Challenges and opportunities in nasal subunt vaccine delivery : mechanistic studies using ovalbumin as a model antigen

Slütter, B.A.

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Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination

Bram Slütter
Wim Jiskoot

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Abstract

Nasal vaccination is a promising, but challenging vaccination strategy. Poor absorption by the nasal epithelium and failure to break nasal tolerance are regarded as important reasons for poor efficacy of nasally applied vaccines. Formulation of the antigen into mucoadhesive nanoparticles, made of N-trimethyl chitosan (TMC) crosslinked with tripolyphosphate (TPP), has been shown to overcome these obstacles. However, although nasally administered antigen loaded TMC/TPP nanoparticles induce a strong humoral response, antibody subtyping indicates a Th2 bias. To design a nasal antigen delivery system capable of inducing stronger Th1 type responses, TPP as a crosslinking agent was replaced by unmethylated CpG DNA, a TLR-9 ligand and a potent inducer of Th1 responses, to prepare ovalbumin (OVA) loaded TMC nanoparticles (TMC/CpG/OVA). Several physicochemical characteristics of TMC/CpG/OVA (size, zetapotential, loading efficiency and antigen release profile) were assessed and compared to TMC nanoparticles prepared by crosslinking with TPP (TMC/TPP/OVA). Mice were nasally administered TMC/TPP/OVA and TMC/CpG/OVA after which antibody responses in serum and nasal washes were assessed and T-cell activation in the spleens determined.

TMC/CpG/OVA showed similar physical properties as TMC/TPP/OVA in terms of particle size (380 nm), zetapotential (+21 mV) and antigen release characteristics. Nasal administration of TMC/CpG/OVA and TMC/TPP/OVA to mice resulted in comparable serum IgG levels (ca. 1000 fold higher than those induced by unadjuvanted OVA) and local secretory IgA levels. Moreover, TMC/CpG/OVA induced a 10 fold higher IgG2a response than TMC/TPP/OVA and enhanced the number of OVA specific IFN-gamma-producing T-cells in the spleen.

In conclusion, OVA loaded TMC nanoparticles, containing CpG as adjuvant and crosslinker, are capable of provoking strong humoral as well as Th1 type cellular immune responses after nasal vaccination.

Introduction

Nasal vaccination has gained much interest over the past decades as it is non-invasive and thereby expected to increase patient compliance. Additionally, vaccination via the nose has been shown to induce, besides systemic humoral (IgG mediated) and cellular responses, local as well as distal secretory immune responses (secretory IgA (sIgA) mediated) [1-3], making the mucosal linings less vulnerable to infection. Moreover, the cross reactivity of sIgA is relatively high compared to IgG antibodies [4, 5], making the induction of local immune responses a promising strategy to target highly variable pathogens, like influenza viruses [6].

Nonetheless, nasal immunization with subunit vaccines is challenging, as residence time in the nasal cavity is limited and therefore the uptake by the nasal epithelium is low. Moreover, the nasal epithelium is renowned for being a rather tolerogenic site [7, 8], making it difficult for subunit antigens to provoke an immune response. Vaccine formulation may be instrumental to successful nasal vaccination. Encapsulation of the antigen into particulate carrier systems has been explored extensively in recent years [9] and holds great promise as particles can be specifically designed to meet the challenges nasal vaccination provide [10]. Among the large variety of particles that can be found in the literature, chitosan based particles are among the most studied ones [11]. Chitosan is a cheap, biodegradable, mucoadhesive polymer. In rodents, particles prepared from chitosan have been shown to effectively induce systemic antibody responses against ovalbumin (OVA) and cholera toxin [12], Hepatitis B surface antigen [13], and Meningococcal C oligosaccharides [14]. More recently chitosan derivatives have been developed, like thiolated chitosans [15] to enhance its mucoadhesiveness and trimethylated chitosans (TMC) [16] to improve its solubility at physiological pH. Especially TMC has been shown to be a very promising nasal vaccine carrier. Nanoparticles prepared from TMC by ionic crosslinking with tripolyphosphate (TPP) increase the nasal residence time of the encapsulated antigen [17], improve the uptake of the antigen by M-cells [18] and additionally promote maturation of dendritic cells (DCs) [9-11]. Consequently, TMC particles loaded with antigens, e.g. tetanus toxoid [12], meningococcal C oligosaccharides [19] or hemagglutinin [20] induce strong systemic as well local antibody responses. Moreover, intranasally administered TMC-coated whole inactivated influenza virus resulted in protection of mice against a challenge with a lethal dose of influenza virus [21]. Nonetheless, a significant drawback of TMC is its tendency to promote a humoral (Th2 type) rather than a Th1 type immune response [20, 22]. Strong Th1 type responses are important for many vaccines that we do not have [23], such as HIV/AIDS and tuberculosis vaccines, underscoring the importance of developing vaccine carrier systems capable of inducing these

