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Research paper

Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice

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Abstract

High molecular weight (Mw) chitosan (CS) solutions have already been proposed as vehicles for nasal immunization. The aim of the present work was to investigate the potential utility of low Mw CS in the form of nanoparticles as new long-term nasal vaccine delivery vehicles. For this purpose, CS of low Mws (23 and 38 kDa) was obtained previously by a depolymerization process of the commercially available CS (70 kDa). Tetanus toxoid (TT), used as a model antigen, was entrapped within CS nanoparticles by an ionic cross-linking technique. TT-loaded nanoparticles were first characterized for their size, electrical charge, loading efficiency and in vitro release of antigenically active toxoid. The nanoparticles were then administered intranasally to conscious mice in order to study their feasibility as vaccine carriers. CS nanoparticles were also labeled with FITC-BSA and their interaction with the rat nasal mucosa examined by confocal laser scanning microcopy (CLSM). Irrespective of the CS Mw, the nanoparticles were in the 350 nm size range, and exhibited a positive electrical charge (+40 mV) and associated TT quite efficiently (loading efficiency: 50–60%). In vitro release studies showed an initial burst followed by an extended release of antigenically active toxoid. Following intranasal administration, TT-loaded nanoparticles elicited an increasing and long-lasting humoral immune response (IgG concentrations) as compared to the fluid vaccine. Similarly, the mucosal response (IgA levels) at 6 months post-administration of TT-loaded CS nanoparticles was significantly higher than that obtained for the fluid vaccine. The CLSM images indicated that CS nanoparticles can cross the nasal epithelia and, hence, transport the associated antigen. Interestingly, the ability of these nanoparticles to provide improved access to the associated antigen to the immune system was not significantly affected by the CS Mw. Indeed, high and long-lasting responses could be obtained using low Mw CS molecules. Furthermore, the response was not influenced by the CS dose (70–200 μg), achieving a significant response for a very low CS dose. In conclusion, nanoparticles made of low Mw CS are promising carriers for nasal vaccine delivery.

Keywords: Chitosan; Nanoparticles; Vaccines; Nasal administration

1. Introduction

Over the last decade, chitosan (CS) has been attracting increasing attention as a biomaterial and as a pharmaceutical excipient for drug delivery because of its favorable biological properties [1]. Besides its low toxicity and susceptibility to biodegradation, CS has shown mucoadhesive properties as well as an important drug penetration enhancement capacity across mucosal barriers [2–6]. Specific reports on the nasal administration of high molecular weight (Mw) CS solutions have shown that CS enhances the nasal absorption of peptide drugs and also antigens [7–10].

As a new mucosal delivery vehicle, as an alternative to high Mw CS solutions, we have recently developed CS nanoparticles. These nanoparticles can be made of a wide range of CS Mws using a very mild and friendly ionic gelation technique [11]. Their size, surface characteristics and in vitro release properties can be controlled by adjusting the formulation conditions and by the incorporation of additional polymer, e.g. poloxamers [12]. A variety of drugs, going from low to high Mw, hydrophilic and

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lipophilic drugs, have been efficiently incorporated into these particles [13]. Furthermore, the ability of these nanoparticles to facilitate the nasal transport of macromolecules has been clearly shown using insulin as a model peptide. More specifically, the plasma glucose levels following nasal administration of insulin-loaded nanoparticles to conscious rabbits were significantly lower than those corresponding to the same dose of CS solutions [14]. These studies led us to accept that the mechanism of action of these CS nanoparticles may be different as compared to that of CS solutions. An additional observation is that the positive effect of CS solutions in promoting drug transport has only been shown for relatively high Mw CS (higher than 70 kDa). A step forward on the evidence of the potential of CS nanoparticles as nasal carriers for macromolecules was very recently shown using tetanus toxoid (TT) as a model antigen. In this study we found that CS nanoparticles (Mw 70 kDa) were able to elicit high and long-lasting IgG immune responses following nasal administration to conscious mice [15]. Consequently, the results suggested that CS nanoparticles were not simply able to facilitate the transport of TT through the nasal epithelium but also to improve its delivery to the immunocompetent cells. However, the mechanism by which this antigen was delivered to the nasal mucosa and the role of the particle's characteristics have not been elucidated until now.

The nasal route holds great promise from the perspective of vaccination due to the particular organization of the nasal mucosa [16]. The nasal mucosa is the first site of contact with inhaled antigens, and the nasal-associated lymphoid tissue (NALT) at the base of the nasal cavity (Waldeyer's ring in humans) is important in the defense of mucosal surfaces. Additionally, the nasal epithelium is leaky and there are underlying blood vessels, cervical lymph nodes and lymphoid cells to which the antigen may have direct access if it can be adequately transported across the epithelium. However, in spite of this attractive configuration, so far the poor transport characteristics of antigens across the nasal barrier have made this modality of vaccination non-viable.

An interesting approach that has been explored in order to improve the transport of antigens across the nasal mucosa has been their encapsulation into polymeric particles [17]. Polymers most frequently used for this application are PLA (poly (lactic acid)) and PLGA (poly (lactic-co-glycolic acid)) [18–22]. These studies showed that, following nasal administration, antigens encapsulated in PLGA microparticles elicit a higher immune response than fluid vaccines. To explain this positive response it was hypothesized that encapsulated antigens are sampled by specialized cells, similar to the microfold (M) cells which overlie the NALT, and then transported to the underlying antigen presenting cells (APCs). More recent work carried out by our group has revealed that the size and surface composition play a significant role in the ability of these biodegradable particles to target the antigen to the APCs. More specifically, we observed that a hydrophilic PEG coating around PLA nanoparticles has a very positive effect on the immune response of the encapsulated antigen [23]. This was justified by the improved stability of the particles in contact with the mucus layer, followed by a greater transport of the antigen encapsulated in PEG-PLA nanoparticles as compared to that of antigen-loaded PLA nanoparticles [15,24].

