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Evaluation of Eudragit-coated chitosan microparticles as an oral immune delivery system

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Abstract

Chitosan microparticles containing ovalbumin (OVA), OVA-containing chitosan microparticles (Chi-OVA), were prepared, coated with Eudragit L100 (ER), and evaluated as oral vaccine. Chi-OVA with an OVA content of 34.4% (w/w) and a mean particle size of 2.3 μm were used for experiments in vitro and in vivo. ER-coated Chi-OVA (ER-Chi-OVA) contained 3.6–20.5% (w/w) OVA and had a particle size of 47.9–161.1 μm. Chi-OVA dissolved readily in JP 14 first fluid, but not in JP 14 second fluid. The release of OVA from Chi-OVA was suppressed extensively in JP 14 second fluid. ER-Chi-OVA did not dissolve in JP 14 first fluid, and the release of OVA was suppressed greatly in JP 14 first and second fluids. OVA solution, Chi-OVA and ER-Chi-OVA (200 and 800 μg OVA/mouse) were administered to Balb/C mice twice at a 1-week interval. At 7 d after the second administration, plasma OVA-specific IgG and fecal OVA-specific IgA levels were measured. OVA-specific IgG tended to be enhanced in Chi-OVA and ER-Chi-OVA, but was the highest in OVA solution. OVA-specific IgA was induced significantly more efficiently by ER-Chi-OVA than the others. These suggested that ER-Chi-OVA should be possibly useful to induce an intestinal mucosal immune response.

Keywords: Chitosan microparticle; Oral vaccine; Ovalbumin; Eudragit L100; Immune response

1. Introduction

Many infections occur via the mucosal surface. However, most of vaccinations are performed by parenteral administration, in which systemic immune responses are induced but mucosal immune responses are not. Parenteral vaccinations require trained personnel and sterilized materials, and infusion often causes non-compliance to patients. Oral vaccinations have no such limitations and can induce both systemic and mucosal immune responses (O'Hagan et al., 1989; Tabata et al., 1996; Trolle et al., 1998; Wikingsson and Sjoholm, 2002; Minato et al., 2003). The induction of mucosal immune responses is also important for the prevention of infection; that is, mucosal immunization can prevent the entry of infectious matter. Thus, much attention

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has been paid to mucosal immunization, especially oral vaccination, as an easy and acceptable approach.

However, the simple oral administration of antigens elicits little immune responses because gastric acid, various hydrolytic enzymes, thick mucus, etc. degrade or remove the ingested antigens (Walker and Taylor, 1978; Fushiki et al., 1985; Kunisawa et al., 2001; Takagi et al., 2003). Therefore, various delivery systems have been investigated in order to make oral immunizations more effective. In particular, microparticules with a diameter of less than 10 μm are reported to show good adjuvant effects (Eldridge et al., 1990; Uchida and Goto, 1994; Tabata et al., 1996; Nakamura et al., 1998). Such particles can protect the antigens from degradation in the stomach and intestine, and deliver them efficiently to the gut associated lymphoid tissue (GALT) located in the lower portion of the small intestine. When such microparticles reach the Peyer's patches in the GALT, they can be taken up by M-cells located between the epithelial cells. The internalization by M-cells appears to be an important initial step of an oral vaccination (Tabata et al., 1996). Also, the mucoadhesive properties and release control of antigens are associated with the effectiveness as well as the protection of the antigens, and the mucoadhesion to the middle and lower part of the small intestine appears to facilitate the delivery of the antigens to Peyer's patches due to closer contact with luminal surface (Kunisawa et al., 2001). Further, the antigens seem to need to be released from the delivered sites, the Peyer's patches; at that time, a gradual release is effective in enhancing the immune response (Uchida and Goto, 1994; Nakamura et al., 1998; Kunisawa et al., 2001). The effect of particle size on the degree of induction and pattern of the immune responses has been reported. PLA or PLGA microspheres with a diameter of 4 μm best induced systemic immune responses (Tabata et al., 1996; Uchida and Goto, 1994), and PLA microspheres of 7 μm best induced the mucosal immune responses (Tabata et al., 1996). Gelatin microspheres with a diameter of a few micrometers also greatly enhanced the mucosal immune response (Nakamura et al., 1998).

Recently, chitosan microparticles have been used for the delivery of antigens to Peyer's patches (Van der Lubben et al., 2001a, 2002). Chitosan itself exhibits mucobioadhesive properties to the mucosal membrane probably because of its cationic and viscous properties, and is considered suitable for the delivery to specific sites of the intestine (Takeuchi et al., 1996; Takishima et al., 2001, 2002). Chitosan microparticles with a diameter of several micrometers were reported to enhance the uptake of a model antigen ovalbumin (OVA) in Peyer's patches (Van der Lubben et al., 2001b,c). However, as chitosan is easily dissolved in the acidic stomach, simple oral administration results in the dissolution or collapse. In this study, OVA-containing chitosan microparticles (Chi-OVA) are prepared, coated with Eudragit L100 (ER) to protect chitosan microparticles from dissolution or collapse under gastric conditions, and characterized in vitro in terms of physical stability and dissolution. Further, systemic and mucosal immune responses are examined in vivo after oral administration to mice.

