Vaccine 28 (2010) 6491–6497

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The Eurocine® L3 adjuvants with subunit influenza antigens induce protective immunity in mice after intranasal vaccination

Pernilla Petersson[a,b], Mona Hedenskog[a], Denise Alves[a], Mia Brytting[a], Ulf Schröder[c], Annika Linde[a,d], Åke Lundkvist[a,d,*]

[a] Swedish Institute for Infectious Disease Control, 171 82 Solna, Sweden
[b] Department of Infectious Diseases, Karolinska Institutet, 171 76 Stockholm, Sweden
[c] Eurocine Vaccines AB, Karolinska Institutet Science Park, 171 77 Stockholm, Sweden[1]
[d] Microbiology, Tumor and Cell Biology Center, Karolinska Institutet, 171 77 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 26 February 2010
Received in revised form 21 June 2010
Accepted 1 July 2010
Available online 15 July 2010

Keywords:
Influenza
Lipid adjuvants
Intranasal vaccination
Mice
Quantitative PCR
Challenge studies

ABSTRACT

The immunogenicity and protective efficacy in mice of intranasally (i.n.) administrated influenza subunit antigens together with lipid-based adjuvants (Eurocine®) were compared to those of subcutaneous (s.c.) immunisation. Influenza hemagglutination inhibition (HAI) and ELISA IgG titers were similar in the group's vaccinated s.c. and after i.n. vaccination with adjuvants. The virus-specific IgA levels in serum were higher after vaccination i.n. with adjuvant than after s.c immunisation. Virus-specific IgA was measurable in nasal washings only after i.n vaccinations, with and without adjuvants. Thus, i.n. vaccination with the endogenous non-toxic, lipid adjuvants induced equal or stronger antibody responses as compared to s.c. immunisation with the same antigen. We further analysed the protective efficacy against virus challenge in a mouse model. A subunit antigen preparation of the A/New/Caledonia/20/99 strain was used for vaccination of NMRI mice with different combinations of adjuvants. The mice were challenged i.n. with 6.5 tissue culture infectious doses$_{50}$ of homologous virus and sacrificed 3 days later. Since the virus is not lethal in mice, the protective efficacy was measured by quantitative, real-time PCR on pulmonary tissue, obtained at autopsy. The mice treated with only adjuvant and the group of naïve mice clearly had the highest mean viral RNA copy numbers (19.200 and 11.000, respectively). All vaccinated groups had significantly lower copy numbers, especially the mice that received the L3A i.n. (–median 120; i.n. L3B – median 2.200; and non-adjuvanted s.c. vaccination – median 2.600). Our findings prompt further investigations of the effect of the formulations in ferrets, monkeys and man.

1. Introduction

The importance of vaccination to prevent excess hospitalisations and mortality due to the yearly influenza outbreaks is continuously increasing. Every year 250,000–500,000 people die from influenza, or from secondary bacterial infections following the primary infection (www.who.org). The existing vaccines against influenza induce a rather short-lived and highly strain-specific immunity and vaccination must therefore be repeated yearly, irrespective of whether antigenic drift or shift has occurred. Thus, there is an urgent need to develop a vaccine that can induce a long-lasting and broader immunity.

These limitations of influenza immunisation and the antigens used have been highlighted in connection to the recent pandemic caused by A(H1N1)v where, as anticipated, the pandemic vaccine could be produced only after it had been thoroughly characterized. Furthermore, in order to secure an acceptable immunological response, adjuvants had to be added to the vaccine. Vaccines against emerging influenza A subtypes may further be less immunogenic than the ones in use today, and the need for adjuvants for pandemic vaccines has been emphasised [1]. Other ways to improve the antigen expression and presentation is by the use of live vaccines, DNA-vaccines or virosomes. Mode of vaccine administration can also influence the immunological response, where nasal administration of influenza vaccines repeatedly has been shown to induce a broader cross-reactivity to various influenza strains than subcutaneous administration [2,3,4]. Induction of virus-specific, secretory, IgA (SIgA) [5] has been discussed as a crucial element in attaining the cross-protection seen after nasal immunisation. Irrespective of antigen and mode of presentation, adjuvants can give further stimulation to the immune response

* Corresponding author at: Swedish Institute for Infectious Disease Control, SE 171 82 Sweden. Tel.: +46 8 457 26 41; fax: +46 8 33 72 72.
E-mail address: ake.lundkvist@smi.se (Å. Lundkvist).
[1] www.eurocine.se.

0264-410X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2010.07.001

Table 1
Experiment no. 1. Immunisation with the A/Panama/2007/99 (H3N2) subunit antigen . The type of vaccination given to the five groups of ten mice, and the median and range of HAI titers of three examinations of pooled serum samples are presented. The pre-immunisation sera were all negative.

