus A/VN/1194/04. The serum of one of these animals displayed cross-reactivity with the heterologous influenza virus A/IND/5/05.

All twelve ferrets that received two immunizations of 3.8 µg HA of NIBRG-14 with CoVaccine HT™ (group T3.8+) developed antibodies against influenza virus A/VN/1194/04 as detected by HI-(GMT 86) and the VN assay (GMT 65) 28 days after the second immunization. The serum antibodies of these twelve animals cross-reacted with the heterologous influenza virus A/IND/5/05 as detected by the VN assay (GMT 38). By HI assay, cross-reactive antibodies were detected in six out of the twelve serum samples (GMT 30).

Vaccine-induced T cell responses

To assess the induction of T cell immunity after vaccination, PBMC of the ferrets collected before the first vaccination, 28 days after the first and 28 days after the second vaccination were labeled with CFSE and subsequently stimulated with WIV NIBRG-14 antigen, PHA or were left untreated (medium control). The number of CFSE low cells was determined in the CD3+CD8- and CD3+CD8+ cell population as shown in figure 1A.

Viral antigen specific proliferation was virtually undetectable with PBMC collected before the immunization of the ferrets. PBMC obtained from mock-vaccinated ferrets or from those vaccinated with CoVaccine HT™ only responded to stimulation with NIBRG-14 antigen very poorly. In contrast, PBMC obtained from ferrets vaccinated with NIBRG-14 proliferated upon in vitro re-stimulation with viral antigen, whereas CSFE low cells were virtually absent in un-stimulated PBMC obtained from any of the ferrets (medium controls). The latter numbers were substracted
from the number of antigen or PHA-stimulated PBMC to assess the number of cells that proliferated upon stimulation. Increased numbers of CFSE<sup>low</sup> CD3+CD8- cells and CFSE<sup>low</sup> CD3+CD8+ cells were observed in PBMC of ferrets after stimulation with PHA compared to the stimulation with medium only. Furthermore, the numbers of CFSE<sup>low</sup> CD3+CD8- and CFSE<sup>low</sup> CD3+CD8+ cells after PHA stimulation did not differ between ferrets from groups S3.8+, S15+, T3.8+, T3.8- and Cve in mock-vaccinated ferrets (data not shown).

Antigen-specific proliferation of CD3+CD8- cells was observed in PBMC collected from ferrets of groups S3.8+, S15+, T3.8+ and T3.8-. The in vitro proliferative response of ferrets from groups S3.8+, S15+ and T3.8+ was significantly stronger than that of mock-vaccinated animals (p-values below 0.01) for all three groups compared to mock-vaccinated group, while differences between ferrets vaccinated twice with WIV NIBRG-14 without CoVaccine HT™ and mock-vaccinated animals approached statistical significance (p=0.08). In addition, the in vitro proliferative response of CD3+CD8- cells obtained from ferrets that received two immunizations of 3.8 µg HA of WIV NIBRG-14 with CoVaccine HT™ compared to ferrets that received 3.8 µg HA of WIV NIBRG-14 twice without CoVaccine HT™ also approached statistical significance (p=0.06) (Fig 1B). With PBMC obtained from ferrets of groups S15+ and T3.8+, a virus-specific proliferative response of CD3+CD8+ cells was observed (Fig 1C), that was significantly stronger than that of mock-vaccinated ferrets (p-values of respectively 0.03 and 0.01). Furthermore, proliferative responses of CD3+CD8+ cells obtained from ferrets that received two immunizations of 3.8 µg HA of WIV NIBRG-14 with CoVaccine HT™ was significantly stronger than that of ferrets that received 3.8 µg HA of WIV NIBRG-14 twice without CoVaccine HT™ (p=0.03). Like CD3+CD8+ cells obtained from mock-vaccinated ferrets, also those from animals vaccinated with CoVaccine HT™ only responded to NIBRG-14 antigen poorly.

