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Intranasally administered Endocine™ formulated 2009 pandemic influenza H1N1 vaccine induces broad specific antibody responses and confers protection in ferrets

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ABSTRACT

Influenza is a contagious respiratory disease caused by an influenza virus. Due to continuous antigenic drift of seasonal influenza viruses, influenza vaccines need to be adjusted before every influenza season. This allows annual vaccination with multivalent seasonal influenza vaccines, recommended especially for high-risk groups. There is a need for a seasonal influenza vaccine that induces broader and longer lasting protection upon easy administration. Endocine™ is a lipid-based mucosal adjuvant composed of endogenous lipids found ubiquitously in the human body. Intranasal administration of influenza antigens mixed with this adjuvant has been shown to induce local and systemic immunity as well as protective efficacy against homologous influenza virus challenge in mice. Here we used ferrets, an established animal model for human influenza virus infections, to further investigate the potential of Endocine™ as an adjuvant. Intranasal administration of inactivated pandemic H1N1/California/2009 split antigen or whole virus antigen mixed with Endocine™ induced high levels of serum hemagglutination inhibition (HI) and virus neutralization (VN) antibody titers that were also cross reactive against distant swine viruses of the same subtype. HI and VN antibody titers were already demonstrated after a single nasal immunization. Upon intratracheal challenge with a homologous challenge virus (influenza virus H1N1/The Netherlands/602/2009) immunized ferrets were fully protected from virus replication in the lungs and largely protected against body weight loss, virus replication in the upper respiratory tract and pathological changes in the respiratory tract. Endocine™ formulated vaccines containing split antigen induced higher HI and VN antibody responses and better protection from body weight loss and virus shedding in the upper respiratory tract than the Endocine™ formulated vaccine containing whole virus antigen.

1. Introduction

Influenza A viruses cause annual seasonal epidemics, sporadic avian influenza virus infections and influenza pandemics such as the H1N1 pandemic virus of 2009–2010 [1]. Seasonal influenza A virus infections cause substantial mortality and morbidity, particularly in high risk groups, such as children younger than age 5, elderly, people with certain chronic medical conditions and immune-compromised individuals [2]. Active immunization is the most cost effective way of limiting influenza related morbidity and mortality. Current split-virion or subunit seasonal influenza vaccines, of which hemagglutinin (HA) is considered the major immunogenic component, are effective against circulating homologous virus strains [3]. Antigenic drift caused by mutations in the HA, necessitates regular updates of the vaccine composition. Furthermore, more pathogenic viruses such as the newly emerged pandemic H1N1 virus of 2009 (pH1N1/09) for which among others,

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relatively young people were at an increased risk, highlight the need for improved influenza vaccines that induce better, more cross-protective, and longer lasting immunity than the current seasonal vaccines do.

Vaccines administered parenterally induce effective systemic immune responses, but only limited local immunity in the respiratory tract. Locally produced specific antibodies, in particular secretory IgA (S-IgA) can provide immunity via their unique capability to neutralize a pathogen before it even passes the mucosal barrier [4,5]. Moreover S-IgA antibodies have been demonstrated to contribute to the establishment of increased cross-protection from influenza [6]. Nasal administration of vaccine has the potential of establishing mucosal immune responses at the first site of natural infection [7]. In addition, nasal administration using a needle free delivery system is non-invasive, simply accessible and painless. The currently licensed nasally administered influenza vaccines are live attenuated influenza vaccines (LAIV). The LAIV vaccine manufactured by Medimmune, sold under the trade name FluMist in the US and Fluenz in Europe, has proven to be effective against seasonal infection and to provide better cross-protection against drifted influenza virus strains than the non-live seasonal vaccines [8–10]. However, the use of LAIV is currently restricted to the age group of 2 to 59 years, thus excluding children below age 2 as well as the elderly, both populations classified as major high risk groups by the WHO [2]. Therefore, nasal administration of an inactivated influenza vaccine that would be safe and protective through systemic and mucosal immunity, would be an attractive alternative to currently used influenza vaccines.

Appropriate adjuvants or carrier systems have shown to be indispensable to ensure effective stimulation of the mucosal immune system when non-replicating split or subunit antigens were used [11]. A mucosal adjuvant would ideally increase the uptake of the antigen through the mucus and mucous membrane and reduce the required antigen dose while eliciting mucosal as well as systemic immunity. Moreover, the adjuvant should ideally not cause adverse side effects. Concerns about the safety of mucosal adjuvants are real, since the reporting of an increased incidence of Bell's palsy syndrome seen after using an intranasally administered inactivated influenza vaccine, adjuvanted with an apparently insufficiently detoxified mutant of the *E. coli* heat labile enterotoxin [12,13]. Nevertheless, research on the design and development of effective and safe intranasal adjuvants is ongoing and several mucosal adjuvants which support influenza immunity are currently under investigation [14–18].

Endocine™, an adjuvant system based on endogenous lipids, was tested in three clinical phase I studies. A nasal diphtheria vaccine formulated with Endocine™ (1 or 4%) was evaluated in a phase I study in 2002, and was found to be safe and tolerable. Subjects receiving the diphtheria vaccine with 4% Endocine™ had a higher increase in neutralization titers compared to subjects receiving unadjuvanted vaccine (unpublished data). An inactivated whole virus influenza vaccine and an HIV vaccine, and was shown to be safe and tolerable in all studies [19,20]. Pre-clinical studies with split virion influenza vaccines showed that Endocine™, (previously known as L3B), significantly increases both local and systemic immune responses after intranasal immunization [21]. Addition of the adjuvant to a subunit influenza antigen given intranasally to mice conferred protection (measured by detection of viral RNA) against homologous virus challenge [22].

To further investigate the potential of Endocine™ to adjuvant inactivated nasal influenza vaccines we used the ferret as a model for influenza. Ferrets are considered to be the most suitable animal model for the different forms of human influenza and are naturally susceptible to infection with all wildtype human influenza A viruses causing clinical changes in ferrets similar to those observed in humans. Also the pathogenesis and antibody responses observed in ferrets are quite similar to those in humans [23,24]. Furthermore ferrets share similarities in lung physiology and airway morphology with humans [25,26] and the pattern of influenza virus attachment and replication in the ferret respiratory tract is largely similar to that in humans [27].

In the current study the efficacy of nasal Endocine™ adjuvanted split virion and whole virus pH1N1/09 candidate vaccines was evaluated using the homologous wildtype H1N1 A/The Netherlands/602/2009 (wt-pH1N1) virus as a challenge. Humoral, hemagglutination inhibiting (HI) and virus neutralizing (VN) antibody responses against homologous and three distant swine H1N1 viruses were evaluated. Efficacy was measured by evaluating clinical, virological and pathology parameters. In addition computed tomography (CT) imaging was performed as a newly developed read out parameter of efficacy by quantifying alterations in aerated lung volumes (ALV) [28,29].

2. Materials and methods

2.1. Vaccines

Vaccine nasal drops: Endocine™ 20 mg/ml formulated inactivated H1N1/California/2009 split virion antigen at 5, 15 and 30 μg HA/0.2 ml and whole virus antigen at 15 μg HA/0.2 ml were provided by Eurocine Vaccines AB (Stockholm, Sweden). Parenteral vaccine: Fluarix®, season 2010/2011, also containing inactivated H1N1/California/2009 (GlaxoSmithKline).

