Comparison of the Mucosal Adjuvant Endocine™ with Two Well-Known Adjuvants: Cholera Toxin and Alum

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Abstract

To enable efficient mucosal vaccination with split or subunit antigens, an adjuvant is often needed. To date, no mucosal adjuvants are approved for human use, however, there are a variety of mucosal adjuvants in development, including the liposome-based adjuvant Endocine™. The aim of this study was to evaluate split influenza antigens together with Endocine™ and in order to assess the potency of Endocine™, the induction of humoral immune responses were compared to those following influenza vaccination with cholera toxin (CT) or aluminum salt (alum). We show that Endocine™ significantly enhances influenza-specific immune responses in intranasally immunized mice compared to non-adjuvanted vaccine. Furthermore, vaccines adjuvanted with Endocine™ evoked comparable serum IgG and virus neutralizing (VN) antibody titers as nasal vaccines adjuvanted with CT. Compared to parenteral vaccination with alum, Endocine™ triggered significantly higher mucosal and serum IgA titers, and similar VN titers. Taken together, these results support further development of Endocine™ as a mucosal adjuvant and as part of a nasal influenza vaccine candidate.

Keywords: Mucosal Adjuvant; Nasal Immunization; Vaccine; Endocine; Influenza; Neutralizing Antibodies

Abbreviations

AFC: Antibody Forming Cell
Alum: Aluminum Salt
CT: Cholera Toxin
CTA: Cholera Toxin A Subunit
CTB: Cholera Toxin B Subunit
HA: Hemagglutinin
HIB: *Haemophilus influenzae* Type B
I.M.: Intramuscular
LT: *Escherichia coli* Heat-Labile Toxin
MDCK: Madin-Darby Canine Kidney
NALT: Nasal Associated Lymphoid Tissue
VN: Virus Neutralizing

Introduction

To strengthen the immune response to a vaccine antigen one can add an immunological helper substance, called an adjuvant. The liposome-based adjuvant Endocine™ has previously been shown to enhance the immune response in mice and ferrets after intranasal vaccination with influenza antigens [1-3], as well as to HIV and BCG antigens [4,5]. Whereas whole inactivated/attenuated viruses as vaccines are able to stimulate an immune response by themselves, split and subunit vaccines are poorly immunogenic, particularly when given at mucosal sites or to children and elderly. Thus, in order for influenza split and subunit vaccines to be administered by the mucosal route, an adjuvant may be added to improve the immune response.

Adjuvants can consist of different compounds, such as mineral salts, lipids, bacterial components, nucleic acid, and saponins, and they exert their effects through different mechanisms. Some adjuvants have immunostimulatory properties, whereas others function as delivery systems. Importantly, adjuvants need to be safe and tolerable. Currently, very few adjuvants have been approved for human use and there are no licensed adjuvants for intranasal administration in humans. In 2000, an intranasal vaccine (NasalFlu) was introduced in Switzerland that contained *Escherichia coli* heat-labile toxin (LT) as an adjuvant [6], but the vaccine was withdrawn after being associated with an increased risk of a form of facial paralysis called Bell´s palsy. The LT adjuvant shares 80% homology with cholera toxin (CT), which is also a potent adjuvant, but can be toxic when used intranasally [7]. However both CT and LT are excellent research tools for the comparison of other adjuvants, due to their strong immune enhancing properties. CT consists of an A (CTA) and a B subunit (CTB). The CTB consists of five monomers and is responsible for the binding to the receptor, while CTA is an enzyme with ADP-ribosylating activity and is believed to contribute to the toxicity of CT [7]. CT has been shown to mainly induce a Th2 response, by stimulating the production of Th2 stimulating cytokines [8,9] and to suppress IL-12 which is important for a Th1 immune response [10].

Aluminum salt (alum), the most widely used adjuvant in human vaccines, is known to be safe and to mainly stimulate a Th2 response, resulting in high antibody titers [11]. The mode of action is still not totally clear, but alum has been suggested to absorb antigens to its surface, have a depot effect and to stimulate immune cells [11-14]. Alum has also been shown to have a cytotoxic effect and thereby stimulate the release of uric acid [11,12]. Although alum is included in many vaccines worldwide, it is not used for mucosal administration or in seasonal influenza vaccines. Many novel adjuvant candidates, also for mucosal use, are in preclinical and clinical development [15-22], but it has proved difficult to find safe and potent adjuvants suitable for human use.