Clinical signs after inoculation with influenza viruses A/VN/1194/04 and A/IND/5/05

From day 3 post inoculation with influenza viruses A/VN/1194/04 and A/IND/5/05 onwards, severe clinical signs were observed in mock-vaccinated ferrets and those vaccinated with CoVaccine HT™ only (group Cve) or with WIV NIBRG-14 only (group T3.8+), and included breathing difficulties, lethargy, decreased appetite and weight loss. In contrast, only mild clinical signs were observed after inoculation with influenza virus A/VN/1194/04 in ferrets immunized once with 3.8 µg NIBRG-14 and CoVaccine HT™ (S3.8+), once with 15 µg NIBRG-14 and CoVaccine HT™ (S15+) or twice with 3.8 µg NIBRG-14 and CoVaccine HT™ (T3.8+). In these groups moderate clinical signs were observed after inoculation with influenza virus A/IND/5/05 (H5N1). One ferret vaccinated with CoVaccine HT™ died in the night between day three and four post inoculation with influenza virus A/VN/1194/04 and one mock-vaccinated ferret succumbed two days post inoculation with influenza virus A/IND/5/05. No mortality was observed in other groups. The relative loss in body weight four days post
inoculation was calculated for each ferret. The mean weight loss of each group was used as a
quantitative clinical indicator of disease upon inoculation with influenza viruses A/VN/1194/04 and
A/IND/5/05. Following inoculation with influenza virus A/VN/1194/04 (Fig 2A), all ferrets lost body
weight, except one animal vaccinated twice with 3.8 µg NIBRG-14 and CoVaccine HT™. The
highest mean weight loss was observed in ferrets of groups S15+ (12%), T3.8- (11%), PBS (11%)
and Cve (13%), while ferrets of groups S3.8+ and T3.8+ lost 3% and 2% of their body weight
respectively, significantly less than mock-vaccinated ferrets (p-values respectively 0.02 and 0.04).
After inoculation with influenza virus A/IND/5/05 (Fig 2B), all ferrets lost body weight. The mean
weight loss of ferrets in groups PBS (mock-vaccinated), T3.8-, S3.8+, S15+, T3.8+ was 13%,
14%, 9%, 10% and 8%, respectively. No statistically significant differences were observed
between groups.

Virus titers in pharyngeal swabs
To assess the extent of virus replication in the upper respiratory tract after inoculation with both
influenza A/H5N1 viruses, pharyngeal swabs were collected before and on day 2 and 4 after
inoculation and tested for the presence of virus. No virus was detected in any of the swabs
collected before inoculation (data not shown). On day 2 and 4 post inoculation with influenza virus
A/VN/1194/04, virus was not detected in pharyngeal swabs obtained from ferrets of groups 3.8+,
S15+ and T3.8+, while those obtained from ferrets of T3.8-, PBS and Cve all tested positive at
day 2 with mean virus titers of $10^{4.4}$, $10^{3.7}$ and $10^{3.4}$ TCID$_{50}$/ml respectively (Fig 3A). Four days
post inoculation these swabs also tested positive with mean virus titers of $10^{4.0}$, $10^{4.9}$, and $10^{5.1}$
TCID$_{50}$/ml, respectively. Following inoculation with influenza virus A/IND/5/05, similar results were
obtained. Two and four days post inoculation, no virus was detected in pharyngeal swabs
collected from ferrets of groups S3.8+ and T3.8+, while virus was detected in only one ferret of
group S15+ on day 2 and 4 post inoculation. All swabs obtained from ferrets of groups T3.8- and
PBS tested positive with mean virus titers of $10^{3.9}$ and $10^{4.3}$ TCID$_{50}$/ml two days post inoculation
and $10^{5.1}$ and $10^{5.5}$ TCID$_{50}$/ml four days post inoculation, respectively (Fig 3B).

