Novel Chitosan Particles and Chitosan-Coated Emulsions Inducing Immune Response via Intranasal Vaccine Delivery

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Purpose. The aim of this study was to prepare a novel vaccine carrier particulate system (nanoparticles and emulsions) with chitosan and to evaluate the effect of this system on the immune response for intranasal delivery.

Methods. Chitosan nanoparticles (NP) and chitosan-coated emulsions (CC-Emul) were prepared by improvement of the method previously reported and by modified ethanol injection methods, respectively. The rats were immunized with the particles adsorbed with ovalbumin (OVA) and cholera toxin (CT) by intranasal (i.n.) and intraperitoneal (i.p.) administration.

Results. NP and CC-Emul could be prepared with particle diameter from about 0.4 μm to 3 μm. IgG induced by i.n. of NP was comparable with that by i.p., and IgA induced by i.n. of 0.4-μm- and 1-μm-size NP was significantly higher than control (OVA and CT). IgG and IgA induced by i.n. of 2-μm-size CC-Emul were significantly higher than those with control.

Conclusions. The novel chitosan particles used simple preparation methods showed high OVA adsorption. When administered intranasally, NP and CC-Emul induced systemic immune response in rats. These findings suggested that CC-Emul and the smaller-size (0.4 μm) NP are effective for targeting to nasal-associated lymphoid tissues (NALTs) in nasal vaccine delivery.

KEY WORDS: chitosan; emulsion; immune response; intranasal; nanoparticle.

INTRODUCTION

Mucosal vaccine delivery is very attractive for inducing a protective immune response because many pathogens invade the body through mucosal surfaces. The main function of mucosa-associated lymphoid tissue is the selective uptake of antigens and the induction of local immune responses (1). In nasal inoculation, particle antigens are mainly taken by the M-cell connected to the nasal-associated lymphoid tissues (NALTs), whereas soluble antigens are mainly absorbed at the nasal epithelium (2,3). Particle antigens will be processed at the NALT and preferentially drain to the antigen-presenting cells (APCs). Because emulsions are directed to lymph, the development of antigen particulate carrier systems (nanoparticle and emulsion) that allow mucosal vaccine delivery is of considerable interest.

Chitosan derived by the deacetylation of chitin, which is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine, has high biodegradability and low toxicity. Chitosan particle delivery system can reduce the clearance rate from the nasal cavity, thereby increasing the contact time of the delivery system with the nasal mucosa (4). Chitosan suspensions or micro- and nanoparticles have been reported to have immune stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cell, promoting resistance to infections by microorganisms, and inducing cytokines (5). Among the various particle properties, the effective particle size on immune responses appears to be a key factor but has not been intensively investigated. The uptake of chitosan micro- (>1 μm) and nanoparticles from the nasal cavity was reported in the past decade (5,6). However, the optimal size of particles remains unclear for intranasal vaccine delivery. Although numerous nanoparticle preparation methods are known, sufficient antigen loading of nanoparticles and emulsions remains a challenge. Therefore, various-size novel chitosan particles and chitosan-coated emulsions loaded with antigen were prepared.

The aim of this study was to prepare and characterize chitosan nanoparticles and chitosan-coated emulsions for adsorptive loading of ovalbumin (OVA) and to evaluate the effect of particle size of chitosan nanoparticles (NP) and chitosan-coated emulsion (CC-Emul) loaded with OVA and cholera toxin (CT) on the immune response for intranasal vaccine delivery.

MATERIALS AND METHODS

Chemicals

Soybean oil and three kinds of chitosan (chitosan 10, chitosan 100, and chitosan 500 with a deacetylation degree of about 80 mol% with molecular weights of 10 kDa, 100 kDa, and 500 kDa, respectively) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). OVA was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA), and CT obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Eight-week-old male Wistar Kyoto Rats (WKY rat) were purchased from Oriental Yeast, Co. Ltd. (Tokyo, Japan). Tween 80 and oleic acid (OA) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Egg phosphatidylcholine (EPC) was from QP Co. Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Preparation of NP