2.2. Ferrets

Healthy female ferrets (*Mustela putorius furo*: outbred), approximately 12 months of age, with body weights of 760-1210 g and seronegative for antibodies against circulating influenza viruses B, A/H1N1, A/H3N2 and A/pH1N1 as demonstrated by hemagglutination inhibition (HI) assays were used. Animals were housed in standard cages, in groups of maximal 8 animals during the pre-immunization phase and in study groups of 6 animals during the immunization phase. The study groups were transferred to negatively pressurized glovebox isolator cages on the day of challenge. During the whole study animals were provided with commercial food pellets and water *ad libitum*. The experimental protocol was approved before start of the experiments by an independent institutional animal ethics committee according to the Dutch law.

2.3. Immunization

Five groups of six ferrets received three intranasal immunizations (droplets: 100 μl in each nostril, using a pipet with filtertip) under anesthesia with ketamine and domitor at days 0, 21 and 42. Groups 3, 4 and 5 were intranasally immunized with 200 μl Endocine™ formulated H1N1/California/2009 split antigen containing 5, 15 and 30 μg HA, respectively. Group 6 was intranasally immunized with 200 μl Endocine™ formulated H1N1/California/2009 whole virus antigen containing 15 μg HA. Control group 1 received 200 μl of saline intranasally. One group of six ferrets (group 2) received two subcutaneous immunizations (days 21 and 42 using 25Gx5/8" needles) with 0.5 ml Fluarix®, season 2010/2011, a non-adjuvanted trivalent influenza vaccine (TIV) that also contains the pH1N1 (15 μg HA) component. Blood samples for serum preparation were collected prior immunization on days 0, 21 and 42 and before challenge on study days 64 and 70.

2.4. Challenge with wild type H1N1 A/The Netherlands/602/09 virus

Four weeks after the last immunization (day 70), all ferrets were challenged with wild-type influenza A/Netherlands/602/2009 (wt-pH1N1) virus as previously described [30]. Briefly, 10^6 50% tissue culture infective doses ($TCID_{50}$) of wt-pH1N1 virus was diluted in 3 ml of PBS and administered via the intratracheal route under anesthesia with a cocktail of ketamine and domitor.

2.5. Procedures and sample collection

Several procedures were performed on the ferrets over the course of the experiment. For implantation of temperature sensors, immunizations, viral challenge and computed tomography (CT) imaging the animals were anesthetized with a cocktail of ketamine (4-8 mg/kg: i.m.; Alfasan, Woerden, The Netherlands) and domitor (0.1 mg/kg: i.m.; Orion Pharma, Espoo, Finland). For sampling (blood, swabs and nasal washes) and euthanasia by exsanguination, the animals were anesthetized with ketamin. Two weeks prior to the start of the experiment, a temperature logger (DST micro-T ultrasmall temperature logger; Star-Oddi, Reykjavik, Iceland) was placed in the peritoneal cavity of the ferrets. This device recorded body temperature of the animals every 10 min. Ferrets were weighed prior to each immunization (days 0, 21 and 42) and on the days of challenge and euthanasia (days 70 and 74). Animals of groups 1, 2 and 4 were monitored by CT imaging on days 64, 71, 72, 73 and 74. Blood samples were collected prior to the immunization on days 0, 21 and 42, on day 64 and before challenge on day 70. Nose and throat swabs were collected prior challenge on day 70 and on each day after challenge.

2.6. HI and VN antibody assays

Serum samples, collected on days 0, 21, 42, 64 and 70 were stored at −80 °C until analysis. Sera were tested in HI and VN assays as previously described [31,32] against H1N1 A/The Netherlands/602/2009 virus and the two distant swine viruses H1N1 A/Swine/Ned/25/80 and H1N1 A/Swine/Italy/14432/76. In addition HI serum antibody titers against the distant virus H1N1 A/New Jersey/08/76 were determined (VN assay for this strain was not possible due to insufficient amount of serum). The antigenic distance from H1N1 A/Netherlands/602/2009 to A/swine Netherlands/25/1980, A/swine/Italy/14432/76 and A/New Jersey/08/1976 is 2.3, 4.4 and 7.7 antigenic units, respectively (unpublished data), on basis of antigenic cartography which allows to quantify HI assay data made with ferret post-infection sera, where 1 antigenic unit corresponds with a 2-fold difference in HI assay titer [33].

2.7. Virus replication in the upper and lower respiratory tract

On days 0, 1, 2, 3 and 4 after challenge, nose and throat swabs were taken from the animals under anesthesia. Four days after challenge, the ferrets were euthanized by exsanguination under anesthesia after which full-body gross-pathology was performed and tissues were collected. Samples of the right nose turbinate and of all lobes of the right lung and the accessory lobe were collected and stored at −80 °C until further processing. Turbinate and lung 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 before dilution.

After collection, nose and throat swabs were stored at −80 °C in the same medium as used for the processing of the tissue samples. Quadruplicate 10-fold serial dilutions of lung and swab supernatants were used to determine the virus titers in confluent layers of MDCK cells as described previously [34].

2.8. Gross-pathology and histopathology

The animals were necropsied according to a standard protocol, as previously described [35]. In short, the trachea was clamped off so that the lungs would not deflate upon opening the pleural cavity allowing for an accurate visual quantification of the areas of affected lung parenchyma. Samples for histological examination of the left lung were taken and stored in 10% neutral-buffered formalin (after slow infusion with formalin), embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE) for examination by light microscopy. Samples were taken in a standardized way, not guided by changes observed in the gross pathology. Semi-quantitative assessment of influenza virus-associated inflammation in the lung was performed as described previously (Table 1) [30]. All slides were examined without knowledge of the identity or treatment of the animals.

2.9. Computed tomography (CT)

A dual-source ultrafast CT system (Somatom Definition Flash; Siemens Healthcare) was used (temporal resolution: 0.075 s, spatial resolution: 0.33 mm, table speed: 458 mm/s; ferret thorax acquisition times ≈0.22 s; enables accurate scanning of living ferrets without the necessity of breath-holding, respiratory gating, or electrocardiogram (ECG)-triggering) as previously described [28,29]. Briefly, all animals of group 1 (saline; infection control), group 2 (TIV; parenteral control) and of group 4 (nasal Endocine™ formulated split antigen, 15 μg HA) were scanned 6 days prior to virus inoculation (day 64) to define the uninfected baseline status of the respiratory system, and after challenge on 1, 2, 3 and 4 days post inoculation (dpi). During *in vivo* scanning the anesthetized ferrets were positioned in dorsal recumbency in a perspex biosafety container of approximately 8.3 l capacity that was custom designed and built (Tecnilab-BMI). The post-infectious reductions in aerated lung volumes were measured from 3-dimensional CT reconstructs using lower and upper thresholds in substance densities of −870 to −430 Hounsfield units (HU).

2.10. Statistical analysis

Differences between the groups immunized with the Endocine™ adjuvanted H1N1/California/2009 vaccine preparations (groups 3–6) were analyzed statistically using the Kruskal–Wallis test. Differences between the sham (saline) immunized control group and the immunized groups were statistically analyzed using the two-tailed Mann–Whitney test.

3. Results

3.1. Immunogenicity of nasal Endocine™ adjuvanted versus parenteral non-adjuvanted inactivated pH1N1/09 vaccines

3.1.1. HI antibody responses

One intranasal immunization with Endocine™ adjuvanted split, or whole virus antigen induced high homologous HI antibody titers: in all ferrets of groups 3 and 5 (5 and 30 μg HA split antigen; titers 160–1120 and 400–3200, respectively) and in 5 out of 6 ferrets of groups 4 and 6 (15 μg HA split and whole virus antigen at; titers ≤5–5760 and 5–1280, respectively). A second immunization

Table 1
Semi-quantitative scoring for histopathological parameters on 4 dpi.