Based on this information, the aim of the present work was to explore further the potential of CS nanoparticles as vehicles for the nasal administration of vaccines. For this purpose, TT was chosen as a model antigen. We first studied the effect of CS dose on the efficacy of CS nanoparticles at eliciting long-lasting immune responses. Then, we investigated the mechanism of interaction of the nanoparticles with the nasal mucosa. Finally, we studied the effect of CS Mw (23, 38 and 70 kDa) on the ability of these nanoparticles to elicit significant immune responses.

2. Materials and methods

2.1. Chemicals and animals

CS, in the form of hydrochloride salt (Protasan® 110 Cl, Mn $>$50 kDa, deacetylation degree: 87%), was purchased from Pronova Biopolymer, S.A. (Norway). Pentasodium tripolyphosphate (TPP) and trehalose were supplied by Sigma (USA). Glucose was provided by Merck (Darmstadt, Germany) and sucrose by Probus S.A. (Barcelona, Spain). Ultrapure water (MilliQ Plus, Millipore Iberica S.A., Spain) was used throughout. Purified TT (Mw 150 kDa, 85–95% monomeric) dissolved in phosphate buffer saline (pH 7.4) was kindly donated by the World Health Organization (WHO). Anti-tetanus monoclonal antibody, purified guinea-pig anti-tetanus immunoglobulin G (IgG) and mouse anti-tetanus immunoglobulin G standard were donated by the National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK), and rabbit anti-guinea pig IgG peroxidase conjugate, goat anti-mouse IgG peroxidase conjugate, goat anti-mouse IgA peroxidase conjugate and the enzyme substrate (ABTS), pilocarpine and pentobarbital were purchased from Sigma S.A. (Madrid, Spain).

Male BALB/c mice (6 weeks old, 22–25 g) from the Central Animals House of the University of Santiago de Compostela (Spain) were used. They were kept in a 12 h light/dark cycle and temperature of 20 ± 2 °C. The animals were allowed access to food and water ad libitum.

2.2. Depolymerization of CS

CS was dissolved to 2 mg/ml in Milli-Q water. CS fragments were generated by sodium nitrite degradation according to Peniston and Johnson [25]. Briefly, varying quantities of 0.1% (w/v) $NaNO_2$ (Probus S.A., Barcelona, Spain) were added to the CS solution at room temperature under magnetic stirring, and the reaction was left overnight

to assure completion of the degradation. We have previously simulated the depolymerization process of CS using Matlab 5.1 (MathWorks, Inc., USA). With this program, the necessary amount of $NaNO_2$ could be predicted in order to obtain approximate Mws of 38 and 23 kDa [26]. These CS fragments and the original (non-depolymerized) polymer were used to prepare the TT-loaded nanoparticles in order to study the influence of Mw CS on the immune response after intranasal administration.

2.3. Preparation and characterization of CS nanoparticles, and determination of the TT association efficiency

CS nanoparticles were prepared according to the procedure previously developed by our group [11] based on the ionotropic gelation of CS with TPP anions. The particles are formed spontaneously upon addition of 1.2 ml of an aqueous TPP solution (0.84 mg/ml) to 3 ml of CS solution (2 mg/ml, 6 mg CS) under magnetic stirring. For the association of TT with CS nanoparticles, TT was incorporated in the TPP solution (600 μg of antigen). Nanoparticles were isolated by centrifugation at 16,000 × *g* on a glucose bed for 40 min. Supernatants were discarded and nanoparticles were resuspended in 5% glucose for their administration.

The particle size and zeta potential of nanoparticles were determined, respectively, by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA) using a Zetasizer® 3000-HS (Malvern Instruments, UK). For the determination of the electrophoretic mobility, samples were diluted with 0.1 mM KCl. Each batch was analyzed in triplicate.

The amount of TT associated with the particles was calculated by the difference between the total amount used to prepare the particles and the amount of TT present in the aqueous phase determined using the micro-bichinchoninic acid (microBCA) protein assay (Pierce®, Rockford, USA). A calibration curve was made using the supernatant of blank nanoparticles.

2.4. In vitro release studies

CS nanoparticles (2 mg) were incubated in 3 ml of trehalose solution (5% w/v) at 37 °C. At predetermined time intervals, the samples were centrifuged, and 2 ml of supernatant was removed. The samples were supplemented with 2 ml of fresh release medium and resuspended. The study was conducted until significant aggregation of the particles occurred (16 days). The presence of trehalose in the incubation medium was required in order to preserve the integrity of the nanoparticles following several centrifugation/resuspension cycles. The amount of antigenically active TT released was determined by an enzyme linked immunosorbent assay (ELISA) as described previously [27]. Results are shown as the distributive percentage of antigenically active TT released with respect to the total amount of protein associated.

2.5. Immunization studies

The immunogenicity of the CS formulations was assessed in BALB/c mice following intranasal immunization. These animal experiments were approved by the Ethical Committee of the University of Santiago de Compostela. Two different doses of TT-loaded CS nanoparticles (Mn 70 kDa) and TT in solution were examined in order to estimate the optimal antigen dose; 30 or 10 μg of antigen (associated with 200 and 70 μg of CS) was given in 20 μl of saline solution (10 μl into each nostril) on days 1, 8 and 15 to four different groups of six mice. All animals were conscious during the administration. Based on the antibody responses achieved, we selected the lowest dose (10 μg) and studied the influence of Mw CS (23, 38 and 70 kDa) on the antibody responses. For this study, four groups of mice ($n = 6–8$) were used, three groups treated with TT-loaded CS nanoparticles of different Mn (23, 38 and 70 kDa) and another group with free toxoid. The administration protocol was the same as indicated above.