Three different size NP (average diameters of about 700 nm, 1300 nm, and 3000 nm for individual batches) were prepared using chitosan 10, chitosan 100, and chitosan 500, respectively, by improvement of methods previously reported by Lubben *et al.* (7). Briefly, 0.25% (v/v) of chitosan 10, chitosan 100, and chitosan 500 solutions were prepared in 2% acetic acid aqueous solution. Then, 1 ml of 10% (w/v) sodium sulfate was added to 100 ml of each chitosan solution. Moreover, to obtain smaller NP, the NP sample prepared by chi-

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ABBREVIATIONS: APC, antigen presenting cell; CC-Emul, chitosan-coated emulsions; CT, cholera toxin; EPC, egg phosphatidylcholin; NALTs, nasal-associated lymphoid tissues; NP, chitosan nanoparticles; OA, oleic acid; OVA, ovalbumin.

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tosan 10 in a diameter of 700 nm was sonicated to reduce the particle size to 300 nm. After preparation of NP with different sizes, samples were centrifuged for 1 h at 48,000 rpm for 300-nm NP and at 10,000 rpm for other-sized NP, respectively. After the pellets were freeze-dried overnight, the 40-mg resultant pellets were resuspended in 1 ml of 0.2% (w/v) Tween 80 solution at pH 11.0 to adjust nanoparticle suspension at pH 6.0. For the administration dosage form, 0.01 mg CT and 20 mg OVA were added to 1 ml of resultant NP and incubated at room temperature overnight.

Preparation of CC-Emul

Emulsions with the size of 0.4 μm and 0.7 μm were prepared by a modified ethanol injection method (8). Briefly, 100 mg of soybean oil, 60 mg of EPC, and 60 mg of OA were dissolved in 5 ml hot ethanol, and then 10 ml Milli Q water was added. After ethanol was removed with part of Milli Q water, 5-ml emulsions formed. Then, 0.25 ml of the aqueous solution dissolved with 2 mg Tween 80 and 2 mg chitosan 10 was added to 0.25 ml of the 0.4-μm-size emulsion (total oil and lipid 44 mg/ml) adjusted to about pH 5.0 by 0.1 M NaOH. Chitosan-coated emulsion (CC-Emul, sized 0.4 μm) was prepared by addition of 20 mg of OVA and 0.01 mg of CT to a final volume of 1 ml of the above emulsions with shaking at room temperature overnight. CC-Emul sized 2 μm was prepared as above except using 0.7-μm-size emulsion and double the amounts of each component and addition of Milli Q water slowly by the modified ethanol injection method. This emulsion was diluted 2-fold before use for CC-Emul sized 2 μm.

The cumulant diameter and ζ-potential of the particles in water were measured by the dynamic and electrophoretic light-scattering method, respectively, using a laser light-scattering instrument (Model ELS-800, Otsuka Electronics, Osaka, Japan).

Determination of OVA Adsorption Amount to NP and CC-Emul

The relative adsorption amount of OVA to NP and CC-Emul was calculated by determining the amount of protein remaining in the supernatant after centrifugation at 48,000 rpm, using bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The adsorption ratio was estimated using the following equation;

$$\text{Adsorption ratio}\,(\%) = \frac{\text{OVA}_{\text{total conc.}} - \text{OVA}_{\text{superntant conc.}}}{\text{OVA}_{\text{total conc.}}} \times 100$$

Immunization Protocol and Enzyme-Linked Immunosorbent Assay

Each group of rats was immunized by i.n. and i.p. with one of the following vaccine formulations on days 0, 14, and 28, following the method of Staats *et al.* (9). Fifty microliters of the various-size NP and CC-Emul were administered as approximately 200 g to rats via one nostril with a polyethylene tube. Control rats received the same concentration of OVA and CT in 0.2% (w/v) Tween 80 Milli Q solution.

Blood samples were collected from the jugular vein–anesthetized rats (receiving pentobarbital at a dose of 50 mg/kg following i.p.) via i.p. and i.n. administration on day 35. Sera were separated by centrifugation at 13,000 rpm for 4 min and were stored at 4°C.

Concentrations of IgG and IgA in serum were measured according to the Rat IgG and IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA). Titration of rat anti-OVA IgG in serum was measured according to the anti-ovalbumin IgG ELISA Kit (Genesis Diagnostics Ltd, Cambridgeshire, UK).