		Group[a]					
		1	2	3	4	5	6
Histopathology	Extent of alveolitis/alveolar damage (score 0–3)	2.08 ± 0.74 (6/6)	1.88 ± 0.54 (6/6)	0.42 ± 0.52 (3/6)	0.08 ± 0.20 (1/6)	0.04 ± 0.10 (1/6)	0.42 ± 0.41 (4/6)
	Severity of alveolitis (score 0–3)	2.04 ± 0.68 (6/6)	1.63 ± 0.31 (6/6)	0.50 ± 0.69 (3/6)	0.08 ± 0.20 (1/6)	0.04 ± 0.10 (1/6)	0.46 ± 0.46 (4/6)
	Alveolar oedema (% slides positive)	29 ± 29 (4/6)	21 ± 19 (4/6)	4 ± 10 (1/6)	0 ± 0 (0/6)	0 ± 0 (0/6)	8 ± 13 (2/6)
	Alveolar hemorrhage (% slides positive)	21 ± 40 (2/6)	17 ± 26 (2/6)	0 ± 0 (0/6)	0 ± 0 (0/6)	0 ± 0 (0/6)	0 ± 0 (0/6)
	Type II pneumocyte hyperplasia (% slide positive)	42 ± 34 (4/6)	46 ± 37 (4/6)	8 ± 20 (1/6)	4 ± 10 (1/6)	0 ± 0 (0/6)	4 ± 10 (1/6)

[a] Group 1 (control, i.n. saline), group 2 (s.c. TIV), group 3 (i.n. Endocine™ adjuvanted split antigen at 5 μg HA), group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA), group 5 (i.n. Endocine™ adjuvanted split antigen at 30 μg HA) and group 6 (i.n. Endocine™ adjuvanted inactivated whole virus antigen at 15 μg HA).
Histopathology. Semi-quantitative scoring for histopathological parameters on 4 dpi. Extent of alveolitis/alveolar damage, score: 0, 0%; 1, 25%; 2, 25–50%; 3, > 50%; severity of alveolitis, score: no inflammatory cells (0); few inflammatory cells (1); moderate numbers of inflammatory cells (2); many inflammatory cells (3); alveolar oedema, alveolar hemorrhage and type II pneumocyte hyperplasia were scored as positive slides (no = 0, yes = 1); All histopathology results are presented as average with standard deviation.

Table 2
Efficacy of Endocine™ formulated 2009 H1N1 vaccines in ferrets demonstrated by clinical, virological and gross-pathology parameters.

		Group[a]					
		1	2	3	4	5	6
Clinical score	Survival	6/6	5/6	6/6	6/6	6/6	6/6
	Fever	1.7 ± 0.6 (6/6)	1.1 ± 0.4 (6/6)	1.3 ± 0.3(6/6)	1.2 ± 0.6(4/5*)	1.1 ± 0.6(6/6)	1.3 ± 0.2(6/6)
	Body weight loss	18.0 ± 4.6 (6/6)	11.5 ± 2.1 (6/6)	−2.2 ± 2.6 (1/6)	1.7 ± 1.5 (4/6)	2.7 ± 3.3 (4/6)	4.7 ± 3.1 (6/6)
Virology	Lung virus load [$\log_{10}TCID_{50}$/g]	5.7 ± 0.5 (6/6)	5.5 ± 0.9 (6/6)	≤1.5 (0/6)	≤1.4 (0/6)	≤1.3 (0/6)	≤1.3 (0/6)
	Turbinates virus load [$\log_{10}TCID_{50}$/g]	7.2 ± 2.4 (6/6)	6.9 ± 1.5 (6/6)	≤1.9 (0/6)	≤1.7 (0/6)	≤1.7 (0/6)	4.1 ± 2.7 (3/6)
	Virus shedding in nasal swabs	2.6 (5/6)	1.2 (4/6)	0.058 (1/6)	0.0 (0/6)	0.0 (0/6)	1.4 (3/6)
	Virus shedding in throat swabs	10 (6/6)	10 (6/6)	0.0 (1/6)	0.14 (1/6)	0.0 (1/6)	4.2 (5/6)
Gross pathology	Affected lung tissue [%]	50 ± 25 (6/6)	37 ± 21 (6/6)	8 ± 4 (5/6)	7 ± 5 (4/6)	7 ± 5 (4/6)	8 ± 4 (5/6)
	Relative lung weight	1.5 ± 0.5	1.3 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.1

[a] Group 1 (control, i.n. saline), group 2 (s.c. TIV), group 3 (i.n. Endocine™ adjuvanted split antigen at 5 μg HA), group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA), group 5 (i.n. Endocine™ adjuvanted split antigen at 30 μg HA) and group 6 (i.n. Endocine™ adjuvanted inactivated whole virus antigen at 15 μg HA).
Clinical Scores. Survival, number of animals that survived up to 4 dpi; fever (^{0}C), maximum temperature increase presented as average with standard deviation, number of animals in which fever was observed in parentheses, (*), body temperature of 1 animal in group 4 was not available due to malfunction of the recorder; % body weight loss between 0 and 4 dpi presented as average with standard deviation, number of animals with body weight loss in parentheses. *Virology*. Virus shedding in nose and throat swab samples, area under the curve (AUC) for titration results 1–4 dpi, number of animals showing 1 or more virus positive swab in parentheses; virus load in lung and turbinates ($\log_{10}TCID_{50}$/g) on 4 dpi presented as average with standard deviation, or the lower limit of detection in case all animals in the group were virus negative, number of animals with lung/turbinate virus in parentheses. *Gross pathology*. % of estimated affected lung parenchyma by visual examination during necropsy on 4 dpi presented as average with standard deviation, number of animals with affected lung in parentheses; lung/body weight ratio ($\times 10^2$) on 4 dpi presented as average with standard deviation.

increased HI antibody titers in all ferrets, irrespective of antigen and antigen dose (groups 3–6, titers 1120–2560, 1120–5760, 640–3840 and 100–2880, respectively) (Fig. 1A). A third intranasal immunization did not substantially boost the HI immune response further (groups 3–6, titers 1280–3840, 1920–4480, 1280–3200 and 160–2560, respectively). The differences in HI antibody titers between the 3 split antigen HA doses (groups 3, 4 and 5) were not significant ($p > 0.05$). However, mean HI antibody titers in group 4 (15 μg HA split antigen) were significantly higher than those in group 6 (15 μg HA whole virus antigen); $p = 0.01$ and $p = 0.02$ after 2 and 3 immunizations, respectively.

Cross-reactive HI antibodies were measured against the distant H1N1 viruses A/Swine/Ned/25/80, H1N1 A/Swine/Italy/14432/76 and H1N1 A/New Jersey/08/76 (Fig. 1B–D, respectively). The highest cross-reactive HI antibody titers were measured in group 4 (15 μg HA split antigen) after 2 immunizations. Cross-reactive HI antibody titers were significantly higher in group 4 (15 μg HA split antigen) than in group 6 (15 μg HA whole virus antigen) against H1N1 A/Swine/Ned/25/80 and H1N1 A/Swine/Italy/14432/76 viruses ($p = 0.0194$ and $p = 0.0292$), but not against H1N1 A/New Jersey/08/76. Of note, the cross-reactive HI antibody profiles against the distant H1N1 viruses A/Swine/Italy/14432/76 and A/New Jersey/08/76 after 2 immunizations (serum sample day 42) were generally in agreement with the calculated antigenic distances that were obtained using post-infection sera. Remarkably, only the cross-reactive HI antibody profile against the distant H1N1 virus A/Swine/Ned/25/80 induced in group 4 (15 μg HA split antigen) was in agreement with the calculated antigenic distance ($p = 0.1269$) whereas these cross-reactive HI responses in the other groups were significantly lower ($p \leq 0.0245$).