responses. The bias of TMC's adjuvant effect toward a Th2 response is not restricted to the nasal administration route, as it is also observed after intradermal [24] and intramuscular administration of TMC-adjuvanted antigen (unpublished data). However, different types of immune responses have been reported after nasal vaccination [25-28], depending on the adjuvant used. As TPP does not act as an adjuvant but solely services as a crosslinking agent to promote TMC nanoparticle formation, we propose it should be possible to substitute TPP with a crosslinking agent that does have an adjuvant effect. Unmethylated CpG DNA is a Toll like receptor 9 ligand and described as a Th1 response-inducing adjuvant, also after nasal administration [23]. Furthermore, phosphate groups on CpG render it negatively charged, which could make CpG a possible crosslinking agent to prepare TMC nanoparticles.

The aim of this paper was to study whether CpG can replace TPP as a crosslinker to prepare ovalbumin (OVA)-containing TMC nanoparticles and whether these new carrier systems are capable of redirecting the TMC-induced Th2 type response towards a more Th1 type response, while maintaining strong systemic and local antibody responses. The TMC/CpG/OVA nanoparticles were compared to TMC/TPP/OVA nanoparticles with respect to their physicochemical characteristics and immunogenicity after nasal administration in mice.

Material and Methods

Materials

Ovalbumin (OVA) was purchased from Calbiochem (Beeston, UK) and CpG DNA (ODN 2006) as well as fluorescein isothiocyanate coupled CpG (CpG-FITC) from InvivoGen (Toulouse, France). N-trimethyl chitosan with a degree of quaternization of 15% was synthesized from 92% deacetylated (MW 120 kDa) chitosan (Primex, Avaldsnes, Norway) and characterized by NMR, as described by Bal et al. [29]. KCl, NaCl, HNa_2PO_4, KH_2PO_4 and bovine serum albumin (BSA) were purchased from Merck (Amsterdam, The Netherlands). Pentasodium tripolyphosphate (TPP), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), Tween 20 and 2-mercapto ethanol were obtained from Sigma-Aldrich (Steinheim, Germany). Goat anti-mouse IgG, IgG1, IgG2a and IgA conjugated with horseradish peroxidase was purchased from Southern Biotech (Birmingham, AL). BDOpteia IFN-γ ELISA kit was bought from Becton Dickinson (Breda, The Netherlands). RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin (P/S) solution, L-glutamine, sodium pyruvate and fluorescein isothiocyanate coupled OVA (OVA-FITC) were acquired from Invitrogen (Breda, The Netherlands), and 70-µm cell strainers from VWR (Amsterdam, The Netherlands).

Nanoparticle preparation

OVA loaded TMC/TPP (TMC/TPP/OVA) nanoparticles were prepared as described before [18]. Briefly, 20 mg TMC and 1 mg OVA were dissolved in 8.3 ml 5 mM Hepes pH 7.4. Under continuous stirring 3.4 ml 0.1% w/v TPP was added to induce ionic complexation into nanoparticles. Particles were collected by centrifugation (10 min, 12000 g), resuspended and washed once with water. OVA loaded TMC/CpG (TMC/CpG/OVA) nanoparticles were prepared in the same way as TMC/TPP/OVA, replacing TPP by CpG. A total amount of 0.9 mg CpG was added to 20 mg TMC and 1 mg of OVA; the addition of more CpG caused aggregation and a dramatic increase of the polydispersity index (PDI), whereas the addition of less CpG reduced the number of particles formed (data not shown). Supernatants were stored for determining the loading efficiency and nanoparticles were stored at 4°C until further analysis. OVA-FITC loaded nanoparticles were prepared by substituting OVA-FITC for OVA.