Virus detection in the lungs
Lungs of ferrets euthanized four days after inoculation and the lungs of the mock-vaccinated
ferret that died two days after inoculation were tested for the presence of infectious virus. After
inoculation with influenza virus A/VN/1194/04, virus was detected in lungs of all ferrets of the
T3.8-, PBS and Cve groups with mean titers of $10^{5.5}$, $10^{6.7}$, $10^{6.5}$ TCID$_{50}$/gram lung respectively
(Fig 3C). The mean virus titer in lungs of ferrets vaccinated twice with 3.8 µg of WIV NIBRG-14
only were (10-fold) lower than mock-vaccinated ferrets, although differences were not significant
(p=0.17). In contrast, after inoculation with influenza virus A/VN/1194/04, no virus was detected in
lungs of ferrets vaccinated once or twice with 3.8 µg WIV NIBRG-14 and CoVaccine HT™ (S3.8+...
and T3.8+), while in only two out of six ferrets vaccinated with 15 µg WIV NIBRG-14 and CoVaccine HT™ (S15+) low titers were detected in the lungs, which was significantly lower than mock-vaccinated animals (p<0.01 for all three groups). After inoculation with influenza virus A/IND/5/05, lungs of all ferrets tested positive by virus isolation, except for one ferret of group T3.8+. The virus titers in lungs of ferrets of group T3.8- were not significantly different from those in lungs of mock-vaccinated ferrets (p=0.99) (titers of $10^{7.4}$ and $10^{7.0}$ TCID₅₀/gram lung respectively). In contrast, the mean lung virus titers of ferrets of groups S3.8+ ($10^{4.7}$ TCID₅₀/gram), S15+ ($10^{5.4}$ TCID₅₀/gram) and T3.8+ ($10^{3.8}$ TCID₅₀/gram) were significantly lower (p<0.01, p<0.01 and p=0.03 respectively) than those of mock-vaccinated ferrets and ferrets of group S3.8- (Fig 3D).

Gross pathologic findings in the lungs of inoculated ferrets

Four days after inoculation with influenza viruses A/VN/1194/04 or A/IND/5/05, ferrets were euthanized and lungs were examined macroscopically and weighed before assessing virus replication and histopathological changes. Macroscopically, dark red and firm consolidated areas were present in lungs of inoculated animals. The percentage of affected lung tissue was estimated and varied significantly between different groups of animals. In lungs obtained from ferrets of groups S3.8+, S15+ and T3.8+ affected areas were significantly smaller than in lungs of mock-vaccinated animals (p-values for all groups <0.01) (Fig 4A and 4B). The extent of consolidation was more pronounced in lungs of ferrets from group S15+ than in those from group S3.8+, especially after inoculation with influenza virus A/IND/5/05, although no significant differences were present between these two groups (p=0.09).

After inoculation with influenza A/VN/1194/04 virus, the mean relative lung weight was highest in groups T3.8- (1.6% of body weight ± 0.4), PBS (1.8% ± 0.2), Cve (1.7% ± 0.2), while that of groups S3.8+ (1.0 ± 0.2), S15+ (1.0 ± 0.2) and T3.8+ (1.0 ± 0.1) was significantly lower than that of mock-vaccinated ferrets (p-values for all groups <0.01) (Fig 4C) Similar results were observed after inoculation with influenza virus A/IND/5/05. Again, the highest relative lung weight was observed in groups T3.8- (2.0 ± 0.3) and PBS (1.7 ± 0.2). The mean relative lung weight of groups S3.8+ (1.1 ± 0.1), S15+ (1.3 ± 0.1) and T3.8+ (1.1 ± 0.1) was significantly lower (p-values <0.01, 0.03 and 0.01 respectively) than that of mock-vaccinated ferrets (Fig 4D).

Histopathologic findings in lungs after inoculation

Upon inoculation with either influenza virus A/VN/1194/04 or A/IND/5/05, ferrets of groups T3.8-, PBS and Cve developed a moderate to severe broncho-interstitial pneumonia. Multifocally in the alveoli, the lumina contained many macrophages and neutrophils mixed with variable numbers of erythrocytes, edema fluid and fibrin; the alveolar septa also contained many macrophages and neutrophils and showed moderate hypertrophy and hyperplasia of type II pneumocytes (Fig 5A...
and B). Multifocally in the bronchioles and bronchi, the walls had loss of epithelium and the lumina contained cell debris. In addition, the walls of bronchioles, and bronchi contained multifocal many macrophages and neutrophils. A few macrophages, lymphocytes, plasmacytes, and occasional neutrophils were present in perivascular and peribronchiolar tissue (Fig 5C). In contrast, upon inoculation with either of the two above viruses, ferrets of groups S3.8+, S15+ and T3.8+ developed only a mild to moderate broncho-interstitial pneumonia. Compared to the above lesion, fewer and smaller areas of lung parenchyma were affected, there were fewer inflammatory cells in alveoli and bronchioles, and there was less severe edema (Fig 5D). However, there was more prominent peribronchiolar and perivascular, and also peribronchial infiltration with many lymphocytes and plasmacytes and occasional macrophages and neutrophils. Also, bronchus-associated lymphoid tissue was present in the lungs of these ferrets (Fig 5E and F). Upon infection with either of the two viruses, ferrets in the S15+ group had more severe broncho-interstitial pneumonia than ferrets in S3.8+ and T3.8+ groups. This increased severity was characterized by both a greater extent of necrosis and inflammation, and by a higher number of inflammatory cells.