2.6. Body fluid and tissue sampling

Blood samples were taken from the tail vein of animals on weeks 2, 4, 12, 18 and 24 post-administration. The serum samples were maintained at −20 °C prior to analysis. Saliva, broncho-alveolar and intestinal lavages were collected on week 24, using a modified procedure described by Eyles et al. [19]. Salivation was induced by i.p. injection of pilocarpine (150 μl, 1 mg/ml PBS). An aliquot of 100 μl of the initial flow of saliva was collected from each mouse. Then, mice were anesthetized i.p. with 1.8 mg of pentobarbital, killed by cervical dislocation and exsanguinated. Broncho-alveolar lavages were obtained by injecting and aspirating 5 ml of ice-cold lavage medium (0.9% w/v NaCl, 0.05% w/v Tween® 20 and 1 mM phenylmethanesulphonyl fluoride (PMSF)) into the trachea to inflate the lungs by means of an intravenous cannula. Lengths (50 cm) of upper small intestine from each mouse were sectioned longitudinally, and the luminal surface was carefully scraped into 4 ml of ice-cold lavage medium (1 mM PMSF, 1 mM iodoacetic acid, 0.001% w/v soybean type 1-5 trypsin inhibitor and 10 mM EDTA).

2.7. ELISA

IgG antibody levels in serum were determined using an ELISA test. First, TT (4 μl/ml) in carbonate buffer (pH 9.6) was added to microplates (Corning, NY, USA) and incubated overnight at 4 °C in a humid container. The wells were washed three times with PBS containing 0.05% (w/v) Tween® 20 (PBST). To minimize non-specific interactions, 100 μl of PBST containing 2.5% (w/v) of

dried skimmed milk powder (PBSTM buffer) was added to the wells and incubated for 1 h at 37 °C in a humid container. After washing the plate three times with PBST, samples along with a reference preparation (in the case of evaluation of IgG, 9 UI/ml) were diluted serially in two-fold steps in PBSTM. The plates were incubated at 37 °C for 2 h in a humid container and washed. Then, 100 μl of anti-mouse IgG peroxidase conjugate was diluted 1:2000 in PBSTM and plates were incubated for another 1 h at 37 °C. The plates were washed and the substrate 0.5 mg/ml ABTS in 0.05 M citric acid (pH 4.0) was added to each well. Following color development (30 min) plates were read at 405 nm on a microplate reader (3550-UV, Biorad, Spain).

2.8. Analysis of saliva, broncho-alveolar and intestinal lavages

Saliva samples were maintained at −20 °C prior to ELISA analysis. Intestinal lavages were sonicated on ice (30 s, 20 W) to extract immunoglobulins from mucin and centrifuged (22,000 × *g*, 40 min, 4 °C). Broncho-alveolar lavages were also centrifuged (22,000 × *g*, 40 min, 4 °C), and the supernatants were rapidly frozen prior to lyophilization. Lyophilized samples were reconstituted with 200 μl of PBS for ELISA assay of specific IgA content as described above for serum. Titer was derived as the maximum dilution of saliva, broncho-alveolar and intestinal lavages giving an OD of 0.1 after correction for background. The results were presented as a mean $\log_{10}$ titer ± SEM per group.

2.9. Statistical analysis

A Kruskal–Wallis test was applied to determine the significance of the difference between IgG level means and an ANOVA test was applied to IgA level means ($P < 0.05$).

2.10. Interaction of CS nanoparticles with the nasal epithelial cells by confocal laser scanning microcopy (CLSM)

With the purpose of investigating the mechanism of interaction of CS nanoparticles with the nasal epithelium, the particles were labeled with FITC-BSA and then administered intranasally to fully awake rats. Two doses of 200 μg of nanoparticles suspended in 20 μl of water were administered with a 5 min time interval to a group ($n = 3$) of animals (10 μl in each nostril). Another group received the equivalent dose of FITC-albumin dissolved in water. Rats were killed by cervical dislocation 5 min after the last administration and 5 ml of formalin 4% (0.1 M PB) was injected into the trachea. Rat nasal mucosas were excised, fixed and finally observed at optical cross-sections of 0.4 μm (Zeiss CLSM 501 equipped with Zeiss Neofluor 40/1.3 objective and Zeiss LSM 510 software, Jena, Germany) at 458 nm.

3. Results and discussion

The main goal of the present work was to investigate the potential interest of low Mw CS nanoparticles as antigen delivery carriers for nasal administration. Using TT as a model antigen, we studied the effect of the CS dose and CS Mw on the efficacy of nanoparticles at eliciting enhanced and long-lasting immune responses. Besides this primary goal, we also expected to obtain information regarding the suitability of the nanoparticles' formation process for the preservation of TT immunogenicity.

As stated in Section 1, a few years ago, we developed CS nanoparticles with the intention of using them to improve the nasal absorption of macromolecules [11]. This idea was partially supported by the reported ability of CS to transiently open the tight junctions between epithelial cells, as observed in Caco-2 cell cultures [2,3]. Indeed, this previous information led us to hypothesize that CS particles containing bioactive macromolecules incorporated in their structure could be a promising approach to concentrate both (CS and the associated macromolecule) at the mucosal surface, thereby improving the transport of the bioactive compound. These nanoparticles could be formed spontaneously by a controlled gelation process without the use of energy sources or organic solvents. More interestingly, they showed a great capacity for the association of proteins [12]. These are very appealing characteristics compared to those of hydrophobic polymers, i.e. PLGA nanoparticles, which can only incorporate a limited amount of protein and require harmful conditions for their preparation [29].