2. Materials and methods

2.1. Materials

Chitosan 10 (Chi), used as chitosan throughout the study, and polysorbate 80 (Tween 80) were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Ovalbumin was obtained from Calbiochem (USA). Eudragit L100 (ER) was obtained from Röhm GmbH (Germany). Sorbitan sesquioleate (SO-15) was purchased from Nikkko Chemicals Co. Ltd. (Japan). The BCA Protein Assay Kit was purchased from Pierce (USA). Mouse IgG and IgA ELISA Quantitation Kits were obtained from Bethyl Laboratories Inc. (USA). 3,3′,5,5′-Tetrametyl benzidine (TMB) Microwell Peroxidase Substrate System was purchased from Kirkegaad & Perry Laboratories Inc. (USA).

2.2. Animals

Female BALB/c mice (6-week-old), being bred under the specific pathogen-free (SPF) conditions, were purchased from Charles River Japan Inc., housed in SPF conditions and used within 1 week after purchase. The animals were kept on the breeding diet MF (Oriental Yeast Co. Ltd., Japan) with water ad libitum at a room temperature maintained at $23 \pm 1\,^{\circ}C$ and a relative humidity of $60 \pm 5\%$. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Tokyo, Japan, and the animal

experiments were performed in compliance with Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

2.3. Preparation of OVA-containing chitosan microparticles (Chi-OVA) and ER-coated Chi-OVA (ER-Chi-OVA)

Chitosan microparticles containing OVA (Chi-OVA) were prepared using both ionic gelation and W/O emulsification–solvent evaporation as follows: Chi-OVA(1):Chi (250 mg) was dissolved in 100 ml of 2% (v/v) acetic acid aqueous solution containing 1% (w/v) Tween 80. Four milliliters of a 10% (w/v) sodium sulfate aqueous solution containing 200 mg of OVA was dripped over 5 min into the Chi solution as it was being stirred with a paddle at 500 rpm and sonicated at 45 kHz and 100 W using a sonicator VS-100 III SUNPAR (Iuchi-seieido, Japan). The stirring and sonication were continued for another 20 min. The precipitate was collected by centrifugation of the mixture at 3000 rpm for 20 min, and washed twice with 50 ml of water by centrifugation. The precipitate was suspended in water and lyophilized to yield Chi-OVA(1). Chi-OVA(2):Chi (100 mg) was dissolved in 5 ml of a 1% (v/v) acetic acid aqueous solution, and 2 ml of aqueous solution containing OVA (200 mg) was added. The Chi solution containing OVA was dripped over 5 min into 200 ml of liquid paraffin containing SO-15 at 1% (w/v), which were being stirred with a paddle at 500 rpm and sonicated at 45 kHz and 100 W using the above sonicator. The stirring and sonication were continued for another 10 min. The W/O emulsion obtained was stirred vigorously with a magnetic stirrer under reduced pressure at 40 °C for 24 h, diethyl ether was added, and the mixture was, further, stirred overnight with a magnetic stirrer. The particles obtained were washed with diethyl ether, ethanol, a 25% (w/w) ammonia aqueous solution, ethanol and diethyl ether in that order. The particles were obtained by filtration and dried in air to yield Ch-OVA(2).

ER-coated Chi-OVA particles (ER-Ch-OVA) were prepared by coating Chi-OVA(2) with ER. Four kinds of ER-Chi-OVA were prepared as follows: ER-Chi-OVA(1):Chi-OVA(2) were suspended in a methanol solution containing an equal amount of ER, and the methanol was evaporated in air so that the suspension became semisolid. The semisolid was sieved using mesh with openings of 600 μm. The obtained semisolid particles were dried in air and ground gently by hand using a mortar and pestle. ER-Chi-OVA(2):Chi-OVA(2) were suspended in a methanol solution containing ER at twice the amount of Chi-OVA(2), and the methanol was evaporated completely in air. The solid obtained was ground by hand using a mortar and pestle. ER-Chi-OVA(3): the particles were prepared in the same manner as ER-Chi-OVA(2) except that the methanol solution contained ER at three times the amount of Chi-OVA(2). ER-Chi-OVA(4): the particles were prepared in the same manner as ER-Chi-OVA(2) except that the methanol solution contained ER at 10 times the amount of Chi-OVA(2).

2.4. Drug content and particle size of Chi-OVA and ER-Chi-OVA

Chi-OVA (3 mg) were dissolved in 2 ml of JP 14 first fluid (pH 1.2) by stirring with a vortex mixer, and 150 μl of the solution was subjected to BCA protein assay using a commercial kit to measure the amount of OVA. In all the BCA protein assays, the standard curve method was used. After the final solution was centrifuged at 3000 rpm for 3 min, the supernatant was measured spectrophotometrically at 562 nm using a Hitachi 220A spectrophotometer (Japan). ER-Chi-OVA(1) (3 mg), ER-Chi-OVA(2 or 3) (5 mg) or ER-Chi-OVA(4) (10 mg) were stirred magnetically in 30 ml of methanol and centrifuged at 3000 rpm for 10 min. The residue was washed with 30 ml of methanol three times, and dried in air. The dried product was dissolved in 2 ml of JP 14 first fluid as shown above, and the amount of OVA in 150 μl of the solution was measured in the same manner as in Chi-OVA. The OVA content was calculated from the amounts of OVA observed and the particles used.

Chi-OVA were coated with platinum at the thickness of 10 nm, and observed using a JEOL JSM-5600LV scanning electron microscope (JEOL, Japan). The Green diameters of 150 particles chosen at random were measured. ER-Chi-OVA were observed with a Real Surface View VE-7800 electron microscope (Keyence, Japan). Further, the Green diameters of 200 ER-Chi-OVA particles chosen at random were measured using an Olympus System Biology BX50 microscope (Olympus, Japan).

