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Vaccination of turkeys against *Chlamydophila psittaci* through optimised DNA formulation and administration

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ABSTRACT

We have demonstrated that vaccination of turkeys with an unformulated DNA vaccine induces significant protection against *Chlamydophila (Cp.) psittaci* infections. Nevertheless, the immunogenicity of the DNA vaccine can still be improved by increasing translation and transfection efficiency. Therefore, the *ompA* codon was adapted to the codon usage in birds, resulting in pcDNA1/MOMP$_{opt}$. To increase gene transfer, polyplexes of pcDNA1/MOMP$_{opt}$–EGFP with different cationic polymers, such as linear and branched polyethyleneimine (lPEI and brPEI) and starburst PAMAM dendrimers, and lipoplexes with cationic DOTAP/DOPE liposomes were created. Transfection of lPEI and brPEI polyplexes with an N/P ratio of 8 resulted in the highest transfection efficiencies, but lPEI polyplexes were completely destroyed following nebulisation. Secondly, we examined the capacity of nebulised or intramuscularly (IM) administered brPEI-pcDNA1/MOMP$_{opt}$ to induce a significant protective immune response in SPF turkeys experimentally infected with 10^8 TCID$_{50}$ of a virulent *Cp. psittaci* strain. Results were compared to IM administration of naked plasmid DNA and to results of non-vaccinated animals. Intramuscular administration of brPEI-pcDNA1/MOMP$_{opt}$ increased the immunogenicity of the *Cp. psittaci* DNA vaccine as compared to IM administration of pcDNA1/MOMP$_{opt}$ or aerosol delivery of brPEI-pcDNA1/MOMP$_{opt}$. Improved immunogenicity was correlated with increased protection. Vaccinated groups were significantly protected against *Cp. psittaci* challenge.

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1. Introduction

The obligate intracellular pathogen *Chlamydophila (Cp.) psittaci* primarily infects birds and is horizontally transmitted through aerosols of nasal secretions and faeces. Initially, the respiratory tract is infected, from where the disease further spreads leading to a systemic infection. Mainly in the poultry industry substantial financial losses result from a decrease in egg-production and the need for antibiotic treatment. Zoonotic transmission occurs in people in close contact with infected birds, the clinical outcome ranging from unapparent to severe flu-like symptoms or pneumonia [1].

Immunisation with a plasmid DNA encoding the Major Outer Membrane Protein (pcDNA1/MOMP) leads to significant protection against severe clinical signs, lesions and bacterial excretion as compared to placebo-vaccinated controls [2]. However, rhinitis (in 43% of the turkeys), pharyngeal excretion (14%) and thoracic (71%) and abdominal (29%) air sac lesions can still be observed. It has been reported that DNA vaccination, using unformulated plasmid DNA (pDNA), shows a low gene transfer efficiency in the host cell and hence a low antigen expression [3]. Therefore, we examined if we could further improve the current pcDNA1/MOMP vaccine. To enhance pDNA delivery into the host cells, cationic liposomes or cationic polymers such as polyethyleneimine (PEI) and dendrimers can be used. These cationic carriers bind the pDNA electrostatically and condense it into positively charged nanoparticles that are more easily taken up by host cells. Furthermore, they protect the pDNA against extracellular nucleases [4]. Several studies have already shown that cationic liposomes, PEI and dendrimers can enhance the transfection efficiency leading to improved gene expression *in vitro* and *in vivo* [5–12]. To optimise transgene expression, different strategies like the use of regulatory elements, Kozak sequences and codon optimisation can be applied [13]. In a recent study performed by Zheng et al. [14], codon optimisation significantly enhanced gene expression and immunogenicity of a *C. muridarum* MOMP-based DNA vaccine. The first aim of this study was to investigate whether the transfection efficiency of pcDNA1/MOMP could be enhanced by forming complexes with cationic liposomes or polymers, in

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addition to improving the translation efficiency of the cloned *ompA* gene by codon optimisation.

Another critical step in the immunisation process is the choice of the vaccine delivery route, which plays a vital role in creating protective immune responses. In experimental studies, the intramuscular route is generally accepted as the 'gold standard'. However, in poultry, antigen-specific protection of mucosae at the respiratory tract surface can be successfully achieved by delivering vaccines as aerosol [15]. Moreover, vaccination by aerosol is a cost effective way of immunising thousands of turkeys at the same time and the vaccine targets the respiratory tract which is not used for consumption. Therefore, the second aim of this study was to examine whether nebulisation has a negative effect on the stability and gene transfer capacity of an optimised *Cp. psittaci* DNA vaccine formulated with cationic polymers (DNA vaccine polyplexes). Only the DNA vaccine polyplexes based on branched polyethyleneimine (brPEI) were not affected by nebulisation. Therefore, this *Cp. psittaci* DNA vaccine polyplex formulation (brPEI-pcDNA1/$MOMP_{opt}$) was used for mucosal (aerosol) and parenteral (intramuscular) DNA vaccination experiments in SPF turkeys and we compared the protective immune response to intramuscular vaccination with pcDNA1/$MOMP_{opt}$ (control). In this way, we tried to examine if the *in vitro* 'accomplished' increased plasmid transfection and *ompA* translation efficiency finally resulted in significantly higher protection of turkeys against *Cp. psittaci* challenge.