Statistical Analysis

All values are expressed as means ± SD. Statistical significance of the data was evaluated by Student's *t* test. A p value of 0.05 or less was considered significant. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

Characterization of NP

Considering the toxicity of Tween 80, we prepared NPs by decreasing the concentration of Tween 80. This method showed the size of NP smaller than the method by Lubben *et al.* (7). The characterization of NP is summarized in Table I. The particle size of NP was increased with increasing molecular weights of chitosan. OVA adsorption into NP decreased the ζ-potentials, but remained positive in ζ-potential (about 25 mV) although the adsorbed ratio of OVA was relatively high at greater than 80%. The release of OVA from NP was not observed during 3 h in PBS solution pH 7.2 at 37°C (data not shown).

Particulate vaccine delivery carrier should be targeted to lymphoid tissue as antigen sampling cells provide access to mucosal lymphoid tissue (7). Because the M-cells connecting lymphoid tissue take up antigens and microparticles smaller than 10 μm, particulate systems for antigen drug delivery require micro-size particles to be taken up by M-cells, neither by epithelial cells, nor by drug release upon arrival at the mucosae. Therefore, it was expected that as the particle size of NP obtained in this study was relatively small and the amount of OVA released from NP was low, vaccination may be effectively achieved.

Characterization of CC-Emul

With the addition of chitosan solution at weight ratios greater than 0.1 of chitosan to total oil and lipids in the emulsions, the average diameters of particles became almost con-

Table I. Characterization of NP Without Cholera Toxin

NP (μm)	OVA*	Average diameter (nm)	ζ-potential (mV)	Adsorbed OVA† (%)
0.4	–	310 ± 3.6	27.3 ± 0.5	
	+	385 ± 8.5	24.7 ± 0.6	84.8 ± 0.3
1	–	692 ± 13.6	28.7 ± 0.9	
	+	1102 ± 80	24.7 ± 0.3	89.1 ± 2.0
2	–	1355 ± 168.2	29.3 ± 3.4	
	+	2048 ± 313	25.4 ± 0.6	88.0 ± 1.1
3	–	3080 ± 147.7	32.2 ± 3.9	
	+	3287 ± 419	25.6 ± 0.4	78.9 ± 0.4

NP, chitosan nanoparticles; OVA, ovalbumin. Data are Mean ± SD (n = 3).

* Chitosan nanoparticles (40 mg) adsorbed OVA (20 mg). NP (0.4 and 1 μm), NP (2 μm), and NP (3 μm) were prepared using chitosan 10, 100, and 500, respectively.

† Calculated from the amount of free OVA determined using the BCA protein assay kit.

Table II. Characterization of Chitosan-Coated Emulsion with Ovalbumin (OVA) and Cholera Toxin (CT)*

Emulsions	Average diameter (nm)	ζ-potential (mV)	Adsorbed OVA† (%)
Emulsion (0.4 μm)	362.1 ± 8.4	−48.8 ± 4.1	
Chitosan-coated‡	362.2 ± 13.0	17.0 ± 0.6	
+ OVA and CT§	391.0 ± 6.6	10.2 ± 1.6	96.6 ± 0.2†
Emulsion (2 μm)	729.6 ± 26.6	−48.6 ± 0.9	
Chitosan-coated	897.3 ± 274.1	14.3 ± 2.3	
+ OVA and CT§	1811.3 ± 15.0	10.3 ± 0.8	105.0 ± 11.4†

Data are mean ± SD (n = 3).
* Chitosan-coated emulsion adsorbed OVA and CT (CC-Emul).
† Calculated from the amount of free OVA determined by BCA protein assay kit.
‡ Chitosan 2 mg, total oil and lipid of emulsion 11 mg per ml.
§ OVA 20 mg, CT 0.01 mg per ml of chitosan-coated emulsion.

stant. Corresponding to the change of size, the ζ-potential of negatively charged emulsions became positive by coating chitosan (data not shown). Therefore, for further experiments, the 0.15 weight ratio of chitosan was used to the total oil and lipids of the emulsion for CC-Emul as the chitosan adsorption may be saturated.