Parenteral, non-adjuvanted trivalent influenza vaccine (TIV) (group 2) displayed relatively limited immunogenicity inducing after two immunizations only in one out of the six ferrets a homologous HI antibody titer ≥40 (titer range 13–70; Fig. 1A) and no cross-reactive HI antibody titers (mean titer <40 (Fig. 1B–D).

3.2. VN antibody responses

VN antibody responses closely paralleled those measured in the HI assays. Homologous VN antibody titers were induced after a single intranasal immunization with Endocine™ adjuvanted split, or whole virus antigen: In 4 out of 6 ferrets of group 3 (5 μg HA split antigen; titers ≤8–64), in 5 out of 6 ferrets of group 4 (15 μg HA split antigen; titers ≤8–724), in all ferrets of group 5 (30 μg HA split antigen; titers 11–627) and in 2 out of 6 ferrets of group 6 (15 μg HA whole virus antigen; titers ≤8–64). A second immunization increased the VN antibody titers in all ferrets, irrespective of the antigen and antigen dose (groups 3–6, titers 64–859, 64–8192, 41–3435 and 32–304) (Fig. 2A). A third immunization was effective in 5 out of 6 animals in group 3 (titers, 362–2436), 2 out of 6 in group 4 (titers, 662–4871), 3 out of 6 in group 5 (titers, 724–4884) and in all animals of group 6 (titers, 113–747). The differences in VN antibody titers between the 3 split antigen HA doses (groups 3, 4 and 6) were not significant ($p > 0.05$). However, mean VN antibody titers in group 4 (15 μg HA split antigen) were significantly higher than in group 6 (15 μg HA whole virus antigen); $p = 0.03$ and $p = 0.01$ after 2 and 3 immunizations, respectively.

Measuring VN antibodies against the distant viruses H1N1 A/Swine/Ned/25/80 and H1N1 A/Swine/Italy/14432/76 showed the highest cross-reactive VN antibody titers in group 4 (15 μg HA split antigen) after 2 immunizations, but the differences were not significant (Fig. 2B and C, respectively). Parenteral, non-adjuvanted TIV (group 2) did not induce VN antibody titers (Fig. 2).

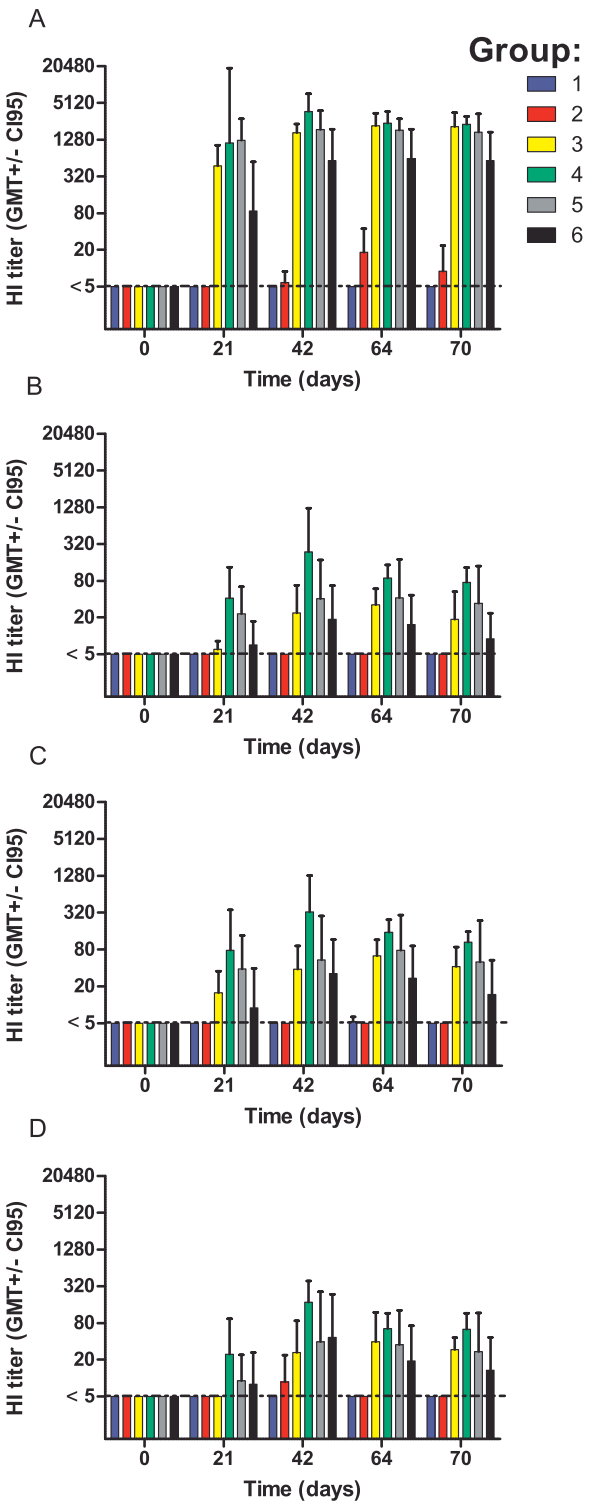

Fig. 1. Development of HI antibody titers against H1N1 A/Ned/602/09 (A), A/Swine/Ned/25/80 (B), A/Swine/Italy/14432/76 (C) and A/New Jersey/08/76 (D). Ferrets of group 1, 3–6 were intranasally inoculated by nasal drops on days 0, 21 and 42 and ferrets of group 2 were subcutaneously injected on days 21 and 42. Blue; group 1 (control, i.n. saline), red; group 2 (s.c. TIV), yellow; group 3 (i.n. Endocine™ adjuvanted split antigen at 5 μg HA), green; group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA), gray; group 5 (i.n. Endocine™ adjuvanted split antigen at 30 μg HA) and black; group 6 (i.n. Endocine™ adjuvanted inactivated whole virus antigen at 15 μg HA). Bars represent geometric mean of 6 animals per group with 95% CI (GMT ± CI95). See text for statistical analysis of the results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