3.1. Formation and characterization of the nanoparticles

The formation of CS nanoparticles occurs spontaneously upon incorporation of the counter anion sodium TPP into a CS solution [11]. In a previous study, we found that the association efficiency of proteins to these nanoparticles varied depending on the solution in which the protein was present. More specifically, proteins with a low p*I*, such as BSA and TT, were better associated with the nanoparticles when dissolved in the alkaline sodium TPP solution [12]. This was attributed to the ionic interaction between the negatively charged protein in the sodium TPP solution and CS upon mixing. In this previous work, we also concluded that other forces, such as hydrogen bonding and hydrophobic forces, could be involved in the association process. In the present study, we aimed to associate a much greater amount of TT within the nanoparticles in the hope of using them for nasal immunization. The particle size, zeta potential and association efficiency of nanoparticles of different CS Mw with a 10% TT theoretical loading are shown in Table 1. The nanoparticles had a similar size (300–350 nm) and a positive surface charge (+40 mV). The association efficiency values were high, in the range of 50–60%, irrespective of the CS Mw. This indicates that

Table 1
Physicochemical properties and association efficiencies of TT to nanoparticles made of CS of different Mw ($n = 9$)

Formulation	Mw (kDa)	Size (nm)	Zeta potential (mV)	Association efficiency (%)
Np CS-23	23	351 ± 23	+38.5 ± 3.2	52.8 ± 1.9
Np CS-38	38	368 ± 68	+43.4 ± 0.8	44.4 ± 5.5
Np CS-70	70	354 ± 27	+37.1 ± 5.9	55.1 ± 3.4

Theoretical loading: 10% w/w.

the entanglement of the protein within the CS chains is not affected by their Mw.

The in vitro release of antigenically active TT from the nanoparticles is shown in Table 2. All formulations exhibited two release phases, a rapid release over the first 2 h followed by a slow release for up to 16 days. The first release phase could be attributed to the release of TT molecules that were lightly associated with CS molecules and located near the surface of the particles, whereas the second phase might correspond to those TT molecules more efficiently entrapped and tightly bound to the CS molecules. Further release could be expected from these particles, but release studies were not continued after this time because of the particle aggregation that took place after a number of centrifugation/resuspension cycles (see Section 2). With respect to the influence of the CS Mw on the release process, results showed a greater amount of TT released at 2 h as the CS Mw decreased. This could be attributed to an easier detachment of low Mw CS molecules and TT molecules located at the surface of the particles. Our previous work studying the release of proteins from CS nanoparticles led us to the conclusion that the chemical structure of the protein, and its interaction with CS in the release medium conditions, were major factors governing the release process [12]. Obviously, the addition of enzymes to the release medium that degrade CS (lysozyme, chitosanase) would have affected the release process. However, under the experimental conditions of this study, the important point to emphasize is that TT is released from the nanoparticles in its antigenically active form. This is a crucial issue since TT is known to be easily degraded in the course of the release studies, when associated with hydrophobic polymers, i.e. PLGA [27]. It is important to emphasize that these data can not be extrapolated to the in vivo scenario since, following nasal administration, the particles do not necessarily undergo a dilution process. Furthermore, the physiological conditions under which TT release may occur in vivo are expected to be quite different from those in vitro. Therefore, the in vitro release data are revealing in that they indicate that TT is released in an active form for extended periods of time.

3.2. Intranasal immunization with two doses of TT-loaded CS nanoparticles

Because of the limited information on the adequate dose of TT for eliciting an immune response following intranasal immunization and in order to simultaneously investigate the effect of the CS dose on the immune response, we immunized four groups of animals with two doses (30 or 10 μg) of TT, either in a free form or associated with CS nanoparticles (70 kDa). Since the same formulation of nanoparticles was used for the two administration protocols, the higher dose of TT was associated with a higher dose of CS (200 and 70 μg of CS, respectively). As shown in Fig. 1, irrespective of the dose, the anti-tetanus IgG levels elicited by the TT-loaded nanoparticles were significantly higher than those corresponding to the fluid vaccine ($P < 0.05$). This trend clearly evidences the adjuvant effect of the nanoparticles. Another remarkable observation was the long-lasting and increasing response over time. Finally, it was interesting to note that neither the dose of TT nor the dose of CS had a significant effect on the IgG responses. It is worthwhile to mention that a control experiment of parenteral immunization using TT-loaded CS nanoparticles could not be performed because these particles as such are not adequate vehicles for injection. Nevertheless, the IgG antibody levels achieved in the present study are comparable to those elicited by the marketed formulation (aluminum phosphate-adsorbed TT) administered parenterally [28]. Similar conclusions could be drawn from the anti-tetanus IgA titers detected in saliva, broncho-alveolar and intestinal lavages at 6 months post-administration (Fig. 2). The results showed that, independent of the dose, the titers were significantly higher for the TT-loaded particles than for the free toxoid ($P < 0.05$). From these data, we concluded that the lower dose of TT (10 μg) and the lower amount of CS (70 μg) were sufficient to stimulate mucosal and humoral immune responses.