Group number	Type of vaccination	Median and range of HAI-titres after second immunisation	Median and range of HAI-titres after third immunisation
1	Subunit antigen + L3B-SO i.n	640 (160–1280)	1280 (640–5120)
2	Subunit antigen + L3B i.n	320 (160–640)	1280 (1280–5120)
3	Subunit antigen + L3A i.n	1280 (320–2560)	2560 (1280–10,240)
4	Subunit antigen i.n	<10 <10	<10 (0–40)
5	Subunit antigen s.c	1280 (320–1280)	2560 (1280–10,240)

[6], and several studies have shown that this is of particular importance in connection to nasal vaccination [3], where limited immune responses are seen when no adjuvants are used [7,8,9].

However, since adjuvants may induce side-reactions, such as the Bell's Palsy seen after using even a detoxified mutant of the *E. coli* heat-labile toxin [10] precautions have to be taken in order to ensure that only well characterized and non-toxic adjuvants, which in themselves preferably do not exert any direct immunological effect, are used.

In addition to a higher and longer long-lasting degree of protection, the addition of an adjuvant to an influenza vaccine might also facilitate a reduction in the amount of antigen needed. WHO has put focus on the low awareness of influenza in non-industrialised countries [11]. For use in such countries, a nasal vaccine would be extremely suitable, since it abolishes the need for syringes and diminishes the need for skilled medical staff to administer the vaccine. It may also reduce the amount of antigen needed.

In this study, we describe how three different adjuvants, containing mixtures of endogenous lipids [9,12] increased the immunogenicity of an egg-grown subunit antigen, when given i.n. to mice. The subunit antigen was chosen since it primarily induces a humoral response as compared to a whole virus or split antigen [13]. Induction of a local and systemic IgA response after i.n. administration and an improvement of this response by addition of an adjuvant were also demonstrated. Further, we proved the protective efficacy of vaccination in NMRI mice against virus challenge by quantification of viral RNA in mice lungs.

2. Materials and methods

2.1. Mice

Eight weeks old female white mice (strain NMRI) were purchased from Scanbur BK, Stockholm, Sweden. Lungs were obtained at autopsy and stored at −20 °C in RNA later (Qiagen Sciences, Hilden, Germany) until RNA extraction. Blood samples were obtained from the tail vein after each vaccination and at sacrifice.

2.2. Vaccination

2.2.1. Three sets of experiments are reported

Egg-grown, monovalent, subunit influenza antigen from the A/Panama/2007/99(H3N2) or A/New Caledonia/20/99 (H1N1) strain, respectively (kindly donated by Dr Jolyon White, Evans Vaccines, UK) were used for immunisation of mice. 1.5 μg of HA was given at each vaccination, either as 5 μL in each nostril of the mice, anaesthetised with isoflurane (Baxter International Inc, US), or diluted to 100 μL in PBS subcutaneously (s.c). All mice were immunised three times with 2 weeks interval. Serum samples were drawn immediately before the first and third immunisation, and at sacrifice 2 weeks after the third immunisation.

In the first set of experiments, using the H3N2 strain, different formulations of the adjuvant (see below) were evaluated. Serum samples were drawn for measurement of specific IgG with an enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HAI), and a method for sampling of respiratory secretions for IgA measurements was evaluated.

In the second set of experiments, using the H1N1 strain subunit antigen, i.n. vaccination with the antigen without adjuvant, with the most efficient adjuvant identified in study 1, only with adjuvant, only with Tris buffer and s.c. vaccinations were compared. Sera were examined by HAI and ELISA for influenza-specific antibodies. To obtain respiratory secretions, the mice were dissected, and trachea perforated with a syringe. The lungs and the nasal cavities were rinsed with 1 mL sterile H_2O, containing a protease inhibitor (Protease Inhibitor Cocktail Tablets, Boehringer Mannheim, Germany). Serum and respiratory secretions were examined for total IgA and antigen-specific IgA with the A/H1 strain used for vaccination.

In the third set of experiments, the H1N1 strain subunit antigen was used for immunisation, followed by virus challenge (see below). The three different groups of mice examined in the three sets of assays are presented in Tables 1–5.

2.3. Adjuvants

The Eurocine© adjuvants are composed of lipids: mono-olein (MO, Danisco Ingredients. Denmark), oleic acid (OA, Kebo AB, Sweden), lauric acid (Kebo AB, Sweden) and soybean oil (SO, Karlshamn AB, Sweden)

Three kinds of adjuvant formulations were included:

L3A 8%: Oleic acid (0.46 g) and lauric acid (0.34 g) was mixed and subsequently sonicated with 9.2 mL of 0.1 M Tris buffer (pH 8.0). The L3A formulation was adjusted to pH 8.0 with 5 M NaOH.
L3B 8%: Mono-olein (0.45 g) and oleic acid (0.35 g) was gently heated and subsequently sonicated with 9.2 mL of 0.1 M Tris buffer (pH 8.0). The L3B formulation was adjusted to pH 8.0 with 5 M NaOH.
L3B-SO 8%: Mono-olein (0.15 g), oleic acid (0.12 g) and soybean oil (0.53 g) was gently heated and subsequently sonicated with 9.2 mL of 0.1 M Tris buffer (pH 8.0). The L3B-SO formulation was adjusted to pH 8.0 with 5 M NaOH.

The 8% lipid formulations were mixed with the antigen at a 1:1 ratio, in order to have a final lipid concentration of 4% in each formulation.