A clear advantage of administering a vaccine nasally is that it stimulates an immune response at the site of pathogen entry, which may prevent or limit infection. Mucosal IgA has been shown to protect against influenza infection and to have cross-reactive properties [23-25]. In addition, nasal vaccination reduces the handling of needles, decreases the risk of transmission of blood-borne diseases, and is less painful. The influenza vaccines on the major markets today are administered with syringe and needle, with the exception of the live attenuated influenza vaccine (FluMist®, MedImmune) which is administered intranasally with a spray device. However, FluMist® is only recommended to healthy people 2-49 or 2-18 years of age in the U.S. and Europe, respectively. Consequently, infants, the elderly and individuals with underlying conditions, which are those that need protection from influenza infection the most, are not able to use this intranasal vaccine. The parenteral influenza vaccines on the market have shown lower efficacy in infants and elderly compared to adults [26-28]. Thus, there is a need to develop efficacious influenza vaccines for these risk groups, and the addition of adjuvants may be necessary.

The aim of the current study was to evaluate the mucosal adjuvant Endocine™ together with influenza antigens and compare the immune responses with those induced by the two well-known adjuvants, CT and alum. Endocine™ is an adjuvant based on the lipids mono-olein and oleic acid [2], which has been shown to be safe and well-tolerated in both preclinical and clinical studies [1-3,29-31]. In ferrets, an intranasal vaccine consisting of the pandemic A/California/2009 (H1N1) antigen adjuvanted with Endocine™ induced high levels of serum hemagglutination inhibition (HI) and virus neutralization (VN) antibody titers [3]. Furthermore, upon intra-tracheal challenge with a homologous strain, the immunized ferrets were fully protected from virus replication in the lungs and upper respiratory tract.

To further evaluate the potency of Endocine™ as a mucosal adjuvant, we compared the induction of humoral immune responses in mice following immunization with influenza antigen adjuvanted with Endocine™, CT, or alum. Split influenza antigens from A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008 were used in the immunizations. Our results demonstrate that Endocine™ significantly enhances influenza-specific serum IgG and VN antibodies, to titers comparable to CT. In addition, we show that Endocine™ induces higher serum and mucosal influenza-specific IgA titers than alum.

Materials and Methods

Mice and ethics statement

Six-week-old BALB/cAJcl mice were purchased from Clea Japan, Inc (Tokyo, Japan). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Mitsubishi Chemical Medience Corporation and were performed in accordance with institutional guidelines for Mitsubishi Chemical Medience animal facility at Kumamoto Institute of Mitsubishi Chemical Medience Corporation in Japan (Permit number: 2012-0328). The mice were maintained under pathogen-free conditions, monitored daily and had free access to water and food. All efforts were made to minimize suffering and if necessary the mice were euthanized in advance.

Study outline

A total of 135 mice were used in the study, five mice/group and influenza strain (Table 1.) To facilitate the compilation of

the large amount of data collected, the groups receiving Endocine™ are compared with non-adjuvanted, CT-vaccinated and alum-vaccinated mice in three figures. Four different parameters (serum IgG, VN titers, nasal wash IgA and serum IgA) were analyzed for each group and influenza strain.

with two-week intervals (Table 1). Blood samples were taken one day before immunization and two weeks after the last immunization. At sacrifice, the cannulate trachea to the nose was washed with 1000 µl PBS (nasal wash sample). All samples were stored at -20°C for further analysis.

Group	Route	Total Volume	Antigen	Days of immunizations	Adjuvant	Number of mice
1. 1 µg HA	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	-	15 (5/strain)
2. 0.01 µg HA + Endocine™	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	Endocine™	15 (5/strain)
3. 0.1 µg HA + Endocine™	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	Endocine™	15 (5/strain)
4. 1 µg HA + Endocine™, 5 µL	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	Endocine™	15 (5/strain)
5. 1 µg HA + Endocine™, 10 µL	Intranasal	10 µL	H1N1, H3N2 or B	Day 0, 14, 28	Endocine™	15 (5/strain)
6. 0.01 µg HA + CT	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	CT	15 (5/strain)
7. 0.1 µg HA + CT	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	CT	15 (5/strain)
8. 1 µg HA + CT	Intranasal	5 µL	H1N1, H3N2 or B	Day 0, 14, 28	CT	15 (5/strain)
9. 1 µg HA i.m.	Intramuscular	100 µL	H1N1, H3N2 or B	Day 0, 14	-	15 (5/strain)
10. 1 µg HA + alum i.m.	Intramuscular	100 µL	H1N1, H3N2 or B	Day 0, 14	Alum	15 (5/strain)

Table 1. Immunization schedule

Adjuvant	Composition	Substance	Amount given/vaccination
Endocine™	Mono-olein and oleic acid	Lipids	5 or 10 µL of 2% Endocine™
Cholera toxin (CT)	CT and CTB	Proteins	1 ng CT and 1 µg CTB
Aluminum (Alum)	Aluminum hydroxide gel $(Al(OH)_3)$	Salt	0.67 mg