In order to understand why CS nanoparticles enhance the immune response to nasally applied TT, we could simply take into account the dose-dependent decrease in the transepithelial resistance of the Caco-2 cell monolayers

Table 2
Distributive percentage of antigenically active TT released in vitro with respect to the total amount of protein associated with nanoparticles made of CS of different Mw (Np CS-23: CS Mw 23 kDa; Np CS-38: CS Mw 38 kDa; Np CS-70: CS Mw 70 kDa) ($n = 3–6$)

Formulation	2 h	1 day	4 days	8 days	12 days	16 days
Np CS-23	25.33 ± 0.93	0.10 ± 0.14	2.64 ± 1.42	2.26 ± 1.16	0.81 ± 0.71	0.24 ± 0.01
Np CS-38	18.10 ± 2.52	3.46 ± 0.14	0.59 ± 0.21	2.41 ± 1.52	0.08 ± 0.03	0.01 ± 0.01
Np CS-70	17.34 ± 0.81	10.28 ± 4.26	0.76 ± 0.74	3.69 ± 0.63	0.01 ± 0.01	0.01 ± 0.01

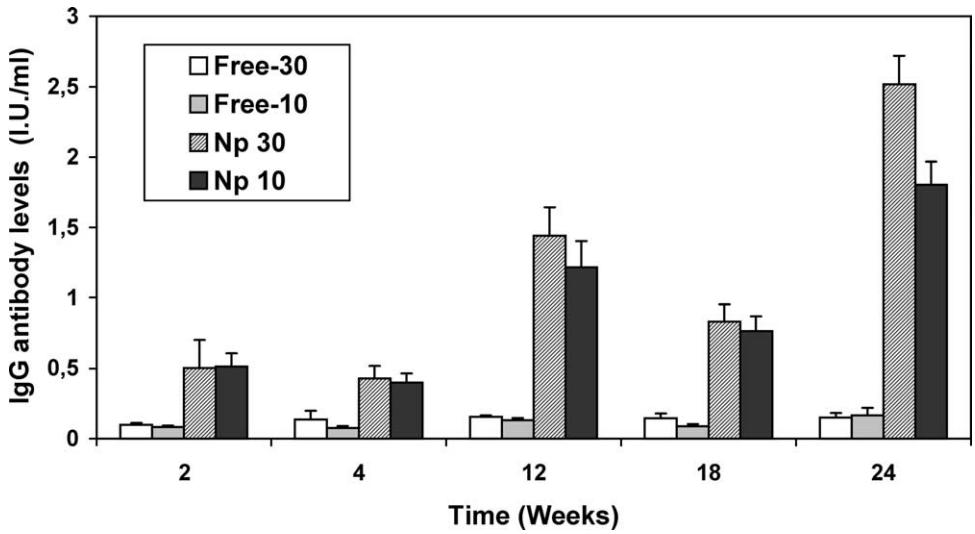

Fig. 1. IgG antibody levels after i.n. administration of two doses of antigen (10 and 30 μg), encapsulated in CS nanoparticles (70 kDa) or in solution in mice (geometric mean ± SEM).

caused by CS [28], and assume that CS nanoparticles interact with the mucus, providing a high concentration of CS to the underlying epithelium while simultaneously delivering the associated toxoid. This penetration enhancement mechanism was previously proposed to explain the adjuvant effect of CS solutions following intranasal administration of Bordetella pertussis proteins [8], subunits influenza virus [9], and diphtheria toxin [10]. Nevertheless, the increasing immune response observed in this study suggests that additional mechanisms, other than merely increasing paracellular transport of the antigen, might be responsible for the positive behavior of CS nanoparticles. On the other hand, in the interpretation of the mechanism of action of CS applied intranasally, we should not forget the initial studies carried out in the 80s aimed at investigating the immunoadjuvant properties of CS [30–32]. For example, in a study performed by Iida et al. [30], it was found that intranasally applied CS induced protective immunity against Senday virus infection, being attributed to the immunostimulatory properties of CS. This information led us to accept the possibility that CS molecules, and even entire particles, might cross the nasal epithelium. In fact, later studies on the effect of CS on Caco-2 cell permeability have led to the conclusion that CS increases cell permeability by affecting paracellular and intracellular pathways [33]. In order to corroborate this hypothesis, we followed the interaction of the particles labeled with FITC-BSA with the rat nasal epithelium by

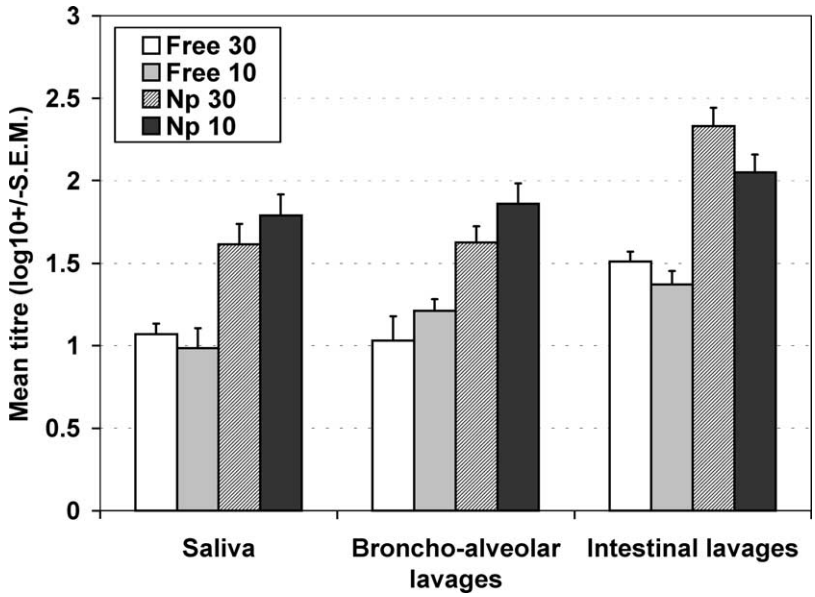

Fig. 2. IgA antibody levels after i.n. administration of two doses of antigen (10 and 30 μg), encapsulated in CS nanoparticles (70 kDa) or in solution in mice. Titers were derived as the maximum dilution of saliva, broncho-alveolar lavages, and intestinal lavages giving an OD of 0.1 after subtraction of background and are presented as the geometric mean titer (GMT) per group.