Size and zetapotential

Particle suspensions were diluted in 5 mM Hepes pH 7.4 until a slightly opalescent dispersion was achieved. Hydrodynamic diameter (average and PDI) and zetapotential were determined with a Nanosizer (Malvern Instruments, Malvern, UK) by dynamic light scattering and laser Doppler electrophoresis, respectively.

Loading efficiency

To determine the loading efficiency, the OVA content of the nanoparticles as well supernatants, was determined using micro bicinchoninic acid (mBCA) protein assay (Pierce, Etten Leur, The Netherlands) according to the manufacturer's instructions. To determine the encapsulation efficiency of CpG, FITC-labeled CpG was included and the amount of CpG-FITC was determined in the supernatant as well as in the particle formulation using fluorescence spectroscopy (FS920 fluorimeter, Edinburgh Instruments, UK; excitation 495 nm, emission 520 nm; band widths 5 nm).

Particle stability and antigen release in vitro

TMC/TPP/OVA-FITC and TMC/CpG/OVA-FITC were diluted to a final particle concentration of 1 mg/ml in 10 mM phosphate buffered saline (PBS) pH 7.4 containing 0.01% Tween 20 and stored in several aliquots at 37°C. At different time points nanoparticle size was determined with DLS after which the dispersions were centrifuged (10 min 14000 g) and supernatants were collected allowing quantification of the released OVA-FITC with fluorescence spectroscopy (excitation 495 nm, emission 520 nm; band widths 5 nm).

Nasal vaccination

Female Balb/c mice (Harlan, Boxmeer, The Netherlands), 6-8 weeks old, received 3 nasal doses of 20 µg OVA or an equivalent dose encapsulated OVA with intervals of 3 weeks. Mice receiving CpG were nasally administered 20 µg (3.1 nmol) of the adjuvant, either as a CpG solution with OVA or as a suspension of TMC/CpG/OVA nanoparticles. Three OVA injections of 20 µg OVA were administered intramuscularly as control. For nasal administration, formulations were applied in a volume of 10 µl PBS, 5 µl per nostril. For i.m. administration, 25 µl of formulation in PBS was injected in the thigh muscle. Blood samples were taken 2 weeks after the final booster dose. After sacrificing the animals, spleens were harvested and nasal washes collected.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Micro titer plates (Nunc, Roskilde, Denmark) were coated with OVA, by incubation of 1 µg/ml OVA in 40 mM sodium carbonate buffer pH 9.4 for 24 hours at 4°C. To reduce aspecific binding, wells were blocked with 1% (w/v) BSA in PBS for 1 hour at 37°C. After extensive washing with PBS serial dilutions of serum ranging from 20 to $2*10^6$ were applied, whereas nasal washes were added undiluted. After incubation for 1.5 hours at 37°C and extensive washing, OVA specific antibodies were detected using HRP conjugated goat anti mouse IgG, IgG1, IgG2a or IgA (1 hour 37°C) and by incubating with 0.1 mg/ml TMB and 30 µg/ml H_2O_2 in 110 mM sodium acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm with an EL808 microplate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany).

T-cell activation study

T-cell activation was studied using a protocol described by Christensen et al. [30]. Single cell suspensions were prepared, by grinding spleens over 70 µm cell strainers and rinsing with spleen medium (RPMI 1640 supplemented with 10% v/v FBS, 1% v/v glutamine, 1% v/v P/S and 0.05 mM 2-mercaptoethanol). Splenocytes were restimulated with 20 µg/ml OVA and maintained for 5 days at 37 °C and 5% CO_2. IFN-γ levels in culture supernatant were determined using a BDOpteia IFN-γ ELISA according to the manufacturer's instructions.