Detection of virus-infected cells by immunohistochemistry

By immunohistochemistry, influenza virus antigen expression of cells in the lung tissue was assessed. After inoculation with influenza A/VN/1194/04 virus, numerous virus-infected cells were present in the lungs of mock-vaccinated ferrets or ferrets vaccinated with 3.8µg HA WIV NIBRG-14 only or CoVaccine HT™ only (Fig 6A and B). The infected cells were predominantly observed in affected areas of the lungs. Inoculated cells were only found sporadically in the lungs of ferrets of groups S3.8+, S15+ and T3.8+ (Fig 6C and D). After inoculation with influenza A/IND/5/05 virus, virus-inoculated cells were present widespread in lungs of ferrets of groups PBS and Cve (Fig 6E and F), while the number of virus-inoculated cells was considerably lower in the lungs of ferrets from groups S3.8+, S15+ and T3.8+ (Fig 6G and H). Influenza virus antigen expression was predominantly observed in alveolar and bronchiolar epithelial cells.
DISCUSSION

In the present study we have demonstrated that the addition of the novel adjuvant CoVaccine HT™ to an inactivated influenza H5N1 vaccine produced by state-of-the-art cell-culture technology makes a promising pandemic influenza vaccine candidate. This vaccine afforded protection against infection with the homologous and an antigenically distinct strain of A(H5N1) virus, even after a single administration of a low antigen dose. The protection of ferrets was associated with the induction of humoral and cell-mediated immune responses in vaccinated animals.

CoVaccine HT™ potentiated the antibody response to the vaccine strain considerably. After two immunizations of 3.8 µg HA of the NIBRG-14 preparation without the adjuvant, virus-specific antibodies were undetectable whereas the addition of CoVaccine HT™ resulted in detectable antibody responses in all ferrets. Even after a single immunization with the adjuvanted vaccine, approximately 50% of the animals developed detectable antibody levels against the homologous vaccine strain. In some ferrets, but especially those vaccinated twice with the CoVaccine HT™-adjuvanted vaccine, antibodies reactive with the heterologous strain A/IND/5/05 were detectable. This is of importance since multiple antigenically distinct A/H5N1 viruses are circulating and it is impossible to predict which clade of these viruses eventually may cause a pandemic. In this light, the induction of broadly reactive antibodies is advantageous and may correlate with protection against infection with heterologous influenza A/H5N1 viruses.

In addition, the induction of T cell responses after vaccination was evaluated. The assessment of virus-specific T cell responses after vaccination or infection of ferrets has been hampered by the lack of reagents necessary for the detection of ferret T cells and their products. Recently, monoclonal antibodies have been defined specific for human cluster of differentiation (CD) antigens that cross-react with those of ferrets lymphocytes (35, 43). Also antibodies specific for ferret IFN-γ have been produced that can be used to detect the production of this cytokine by ferret T cells, for example by ELISPOT assay (28). However, although reagents are becoming available, assays for the detection of virus-specific T-cells have not been described so far to our knowledge. Using monoclonal antibodies cross-reactive with ferret CD3 and CD8 (35, 43) we developed a T cell proliferation assay that allowed discriminating proliferation of CD8+ and CD8- virus-specific T cells. Using this assay, it was shown that the use of CoVaccine HT™ significantly improved the virus-specific T cell response after vaccination of ferrets. Even after a single immunization with the adjuvanted vaccine substantial T cell responses were observed. Strong CD8-, presumably CD4+, T cell response, were detected that could be at the basis for the improved immunogenicity of the vaccine in term of antibody responses. After vaccination with the CoVaccine HT™-adjuvanted vaccines, a virus-specific CD8+ T cell response was observed, which could be the result of cross-priming in vivo (3). Previously we were unable to detect virus-
specific CD8+ T cell responses in mice after vaccination with CoVaccine HT™-adjuvanted vaccine (5). However, in the mouse model CD8+ T cell responses were assessed ex vivo using tetramers detecting the responses to a single epitope only. It is likely that the proliferation and amplification of virus specific T cells in vitro increased the sensitivity of the detection of these cells in the current study. In the mouse model it was demonstrated that, at least partially, the adjuvant effect of CoVaccine HT™ was mediated through TLR-4 signaling (5). At present it is unclear whether this mode of action of the adjuvant also played a role in the improved immunogenicity of the vaccine in the ferret model. The availability of assays to detect virus-specific T cell responses may aid the elucidation of correlates of protection against influenza in the ferret model and future rational vaccine design.