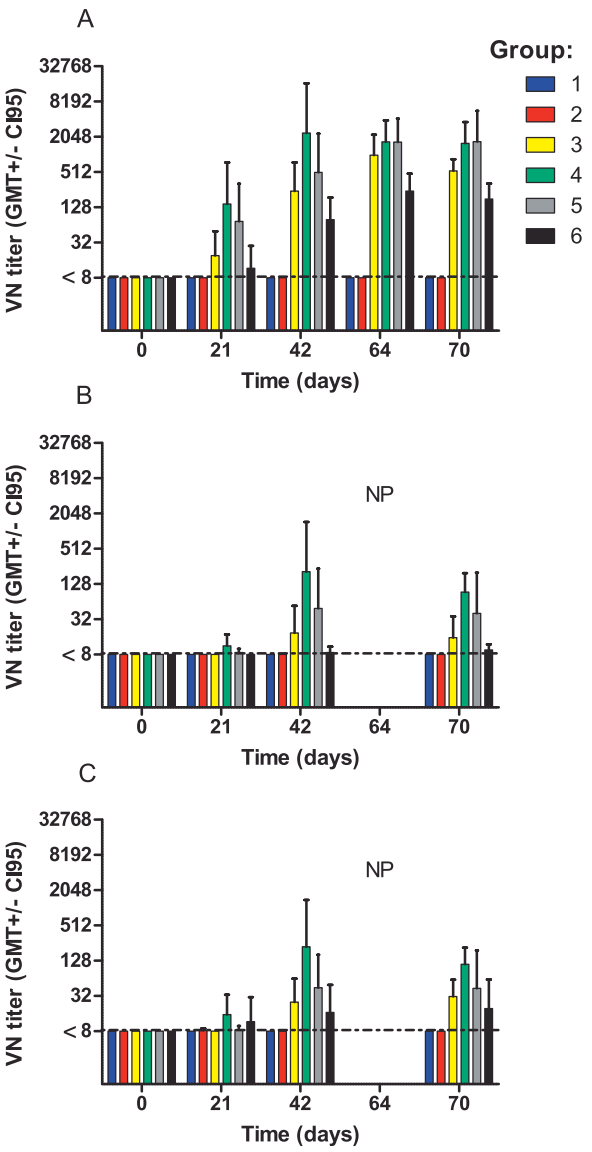

Fig. 2. Development of VN antibody titers against H1N1 A/Ned/602/09 (A), A/Swine/Ned/25/80 (B) and A/Swine/Italy/14432/76 (C). Ferrets of group 1, 3–6 were intranasally inoculated by nasal drops on days 0, 21 and 42 and ferrets of group 2 were subcutaneously injected on days 21 and 42. Blue; group 1 (control, i.n. saline), red; group 2 (s.c. TIV), yellow; group 3 (i.n. Endocine™ adjuvanted split antigen at 5 μg HA), green; group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA), gray; group 5 (i.n. Endocine™ adjuvanted split antigen at 30 μg HA) and black; group 6 (i.n. Endocine™ adjuvanted inactivated whole virus antigen at 15 μg HA). Bars represent geometric mean of 6 animals per group with 95% CI (GMT ± CI95). NP = not performed. See text for statistical analysis of the results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. *Protective efficacy of inactivated nasal Endocine™ adjuvanted versus parenteral non-adjuvanted pH1N1/09 vaccines against a homologous challenge*

3.3.1. *Clinical signs*

Challenge with the homologous wt-pH1N1 was performed four weeks after the last immunization. All ferrets of groups 3–6 (i.n. Endocine™ adjuvanted pH1N1/09 vaccines) as well as control group 1 (i.n. saline) survived the follow-up of 4 days post inoculation (dpi), when they were euthanized. The observation that intratracheal infection with 10^6 $TCID_{50}$ of wt-pH1N1 was not lethal in the naïve control ferrets is in accordance with earlier observations with this virus in ferrets [35,30,36]. However, 1 out of 6 ferrets of control group 2 (s.c. TIV) was found dead on 4 dpi. Pathology revealed that this animal suffered from acute extensive pneumonia, which was the most probable cause of death since no other lesions were evident at necropsy.

Fever was observed in all groups (Table 2). Ferrets of control group 1 displayed the highest fever (mean maximum temperature increase of 1.7 °C), but the differences between control group 1 and the immunized groups (mean maximum temperature increase of 1.1–1.3 °C) were not significant.

Intranasal immunization with Endocine™ adjuvanted split antigen prevented body weight loss in 5 out of 6 ferrets of group 3 (5 μg HA), 2 out of 6 ferrets of group 4 (15 μg HA) and 2 out of 6 ferrets of group 5 (30 μg HA) (Table 2). Body weight loss was most pronounced in control groups 1 (i.n. saline) and 2 (parenteral TIV) and with a mean body weight loss of 18.0% and 11.5%, respectively, significantly higher than in the immunized groups 3 (−2.2%), 4 (1.7%), 5 (2.7%) and 6 (4.7%).

3.4. *Virus load in lung and upper respiratory tract*

All ferrets of control groups 1 (i.n. saline) and 2 (parenteral TIV) showed high titers of replication competent virus in lung (mean titers; 5.7 and 5.5 $\log_{10}TCID_{50}$/gram tissue, respectively) and nasal turbinates (mean titers: 7.2 and 6.9 $\log_{10}TCID_{50}$/gram tissue, respectively) (Table 2). Ferrets of groups 3, 4 and 5 (i.n. Endocine™ adjuvanted split antigen pH1N1/09 vaccines) had no detectable infectious virus in their lungs and nasal turbinates. Ferrets of group 6 (i.n. Endocine™ adjuvanted whole virus at 15 μg HA) had no detectable infectious virus in their lungs and with a mean titer of 4.1 $\log_{10}TCID_{50}$/gram tissue a significantly lower virus titer in the nasal turbinates as compared to control group 1 ($p = 0.02$).

Intranasal immunization with Endocine™ adjuvanted pH1N1/09 vaccines reduced virus titers in swabs taken from the nose and throat as compared to saline or TIV administration. Virus loads expressed as area under the curve (AUC) in the time interval of 1–4 dpi, in nasal and throat swabs are shown in Table 2. Virus loads in nasal swabs of groups 3, 4 and 5 (i.n. Endocine™ adjuvanted split antigen at 5, 15 and 30 μg HA, respectively), but not of groups 2 and 6 were significant lower than in group 1 (group 1 versus groups 3–5; $p \leq 0.03$). Virus loads in throat swabs of group 1 and 2 were comparable and significant higher than in groups 3, 4, 5 and 6 ($p \leq 0.03$).

3.5. *Gross-pathology and histopathology*

Reduced virus replication in groups intranasally immunized with the Endocine™ adjuvanted pH1N1/09 vaccines corresponded with a reduction in gross-pathological changes of the lungs (Table 2).

The macroscopic post-mortem lung lesions consisted of focal or multifocal pulmonary consolidation, characterized by well delineated reddening of the parenchyma. All ferrets in control group 1 (i.n. saline) and group 2 (parenteral TIV) showed affected lung tissue with a mean percentage of 50% and 37%, respectively, and corresponded with a mean relative lung weight (RLW) of 1.5 and 1.3, respectively (Table 2). In contrast, lungs in groups 3–6 (i.n. Endocine™ adjuvanted pH1N1/09 vaccines) were much less affected with mean percentages of affected lung tissue of 7–8%. The RLWs in these four Endocine™-vaccinated groups were in line with these observations (in a close range of 0.8 to 0.9).