2.5. In vitro release from Chi-OVA and ER-Chi-OVA

Chi-OVA (10 mg) were suspended in 5 ml of JP 14 second fluid (pH 6.8), and incubated at 37 °C and 90 rpm. At 1, 3, 6 and 24 h after the start of the incubation, 300 μl of each medium was taken, centrifuged at 3000 rpm for 10 min, and 150 μl of the supernatant was used for the measurement of the concentration of OVA released. The remaining supernatant and precipitate were returned to the incubated medium. The OVA concentration was measured by BCA protein assay. In that assay, the final sample was obtained after centrifugation at 3000 rpm for 3 min, and measured spectrophotometrically at 562 nm.

ER-Chi-OVA (60 mg) were suspended in 5 ml of JP 14 first fluid and incubated at 37 °C and 90 rpm. At 1 h after the start of the incubation, the suspension was centrifuged at 3000 rpm for 10 min, and 150 μl of the supernatant was taken for measurement of the concentration of the OVA released. After the remaining supernatant was discarded, JP 14 second fluid (5 ml) was added to the precipitate. The suspension was incubated at 37 °C and 90 rpm. At 1, 3, 6 and 24 h after the start of the incubation in JP 14 second fluid, 300 μl of the medium was taken and centrifuged at 3000 rpm for 10 min, and 150 μl of the supernatant was used for the measurement of the concentration of OVA released. The remaining supernatant and precipitate were returned to the incubated medium. BCA protein assay was used to measure the concentration of OVA. In that assay, the final sample was centrifuged at 3000 rpm for 3 min, and the upper solution was measured spectrophotometrically at 562 nm.

2.6. Immunization

The mice were separated in metabolic cages to be bred with one per cage, and starved for 24 h. Then, each sample was administered as follows. ER-Chi-OVA: ER-Chi-OVA(1) corresponding to 200 μg or 800 μg of OVA were suspended in 80 μl of a 20% (w/v) polyethylene glycol 20,000 aqueous solution, and placed in a Teflon tube attached to the end of a syringe. After the suspension was administered into the stomach, 80 μl of the 20% (w/v) polyethylene glycol 20,000 aqueous solution was administered. Then, 80 μl of normal saline was, further, administered to avoid a loss of administration amount. Chi-OVA: Chi-OVA(2) corresponding to 200 μg or 800 μg of OVA were suspended in 80 μl of a 0.05 M carbonate buffer (pH 9.6) containing polyethylene glycol 20,000 at 20% (w/v), and placed in a Teflon tube attached to the end of a syringe. After the suspension was administered into the stomach, 80 μl of the 0.05 M carbonate buffer containing polyethylene glycol 20,000 at 20% (w/v) was administered. Further, 80 μl of 0.05 M carbonate buffer (pH 9.6) was administered to avoid a loss of administration amount. OVA solution: OVA (200 μg or 800 μg) dissolved in 80 μl of the 0.05 M carbonate buffer (pH 9.6) containing polyethylene glycol 20,000 at 20% (w/v) was administered into the stomach, and then 80 μl of 0.05 M carbonate buffer (pH 9.6) was, further, administered to make the sample completely ingested. Control: Untreated mice were used as the control. This immunization was done twice at a 1-week interval. At 6 d after the second immunization, mice of all the groups were separated in metabolic cages to be bred with one per cage. After 24 h, a blood sample was taken from the heart using a heparinized syringe, and the plasma was obtained by centrifugation at 3000 rpm for 5 min. The plasma was used for the measurement of OVA-specific IgG. At the same time, the feces were recovered, suspended in phosphate-buffered saline, pH 7.4, (PBS) at concentration of 100 mg/ml, homogenized using a Teflon pestle and centrifuged at 3000 rpm for 5 min. The supernatant was used as a fecal extract for the measurement of OVA-specific IgA.

2.7. Assay by ELISA

The OVA-specific IgG in plasma and the OVA-specific IgA in feces were examined by ELISA. OVA solution (10 μg/ml) was prepared using 0.05 M carbonate buffer of Na_2CO_3–$NaHCO_3$, pH 9.6, which was also used in Section 2.6, and 100 μl of the solution was added to each well of the ELISA plate and incubated overnight at 4 °C. The plate was then washed three times using 0.05 M Tris–HCl buffer, pH 8, containing NaCl at 0.14 M (TBS) with Tween 20 at 0.05% (w/v) (TBS-T). Two hundred microliters of TBS with bovine serum albumin at 1% (w/v) was added to each well and incubated at 37 °C for 1 h,

and the plate was washed three times using TBS-T. After the plasma and fecal extract were diluted using TBS with bovine serum albumin at 1% (w/v), the diluted samples were added to the plate at 200 μl/well and incubated at 37 °C for 2 h. Then, the plate was washed four times using TBS-T. TBS-T containing the goat anti-mouse IgG-horseradish peroxidase conjugate or the goat anti-mouse IgA–horseradish peroxidase conjugate was added to the plate at 100 μl/well and incubated at 37 °C for 2 h. Then, the plate was washed four times using TBS-T. A TMB/H_2O_2 mixture prepared according to the direction by TMB Microwell Peroxidase Substrate System (Kirkegaad & Perry Laboratories Inc., USA) was added to the plate at 100 μl/well and incubated at room temperature for 30 min. Then, 1 M phosphoric acid aqueous solution was added to each well at 100 μl/well, and each well was measured for optical density at 450 nm using a Toso MPR A4i micro plate reader (Toso Co., Japan) within 30 min. OVA-specific IgG responses were compared using the optical densities given by samples obtained with dilution of the plasma to eight-fold volume. OVA-specific IgA responses were evaluated by comparison of the optical densities given by the original fecal extract. Further, the endpoint titers were calculated as the reciprocal $\log_2$ of the last dilution that gave a mean optical density at 450 nm being 0.2 greater than that obtained with non-immunized mice (control).