The ultimate goal of vaccination of course is to protect against the development of severe disease upon exposure to the pathogen. Since the breadth of protective immunity is considered an important property of vaccines, vaccinated ferrets were inoculated with two antigenically distinct influenza A/H5N1 viruses, the homologous clade 1 virus A/VN/1194/04 and the heterologous clade 2.1 virus A/IND/5/05. With the vaccines containing CoVaccine HT™, a dramatic reduction of clinical signs, gross pathological and histopathological changes and virus replication in the upper and lower respiratory tract after inoculation with influenza virus A/VN/1194/04 was achieved. In contrast, two immunizations with unadjuvanted NIBRG-14 vaccine failed to induce protective immunity. Even after a single administration of a vaccine containing CoVaccine HT™ and as little as 3.8 µg HA, ferrets were fully protected against the homologous virus A/VN/1194/04. Vaccination was somewhat less effective against the development of clinical signs after infection with the heterologous virus A/IND/5/05, although still a significant reduction of virus replication in the upper and lower respiratory tract was achieved, even after a single administration of a low dose of antigen and CoVaccine HT™. In previous studies, it was demonstrated that with two immunizations with a low vaccine dose in combination with the adjuvant AS03, protection could be achieved against the virus A/IND/5/05, but this vaccine also failed to induce sterilizing immunity in ferrets against this heterologous challenge virus (1). This indicates that it is inherently difficult to induce sterilizing immunity against viruses that are antigenically distinct. Our findings are in concordance with the results obtained in other studies that show that immunogenicity of otherwise poorly immunogenic inactivated H5N1 vaccines can be increased dramatically with the use of adjuvants (38-39). Especially promising results have been obtained with the adjuvants MF59 and AS03, which are both currently licensed for use in prepandemic H5N1 vaccines and in vaccines against the current influenza pandemic A/H1N1(2009) outbreak (17).

It is of interest to note that the use of a higher vaccine dose (15 µg of HA) did not result in the induction of higher antibody titers or increased protection against infection. In contrast, a single administration of the adjuvanted vaccine containing 15 µg seemed less effective in preventing...
virus replication, weight loss and the development of (histo)pathological changes after inoculation with influenza viruses A/VN/1194/04 or A/IND/5/05 than the use of the vaccine containing only 3.8 µg of HA. The mechanism of this phenomenon is poorly understood, but it suggests that the ratio between the amount of viral antigen and adjuvant can affect the outcome of vaccination and the protective efficacy of the vaccine.

In general, the protection against infection with both challenge viruses correlated with the induction of virus specific HI and VN antibodies. However, a number of animals vaccinated once with adjuvanted-vaccine were protected against infection in the absence of detectable virus-specific antibodies. This discrepancy also has been observed in other studies that evaluated candidate H5N1 vaccines (1, 20-21, 26). It is possible that lower, undetectable levels of virus specific antibodies are already protective, or that priming of B cells for a secondary antibody response contributes to protective immunity. Alternatively, the induction of virus specific T cell immunity could have contributed to protection against infection. However, in the present study, the induction of virus specific T cells correlated only partially with the protection against infection.

For example, an individual ferret (S15+ group) was protected against infection but failed to develop a robust virus-specific T cell response, whilst another (T3.8- group) was unprotected despite a strong T cell response. Furthermore, the magnitude of the antibody response upon vaccination did not correlate with the CD3+CD8- cell response.