Statistics

Serum antibody titers were analyzed with a Kruskal-Wallis test with Dunn's post-test. Antibody levels in nasal washes as well as splenocyte responses were analyzed with a one-way ANOVA with Bonferroni post-test. Statistics were performed using GraphPad 5.0 for Windows.

Table 1: Particle characteristics

Nanoparticles	Size (nm)	PDI	Zetapotential (mV)	Loading efficiency OVA (%)	Loading efficiency CpG (%)	Burst release (%)
TMC/TPP/OVA	314 +/- 31	0.12 +/- 0.09	18.2 +/- 1.8	63 +/- 6	-	25 +/- 2
TMC/CpG/OVA	304 +/- 22	0.20 +/- 0.11	20.9 +/- 2.0	52 +/- 7	56 +/- 5	46 +/- 1*

*Values represent mean of 3 individually prepared batches ± standard deviation. Burst release was defined as the percentage of OVA release after 1 h in PBS. * p<0.001 compared to TMC/TPP/OVA.*

Results and discussion

Nanoparticle characterization

The characteristics of the TMC/CpG/OVA nanoparticles and the TMC/TPP/OVA nanoparticles were comparable in size and zetapotential (Table 1). Both particle types showed an average hydrodynamic diameter of ca. 300 nm, were fairly monodisperse (PDI 0.1-0.2) and had a positive zetapotential of about +20 mV. Moreover, changing the crosslinker did not alter the loading efficiency (Table 1) and the release pattern (data not shown), as both particle showed a burst release followed by no release over 48 hours. TMC/CpG/OVA did show a significantly higher burst under physiological conditions ($p<0.001$ Student's t-test). This may be related to the higher amount and charge density of TPP compared to CpG, allowing a stronger interaction with TMC. Similar large burst releases (>50%) have been observed for even less densely negatively charged polymers like dextran sulfate and hyaluronic acid (Verheul *et al.* unpublished results).

These results indicate that ionic crosslinking of TMC is just as easily achieved with other phosphate group-bearing entities as with TPP and TMC/CpG/OVA nanoparticles appear to be physically very similar to TMC/TPP/OVA nanoparticles.

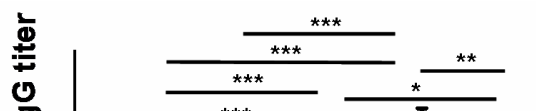
Nasal vaccination

Nasal vaccination with subunit antigen is challenging as only a very limited amount of soluble antigen will be taken up by the nasal epithelium and subsequently be processed by DCs. This is reflected in the observation that nasal administration of a solution of plain OVA

resulted in negligible antibody titers, whereas intramuscular injection of the same dose of OVA induced high IgG titers after a booster dose (Figure 1). Coadministration of OVA with TMC, positively affected the IgG response ($p<0.001$ compared to OVA after prime as well as booster dose), as reported before for TMC mixed with other antigens [20, 21]. TMC can enhance the uptake of antigens through the nasal epithelium [31, 32] as it opens tight junction [33, 34] and can prolong the disposition of antigen in the nasal cavity [35]. The addition of CpG as an adjuvant resulted in enhanced antibody titers ($p<0.05$), but to a significantly lesser extent as TMC ($p<0.001$). Possible reasons for the weaker adjuvant effect of soluble CpG as compared to TMC are: CpG probably does not prolong the nasal residence time of the antigen, the adjuvant itself resides in the nasal cavity for only a short period of time and it may not be taken up by the nasal epithelium as efficiently as TMC.

An even better approach than the application of solutions seems a particulate delivery system comprising the antigen and TMC, as both TMC/TPP/OVA and TMC/CpG/OVA vaccinated mice showed significantly enhanced IgG titers compared to OVA alone or a mixture of soluble TMC and OVA ($p<0.05$ after a priming dose). As the size of these nanoparticles would inhibit rather than induce intercellular transport through the tight junctions between nasal epithelial cells, nanoparticles promote the immunogenicity of the antigen in a different way. The mere particulate structure could favor uptake by M-cells [18, 36-38], allowing antigen access to the subepithelial space. There, multimerization of epitopes of the particle's surface could contribute to an improved uptake by DCs and B-cells [39, 40].