2.8. Statistical analysis

The results were compared using ANOVA followed by Scheffe's *F* post hoc test. A significant difference was set at $P < 0.05$.

3. Results

3.1. Particle characteristics of Chi-OVA and ER-Chi-OVA

Chi-OVA were prepared by ionic gelation and emulsification–solvent evaporation. Although an aqueous suspension of Chi-OVA was obtained by ionic gelation using sodium sulfate, the resulting microparticles (Chi-OVA(1)) tended to aggregate after lyophilization of the aqueous suspension, which was observed with a scanning electron microscope. Therefore, their particle size is not described in Table 1. Chi-OVA(1) exhibited a very low OVA content and encapsulation efficiency. On the other hand, Chi-OVA prepared by the water-in-oil method (Chi-OVA(2)) showed good granulation. The particle shape was irregular, but somewhat spherical (Fig. 1A). The mean particle diameter was 2.3 μm with a size distribution of 1–5 μm. The mean OVA content was 34.4% (w/w), and the encapsulation efficiency was more than 50% (Table 1). Thus, Chi-OVA(2) was used in the subsequent experiment.

ER-Chi-OVA were prepared at different combination ratios of Chi-OVA to ER. As shown in Table 2, the drug content was almost proportional to the ratio of Chi-OVA. Encapsulation efficiencies were 95.3–119.2%, indicating that the ideal drug content was well achieved. All the ER-Chi-OVA microparticles were much larger than Chi-OVA. ER-Chi-OVA(2–4), prepared by simply grinding the dried mixture of Chi-OVA and ER, showed a larger size with the increase in amount of ER. ER-Chi-OVA(1), obtained by passage through mesh and subsequent grinding, were larger than ER-Chi-OVA(2). The particle shape of ER-Chi-OVA(1) was irregular, but rugged ellipsoid as shown in Fig. 1B. As ER-Chi-OVA(1) had the

Table 1
Particle characteristics of Chi-OVA prepared by different methods

Formulation	Chi (mg)	OVA (mg)	Preparative method	OVA content (%, w/w)[a]	Particle diameter (μm)[b]	Encapsulation efficiency (%)[c]
Chi-OVA(1)	250	200	Ionic gelation	1.3 ± 0.3	–	2.9
Chi-OVA(2)	100	200	Emulsification–solvent eveporation	34.4 ± 2.8	2.3 ± 1.0	51.6

[a] The results are expressed as the mean ± S.D. ($n = 3$).
[b] The results are expressed as the mean ± S.D. ($n = 150$).
[c] The results are calculated using the mean values.

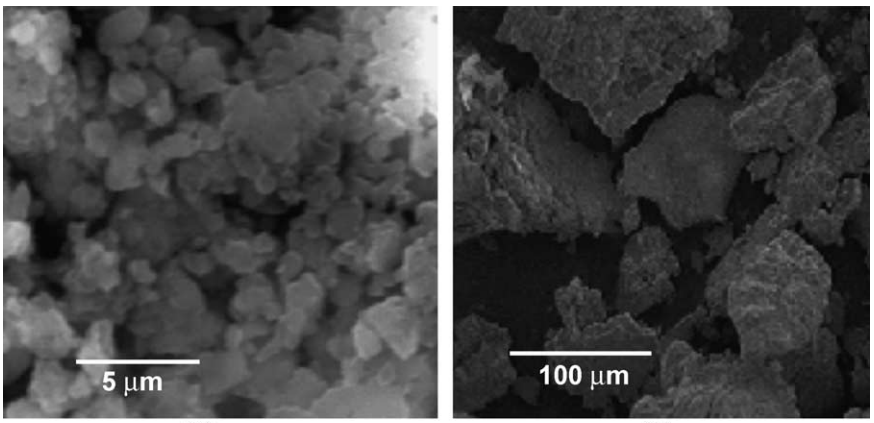

Fig. 1. Scanning electron micrographs of (A) Chi-OVA(2) and (B) ER-Chi-OVA(1). The lengths of the white bars in (A) and (B) express 5 and 100 μm, respectively.

highest drug content, they were used in the subsequent experiment.

3.2. In vitro release from Chi-OVA and ER-Chi-OVA

Chi-OVA dissolved easily in JP 14 first fluid but not in JP 14 second fluid. Therefore, the release studies were performed using only JP 14 second fluid. Fig. 2 shows the in vitro release of OVA from Chi-OVA(1–2) in JP 14 second fluid. Chi-OVA(1) exhibited an initial burst of approximately 10%, then the drug was hardly released for 23 h. On the other hand, Chi-OVA(2) exhibited an initial release of less than 2%, then OVA was released very slowly. At 24 h after the release test, nearly 5% was released. Thus, the release of OVA from Chi-OVA(2) was suppressed extensively in JP 14 second fluid.