The CoVaccine HT™-adjuvanted vaccines were well tolerated by the ferrets. Only some mild local reactions were observed at the site of vaccination, consisting of local erythema for one day. Data collected from temperature loggers that were placed in the abdominal cavity before the start of the experiment with ferrets that were challenged with influenza A/VN/1194/04 indicated that the CoVaccine HT™ adjuvant NIBRG-14 vaccine caused a rise of body temperature for 1-2 days. It should be noted however, that these side effects are not uncommon after administration of adjuvanted influenza vaccines (23, 42).

Collectively, we conclude that CoVaccine HT™ is a promising adjuvant for the use in (pandemic) influenza vaccines. In the present study we have shown that its use in ferrets increased the immunogenicity of a cell-culture produced inactivated H5N1 vaccine. It increased both virus-specific T cell and antibody responses, resulting in protection against infection with the homologous and an antigenically distinct virus, even after a single administration of as little as 3.8 µg of HA antigen. These favorable properties justify the further clinical development of this influenza vaccine candidate.
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REFERENCES


### Table 1. Overview of vaccination regimens in this study

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Immunizations</th>
<th>WIV NIBRG-14 (µg HA-content)</th>
<th>CoVaccineHT (2mg SFASE)</th>
</tr>
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<tbody>
<tr>
<td>S3.8+</td>
<td>1</td>
<td>3.8</td>
<td>+</td>
</tr>
<tr>
<td>S15+</td>
<td>1</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>T3.8+</td>
<td>2</td>
<td>3.8</td>
<td>+</td>
</tr>
<tr>
<td>T3.8-</td>
<td>2</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td>PBS</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CVE</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Antibody responses four weeks after the second immunization

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody titers against A/VN/1194/04</th>
<th>Antibody titers against A/IND/5/05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI GMT Responders VN GMT Responders</td>
<td>HI GMT Responders VN GMT Responders</td>
</tr>
<tr>
<td></td>
<td>(sd) (sd)</td>
<td>(sd) (sd)</td>
</tr>
<tr>
<td>S3.8+</td>
<td>24 (22) 7/12 9 (4) 8/12 &lt;10 1/12 &lt;10 2/12</td>
<td></td>
</tr>
<tr>
<td>S15+</td>
<td>13 (12) 5/12 9 (6) 5/12 &lt;10 1/12 &lt;10 1/12</td>
<td></td>
</tr>
<tr>
<td>T3.8+</td>
<td>86 (21) 12/12 65 (23) 12/12 30 (30) 6/12 38 (21) 12/12</td>
<td></td>
</tr>
<tr>
<td>T3.8-</td>
<td>&lt;10 0/11 &lt;10 0/11 &lt;10 0/11 &lt;10 0/11</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
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<td></td>
</tr>
<tr>
<td>Cve</td>
<td>&lt;10 0/6 &lt;10 0/6 &lt;10 0/6 &lt;10 0/6</td>
<td></td>
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</tbody>
</table>
**LEGENDS**

**Fig 1. T cell immune responses after vaccination of ferrets.** The proliferation of CD3+CD8- and CD3+CD8+ cells was measured by a CFSE dilution assay. As an example the results obtained with PBMC of two ferrets are shown (A), one mock-vaccinated ferret and one ferret vaccinated twice with 3.8µg HA of WIV and CoVaccine HT™ (T3.8+). The scale of the y-axis differs between graphs. PBMC of the non-immune ferret respond to PHA only, while PBMC of the immune ferret responded to stimulation with WIV NIBRG-14 and with PHA. The number of CD3+CD8- cells responding to WIV NIBRG-14 was calculated by subtracting the mean number of CD3+CD8-CFSE<sup>low</sup> cells incubated with medium from the mean number of CD3+CD8-CFSE<sup>low</sup> cells incubated with WIV NIBRG-14 (B). The same procedure was used to calculate the number of responding CD3+CD8+ cells (C). The number of ferrets of each group tested in this assay differed between the respective groups, group Cve (n=6), group T3.8- (n=9), groups T3.8+ and PBS (n=10) and groups S3.8+ and S15+ (n=12). Significant differences compared to the mock-vaccinated group are indicated by one (p<0.05) or two asterisks (p<0.01).