Table 2. Adjuvants used for immunization

Vaccination and sampling

The mice (5 per group) were vaccinated with monovalent split influenza vaccine formulated with or without adjuvant (Endocine™, CT + CTB, or alum). During vaccination, the mice were anaesthetized with a 50 µL/head of mixture (10:2.2, v/v) of ketamine hydrochloride (Ketaral®; Daiichi-Sankyo) and xyladine hydrochloride (Ceractal®; Bayer). Three monovalent vaccine bulks were used for vaccinations; A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) and B/Brisbane/60/2008 (BIKEN, Kagawa, Japan). Mice were given either 2.5 µL (group 1-4 and 6-8) or 5 µL (group 5) of the vaccine in each nostril, containing a total of 0.01, 0.1 or 1 µg hemagglutinin (HA) (Table 1). Mice given intramuscular injections (50 µL/leg, in total 100 µL/mouse) received 1 µg HA. The intranasally immunized mice received three immunizations with two-week intervals (day 0, 14 and 28), and mice given injections received two immunizations

Adjuvants

The adjuvants used in this study have previously been described [2, 4, 11, 30, 32-34]. Endocine™ (Eurocine Vaccines AB, Stockholm, Sweden) consists of the lipids mono-olein and oleic acid (Table 2.). The final concentration of Endocine™ when mixed with influenza antigens was 2% based on previous experience [1-3, 29, 30]. The CT adjuvant consisted of 1 µg CTB (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1 ng CT (Wako Pure Chemical Industries, Ltd.) per dose diluted in PBS. The alum adjuvant was given in the form of aluminum hydroxide gel (Sigma-Aldrich, S:t Louis, Mo). Alum was mixed with antigen and rotated for more than one hour, and 0.67 mg was used per mouse. The adjuvant concentrations used were chosen based on previous experience and published literature [35-40].

Determination of influenza-specific antibody titers by ELISA

All sera and nasal wash samples were individually analyzed by ELISA. In brief, 96-well ELISA plates were coated with 1 μg HA/mL of monovalent split HA solution (BIKEN) and incubated overnight at 4°C. Plates were blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) overnight at 4°C. Sera and nasal wash samples were diluted in 0.4% Block Ace and added to the plate. For IgG and IgA measurements, goat anti-mouse IgG (H+L) HRP conjugate, goat anti-mouse IgG1 HRP conjugate, and goat anti-mouse IgA HRP conjugate (all from Southern Biotech, Birmingham, AL, US) were used. To develop the reaction, TMB was used according to the manufacturer's protocol (KPL, Gaithersburg, MD, US). ELISA titers of each sample were determined by maximum dilution ratio which gave an absorbance at least 2.5 times higher than buffer blank. The samples were analyzed for specific antibodies against the antigens which the mice had been immunized with (split A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) or B/Brisbane/60/2008).

Virus neutralizing (VN) assay

The virus neutralization assay was performed as previously described [41-43], with some modifications. In brief, Madin-Darby Canine kidney (MDCK) cells were grown to confluency in a F96-well tissue culture plate (BD Falcon, New Jersey, US) in GIBCO® Eagle MEM (Invitrogen, N.Y, USA) and 10% FCS (Invitrogen, N.Y, USA) at 37°C humidified incubator with 5% CO_2. The serum samples were diluted 1:3 in RDE II (Denka-Seiken CO, Japan) solution and inactivated at 56°C for 1 hour before use. Serially diluted sera and a final infecting titer of 100 $TCID_{50}$/mL of virus in VDM media (GIBCO® Eagle MEM, 10mM HEPES (Invitrogen), 1% Penicillin-Streptomycin (Nacalai Tesque, Japan), 0.2% bovine albumin (Sigma-Aldrich) and 20 μg/mL trypsin (Nacalai Tesque)) were incubated for 30 minutes at 37°C, after which they were added to MDCK cells. After four days of incubation in a 37°C humidified incubator with 5% CO_2, the plates were fixed with 10% Mildform® 20 NM (Wako Pure Chemical Industries, Ltd.) for 30 minutes at room temperature. The cells were stained with Naphtol Blue Black Solution (Naphtol Blue Black 0.5g (Sigma-Aldrich), Sodium Acetate 0.5g, Acetic Acid 45 mL and distilled water 455 mL) for 30 minutes in room temperature. The cells were destained with tap water and then dried. Subsequently, 0.1 M NaOH (Wako Pure Chemical Industries, Ltd.) was added to the cells, and plates were read at 630 nm in a microplate reader (SpectraMax190, CA, US). VN titers of each serum sample were determined by maximum dilution ratio which gave higher absorbance than average of positive and negative controls.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, US). To test for significant differences between two groups (Endocine™- and CT-adjuvanted vaccines of the same HA dose), the Mann Whitney U test was used. A p-value of <0.05 was considered statistically significant. To compare ELISA titers between more than two groups (non-adjuvanted vaccine, Endocine™- and alum-adjuvanted vaccine), the Kruskal-Wallis test was first used to test if there was a significant difference among any study groups, and if so, the Mann Whitney U test was used. A p-value of <0.05 was considered statistically significant.