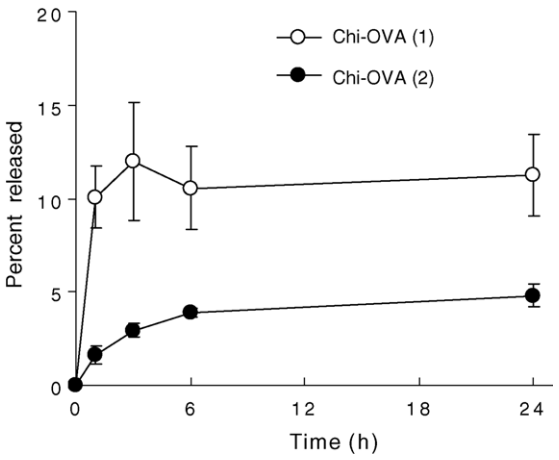

Fig. 2. Release profiles of OVA from Chi-OVA in JP 14 second fluid (pH 6.8) at 37 °C. Each point represents the mean ± S.D. ($n = 3$).

The release of OVA from ER-Chi-OVA(1–4) was examined in JP 14 first fluid for the first 1 h and in JP 14 second fluid for the following 24 h. As the amount of microparticles used in the release tests was constant, 60 mg, the amount of OVA released increased with the increase in the OVA content (Fig. 3A). A small amount of OVA was released in the incubation in JP 14 first fluid. After changing the incubation medium to JP 14 second fluid, OVA was slightly released in JP 14 second fluid. The percentages of OVA released in the release tests for 25 h was in the range of 7–10% for all the ER-Chi-OVA. Thus, OVA was retained well in Chi-OVA under these test conditions. It was visually observed that ER dissolved after changing JP 14 first fluid to JP 14 second fluid.

3.3. In vivo immune responses

Formulations were administered twice at a 1-week interval. Samples were taken at a 1-week after the second administration. Fig. 4 shows the plasma OVA-specific IgG response of each formulation, in which the optical densities at 450 nm were compared among the samples that were obtained with the dilution of the

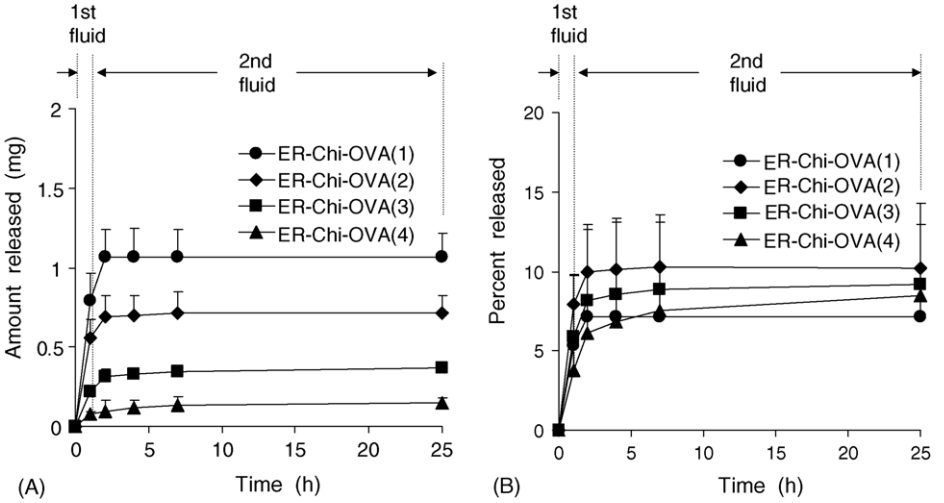

Fig. 3. Release profiles of OVA from ER-Chi-OVA in JP 14 first fluid (pH 1.2) for the initial 1 h and in JP 14 second fluid (pH 6.8) for the next 24 h at 37 °C. The results are expressed in (A) the amount released and (B) percent released. Each point represents the mean ± S.D. ($n = 3$).

plasma to eight-fold volume. ER-Chi-OVA exhibited a significantly higher response than the control at 200 μg OVA/mouse, and tended to be more effective than Chi-OVA. Chi-OVA also exhibited a greater systemic immune response than control. As to the OVA solution (200 and 800 μg OVA/mouse), even the sample obtained by diluting the plasma to 256-fold or more volume displayed a larger mean OVA-specific IgG level than the sample for ER-Chi-OVA or Chi-OVA obtained by diluting plasma to eight-fold volume, though the OVA-specific IgG level for the OVA solution was deviated greatly. Therefore, the data for the OVA solution were not described. All the samples except the control tended to exhibit a greater immune response at 800 μg OVA/mouse than at 200 μg OVA/mouse. However, significant difference was not detected at 800 μg OVA/mouse.