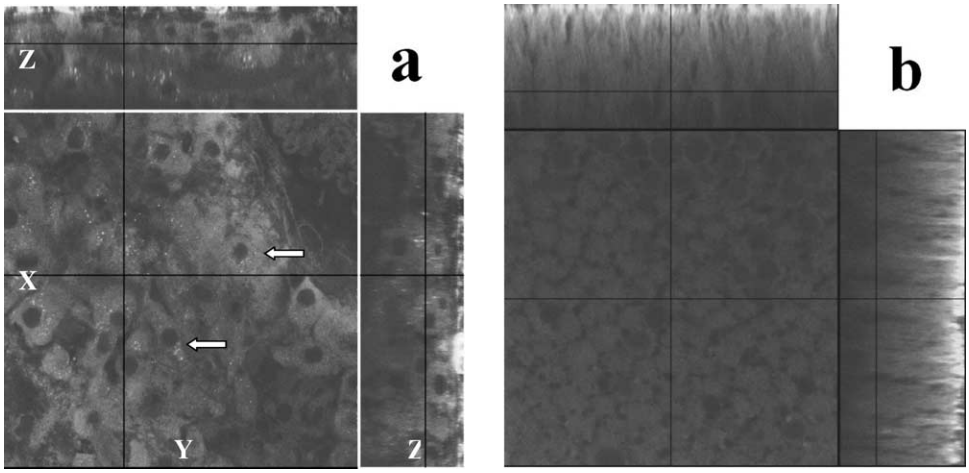

Fig. 3. Confocal laser scanning micrographs of the nasal rat tissue excised following administration of (a) a suspension of FITC-BSA-loaded CS nanoparticles or (b) FITC-BSA solution. The white arrows show the intracellular localization of the particles.

confocal microscopy. The images of the *x*,*z* and *y*,*z* CLSM sections (Fig. 3) show discrete granules throughout the cytoplasm of the epithelial cells, excluding the nuclei. These results indicate that nanoparticles are internalized into the nasal epithelium cells and, consequently, it is reasonable to suggest that they could be further transported to the submucosa layer. The observation that CS nanoparticles may cross the nasal mucosa, thereby transporting the associated antigen and delivering it for extended periods of time, would explain the increasing and long-lasting responses observed in our study. This long-term delivery would also agree with the sustained antigen release observed in vitro.

Besides the transport of the nanoparticle-associated antigen across the regular nasal epithelial cells, there is a possibility that CS nanoparticles could be taken up by the NALT cells. Some preliminary studies using fluorescent microscopy revealed an intense fluorescence in this specific region following nasal administration of CS fluorescent nanoparticles to mice. However, more detailed studies need to be carried out in order to further corroborate the preferable uptake of the particles by the NALT cells.

3.3. Intranasal immunization with a low dose (10 μg) of TT-loaded CS nanoparticles: effect of CS Mw

Based on the data discussed above, the low doses of CS (70 μg) and TT (10 μg) were selected for the subsequent step, aimed at elucidating whether or not the Mw of CS could affect the anti-tetanus systemic and mucosal responses. Fig. 4 shows the IgG antibody levels after intranasal administration of TT in different formulations of CS nanoparticles (Mw 23, 38 and 70 kDa) and in solution. The general trend is that CS nanoparticles generated IgG levels significantly higher ($P < 0.05$) than those elicited by the fluid vaccine. When comparing statistically the responses elicited at each individual time

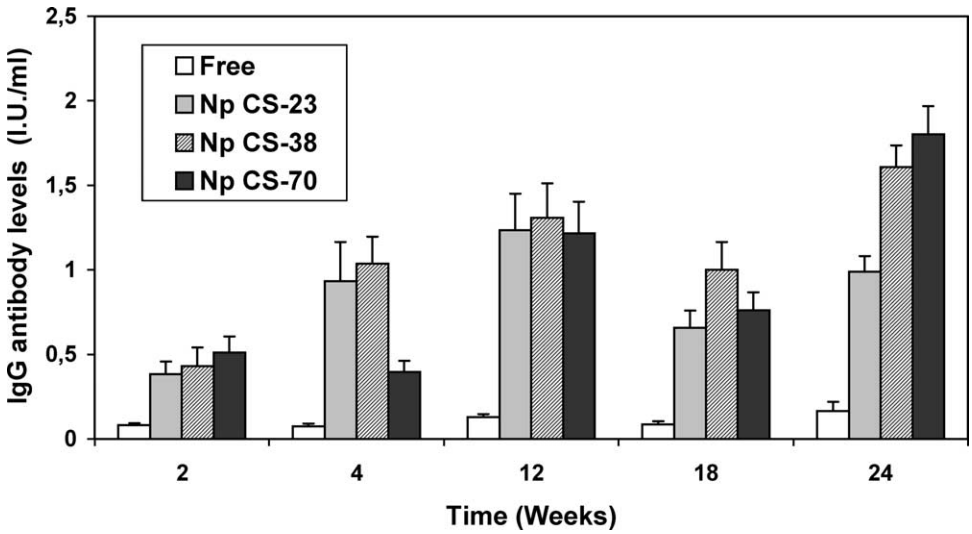

Fig. 4. IgG antibody levels after i.n. administration of 10 μg of TT free or encapsulated in CS nanoparticles (CS Mw: 23, 38 and 70 kDa) in mice (geometric mean ± SEM).