After the addition of chitosan to the emulsion, the average diameters of the emulsions increased slightly, while the ζ-potential of the emulsions sized 0.4 μm and 0.7 μm with about −49 mV became 17 mV and 14.3 mV, respectively (Table II). Furthermore, after the addition of OVA and CT, the 362-nm and 897-nm approximate diameters of CC-Emul were increased to 391 nm and 1811 nm, respectively, and their ζ-potential decreased to about 10 mV. The adsorbed ratio of OVA to CC-Emul was nearly 100% (Table II). These results suggested that adequate amounts of chitosan to bind OVA completely existed on the surface of the emulsions. These CC-Emul represent CC-Emul sized 0.4 μm and 2 μm, respectively, from the final size of emulsions. The OVA released from CC-Emul was not detected after 3 h incubation in PBS solution pH 7.2 at 37°C (data not shown). Relatively smaller CC-Emul particles appeared to be more stable than larger ones.

Immunization Following i.p. and i.n. Administration of NP

Figure 1 shows the immune activity of antibody at 35 days after the first immunization following i.p. and i.n. administration of NP. NP following i.p. administration induced significantly higher IgG than control (Fig. 1A). Chitosan has a positive charge and adjuvant activity after i.p. administration in mice (10). Our finding may correspond to the report; the positively charged liposomes are effectively transferred to APC rather than negatively charged ones by i.p. administration (11).

NP following i.n. administration induced significantly higher IgG antibody response compared with control (Figs. 1A and 1B). Production of anti-OVA IgG obtained in this immunization study was comparable to that of IgG in the blood (data not shown). NP sized 0.4 μm and 1 μm showed significantly higher production of IgA compared with the 3-μm-size NP. The uptake of chitosan microparticles has previously been reported (6), but not that of nanoparticles. The reason might be due to the recongnition of NPs by M cells.

Microparticles are retained in the M-cells and induce mucosal immunity, whereas nanoparticles can also be taken up from the NALTs and also induce systemic immunity. Uchida *et al.* reported that 4-μm-size synthetic polymer particles showed higher IgG antibody response than 1.3-μm-size ones for oral administration (12). Jung *et al.* (13) reported that the oral and i.n. administration of the about 0.5-μm-size synthetic polymer particles with negative ζ-potential increased IgG, but those of particles >1 μm did not. However, in the current results, there was no significant difference in the IgG production between the different-size NP following i.n. administration.

Immunization Following i.p. and i.n. Administration of CC-Emul

Although i.p. application of CC-Emul did not increase IgG production compared with control (Fig. 2A), IgA levels