The mucosal OVA-specific IgA responses are shown in Fig. 5, in which the optical densities at 450 nm were compared for the original fecal extracts. At 200 μg OVA/mouse, ER-Chi-OVA displayed a significantly higher level of IgA than the control, OVA solution and Chi-OVA. Also, at 800 μg OVA/mouse, OVA-specific IgA responses tended to increase in the order

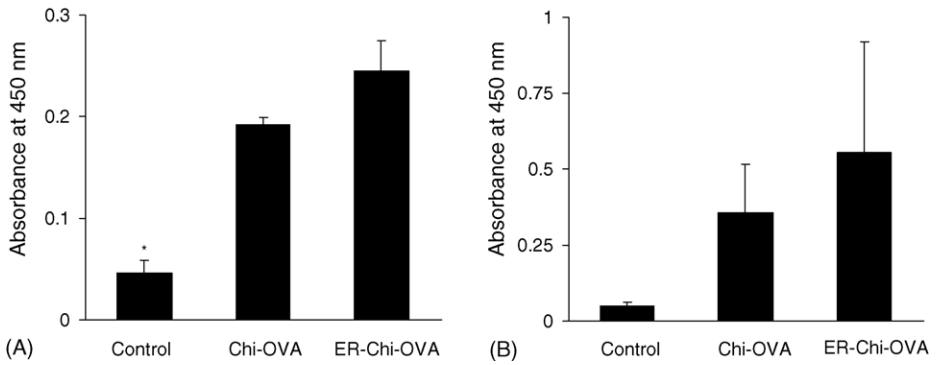

Fig. 4. Plasma OVA-specific IgG responses induced by oral administration of several samples at (A) 200 μg OVA/mouse and (B) 800 μg OVA/mouse. Chi-OVA(2) and ER-Chi-ER(1) were used as Chi-OVA and ER-Chi-OVA, respectively. The results are expressed as the optical densities at 450 nm given by the samples obtained with the dilution of the plasma to eight-fold volume. Each column represents the mean ± S.E. ($n = 3$). The OVA solution is not shown due to a much higher response being out of range, and described in the text. $^*P < 0.05$ vs. ER-Chi-OVA.

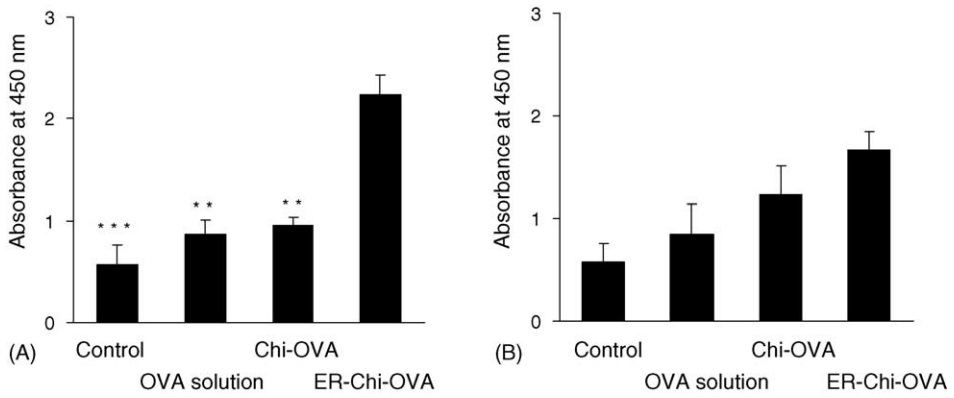

Fig. 5. Fecal OVA-specific IgA responses induced by oral administration of several samples at (A) 200 μg OVA/mouse and (B) 800 μg OVA/mouse. Chi-OVA(2) and ER-Chi-ER(1) were used as Chi-OVA and ER-Chi-OVA, respectively. The results are expressed as the optical densities at 450 nm given by the original fecal extract. Each column represents the mean ± S.E. ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. ER-Chi-OVA.

ER-Chi-OVA > Chi-OVA > OVA solution > control. However, no significant difference was observed among the samples at 800 μg OVA/mouse.

The endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an mean optical density at 450 nm being 0.2 greater than that obtained with non-immunized mice (Table 3). This also indicated that the OVA-specific IgG level tended to be greater with the increase in the dose. As to the OVA-specific IgA level, ER-Chi-OVA exhibited a higher titer at a dose of 200 μg OVA/mouse than 800 μg OVA/mouse, while the titers of OVA solution and Chi-OVA were hardly changed in both the doses.

4. Discussion

The purpose of the present study is to develop an oral mucosal immune drug delivery system using chitosan which is less toxic, mucoadhesive and biodegraded by the intestinal bacteria. A well-characterized protein antigen OVA was used as a model antigen. The potential for immunization is dependent on the physicochemical and biological characteristics of the system. In microparticulte systems, the particle size and release profiles of antigens play an essential role in the immunization potentials. The microparticles with a diameter of several micrometers appeared to enhance the immunization potential. When PLGA or PLA microspheres were used as a carrier of the antigen OVA, the microspheres with a diameter of 4 μm induced the best systemic immune responses (Uchida and Goto, 1994; Tabata et al., 1996), and PLA microspheres with a diameter of 7 μm induced the best mucosal immune responses (Tabata et al., 1996). These correlated with the uptake by the Peyer's patches and subsequent biodisposition and release of OVA. The internalization of an antigen into M-cells existing in the Peyer's patches is the initial step of the oral vaccination. Therefore, the delivery efficiency of the microparticles to the Peyer's patches is considered important for the elevation of immune responses. Further, the release of OVA is essentially related to the immunization potential. Both a quick release of OVA and a complete suppression of OVA release are inadequate for the induction of immune responses (Uchida and Goto, 1994; Nakamura et al., 1998; Kunisawa et al., 2001). A long-term immune response was observed in the particulate systems releasing OVA gradually (Uchida and Goto, 1994; Tabata et al., 1996; Nakamura et al., 1998). Especially, the gradual release of OVA from the Peyer' patches appears to be important for a mucosal immune response (Tabata et al., 1996).