The pulmonary consolidation corresponded with an acute broncho-interstitial pneumonia at microscopic examination. It was characterized by the presence of inflammatory cells (mostly macrophages and neutrophils) within the lumina and walls of alveoli, and swelling or loss of lining pneumocytes. In addition protein rich oedema fluid, fibrin strands and extravasated erythrocytes

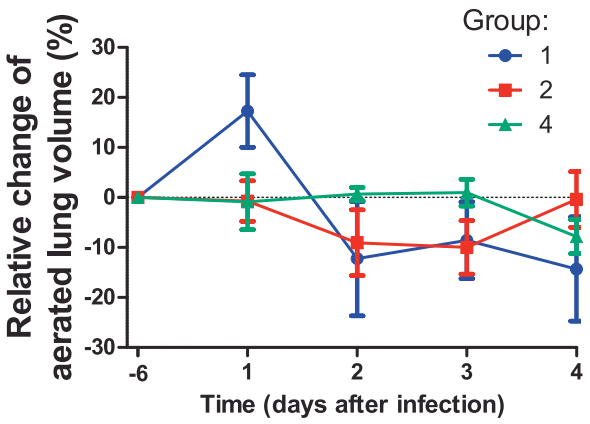

Fig. 3. Changes in aerated lung volume after infection with H1N1 A/Netherlands/602/2009. Blue; group 1 (control, i.n. saline), red; group 2 (s.c. TIV), green; group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA). The aerated lung volume was calculated using lower and upper thresholds in substance densities of −870 to −430 Hounsfield units (HU) for the analysis of 3D-reconstructions of the lung. The percentage change of aerated lung volume was calculated using the individual base line aerated lung volumes of day −6 against the aerated lung volumes of the different days after infection. These data are expressed as mean ± SEM. Animals were intratracheally challenged with 10^6 $TCID_{50}$ H1N1 A/The Netherlands/602/2009 on day 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in alveolar spaces and type II pneumocyte hyperplasia were generally observed in the more severe cases of alveolitis. The histological parameters that were scored are summarized in Table 1. The most severe alveolar lesions were found in the control groups 1 (i.n. saline) and 2 (parenteral TIV). All parameters of alveolar lesions scored lowest in group 5, but in fact the differences between the groups 3–6 were not significant.

3.6. Computed tomography (CT)

The development of pulmonary lesions was investigated by means of CT in ferrets of group 1 (i.n. saline), group 2 (s.c. TIV) and group 4 (i.n. Endocine™ adjuvanted split antigen at 15 μg HA), largely as described previously [29]. Consecutive *in vivo* imaging with CT scanning showed that ferrets of group 4 were largely protected against the appearance of pulmonary ground-glass opacities. Post infection reduction in aerated lung volumes (ALV) were measured from 3D CT reconstructs using lower and upper thresholds in substance densities of −870 to −430 HU. Ferrets of control group 1 showed a temporal significant increase in ALV on 1 dpi, as compared to both immunized groups 2 and 4 (Mann Whitney, two-tailed, $p = 0.05$) (Fig. 3). Subsequently, the ferrets of group 1 showed a decrease of ALV at 2 dpi, which remained low on 3 and 4 dpi (group mean ALV ranging from 17.3 to −14.3%). Ferrets of group 4 were protected against major alterations in ALV (group mean ALV ranging from 0.95 to −7.8%), whereas ferrets of group 2 showed an intermediate decrease of ALV (group mean ALV ranging from 2.7 to −10.0%).

4. Discussion

Nasal influenza vaccines composed of inactivated pH1N1/09 split or whole virus antigen mixed with Endocine™ adjuvant induced high antibody titers in influenza naïve ferrets and protection against homologous challenge. Nasal Endocine™ formulated inactivated pH1N1/09 influenza vaccines induced high levels of serum HI and VN antibodies, which were in the same order of magnitude as antibody levels found in influenza naïve ferrets intranasally immunized with monovalent H1N1/California/2009 live attenuated influenza vaccines (LAIVs) [37–39] or in influenza naïve ferrets intramuscularly immunized with AS03 adjuvanted H1N1/California/2009 split vaccines [39]. The results in control ferrets parenterally immunized with non-adjuvanted seasonal TIV were similar to those seen in naïve controls (i.n. saline). The parenteral non-adjuvanted seasonal TIV did not induce protective HI and VN antibody titers in influenza naïve ferrets, which is in accordance with the general observation that non-adjuvanted inactivated influenza vaccines and in particular split antigen vaccines are weakly immunogenic in influenza naïve ferrets [39–41].

The influenza naïve ferret model may be considered a representative pre-clinical animal model for influenza vaccine efficacy in influenza naïve individuals. A study on prevalence of antibodies against seasonal influenza A and B viruses in children in The Netherlands showed that children between 2 and 3 years of age have the highest attack rate [42]. In addition it was shown that the seroprevalence of antibodies to influenza viruses was higher in children 1 to 6 months of age than in children 7 to 12 months to age, reflecting the window of maternal antibodies. During the time when maternal antibodies are helping protect children against infections the nasopharyngeal tonsil (adenoid) develops in children [43]. The adenoid, which is part of the lymphoid tissue of Waldeyer's ring, is active in early childhood up till the time of adolescence, and has been reported to be functionally comparable to nasal-associated lymphoid tissue (NALT) in rodents [44]. Several studies have suggested that NALT/Waldeyer's ring is a mucosal inductive site for humoral and cellular immune responses in the upper respiratory tract [45], and that tonsils and adenoids might function as effector sites of adaptive immunity [46]. Since the adenoid is unique to children and strategically placed exposed to both alimentary and airborne antigens, nasal vaccines have an especially interesting potential in children. Vaccination of children older than 6 months against seasonal influenza is either recommended, or considered by several public health authorities [47,48]. This is based on studies, which demonstrate that annual vaccination of children is beneficial and usually cost-effective [49,47,50]. Children in the age of 6–24 months who have not experienced an influenza virus infection will most likely benefit from vaccination. Still many European health authorities are reluctant to include influenza vaccination in their national vaccination programs. Doubts about the efficacy of available influenza vaccines most likely plays a substantial role in the decision making progress [51,52]. The possibility of preventing influenza in children aged 6–24 months by means of available vaccines still remains an open question. For children of this age group LAIVs are not licensed due to an increase in hospitalization and wheezing post administration and adjuvanted seasonal TIVs have not yet been licensed for this age group, because of lack of adequate safety data [51]. Consequently, there is a continuing need to design and develop a new generation of broadly protective and safe vaccines, especially for this age category.

The anionic adjuvant Endocine™ was developed specifically to formulate intranasal vaccines. Endocine™ is composed of endogenous lipids found ubiquitously in the human body and has been tested successfully in clinical trials with diphtheria, influenza and HIV [19–21] (and unpublished data). The results of these trials showed that Endocine™ is safe and tolerable in humans, and in the influenza trial the Endocine™ adjuvanted whole virus vaccine fulfilled the EMA/CHMP HAI criteria for a seasonal influenza vaccine. Moreover, influenza-specific IgA was measured in nasal swabs and it was shown that the Endocine™ adjuvanted vaccine induced a significantly higher fold-increase in nasal IgA compared to the mock vaccine with Endocine™ alone [19]. In line with these observations, no adverse effects of the administration of Endocine™ were noted in pre-clinical toxicology or efficacy studies (unpublished data). The two components of Endocine™, monoolein (monoglyceride) and oleic acid (fatty acid), are metabolites generated in mammalians when lipids (triglycerides) are mobilized and energy needed.