2. Material and methods

2.1. Vaccine construction

To enhance the expression of MOMP in turkey cells, the coding sequence of the *ompA* gene was adapted and optimised to the codon usage in birds (GenScript Corporation, New Jersey, USA) in order to increase the codon adaptation index (CAI) as described by Sharp and Li [16]. The CAI was calculated (http://www.evolvingcode.net/codon/cai/cai.php) based on the most frequent codon usage in chickens and turkeys. EGFP was cloned downstream from the codon optimised $ompA_{opt}$ into the EcoRV restriction site of pcDNA1, resulting in the final construct: pcDNA1/$MOMP_{opt}$–EGFP. Plasmid DNA was propagated in *Escherichia coli* MC1061/P3, purified using the EndoFree® Plasmid Giga kit (Qiagen, Venlo, The Netherlands) and dissolved in 20 mM Hepes buffer (pH 7.4). Following purification, a PCR reaction on the plasmid was performed with vector associated SP6 and T7 primers to amplify the fusion construct cloned into the multicloning site of pcDNA1. Amplified PCR products of the appropriate size were selected for full length sequencing (VIB Genetic Service Facility, Antwerp, Belgium), using pcDNA1 SP6 and T7 priming sites.

To verify increased expression of the codon optimised *ompA*, DF-1 cells (chicken embryo fibroblasts; ATCC: CRL-12203) were transfected with pcDNA1/MOMP and pcDNA1/$MOMP_{opt}$–EGFP using Polyfect® transfection reagent (Qiagen). Expression of MOMP and $MOMP_{opt}$ was confirmed by indirect immunofluorescence staining. Briefly, transfected DF-1 cells were incubated at 37 °C and 5% CO_2 for 48 h. Subsequently, cells were fixated with ice-cold methanol. MOMP and $MOMP_{opt}$ were visualised by use of a polyclonal anti-MOMP antibody [17] in combination with an Alexa Fluor 546 labelled goat–anti-rabbit antibody (Molecular Probes, Invitrogen, Merelbeke, Belgium). Both antibodies were diluted 1/200 in PBS + 1% BSA and incubated for 1 h at 37 °C. Transfected and stained DF-1 cells were analyzed using a fluorescence microscope (Nikon Eclipse TE 2000-E) equipped with excitation filters of 528–553 nm for Alexa Fluor (red fluorescence) and 465–495 nm for EGFP (green fluorescence).

2.2. Polymers and lipids

Branched polyethylenimine (brPEI) (25 kDa) and Starburst PAMAM dendrimers of generation 2 (G2) and generation 5 (G5) were purchased from Sigma (Bornem, Belgium). Linear polyethylenimine (lPEI) (22 kDa) was kindly provided by Prof. Ernst Wagner (LMU, Munich, Germany). The lipids DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA).

DOTAP/DOPE liposomes (molar ratio of 1/1) were prepared by dissolving appropriate amounts of lipids in chloroform in a round bottom flask. The solvent was removed by rotary evaporation at 40 °C followed by purging the flask with nitrogen for 30 min at room temperature (RT). Lipids were hydrated by adding 20 mM Hepes buffer (pH 7.4). Glass beads were added and swirled to facilitate detachment of the lipid layer from the wall of the flask. The formed dispersion was stored overnight at 4 °C and subsequently extruded 11 times using 2 stacked 100 nm polycarbonate membrane filters (Whatman GmbH, Dassel, Germany).

Lipoplexes (i.e. complexes between cationic liposomes and pDNA) were prepared at +/− charge ratios of 4, 6 and 8. Plasmid DNA was first diluted in Hepes buffer to a concentration of 0.413 μg/μl. Subsequently, appropriate volumes of liposomes (5 mM DOTAP/5 mM DOPE) were added resulting in the desired charge ratio. Immediately after adding the liposomes, Hepes buffer was added to a final concentration of plasmid DNA of 0.126 μg/μl. Lipoplexes were vortexed and incubated for 30 min at RT before use.

Complexes with lPEI and bPEI were prepared at N/P ratios of 5, 8, 10, 12, 15, 18 and 20. Plasmid DNA was first diluted in Hepes buffer to a concentration of 0.5 μg/μl. Subsequently, appropriate volumes of lPEI and brPEI were dissolved in Hepes buffer and an equal volume of pDNA was added. Immediately after adding the DNA to the PEI polymers, Hepes buffer was added until the final concentration of plasmid DNA was 0.126 μg/μl. Polyplexes were vortexed and incubated for 30 min at RT before use.

Complexes with starburst PAMAM dendrimers G2 and G5 were prepared at N/P ratios of 1, 4, 5, 10 and 20. Plasmid DNA was first diluted in Hepes buffer to a concentration of 0.5 μg/μl. Subsequently, appropriate volumes of starburst PAMAM dendrimers G2 and G5 were dissolved in Hepes buffer and an equal volume of plasmid DNA was added. Immediately after adding the DNA to the dendrimers, Hepes buffer was added until a final concentration of plasmid DNA of 0.126 μg/μl. Complexes were vortexed and incubated for 30 min at RT before use.

Size and zeta potential (ζ) of the complexes were measured by dynamic light scattering (Autosizer 4700, Malvern, Hoeilaart, Belgium) and particle electrophoresis (Zetasizer 2000, Malvern), respectively. Before each measurement, 950 μl Hepes buffer was added to 50 μl of the lipoplexes or polyplexes.

2.3. Cytotoxicity for BGM cells

Toxicity of the lipoplexes and polyplexes was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay after transfecting the different complexes in the BGM cell line, which are kidney epithelial cells from the African Green Monkey (ATCC: CCL-26). Briefly, BGM cells were seeded in 96-well plates (100 μl/well; 3×10^5 cells/ml) and transfected 24 h later by pipetting the complexes into the culture medium (MEM supplemented with 10% FCS, 1% vitamins, 1% L-glutamin, 1% streptomycin and 2% vancomycin, all products from Invitrogen). Cytotoxicity of all lipoplexes and polyplexes was tested in duplicate after 24 and 48 h of incubation with the complexes by adding MTT (10 μl, 0.5 mg/ml) to the cells. The MTT assay was performed

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