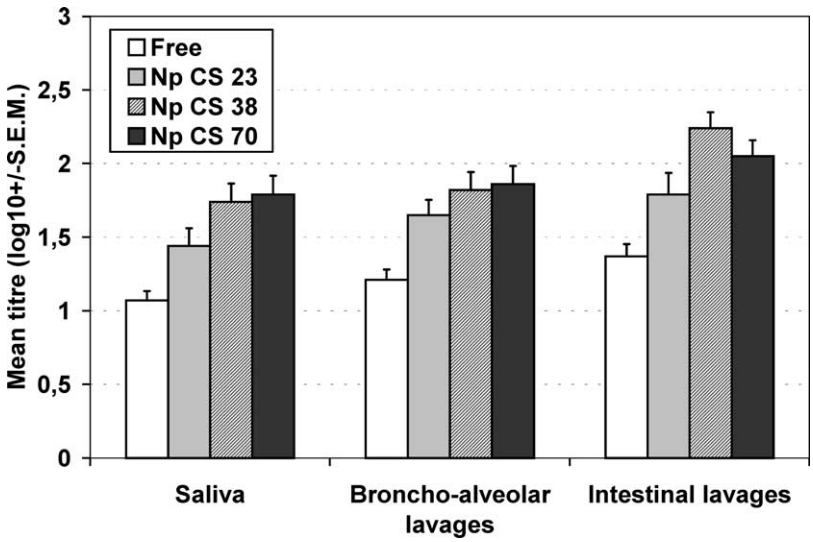

Fig. 5. IgA antibody levels after i.n. administration of 10 μg of TT free or encapsulated in CS nanoparticles (CS Mw of 23, 38 and 70 kDa). Titers were derived as the maximum dilution of saliva, broncho-alveolar lavages, and intestinal lavages giving an OD of 0.1 after subtraction of background and are presented as the geometric mean titer (GMT) per group.

point, we found that the IgG levels for CS 70 kDa nanoparticles were always significantly higher than those corresponding to the fluid vaccine, with the exception of those shown at 1 month. Similarly, the IgG concentration for CS 23 kDa nanoparticles at 6 months was not significantly different from that elicited by the fluid vaccine. In other words, the observed tendency for the lower Mw CS nanoparticles was to elicit a greater response at earlier times, reaching a maximum at week 12, and for the higher Mw nanoparticles to provide a slower, yet increasing, immune response for up to 24 weeks. Nevertheless, when the statistical analysis was only performed on the data corresponding to the nanoparticles (excluding control solutions) no significant effect of the CS Mw could be concluded. Therefore, these statistical analyses led us to propose that the CS Mw has only a minor effect on the efficacy of CS nanoparticles as TT delivery systems.

The results of IgA anti-tetanus levels in saliva, broncho-alveolar and intestinal lavages are shown in Fig. 5. As was the case with the humoral response, there were no significant differences between the IgA levels produced by the nanoparticles made of different CS Mw, these levels being, in all cases, significantly higher than those corresponding to the free toxoid.

It is not possible to relate the efficacy of the nanoparticles with the effect of the CS Mw on the permeability enhancing properties of CS solutions since this effect has not been definitely identified. It is believed that this effect may vary depending on the physiological and biological characteristics of the mucosal surface, as well as on the type, deacetylation degree of CS and presentation form of CS (solution vs. particles). Indeed, Schipper et al. [4] observed that the effect of the Mw on the ability of CS to increase membrane permeability on Caco-2 cell cultures was dependent on the deacetylation degree. In contrast, this effect was not observed by Aspden et al. [7] when studying the nasal absorption promoting effect of CS. Besides the differences in the CS types used in the reported studies, it should be noted that the experiments performed on CS solutions have only compared the behavior of relatively high Mw CSs (Mw higher than 70 kDa).

The results of the present study led us to suggest that the mode of action of CS nanoparticles is not significantly affected by the CS Mw. However, the greater response observed for low Mw CS particles at earlier times and for higher Mw CS particles at later times could be related to the inherent immunostimulatory properties of CS or to a different release rate of TT from low vs. high Mw CS nanoparticles. This speculation is contingent upon the assumption that CS molecules and/or CS nanoparticles cross the nasal mucosa and reach some specific body compartments from which there is a prolonged delivery of CS and the antigen. Clearly, these mechanisms need to be further investigated.

4. Conclusions

In light of these results, low Mw CS nanoparticles could be considered as promising new nasal vaccine delivery systems able to provide high and long-lasting mucosal and humoral immune responses.