The Chi microparticles containing OVA (Chi-OVA) were prepared with two methods, that is, ionic gelation using sodium sulfate and W/O emulsification–solvent evaporation using surfactant SO-15. When Chi-OVA were prepared with ionic gelation, the OVA content was very low, and the small microparticles were not obtained well after lyophilization (Table 1). Since OVA

is water-soluble, its incorporation into ionic gel might be difficult. Further, as the ionic Chi–sulfate complex swelled in aqueous medium, ionic gel particles were probably entangled together, resulting in aggregation during the lyophilization process. On the other hand, Chi-OVA prepared with emulsification–solvent evaporation (Chi-OVA(2)) exhibited limited aggregation, leading to a mean diameter of 2.3 μm. As the microparticles were dried after washing with organic solvent in emulsification–solvent evaporation, their aggregation was considered to be suppressed (Fig. 1A). Further, the incorporation of OVA into Chi-OVA was achieved efficiently using emulsification–solvent evaporation (Table 1). Chi-OVA dissolved easily in JP14 first fluid within several minutes by stirring at room temperature when the drug content was determined. However, the release of OVA from Chi-OVA in JP 14 second fluid was suppressed to a large extent, which was considered based on the less soluble properties of Chi under neutral and basic pH conditions and the high molecular weight of OVA (Fig. 2). The suppressed release in Chi-OVA was considered to be advantageous for protection from hydrolytic enzymes and prolonged release of OVA. Thus, Chi-OVA(2), obtained by the emulsification–solvent evaporation method, were considered to be useful for oral vaccination and used in the in vivo experiment.

ER-Chi-OVA were prepared by the suspension of Chi-OVA(2) in the ER methanol solution, subsequent evaporation and grinding of the solid obtained. When the ratio of ER to Chi-OVA was changed in several formulations, Chi-OVA were well mixed with ER, and recovered efficiently in all the cases (Table 2). ER-Chi-OVA(2–4) were prepared by simply grinding the solid of the mixture of Chi-OVA and ER. As the ratio of ER to Chi-OVA increased, the particles became larger. The increase of ER appeared to make the particles irrefrangible. When the ratio of ER to Chi-OVA was small in the preparation of ER-Chi-OVA, simple grinding might break down Chi-OVA themselves and make the integrity of the ER coating lost. Therefore, in the preparation of ER-Chi-OVA(1), a semisolid of the mixture of Chi-OVA(2) and ER, obtained by incomplete evaporation of the methanol, was first sieved with a mesh, dried in air, then the dried particles were ground gently. This different preparative procedure made ER-Chi-OVA(1) larger than ER-Chi-ER(2) although the ratio of ER to Chi-OVA was less in ER-Chi-OVA(1) than in ER-Chi-ER(2). The SEM of ER-Chi-OVA(1) indicated that Chi-OVA were masked well with ER (Fig. 1B). The release of OVA from ER-Chi-OVA was suppressed in JP 14 first and second fluids (Fig. 3). The small release in JP 14 first fluid suggested that the ER coating might not be necessarily complete. However, the shape and size of the ER-Chi-OVA were maintained during the incubation in the first fluid. Therefore, some Chi-OVA located near the surface of ER-Chi-OVA might allow the release of OVA from ER-Chi-OVA in JP 14 first fluid. Further, the particles were observed visually to be finer after the dissolution medium was changed from JP 14 first fluid to JP 14 second fluid. ER-coated another drug-containing chitosan microparticles with several dozen micrometers were found make the original Chi microparticles regenerated relatively easily in JP 14 second fluid (data not shown). These indicated that the ER coated could dissolve readily in JP 14 second fluid and suggested that Chi-OVA could be regenerated from ER-Chi-OVA in the lower small intestine, though the regeneration of Chi-OVA from ER-Chi-OVA in vivo will need to be checked to make clear such phenomena.

Table 2
Particle characteristics of ER-Chi-OVA prepared by different methods

Formulation	Chi-OVA[a]:ER (w/w)	OVA content (%, w/w)[b]	Particle diameter (μm)[c]	Encapsulation efficiency (%)[d]
ER-Chi-OVA(1)	1:1	20.5 ± 4.7	72.2 ± 23.1	119.2
ER-Chi-OVA(2)	1:2	12.2 ± 0.5	47.9 ± 11.5	106.4
ER-Chi-OVA(3)	1:3	8.2 ± 2.1	97.0 ± 19.3	95.3
ER-Chi-OVA(4)	1:10	3.6 ± 0.3	161.1 ± 40.4	115.0

[a] Chi-OVA(2) were used as Chi-OVA.
[b] The results are expressed as the mean ± S.D. ($n = 3$).
[c] The results are expressed as the mean ± S.D. ($n = 200$).
[d] The results are calculated using the mean values.

Table 3
OVA-specific antibody responses induced by oral administration

Susbstance	Dose (μg/mouse)	Plasma anti-IgG titer (reciprocal $\log_2$)	Fecal anti-IgA titer (reciprocal $\log_2$)
OVA solution	200	>7[a]	1
	800	>7[a]	0
Chi-OVA	200	<3[b]	0
	800	3	3
ER-Chi-OVA	200	<3[b]	5
	800	5	3

[a] In the dilution extent greater than 128, the mean optical density at 450 nm was 0.2 greater than that of control (no-immunized mice).
[b] In the dilution extent of 8, the mean optical density at 450 nm was not 0.2 greater than that of control (no-immunized mice).