**Fig 2. Weight loss after inoculation with influenza A/VN/1194/04 and A/IND/5/05 viruses.** The loss of body weight four days p.i. was determined for each ferret relative to the body weight at the day of inoculation (%). For each group, the mean weight loss ± s.d. after inoculation with influenza A/VN/1194/04 (A) and A/IND/5/05 (B) was calculated. Bars represent mean weight loss of each group with standard deviations. Significant differences compared to the mock-vaccinated group are indicated by an asterisk (p<0.05).

**Fig 3. Virus titers of pharyngeal swabs and lungs after inoculation with influenza A/VN/1194/04 and A/IND/5/05 virus.** Virus titers were determined in pharyngeal swabs obtained 2 (grey bars) and 4 days (black bars) after inoculation with influenza A/VN/1194/04 (A) and influenza A/IND/5/05 (B). Virus titers are expressed as TCID<sub>50</sub> per milliliter (log10). The plus sign indicates that no virus was detected in pharyngeal swabs obtained from ferrets of groups S3.8+, S15+ and T3.8+ after inoculation with influenza A/VN/1194/04 virus and from ferrets of groups S3.8+ and T3.8+ after inoculation with influenza A/IND/5/05 virus resulting in a mean virus titer below the cut-off value. Lung virus titers were determined four days after inoculation with influenza A/VN/1194/04 (C) and influenza A/IND/5/05 (D) virus. Virus titers are expressed as the TCID<sub>50</sub> per gram lung (log10). The dotted line indicates the cut-off value for obtaining a positive result. Significant differences compared to the mock-vaccinated group are indicated by one (p<0.05) or two asterisks (p<0.01).
Fig 4. Gross pathological changes after inoculation with influenza A/H5N1 viruses. The percentage lung tissue displaying consolidation at necropsy of the ferrets was estimated for all ferrets of each group after inoculation with influenza virus A/VN/1194/04 (A) and A/IND/5/05 (B). Lungs of all ferrets of each group were also weighed after necropsy and the weight of the lungs was related to the body weight of the ferrets four days after inoculation which is shown as a percentage for ferrets inoculated with influenza virus A/VN/1194/04 (C) and A/IND/5/05 (D). Bars represent relative lung weight of each group with standard deviations (BW = body weight). Significant differences compared to the mock-vaccinated group are indicated by one (p<0.05) or two asterisks (p<0.01).

Fig 5. Examples of histopathologic findings in lungs after inoculation with influenza A/VN/1194/04 and A/IND/5/05. Histopathologic changes in the lungs of ferrets of groups T3.8-, Cve and PBS inoculated with either influenza virus A/VN/1194/04 or influenza A/IND/5/05 included multiple large areas of severe alveolitis with proteinaceous fluid (edema) (A) and the presence of an inflammatory infiltrate consisting mainly of neutrophils and macrophages, cellular debris and erythrocytes (B). In the bronchioles, there was multifocal loss of epithelial cells and cellular debris (C). In lungs of ferrets of groups S3.8+, S15+ and T3.8-, only a mild to moderate alveolitis was observed with inflammatory infiltrate mainly in the alveolar septa (D). Perivascular (E) and peribronchiolar (F), infiltrates were present consisting mainly of lymphocytes and plasma cells and some macrophages and neutrophils. Pictures were made from lungs of ferrets inoculated with influenza A/VN/119/04. H&E staining, magnification 20x.

Fig 6. Detection of virus-infected cells by immunohistochemistry. By immunohistochemistry, the presence of productively infected cells in the lung tissue was assessed. Cells that are positive for the presence of viral antigen show a deep red staining in the nucleus. Four days after inoculation with influenza A/VN/1194/04 (A+B) virus and influenza A/IND/5/05 (E+F), multiple virus-infected cells were present in both alveoli and bronchioles of lungs of ferrets of groups T3.8-, PBS, Cve. Inoculated cells were only found sporadically in the lungs of ferrets of groups S3.8+, S15+ and T3.8+. After inoculation with influenza A/IND/5/05 virus, the number of inoculated cells was reduced in the lungs of ferrets from groups S3.8+, S15+ and T3.8+ (G+H), most inoculated cells were present in areas with inflammatory infiltrate (see insert).