Results

Endocine™ strongly enhances influenza-specific immune responses and shows dose-sparing effects in intranasally immunized mice

In the current study, BALB/cAJcl mice were immunized with influenza antigens formulated with or without adjuvant, with the main objective to compare the adjuvant Endocine™ with the well-known adjuvants CT and alum. The intranasally immunized mice received three immunizations, and the parenterally immunized mice received two injections (Table 1). Our results showed that Endocine™-vaccinated mice that received 1 μg HA had significantly higher serum IgG titers already after the first dose compared to mice receiving the non-adjuvanted vaccine, irrespective of influenza strain (Figure 1A-C). After the second dose, the 0.1 μg HA Endocine™-vaccinated mice showed significantly higher serum IgG titers than the mice receiving a 10X higher antigen dose of the non-adjuvanted vaccine, irrespective of influenza strain (Figure 1A-C). The IgG titers were not significantly induced by the third vaccination and similar IgG titers were induced in the 0.1 and 1 μg HA Endocine™-vaccinated mice. Importantly, significantly higher VN titers were detected in Endocine™-vaccinated mice, even in mice vaccinated with a 10X lower antigen dose (0.1 μg HA), compared to the non-adjuvanted vaccine (Figure 1D). Mice vaccinated with a 100X lower HA dose in combination with Endocine™ induced similar VN titers as mice receiving 1μg of non-adjuvanted HA antigen (Figure 1D).

Nasal wash IgA and serum IgA end titers were also significantly enhanced by Endocine™ when formulated with antigen from any of the three influenza strains (Figure 1E-F). The serum IgA pre-vaccination titers were below 20 for all groups and strains, except for the H1N1-specific IgA in the non-adjuvanted group (data not shown). Influenza A specific IgA in serum was induced after one vaccination in the 1 μg HA + Endocine™, 10 μL group and after two vaccinations with the 0.1 and 1 μg HA + Endocine™, 5 μL groups (data not shown).

These results show that Endocine™ has a dose-sparing effect as even with a ten times lower antigen dose (0.1 μg HA), the Endocine™-formulated vaccine stimulated higher serum IgG, VN, and nasal IgA titers than the non-adjuvanted vaccine, independent of influenza strain (Figure 1A-E). Overall, when a 100X lower antigen dose was used (0.01 μg HA) with Endocine™, the immune responses were similar to those induced by the non-adjuvanted vaccine.

A difference in humoral immune responses was observed for the two different volumes of the 1 μg HA dose mixed with Endocine™. By administrating the vaccine in a larger volume (5 μL/nostril instead of 2.5 μL/nostril), more serum and mucosal antibodies, and higher VN titers were evoked in response to all three influenza strains (Figure 1A-F).

Endocine™-adjuvanted influenza vaccine elicits comparable serum IgG and VN titers as a CT-adjuvanted influenza vaccine