Besides a systemic antibody response, both TMC nanoparticles also induced a potent mucosal immune response, indicating effective uptake of OVA by local B-cells. Nasal washes of both TMC/TPP/OVA and TMC/CpG/OVA vaccinated mice contained comparable elevated levels of sIgA ($p<0.05$) (Figure 2), whereas no significant sIgA elevation was detected in nasal washes after vaccination with plain OVA solution. Solutions of OVA with adjuvant (TMC or CpG) also showed an increase in sIgA levels but significantly lower than the sIgA levels induced by TMC/CpG/OVA ($p<0.05$). Although local antibodies are not often used as a correlate of protection, the interest in sIgA is increasing. sIgA is recognized as an important factor in mucosal homeostasis [41] and is capable of inducing M-cell transport of neutralized antigen [42], thereby delivering the antigen to local DCs [43]. Antigen specific sIgA at mucosal surfaces could therefore protect the host from future infection by directly neutralizing the pathogen, but also by acting as an early warning signal for the immune system. Furthermore, sIgA production after nasal vaccination is not restricted to the upper airways, as via a system called the common mucosal immune system [44], sIgA antibodies can be detected also in other mucosal secretions.



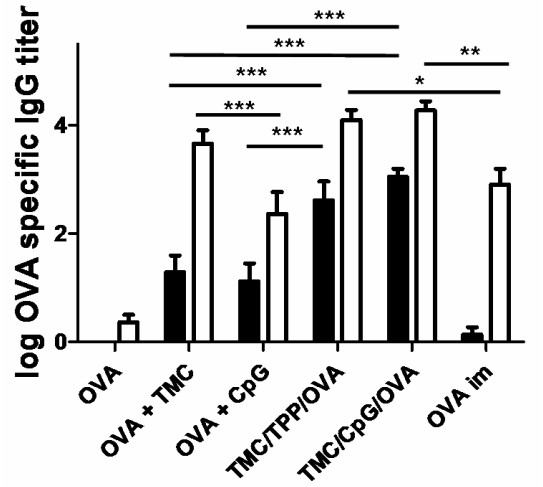


***Figure 1**: OVA specific serum IgG titers in serum after a priming (black bars) and a booster dose (white bars). Mice received 3 doses of 20 µg OVA nasally or intramuscularly (OVA im). The 2nd boost did not further increase IgG levels and is not shown for reasons of clarity. All formulations except for OVA im after priming were significantly higher than OVA (p<0.01). Bars represent mean n=8 ± SEM. * p<0.05, **p<0.01, ***p<0.001*

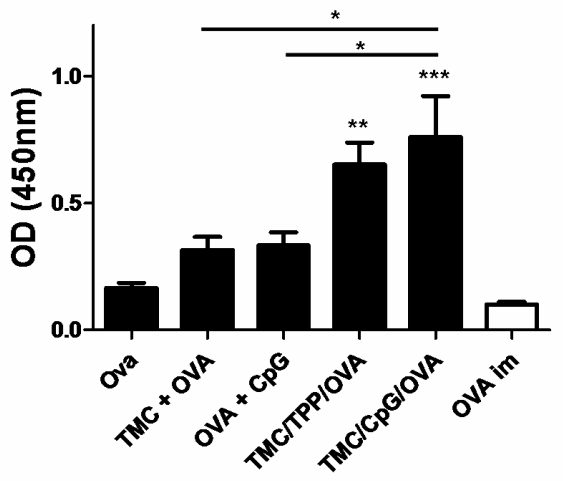


***Figure 2**: OVA specific IgA levels in nasal washes of nasally immunized Balb/c mice. Bars represent mean n=8 ± SEM. *p<0.05 compared to TMC/CpG/OVA, **p<0.01 compared to OVA ***p<0.001 compared to OVA.*