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References

[1] O. Felt, P. Buri, R. Gurny, Chitosan: a unique polysaccharide for drug delivery, Drug Dev. Ind. Pharm. 24 (11) (1998) 979–993.
[2] P. Artursson, T. Lindmark, S.S. Davis, L. Illum, Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2), Pharm. Res. 11 (9) (1994) 1358–1361.
[3] G. Borchard, H.L. Lueßen, G.A. De Boer, J. Coos Verhoef, C.M. Lehr, H.E. Junginger, The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption III: effects of chitosan glutamate and carbomer on epithelial tight junctions in vitro, J. Control. Rel. 39 (3) (1996) 131–138.
[4] N.G.M. Schipper, K.M. Varum, P. Artursson, Chitosan as absorption enhancers for poorly absorbable drugs 1: influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelia, Pharm. Res. 13 (11) (1996) 1686–1692.
[5] N.G.M. Schipper, S. Olsson, A.J. Hoostraate, A.G. De Boer, K.M. Varum, P. Artursson, Chitosan as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement, Pharm. Res. 14 (7) (1997) 923–929.
[6] N.G.M. Schipper, K.M. Varum, P. Stenberg, G. Ockind, H. Hennernäis, P. Artursson, Chitosan as absorption enhances for poorly absorbable drugs 3: influence of mucus on absorption enhancement, Eur. J. Pharm. Sci. 8 (4) (1999) 335–343.
[7] T.J. Aspden, L. Illum, O. Skaugrud, Chitosan as a nasal delivery system: evaluation of insulin absorption and effect on nasal membrane integrity using rat model, Eur. J. Pharm. Sci. 4 (1) (1996) 23–31.
[8] I. Jabbal-Gill, A.N. Fisher, R. Rappuoli, S.S. Davis, L. Illum, Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice, Vaccine 16 (20) (1998) 2039–2046.
[9] A. Bacon, J. Makin, P.J. Sizer, I. Jabbal-Gill, M. Hinchcliffe, L. Illum, S. Chatfield, M. Roberts, Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens, Infect. Immun. 68 (10) (2000) 5764–5770.
[10] E.A. McNeela, D. O'Connor, I. Jabbal-Gill, L. Illum, S.S. Davis, M. Pizza, S. Peppoloni, R. Rappuoli, K.H.G. Mills, A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM_{197}) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery, Vaccine 19 (10) (2001) 1188–1198.
[11] P. Calvo, C. Remunan-López, J.L. Vila-Jato, M.J. Alonso, Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers, J. Appl. Pol. Sci. 63 (1) (1997) 125–132.
[12] P. Calvo, C. Remuñan-López, J.L. Vila-Jato, M.J. Alonso, Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines, Pharm. Res. 14 (10) (1997) 1431–1436.
[13] K. Janes, P. Calvo, M.J. Alonso, Polysaccharide colloidal particles as delivery systems for macromolecules, Adv. Drug Del. Rev. 47 (1) (2001) 83–97.
[14] R. Fernández-Urrusuno, P. Calvo, C. Remuñan-López, J.L. Vila-Jato, M.J. Alonso, Enhancement of nasal absorption of insulin using chitosan nanoparticles, Pharm. Res. 16 (10) (1999) 1576–1581.
[15] A. Vila, A. Sánchez, M. Tobío, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, J. Control. Rel. 78 (1) (2002) 15–24.
[16] C.D. Partidos, Intranasal vaccines: forthcoming challenges, Pharm. Sci. Technol. Today 3 (8) (2000) 273–280.
[17] D. Lemoine, M. Francotte, V. Préat, Nasal vaccines from fundamental concepts to vaccine development, Stp Pharma Sci. 8 (1) (1998) 5–18.
[18] A. Almeida, H.O. Alpar, R.W. Brown, Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly(L-lactic acid) microspheres in rats, rabbits and guinea-pigs, J. Pharm. Pharmacol. 45 (3) (1993) 198–203.
[19] E. Eyles, G.J.E. Sharp, E.D. Williamson, I.D. Spiers, H.O. Alpar, Intranasal administration of poly-lactic acid microsphere co-encapsulated Yersinia pestis subunits confers protection from pneumonic plague in the mouse, Vaccine 16 (20) (1998) 698–707.
[20] E. Eyles, E.D. Williamson, H.O. Alpar, Immunological responses to nasal delivery of free and encapsulated tetanus toxoid: studies on the effect of vehicle volume, Int. J. Pharm. 189 (1) (1999) 75–79.
[21] C. Yan, W.L. Rill, R. Malli, J. Hewetson, H. Naseem, R. Tammariello, M. Kende, Intranasal stimulation of long-lasting immunity against aerosol ricin challenge with ricin toxoid vaccine encapsulated in polymeric microspheres, Vaccine 14 (11) (1996) 1031–1038.
[22] A. Moore, P. Mcguirk, S. Adams, W.C. Jones, J.P. Mcgee, D.T. O'Hagan, K.H. Mills, Immunization with a soluble recombinant protein entrapped in biodegradable microparticles induces HIV-specific CD8 + cytotoxic T lymphocytes and CD4 + Th1 cells, Vaccine 13 (18) (1995) 1741–1749.
[23] A. Vila, A. Sánchez, C. Pérez, M.J. Alonso, PLA-PEG nanospheres: new carriers for transmucosal delivery of proteins and plasmid DNA, Polym. Adv. Technol. 13 (1) (2002) 1–8.
[24] M. Tobío, R. Gref, A. Sánchez, R. Langer, M.J. Alonso, Stealth PLA-PEG nanoparticles as protein carriers for nasal administration, Pharm. Res. 15 (2) (1998) 270–275.
[25] Q.P. Peniston, E.L. Johnson, Process for depolymerization of chitosan. U.S. Patent, 3,922,260 (1975).
[26] K.A. Janes, M.J. Alonso, Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization, J. Appl. Pol. Sci. 88 (12) (2003) 2769–2776.
[27] M. Tobío, M.J. Alonso, Study of the inactivation process of the tetanus toxoid in contact with PLGA degrading microspheres, Stp Pharma Sci. 8 (5) (1998) 303–310.
[28] M. Tobío, S.P. Schwendeman, Y. Guo, J. McIver, R. Langer, M.J. Alonso, Improved immunogenicity of a core-coated tetanus toxoid delivery system, Vaccine 18 (6) (2000) 618–622.
[29] D. Blanco, M.J. Alonso, Development and characterization of protein-loaded poly (lactide-co-glycolide) nanospheres, Eur. J. Pharm. Biopharm. 43 (3) (1997) 285–294.
[30] J. Iida, T. Une, C. Ishihara, K. Nishimura, S. Tokura, N. Mizukoshi, I. Azuma, Stimulation of non-specific host resistance against Sendai virus and Escherichia coli infections by chitin derivatives in mice, Vaccine 5 (4) (1987) 270–274.
[31] K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura, I. Azuma, Adjuvant activity of chitin and its derivatives, Vaccine 2 (1) (1984) 93–98.
[32] K. Nishimura, S. Nishimura, N. Nishi, F. Numata, Y. Tone, S. Tokura, I. Azzuma, Adjuvant activity of chitin derivatives in mice and guinea-pigs, Vaccine 3 (5) (1985) 379–384.
[33] V. Dodane, M.A. Khan, J.R. Merwin, Effect of chitosan on epithelial permeability and structure, Int. J. Pharm. 182 (1999) 21–32.