In all cases but one, no significant differences in
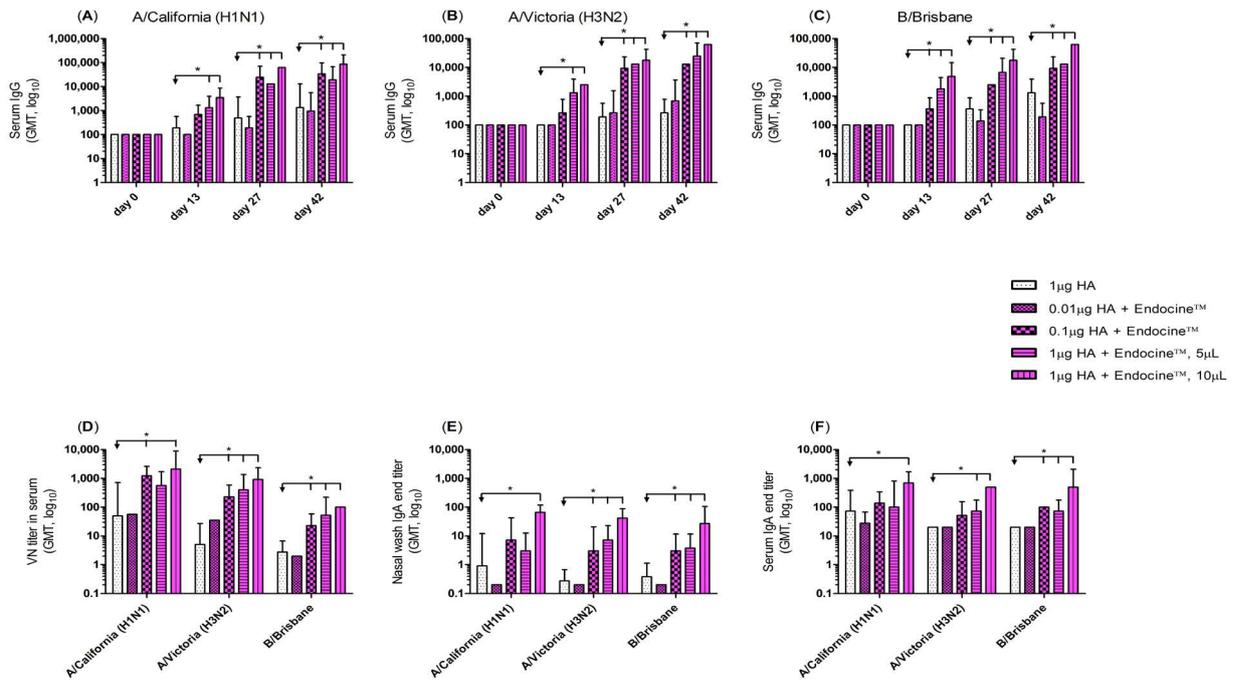
Figure 1. Endocine™ significantly enhances influenza-specific immune responses in intranasally immunized mice. Mice (n=5 per group) were immunized three times with antigen from A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) or B/Brisbane/60/2008, with or without the adjuvant Endocine™. Serum IgG responses were evaluated against; (A) A/California/7/2009 (H1N1), (B) A/Victoria/210/2009 (H3N2) and (C) B/Brisbane/60/2008. VN titers (D), nasal wash IgA (E), and serum IgA end titers (F) were also evaluated. Data shown represent geometric mean titers with 95% Cl. Statistically significant differences compared to the non-adjuvanted vaccine are indicated, *p<0.05.
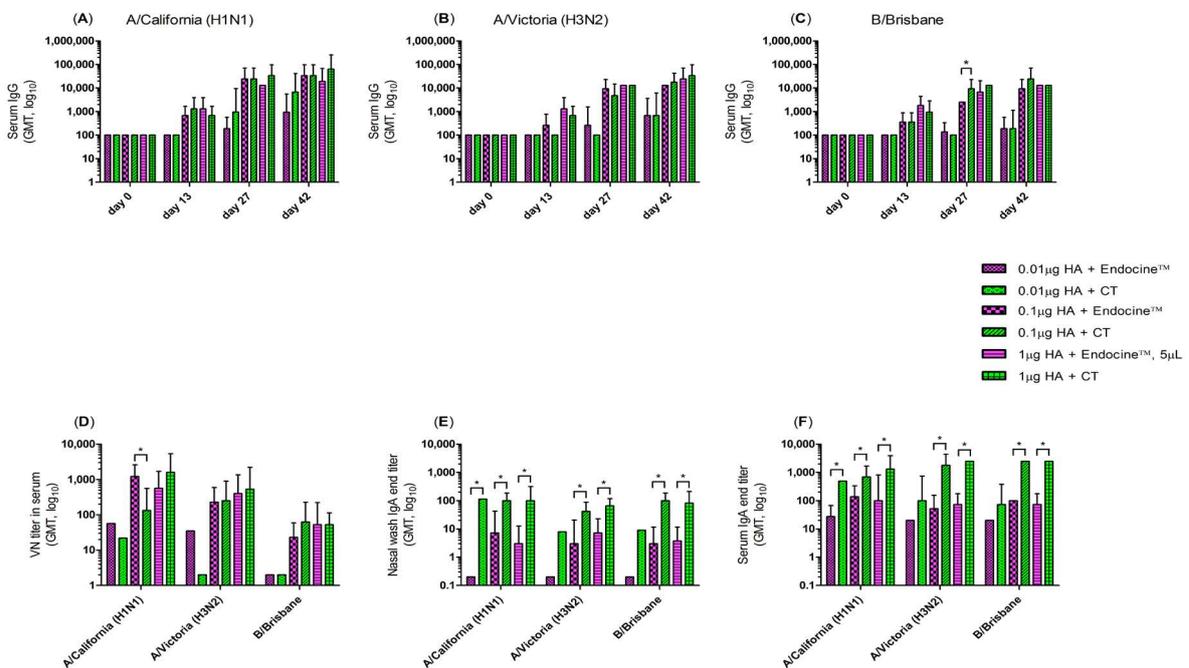
Figure 2. Influenza-specific immune responses in mice vaccinated with Endocine™- versus CT-formulated intranasal vaccines. Mice (n=5 per group) were immunized three times with antigen from A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) or B/Brisbane/60/2008 with the adjuvant Endocine™ or cholera toxin (CT). Serum IgG responses were evaluated against; (A) A/California/7/2009 (H1N1), (B) A/Victoria/210/2009 (H3N2) and (C) B/Brisbane/60/2008. VN titers (D), nasal wash IgA (E) and serum IgA end titers (F) were also evaluated. Data shown represent geometric mean titers with 95% Cl. Statistically significant differences between Endocine™- and CT-vaccinated mice (of the same dose and influenza strain) are indicated, *p<0.05.
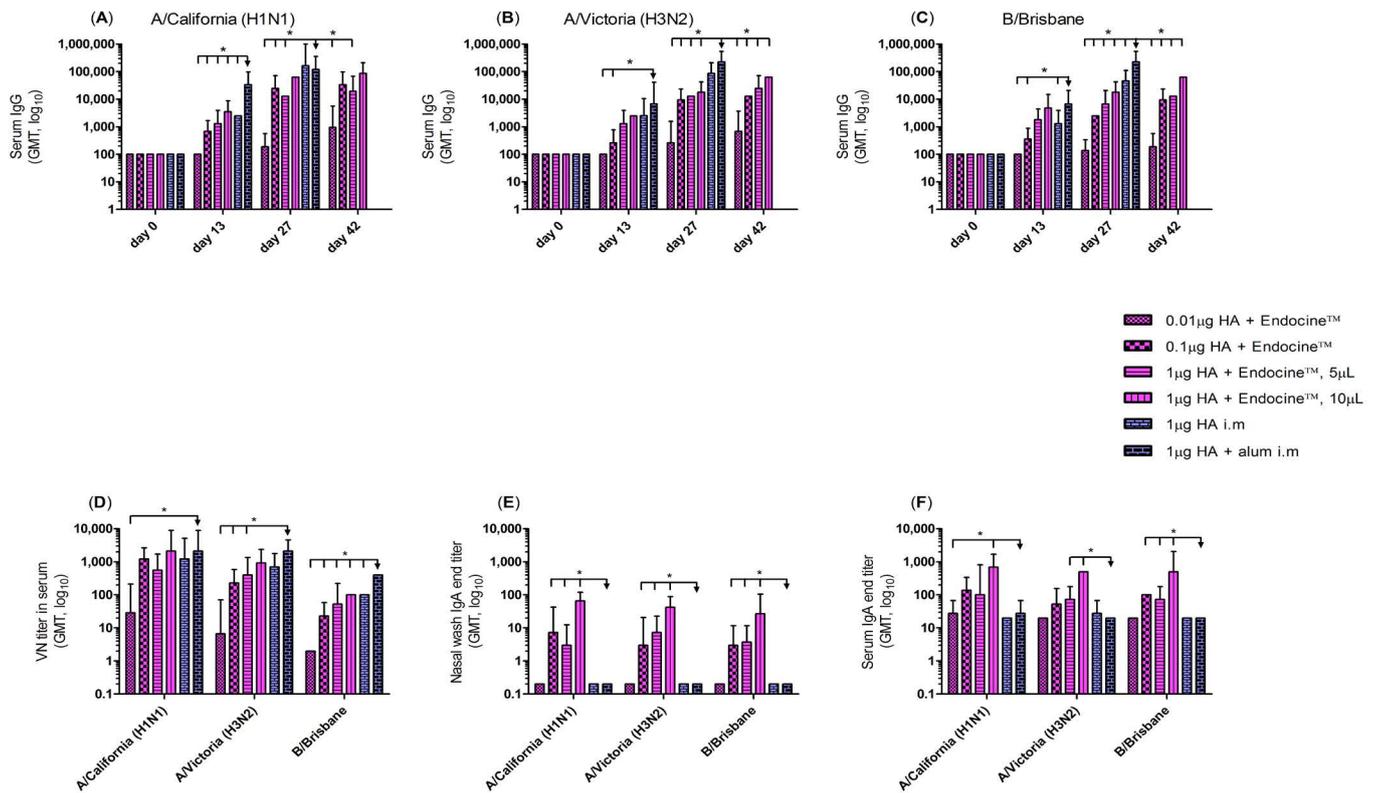
Figure 3. Influenza-specific immune responses in mice vaccinated with Endocine™- versus alum-formulated vaccines. Mice (n=5 per group) were immunized with antigen from A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) or B/Brisbane/60/2008, with the adjuvant Endocine™ (three immunizations) or alum (two immunizations). Serum IgG responses were evaluated against; (A) A/California/7/2009 (H1N1), (B) A/Victoria/210/2009 (H3N2) and (C) B/Brisbane/60/2008. VN titers (D), nasal wash IgA (E) and serum IgA end point titers (F) were also evaluated. Data shown represent geometric mean titers with 95% Cl. Statistically significant differences between Endocine™ and alum-vaccinated mice as well as between non-adjuvanted i.m vaccine and alum-adjuvanted vaccine are indicated, *p<0.05.

