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Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine

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ABSTRACT

The nose is a promising immunization site and intranasal (i.n.) vaccination studies with whole inactivated influenza virus (WIV) adjuvanted with N,N,N-trimethylchitosan (TMC–WIV) have shown promising results. In this study, the influence of TMC on the i.n. delivery of WIV was studied in mice by comparing the nasal residence time and the specific location in the nasal cavity of WIV and TMC–WIV. Additionally, the local toxicity profile of the WIV formulations was assessed. *In vivo* fluorescence imaging was used to study the nasal residence time and the fate of the bulk vaccine in mice that received vaccines fluorescently labeled with IRDye800CW®. An immunohistochemical (IHC) staining method for nasal cross-sections was developed to visualize the antigen in the nasal cavity. Therefore, mice were sacrificed at different time points after vaccination with various vaccine formulations and nasal cross-sections were made. The local toxicity was assessed using hematoxylin and eosin staining for the nasal cross-sections. No significant differences in the nasal residence time between WIV and TMC–WIV were observed. However, IHC revealed a striking difference in the location and distribution of WIV in the nasal cavity. When formulated as plain WIV, positive staining was mainly found in the nasal cavity, presumably in mucus blobs. TMC-coated WIV, on the other hand, was mostly present as a thin layer on the epithelial surfaces of the naso- and maxilloturbinates. This difference in staining pattern correlates with the observed differences in immunogenicity of these two vaccines and indicates that TMC–WIV results in a much closer interaction of WIV with the epithelial surfaces than WIV alone, potentially leading to enhanced uptake and induction of immune responses. This study further shows that both WIV and TMC–WIV formulations induce minimal local toxicity. Taken altogether, these results provide more insight in the mode of action and safety of TMC and justify further research to develop TMC-adjuvanted nasal vaccines.

1. Introduction

Intranasal (i.n.) vaccination offers several advantages to the classical intramuscular (i.m.) route of vaccination. The simple, needle-free administration does not require trained personnel and i.n. immunization has the potential to evoke both mucosal and systemic immune responses. The major drawback of this route of administration is the relatively poor immunogenicity of nonadjuvanted vaccines when compared to the i.m. route. This is likely the result of inefficient delivery of antigens to the immune system via the nasal cavity, as illustrated by the strong immune responses that the same vaccines elicit after i.m. administration [1].

The delivery of vaccine antigens is impaired by the mucociliary clearance, which removes inhaled substances like dust and bacteria and viruses, entrapped in mucus, from the nasal cavity towards the throat, preventing potentially harmful substances that can penetrate the nasal epithelium.

Several strategies have been explored to improve the efficacy of i.n. vaccination [2]. In the case of influenza vaccines, the use of a live attenuated influenza virus vaccine, which replicates only locally in the upper respiratory tract, has been successful [3]. Furthermore, the use of adjuvants in combination with killed vaccines has shown promising results (as reviewed in [2]). Besides immunostimulatory adjuvants like TLR-ligands, toxin-derivatives and cytokines, the use of

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mucoadhesive polymers is of special interest in nasal vaccination as it may improve the delivery of i.n. administered antigens [4].

Promising results were obtained using N,N,N-trimethylchitosan (TMC), a water soluble derivative of chitosan, as an adjuvant in nasal vaccines [5–8]. TMC is a cationic mucoadhesive polymer that can be characterized by the degree of quaternization (DQ), the degree of O-methylation (DOM) and the degree of acetylation (DAc).

We prepared formulated whole inactivated influenza virus (WIV) with various TMCs to obtain positively charged TMC-coated WIV (TMC–WIV) formulations that induced significantly higher immune responses and provided protection against challenge with live influenza virus [7]. Furthermore, we showed that the DQ and DOM of TMC did not significantly influence the immunogenicity of TMC–WIV formulations [8].

It is generally believed that the mode of action of mucoadhesive adjuvants, including TMC in i.n. WIV vaccines, is the improvement of the mucoadhesion of the antigen, leading to an increased nasal residence time and/or an altered interaction of the antigen with the mucosal surfaces in the nasal cavity. However, the relationship between mucoadhesiveness, nasal residence time and immunogenicity has not been properly investigated *in vivo*. Mostly, *in vitro* methods have been employed to describe the mucoadhesive properties of polymers and vaccine formulations [9], including TMC [10] which is moderately mucoadhesive.

Recent advances in probe design and instrumentation have enabled *in vivo* fluorescence imaging of fluorescently labeled antigens using near infrared (NIR) fluorescence spectroscopy with improved, submillimeter spatial resolution [11]. NIR fluorescence imaging is a cheap and practical alternative to nuclear imaging.

Another approach to study the mechanism of action of TMCs as mucosal vaccine adjuvants is using microscopy techniques (e.g. [12–15]). The administered antigen can be visualized in isolated nasal cross-sections by immunohistochemical (IHC) staining with antigen specific antibodies.

Using microscopic techniques, the local toxicity of i.n. administered vaccines and vaccine adjuvants can also be assessed. The safety of vaccines is the key in the development of nasal vaccine adjuvants, as evidenced by the withdrawal of an inactivated i.n. influenza vaccine with LT as an adjuvant from the market because of adverse effects [16]. Although various TMCs have different *in vitro* cell toxicities, they are considered relatively nontoxic *in vitro* [17–20]. Nevertheless, the local toxicity of TMC solutions and TMC–WIV formulations may be different after i.n. administration and should therefore be determined.