Monoolein is composed of glycerol and oleic acid and is a nontoxic, biodegradable and biocompatible material which is included in the FDA Inactive Ingredients Guide and in nonparenteral medicines licensed in the United Kingdom [53]. Oleic acid has been described as being the most abundant fatty acid in human adipose tissue and it is abundantly present in mammalian tissues including tissues from rat, chicken, pig and cow [54,55]. Both oleic acid and monoolein and are classified as GRAS (generally recognized as safe) by the FDA, US. A study in mice showed that Endocine™ mixed with a commercially available trivalent split influenza vaccine (Vaxigrip) significantly ($p < 0.003–0.05$) improved the humoral (HI, VN) and cellular (IFNγ and IL-2 secreting cells) immunity upon nasal administration [21]. Furthermore, intranasal immunization with the Endocine™ formulated vaccine significantly increased the H1N1-specific IgA levels both in serum and nasal washings [21]. In the present study, we have shown that Endocine™ formulated inactivated pH1N1/09 influenza vaccines administered as nasal drops induced a protective systemic immune response in influenza naïve ferrets. Serum HI antibody titers of ≥40 (GMT) were already measured after one immunization, even at the lowest antigen dose of 5 μg HA split antigen. All animals in this study received three nasal immunizations, but optimal serological responses were already measured after two immunizations and the third immunization proved to be redundant for antibody induction. Endocine™ formulated split antigen at an antigen dose of 15 μg HA induced significant higher HI and VN antibody titers than Endocine™ formulated whole virus at the same HA antigen dose. In general inactivated whole virion vaccines are more immunogenic than split/subunit vaccines [56]. However, it has been shown that whole virion vaccines may be more effective without an additional adjuvant [57], and it was mentioned that the neutralizing activity of an adjuvanted whole virion H5N1 vaccine was lower than that of an adjuvanted split-virion H5N1 vaccine [58]. The intratracheal route of virus inoculation establishes a reproducible severe pneumonia in the ferret model [36]. Ferrets immunized with nasal Endocine™ formulated vaccines, but not ferrets immunized with parenteral TIV were protected from severe pneumonia. Protection from pneumonia corresponded with the absence of detectable virus replication in the lung and absent or significantly reduced virus replication in the upper respiratory tract. Also the previously developed CT-scanning [14,15,28,29], confirmed that nasal Endocine™ formulated vaccine, but not parenteral TIV protected the ferrets from severely affected and inflamed lungs and marked alterations in ALVs.

Current candidate influenza vaccine design has a strong focus on mucosal immunity and the crucial role of mucosal adjuvants in the development of effective inactivated or subunit nasal vaccines [14–18]. Adjuvanted nasal vaccines may have the advantage to induce systemic as well as mucosal immunity, including specific secretory IgA (S-IgA) [6]. Locally produced antibodies, particularly S-IgA have been demonstrated to play an important role in responses to natural infection. Pre-existing S-IgA antibodies can prevent infection by neutralizing influenza virus before it passes the mucosal barrier, can effectively prevent infection of epithelial cells and have been shown to contribute to the establishment of cross-protection [59]. In the present ferret study, nasal wash and swab samples were collected for detection of antibodies against influenza. Interestingly, the nasal wash procedure clearly yielded higher antibody titers than the nasal cotton swabs. Endocine™ formulated split antigen (15 μg HA) induced significantly ($p < 0.05$) higher nasal Ig titers in nasal wash samples after two immunizations compared to the parenteral vaccine (manuscript in preparation). Furthermore, the present study showed that the Endocine™ formulated inactivated pH1N1/09 influenza vaccines administered nasally induced broad specific systemic antibody responses in naïve ferrets. The Endocine™ formulated split antigen (15 μg HA) vaccine induced cross reactive HI antibody titers of >40 (GMT) against distant viruses of swine origin already after one immunization and both HI and VN cross reactive titers>200 (GMT) was achieved after two immunizations.

Overall this study shows the feasibility to induce protective systemic immunity after intranasal administration of relatively low doses inactivated pH1N1/09 antigens when formulated with Endocine™. This promising data not only justifies additional studies in the ferret model to define the number of immunizations needed for protection, as well as evaluation of optimal antigen type and dose, but also planning for further clinical studies in different age groups, including children and the elderly.

Conflict of interest statement

The authors, KS, EVK and GvA are full time and AO part time employed by Erasmus MC spin-off company ViroClinics BioSciences B.V. The authors AKM, JH, AL and HA are affiliated with Eurocine Vaccines AB, Karolinska Institute Science Park.

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References

[1] Cohen J. Swine flu outbreak. Past pandemics provide mixed clues to H1N1's next moves. Science 2009;324:996–7.
[2] Vaccines against influenza WHO position paper—November 2012. Wkly Epidemiol Rec 2012;87:461–476.
[3] Beyer WE, McElhaney J, Smith DJ, Monto AS, Nguyen-Van-Tam JS, Osterhaus AD. Cochrane re-arranged: support for policies to vaccinate elderly people against influenza. Vaccine 2013;31:6030–3.
[4] Renegar KB, Small PA, Boykins LG, Wright PF. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. J Immunol 2004;173:1978–86.
[5] Stokes CR, Soothill JF, Turner MW. Immune exclusion is a function of IgA. Nature 1975;255:745–6.
[6] Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 1984;14:350–6.
[7] Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens. J Immunol 2009;183:6883–92.
[8] Carter NJ, Curran MP. Live attenuated influenza vaccine (FluMist(R); Fluenz): a review of its use in the prevention of seasonal influenza in children and adults. Drugs 2011;71:1591–622.
[9] Dhere R, Yeolekar L, Kulkarni P, Menon R, Vaidya V, Ganguly M, et al. A pandemic influenza vaccine in India: from strain to sale within 12 months. Vaccine 2011;29(Suppl 1):A16–21.
[10] Kreijtz JH, Fouchier RA, Rimmelzwaan GF. Immune responses to influenza virus infection. Virus Res 2011;162:19–30.
[11] Slutter B, Hagenaars N, Jiskoot W. Rational design of nasal vaccines. J Drug Target 2008;16:1–17.
[12] Fujihashi K, Koga T, van Ginkel FW, Hagiwara Y, McGhee JR. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine 2002;20:2431–8.
[13] Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004;350:896–903.
[14] Even-Or O, Joseph A, Itskovitz-Cooper N, Samira S, Rochlin E, Eliyahu H, et al. A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS) II. Studies in mice and ferrets and mechanism of adjuvanticity. Vaccine 2011;29:2474–86.
[15] Hamouda T, Sutcliffe JA, Ciotti S, Baker Jr JR. Intranasal immunization of ferrets with commercial trivalent influenza vaccines formulated in a nanoemulsion-based adjuvant. Clin Vaccine Immunol 2011;18:1167–75.