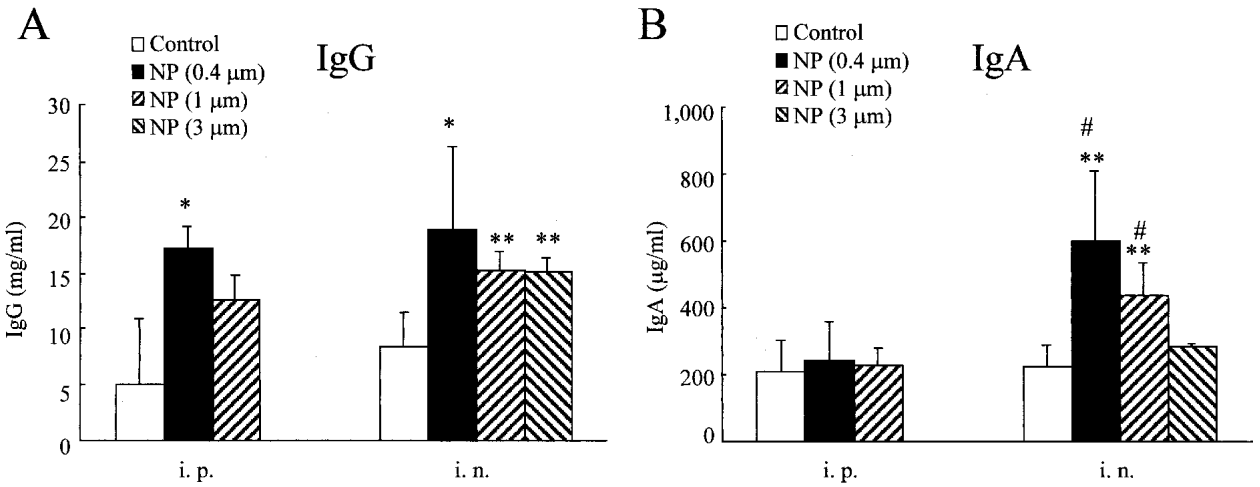

Fig. 1. The (A) IgG and (B) IgA concentrations from rats (n = 3 to 5) immunized by i.p. and i.n. on days 0, 14, and 28 with OVA (5 mg/kg) and CT (2.5 μg/kg) alone for control and NP in Milli Q water. The particle diameter of NP is approximately 0.4 μm, 1 μm, and 3 μm. The sera were collected on day 35. Data are mean ± SD. *$p < 0.05$ and **$p < 0.01$ compared with control, and #$p < 0.05$ compared with NP (3 μm).

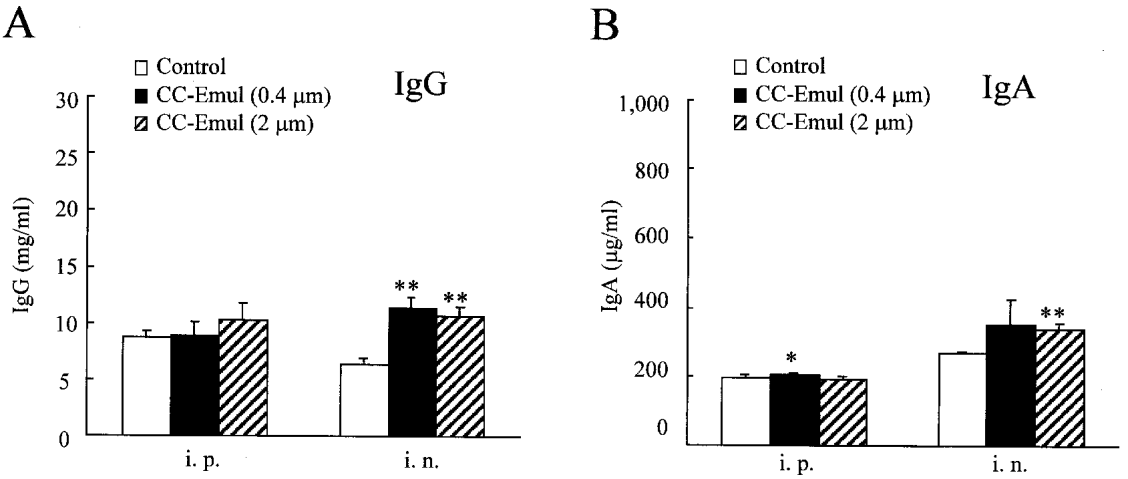

Fig. 2. The (A) IgG and (B) IgA concentrations from rats (n = 3 to 5) immunized by i.p. and i.n. on days 0, 14, and 28 with OVA (5 mg/kg) and CT (2.5 μg/kg) alone for control and CC-Emul in Milli Q water. The particle diameter of CC-Emul is approximately 0.4 μm and 2 μm. The sera were collected on day 35. Data are mean ± SD *$p < 0.05$ and **$p < 0.01$ compared with control.

were similar to those by NP (Fig. 2B). Intranasal administration of CC-Emul sized 2 μm showed significantly higher IgG and IgA antibody responses compared with control. Antibody responses after i.n. administraion between NP and CC-Emul were not different, and each antibody reached similar levels in both particles. No significant difference of IgG and IgA productions was seen between 0.4-μm- and 2-μm-size CC-Emul. This finding corresponds well to NP.

CC-Emul after i.p. administration did not increase IgG production. The reason was not clear, but basically emulsions are easily transferred to lymphoid tissue, so this might render the effect of particle size for immunization by CC-Emul irrelevant. Nevertheless, further experiments are needed to elucidate the difference in the production of antibody by formulations.

CONCLUSIONS

The novel chitosan particles (nanoparticles and emulsions) used simple preparation methods showed high OVA adsorption. OVA was hardly released from each formulation, suggesting that the developed particulate vaccine system may retain antigen on particles until uptake into mucosal membrane. Moreover, chitosan nanoparticles and emulsions administered i.n. induced a significantly higher immune response compared with control, and also, this response was comparable with i.p. injection. From these findings, the chitosan particulate system for nasal vaccine delivery could be a promising candidate.

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