The major important difference between the effects of TMC/TPP/OVA nanoparticles and TMC/CpG/OVA nanoparticles appeared to be the type of response elicited (Figure 3). TMC/TPP/OVA caused a predominant IgG1 response ($p<0.05$), whereas TMC/CpG/OVA vaccinated mice showed a decreased IgG1/IgG2a ratio, indicating that the inclusion of CpG into TMC nanoparticle promoted a Th1 response. Similarly, coadministration of TMC led to an increased IgG1/IgG2a ratio indicating a shift towards Th2, whereas the addition of CpG to OVA decreased the IgG1/IgG2a ratio. The Th1-inducing effect of nasally administered CpG has been observed before [45, 46] and TMC/CpG/OVA nanoparticles seem to exert a similar effect. This was confirmed by the T-cell activation study, showing that splenocytes from TMC/CpG/OVA immunized mice produced large quantities of IFN-γ after restimulation with OVA (Figure 4), even more than mice immunized with a solution of OVA and CpG. Splenocytes from mice vaccinated with a solution of OVA and TMC or TMC/TPP/OVA did not produce more IFN-γ than

splenocytes from naïve mice. So, changing the crosslinker from TPP to CpG strongly shifted the T-cell polarization towards the Th1 direction.

Overall, TMC/CpG particles seem to be capable of eliciting strong humoral responses, both local (sIgA) and systemic (IgG, IgG1, IgG2a), as well as a Th1 type response, making them a promising vaccine carrier for nasally applied OVA and, most likely, a wide variety of other antigens for which a Th1 type immune response is needed.

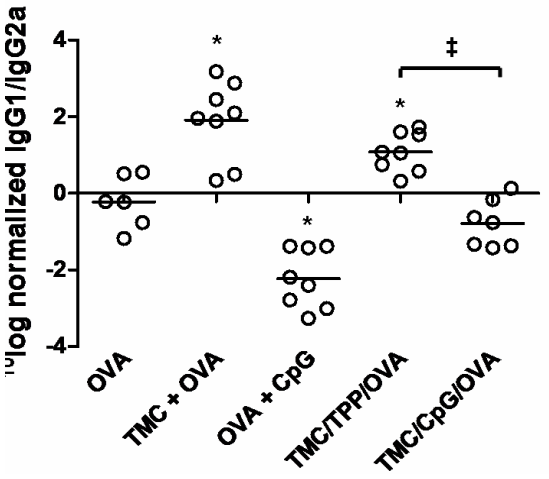


***Figure 3**: Serum IgG1/IgG2a levels normalized for the average OVA IgG1/IgG2a ratio. * p<0.05 compared to OVA. ‡ p<0.05.*

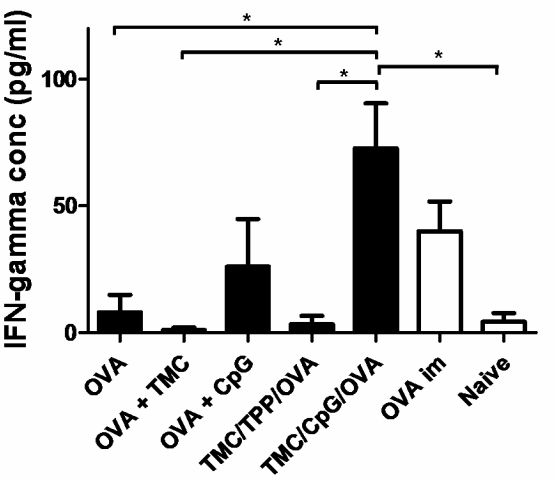


***Figure** 4: IFN-γ production by splenocytes restimulated with OVA. Values represent mean n=5 ± SEM. * p<0.05*

Conclusion

TMC/TPP/OVA nanoparticles have previously been shown to be very effective nasal vaccine carriers. Replacing TPP by CpG as a crosslinking agent to obtain TMC/CpG/OVA nanoparticles modulated the immune response towards a Th1 response after nasal vaccination, while maintaining the strong systemic and local antibody responses observed with TMC/TPP nanoparticles. TMC/CpG nanoparticles therefore are an interesting nasal delivery system for vaccines requiring broad humoral as well as strong Th1 type cellular immune responses.

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