[16] Yu S, Tang C, Shi X, Yang P, Xing L, Wang X. Novel Th1-biased adjuvant, SPO1, enhances mucosal and systemic immunogenicity of vaccines administered intranasally in mice. Vaccine 2012;30:5425–36.
[17] de Haan A, Haijema BJ, Voorn P, Meijerhof T, van Roosmalen ML, Leenhouts K. Bacterium-like particles supplemented with inactivated influenza antigen induce cross-protective influenza-specific antibody responses through intranasal administration. Vaccine 2012;30:4884–91.
[18] Das SC, Hatta M, Wilker PR, Myc A, Hamouda T, Neumann G, et al. Nanoemulsion W805EC improves immune responses upon intranasal delivery of an inactivated pandemic H1N1 influenza vaccine. Vaccine 2012;30:6871–7.
[19] Hinkula J, Falkeborn T, Pauksens K, Maltais A-K, Lindberg A, Stittelaar K, et al. A nasal influenza vaccine with unique safety profile and robust immunogenic properties. In: Abstract for the fourth international conference on: modern vaccines adjuvants & delivery systems. 2012.
[20] Brekke K, Lind A, Wendel-Hansen V, Sommerfelt M, Schroder U, Kaksaas I, et al. Immunotherapy of HIV-infected patients. Safety and immunogenicity of intranasal administration of Vacc-4x with Endocine as adjuvant. In: Abstract presented at the seventh conference on global health and vaccination research: innovation for global health. 2012.
[21] Falkeborn T, Brave A, Larsson M, Akerlind B, Schroder U, Hinkula J, Endocine. N3OA and N3OASq; three mucosal adjuvants that enhance the immune response to nasal influenza vaccination. PLoS One 2013;8:e70527.
[22] Petersson P, Hedenskog M, Alves D, Brytting M, Schroder U, Linde A, et al. The Eurocine L3 adjuvants with subunit influenza antigens induce protective immunity in mice after intranasal vaccination. Vaccine 2010;28:6491–7.
[23] Maher JA, DeStefano J. The ferret: an animal model to study influenza virus. Lab Anim (NY) 2004;33:50–3.
[24] Belser JA, Katz JM, Tumpey TM. The ferret as a model organism to study influenza A virus infection. Dis Model Mech 2011;4:575–9.
[25] van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 2007;171:1215–23.
[26] Plopper CG, Hill LH, Mariassy AT. Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung. III. A study of man with comparison of 15 mammalian species. Exp Lung Res 1980;1:171–80.
[27] van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. H5N1 virus attachment to lower respiratory tract. Science 2006;312:399.
[28] Veldhuis Kroeze EJ, van Amerongen G, Dijkshoorn ML, Simon JH, de Waal L, Hartmann IJ, et al. Pulmonary pathology of pandemic influenza A/H1N1 virus (2009)-infected ferrets upon longitudinal evaluation by computed tomography. J Gen Virol 2011;92:1854–8.
[29] Veldhuis Kroeze EJ, Stittelaar KJ, Teeuwsen VJ, Dijkshoorn ML, van Amerongen G, de Waal L, et al. Consecutive CT in vivo lung imaging as quantitative parameter of influenza vaccine efficacy in the ferret model. Vaccine 2012;30:7391–4.
[30] Munster VJ, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, et al. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science 2009;325:481–3.
[31] Palmer D, Dowle W, Coleman M, Schild G. Hemagglutination inhibition test. In: Advanced laboratory techniques for influenza diagnosis: procedural guide. Atlanta, GA: U.S. Department of Health Education; 1975. p. 25–62.
[32] Frank AL, Puck J, Hughes BJ, Cate TR. Microneutralization test for influenza A and B and parainfluenza 1 and 2 viruses that uses continuous cell lines and fresh serum enhancement. J Clin Microbiol 1980;12:426–32.
[33] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004;305:371–6.
[34] Rimmelzwaan GF, Baars M, Claas EC, Osterhaus AD. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. J Virol Methods 1998;74:57–66.
[35] van den Brand JM, Stittelaar KJ, van Amerongen G, Reperant L, de Waal L, Osterhaus AD, et al. Comparison of temporal and spatial dynamics of seasonal H3N2, pandemic H1N1 and highly pathogenic avian influenza H5N1 virus infections in ferrets. PLoS One 2012;7:e42343.
[36] van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, et al. Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. J Infect Dis 2010;201:993–9.
[37] Chen GL, Min JY, Lamirande EW, Santos C, Jin H, Kemble G, et al. Comparison of a live attenuated 2009 H1N1 vaccine with seasonal influenza vaccines against 2009 pandemic H1N1 virus infection in mice and ferrets. J Infect Dis 2011;203:930–6.
[38] Stittelaar KJ, Veldhuis Kroeze EJ, Rudenko L, Dhere R, Thirapakpoomanunt S, Kieny MP, et al. Efficacy of live attenuated vaccines against 2009 pandemic H1N1 influenza in ferrets. Vaccine 2011;29:9265–70.
[39] Baras B, de Waal L, Stittelaar KJ, Jacob V, Giannini S, Kroeze EJ, et al. Pandemic H1N1 vaccine requires the use of an adjuvant to protect against challenge in naive ferrets. Vaccine 2011;29:2120–6.
[40] Baras B, Stittelaar KJ, Kuiken T, Jacob V, Bernhard R, Giannini S, et al. Longevity of the protective immune response induced after vaccination with one or two doses of AS03A-adjuvanted split H5N1 vaccine in ferrets. Vaccine 2011;29:2092–9.
[41] Pearce MB, Belser JA, Gustin KM, Pappas C, Houser KV, Sun X, et al. Seasonal trivalent inactivated influenza vaccine protects against 1918 Spanish influenza virus infection in ferrets. J Virol 2012;86:7118–25.
[42] Bodewes R, de Mutsert G, van der Klis FR, Ventresca M, Wilks S, Smith DJ, et al. Prevalence of antibodies against seasonal influenza A and B viruses in children in Netherlands. Clin Vaccine Immunol 2011;18:469–76.
[43] Jaw TS, Sheu RS, Liu GC, Lin WC. Development of adenoids: a study by measurement with MR images. Kaohsiung J Med Sci 1999;15:12–8.
[44] Boyaka PN, Wright PF, Marinaro M, Kiyono H, Johnson JE, Gonzales RA, et al. Human nasopharyngeal-associated lymphoreticular tissues. Functional analysis of subepithelial and intraepithelial B and T cells from adenoids and tonsils. Am J Pathol 2000;157:2023–35.
[45] Zuercher AW, Coffin SE, Thurnheer MC, Fundova P, Cebra JJ. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. J Immunol 2002;168:1796–803.
[46] Brandtzaeg P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. Int J Pediatr Otorhinolaryngol 2003;67(Suppl 1):S69–76.
[47] Usonis V, Anca I, Andre F, Chlibek R, Ivaskeviciene I, Mangarov A, et al. Central European Vaccination Advisory Group (CEVAG) guidance statement on recommendations for influenza vaccination in children. BMC Infect Dis 2010;10:168.
[48] Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP)—United States, 2012–13 influenza season. MMWR Morb Mortal Wkly Rep 2012;61:613-618.
[49] Heikkinen T, Booy R, Campins M, Finn A, Olcen P, Peltola H, et al. Should healthy children be vaccinated against influenza? A consensus report of the Summits of Independent European Vaccination Experts. Eur J Pediatr 2006;165:223–8.
[50] Principi N, Esposito S. Pediatric influenza prevention and control. Emerg Infect Dis 2004;10:574–80.
[51] Esposito S, Tagliabue C, Tagliaferri L, Semino M, Longo MR, Principi N. Preventing influenza in younger children. Clin Microbiol Infect 2012;18(Suppl 5):42–9.
[52] Yin JK, Salkeld G, Heron L, Booy R. How to better inform the decision making about universal influenza vaccination in children. J Ped Infect Dis 2012;7:69–73.
[53] Ganem-Quintanar A, Quintanar-Guerrero D, Buri P. Monoolein: a review of the pharmaceutical applications. Drug Dev Ind Pharm 2000;26:809–20.
[54] Bourre JM, Dumont OL, Clement ME, Durand GA. Endogenous synthesis cannot compensate for absence of dietary oleic acid in rats. J Nutr 1997;127:488–93.
[55] Millican RC, Brown JB. The isolation and properties of some naturally occuring octadecenoic (oleic) acids. J Biol Chem 1944;154:437–50.
[56] Tetsutani K, Ishii KJ. Adjuvants in influenza vaccines. Vaccine 2012;30:7658–61.
[57] Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N Engl J Med 2008;358:2573–84.
[58] Leroux-Roels I, Borkowski A, Vanwolleghem T, Drame M, Clement F, Hons E, et al. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet 2007;370:580–9.
[59] van Riet E, Ainai A, Suzuki T, Hasegawa H. Mucosal IgA responses in influenza virus infections; thoughts for vaccine design. Vaccine 2012;30:5893–900.