influenza-specific IgG responses could be detected between Endocine™- and CT-vaccinated mice, using the same antigen dose and influenza strain, regardless of time point (Figure 2A-C). Similar VN titers were seen in Endocine™ and CT vaccinated mice (Figure 2D). By contrast, mice immunized with the CT-adjuvanted vaccine responded with significantly higher influenza-specific IgA titers in both nasal wash (Figure 2E) and in serum (Figure 2F) compared to mice immunized with the Endocine™-adjuvanted vaccine. As shown in Figure 1E and F, the increase of antigen from 0.1 µg to 1 µg HA with Endocine™ did not enhance the IgA titers, however the increase in volume did increase the IgA titer, in both nasal wash and serum, to similar levels induced by the CT-adjuvanted vaccine.

Endocine™-adjuvanted vaccine induces significantly higher serum and mucosal IgA titers than an alum-adjuvanted parenteral vaccine

Intramuscular administration of the alum-adjuvanted vaccine stimulated a significantly higher serum IgG response than the Endocine™-adjuvanted intranasal vaccine against all three influenza strains (Figure 3A-C). Already after the first dose, alum-vaccinated mice generated the highest levels of IgG antibodies (Figure 3A-C). There were no significant differences in VN end titers between mice vaccinated with split vaccine of type A/H1N1 formulated with

Endocine™ or alum, even when the Endocine™-adjuvanted vaccine was given at a ten times lower HA dose (Figure 3D). However, significantly higher VN titers were seen in the alum-vaccinated mice against A/H3N2 and influenza B. The Endocine™-adjuvanted intranasal vaccines (group 3, 4 and 5) induced significantly higher IgA titers in nasal wash (Figure 3E) and in serum (Figure 3F) compared to the injected alum-adjuvanted vaccines, even at a ten times lower antigen dose. Comparison of the groups already after the second dose, showed that the 1 µg HA Endocine, 10µL group, showed significantly higher serum IgA titers than the alum-adjuvanted vaccine (data not shown).

Alum increased IgG and VN titers compared to non-adjuvanted parenteral vaccination, however, the increase was only significant for the B/Brisbane strain (Figure 3A-D). No IgA was induced after parenteral vaccination either with or without alum (Figure 3E-F).

Discussion

Similar to previous studies [1-3,19,44], this study shows that in order to use less immunogenic antigens as mucosal vaccines, an adjuvant is required to evoke an efficient immune response. Currently, there are no adjuvants approved for intranasal administration, but several adjuvants are in development [15,17-19,22,44,45]. CT and alum are two well-

known adjuvants, and the aim of the current study was to compare the novel mucosal adjuvant Endocine™ with these adjuvants. This study shows that Endocine™-adjuvanted nasal vaccines induce VN and IgG titers comparable to CT-adjuvanted vaccines. In addition, we show that Endocine™-adjuvanted intranasal vaccines induce significantly higher serum and nasal IgA end titers than alum-adjuvanted parenteral vaccines. This difference was significant even when a 10-times lower antigen dose was used with Endocine™, however, the intranasal vaccine was delivered three times and the parenteral vaccine twice.

CT is a potent mucosal adjuvant derived from Vibrio cholerae, the causative agent of the diarrheal disease cholera. As CT is toxic it is common to use the Cholera toxin B subunit (CTB) and a trace amount of cholera toxin (CT), as we have done in this study, to reach a good balance between toxicity and immunogenicity [37,46]. CT is known to efficiently stimulate the immune system and has previously been shown to enhance serum IgG and nasal IgA levels after intranasal immunization [7,47-49]. However CT which shares similarities with LT, have been shown to bind to ganglioside GM1, which has been suggested to be the cause of adverse events like Bell's palsy when used intranasally [6,50,51]. The symptoms of Bell´s palsy occur when the facial nerve is swollen, inflamed or compressed [52]. The disrupted nerve affects eye movement, tear and saliva glands as well as taste sensation. Thereby it is of importance to think of the closeness to the olfactory nerve and the brain when working with nasal adjuvants. Despite the fact that significant efforts have been made to identify non-toxic derivatives and mutants of CT and LT, none of these have been approved for clinical use. However some have shown good immune responses and acceptable safety profiles [7,34,47]. In a phase I/II study with Endocine™, the adverse events were mild, transient and local [29]. Common adverse events seen after alum vaccination are pain at injection site, malaise and headache [38,53].

Our results showed that whereas Endocine™ stimulated similar levels of serum IgG, serum IgG1 (data not shown) and VN titers as CT, the CT-adjuvanted vaccine induced significantly higher serum and nasal IgA titers. Nevertheless, Endocine™ significantly enhanced the production of influenza-specific IgA, both locally and systemically, compared to the non-adjuvanted intranasal vaccine. Notably, the Endocine™-formulated vaccines induced similar neutralization titers as the non-adjuvanted intranasal vaccine at a 100-fold lower HA dose (Figure 1D), showing that Endocine™ has a dose-sparing effect and greatly potentiates the immune response to influenza antigens.