The aim of this study was to compare the nasal residence time, the specific location in the nasal cavity and the local toxicity of WIV and TMC–WIV. Therefore, an *in vivo* fluorescence imaging approach was used and an IHC staining protocol was developed.

2. Materials and methods

2.1. Materials

Purified, cell culture-grown (Madin–Darby Canine Kidney (MDCK) cells), β-propiolactone (BPL)-inactivated influenza A/PR/8/34 virus (WIV), and polyclonal anti-WIV rabbit serum were from Nobilon International BV, (Boxmeer, The Netherlands). Alexa Fluor® 488-labeled goat anti-rabbit IgG and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Invitrogen (Breda, The Netherlands). Osteosoft decalcifier (10% EDTA) and ethanol were from Merck Serono BV (Schiphol, The Netherlands). Xylene was obtained from Mallinckrodt Baker (Deventer, The Netherlands). Citric acid, Triton-X, n-octyl-β-D-glucopyranoside (OG), trypsin from bovine pancreas, normal goat serum and normal rabbit serum were obtained from Sigma (Zwijndrecht, The Netherlands). Phosphate buffered saline (10 mM phosphate buffer, pH 7.4 150 mM NaCl) (PBS) was purchased from Braun (Melsungen, Germany). IRDye800CW®-labeled epidermal growth factor (EGF) and the IRDye800CW® protein labeling kit were from LI-COR Biosciences (Lincoln, NE, USA). Fixative (4% formalin in phosphate buffer pH 7.0) was purchased from Klinipath (Duiven, The Netherlands). All other chemicals used were of analytical grade.

2.2. Properties of N,N,N-trimethylchitosans

In the *in vivo* fluorescence imaging studies we used *O*-methyl free TMC with a DQ of 43% (TMC43) to compare the nasal residence time of TMC-coated WIV and plain WIV. For the immunostaining and toxicity studies, O-methyl free TMC68 and O-methylated TMCs (TMC–OM) with DQs of 15% (TMC–OM15) and 37% (TMC–OM37) were also included in the study. The various TMCs were produced and characterized as described earlier [7,17]. The polymer characteristics of the TMCs are summarized in Table 1.

2.3. Formulation of TMC–WIV

WIV in PBS was pelleted by centrifugation at 22,000 × *g* for 30 min at 4 °C and resuspended in 5 mM HEPES buffer (pH 7.4) at a final concentration of 2.5 mg total protein/ml. The amount of hemagglutinin (HA) was approximately 35% of the total protein content, as determined previously [21].

For the *in vivo* fluorescence imaging experiments, WIV was labeled with the NIR fluorescent probe IRDye800 CW® according to the instructions provided by the manufacturer. Briefly, the pH of the WIV suspension with a final protein concentration of approximately 1 mg/ml was raised to pH = 8.5 with 1 M K_2PO_4, pH 9. To 450 µl of this suspension, 15 µl of IRDye800CW® reactive dye solution (4 mg/ml in DMSO) was added and the mixture was incubated for 2 h in the dark at room temperature while shaking. Next, labeled WIV was separated from the free label using a size-exclusion *Zeba*™ desalting spin column. The purified IRDye800CW®-labeled WIV was then centrifuged for 45 min at 22,000 × *g* at 4 °C and resuspended in 100 µl 5 mM HEPES buffer. The protein concentration was determined by a micro BCA protein assay from Pierce, part of Thermo Fisher Scientific (Rockford, IL, USA) using bovine serum albumin (BSA) as a reference protein and the IRDye800CW®-labeled WIV suspension was adjusted with approximately 50 µl 5 mM HEPES to a WIV concentration of 2.5 mg/ml.

The TMC–WIV vaccines were prepared by simply adding equal volumes of a TMC solution (in 5 mM HEPES, pH 7.4) to a WIV dispersion at a 1:1 w/w ratio using a Gilson pipette while gently mixing for 5 s. A volume of 10 µl (5 µl to each nostril) of TMC–WIV formulations was administered to mice at a final WIV concentration of 1.25 mg/ml.

Imaging and local toxicity studies after i.n. administration of WIV formulations in mice.

All animal experiments described in this study were conducted according to the guidelines provided by the Dutch Animal Protection Act and were approved by a Committee for Animal Experimentation.

Table 1
Physicochemical characteristics of the TMCs used in this study.

Polymer	Mn (kDa)	Mw (kDa)	DQ (%)	DAc (%)	DOM-6 (%)	DOM-3 (%)
TMC43	36	75	43	17	–	–
TMC68	39	84	68	17	–	–
TMC–OM15	44	94	15	16	6	4
TMC–OM37	30	63	37	4	14	9

Abbreviations: Mn = number average molecular weight; Mw = weight average molecular weight; DQ = degree of quaternization; DAc = degree of N-acetylation; DOM-6 = degree of O-6 methylation; DOM-3 = degree of O-3 methylation.
Formulation of TMC–WIV.