Parenteral vaccination is known to elicit a strong systemic antibody response, but is a poor inducer of effective immunity at mucosal surfaces [54]. A study by Sealy et al. showed that when an injected vaccine (Fluvirin® or Fluzone®) was used, antibody forming cells (AFCs), producing mainly IgG, are located predominantly in the spleen and bone-marrow and are associated with long-term serum antibody responses [55]. On the other hand, when intranasal immunization (FluMist®) was used, both acute and long-life AFCs were stimulated and they were mainly located in the diffuse nasal associated lymphoid tissue (NALT) and lungs, with IgA as the main isotype. A major advantage of intranasal vaccination is that it induces a first line of defense against pathogens in the form of mucosal/secretory IgA. Secretory IgA antibodies reduce the attachment of influenza viruses to host cells and inhibits their internalization [56], and is also important for clearance and prevention of re-infection with influenza virus [57]. In addition, secretory IgA has been shown to be more long-lived than IgG in the mucosa [55], and it can neutralize viruses inside cells without causing tissue damage [57]. Interestingly, intranasal administration stimulates the production of mucosal IgA at higher levels than parenteral administration, both in humans and in murine models [17-19,58,59]. Importantly, intranasal administration of inactivated antigens without an adjuvant generates a poor IgA response, as was also observed in this study. Our results show that even though parenteral vaccination with alum generates potent IgG already after one dose, and VN end titers, it does not stimulate the induction of influenza-specific IgA in serum or nasal wash. By contrast, the Endocine™-formulated vaccine stimulated both systemic and mucosal immunity. Even though alum was only given twice and the Endocine™-groups received three doses, a third dose would probably not have raised the IgA titers in parenteral vaccinated mice, since there were no detectable IgA after the second dose. The adjuvant effects of alum were discovered during the 1920s, and has been used in numerous injectable human vaccines, such as hepatitis A and B, human papillomavirus (HPV), Haemophilus influenzae type B (HIB), and pneumococcal vaccines [11,12]. Alum has an excellent safety profile and ability to enhance humoral immune responses [60], however, it is not an optimal adjuvant for nasally administered vaccines or seasonal influenza vaccines [61,62]. However alum is used in parenterally administered H5N1 licensed influenza vaccines [38]. At the antigen dose administered in this study there were few significant differences in influenza-specific immune responses between intramuscular injection with or without alum.

The Endocine™-adjuvanted vaccine given in 5 μL per nostril induced higher antibody titers compared to the same vaccine dose administered in 2.5 μL per nostril. If a larger volume results in a longer retention time of the formulation in the nostril, or in a greater vaccine coverage of the nasal mucosa, it could possibly allow for a more efficient uptake of antigens. Another explanation might be that with a larger volume, there is a higher possibility that the vaccine formulation reaches the bronchioles or lungs, and exposure of these tissues to vaccine antigens could increase the immune response to the vaccine. A study by Minne et al showed that when using a larger volume (50 μL) for intra nasal vaccination in mice, some vaccine ended up in the trachea and the central airways [63]. The study showed that when the intranasal vaccine reached the deeper lungs, the systemic immune response was as high as after intramuscular injection, with the addition of the intranasal vaccination also inducing mucosal IgA and a local response towards a Th1 immunity [63].

Intranasal vaccination with mucosal adjuvants has recently been reviewed as particularly efficient in providing broader immunity, also at distal mucosal sites, and it has been suggested that the nasal mucosa may be the preferred route of delivery for future influenza vaccines [64]. By developing safe adjuvants for mucosal use, a wider range of antigens can be used as mucosal vaccine components. By themselves, most antigens derived from viruses and bacteria are after inactivation of the pathogen not immunogenic enough, but

by adding an adjuvant, a potent mucosal immune response can be achieved. Nasal vaccination is interesting not only against influenza virus, but also against other pathogens which infect through the mucosa and to which no approved vaccine yet exist.

The results from the current study demonstrate that intranasal administration of Endocine™-adjuvanted influenza vaccines induce a similar systemic humoral immunity as CT and higher mucosal immunity compared to alum. In conclusion, the results from this study and earlier studies support continued development and evaluation of the mucosal adjuvant Endocine™, since it induces both systemic and mucosal immunity, and has a promising tolerability profile.

Acknowledgments

Conflicts of Interest

TF, JH and AKM are affiliated with Eurocine Vaccines AB, Karolinska Institute Science Park. NA, MH and MA are affiliated with Mitsubishi Tanabe Pharma Corporation.

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