The induction of OVA-specific IgG was compared based on the optical densities given by the samples obtained by diluting the plasma to eight-fold volume, and that of OVA-specific IgA was evaluated from the optical densities presented by the original fecal extract (Figs. 4 and 5). Further, the endpoint titers were calculated as the reciprocal $\log_2$ of the last dilution that gave a mean optical density at 450 nm being 0.2 greater than that obtained with non-immunized mice (Table 3). The reason for using a mean optical density in the calculation of endpoint titers was due to the large deviation of individual values.

As to OVA-specific IgG, as the OVA solution overall displayed much higher response than others, though the individual values were deviated largely. Thus, the dilution extent of the plasma was started from 8 in the determination of OVA-specific IgG (Fig. 4). In many papers reported, OVA solution gave only slight induction of systemic immune response (Kunisawa et al., 2001; Uchida and Goto, 1994; Uchida et al., 1994; Tabata et al., 1996), which was different from the present result. One of the reasons is to use a larger amount of OVA (200 and 800 μg OVA/mouse) in the present study. The larger amount of OVA seems to elevate the systemic immune response of OVA solution (Uchida and Goto, 1994; Uchida et al., 1994). The difference in a solvent used is considered as another reason. Namely, 0.05 M carbonate buffer of pH 9.6 containing polyethylene glycol 20,000 at 20% (w/v) was used as a solvent in the present study, while saline or PBS were used in other reports. The alkaline buffer might neutralize the stomach acid and suppress the peptic degradation of OVA (Walker and Taylor, 1978; Fushiki et al., 1985; Takagi et al., 2003). However, since Kunisawa et al. (2001) reported that the duodenal administration of OVA solution in PBS hardly induced OVA-specific IgG, polyethylene glycol 20,000 might also be associated with the induction of OVA-specific IgG by the OVA solution. Since the localization of OVA in the lower small intestine and sustained supply of OVA appears to be related to the OVA-specific IgG response (Kunisawa et al., 2001), polyethylene glycol 20,000 might contribute to such localization or sustained supply. Actually, when OVA solution in saline (100 μg OVA/mouse) was administered in the same manner, OVA-specific IgG was approximately two-fold greater in OVA solution (100 μg OVA/mouse) than control. However, the present high OVA-specific IgG response was difficult to explain. Therefore, this point will have to be studied further. ER-Chi-OVA and Chi-OVA tended to show a higher OVA-specific IgG response than control, and ER-Chi-OVA had a tendency to exhibit a greater OVA-specific IgG response than Chi-OVA (Fig. 4). ER-Chi-OVA were considered to protect Chi-OVA until delivered to the lower intestine, while Chi-OVA were considered to adhere to gastrointestinal mucosa before reaching GALT, leading to the lower expression of OVA-specific IgG in Chi-OVA than in ER-Chi-OVA.

As to the expression of OVA-specific IgA, the OVA solution was less effective than Chi-OVA and ER-Chi-OVA, different from the results in OVA-specific IgG. The systemic and mucosal responses appear to be influenced differently by antigen biodistribution and its release time period etc. (Tabata et al., 1996; Kunisawa et al., 2001). The prolonged retention and sustained supply of OVA in Peyer's patches appears to be important for elevation of OVA-specific IgA (Tabata et

al., 1996; Kunisawa et al., 2001). That was considered as one of the reasons for the lower induction potency of the OVA solution. At the dose of 200 μg OVA/mouse, ER-Chi-OVA induced significantly more OVA-specific IgA than others. Further, the endpoint titer was achieved most greatly by ER-Chi-OVA with 200 μg OVA/mouse (Table 3). This suggested that Chi-OVA would reach GALT more efficiently by administration of ER-Chi-OVA. Chi-OVA appeared not to be delivered efficiently to the target sites, which was considered due to the widespread gastro-intestinal mucoadhesion as described above. The dissolution in the stomach and mucoadhesive properties of Chi microparticles were considered to prevent Chi-OVA from moving to the lower intestine. At the dose of 800 μg OVA/mouse, although ER-Chi-OVA tended to exhibit the best induction of OVA-specific IgA, the induction potency of ER-Chi-OVA was not elevated as compared with that at 200 μg OVA/mouse, and no significant difference was observed among the samples. Since a large amount of particles were administered at 800 μg OVA/mouse, the saturation of the uptake by M-cells or limited access of the particles to Peyer's patches might have occurred, leading to the suppression of the elevation of OVA-specific IgA at a higher dose of 800 μg OVA/mouse. As discussed above, in ER-Chi-OVA, the ER coat is expected to prevent Chi-OVA from dissolving at an stomach acidic pH and to allow Chi-OVA to be regenerated in them to dissolve gradually in the lower intestine. According to Van der Lubben et al. (2002), Chi microparticles with several micrometers can be taken up well by Peyer's patches. Thus, Chi-OVA regenerated around the GALT from ER-Chi-OVA are considered to be taken up by Peyer's patches and release OVA for a long period, resulting in the elevation of OVA-specific IgA. The detailed biological fates such as histological distribution of the microparticles and in vivo release profiles of OVA will have to be examined in order to elucidate the detailed mechanism and to refine the present system.

5. Conclusion

Emulsification–solvent evaporation gave Chi-OVA which had a size adequate for uptake by Peyer's patches and a high OVA content. The coating with ER protected Chi-OVA from the dissolution in JP 14 first fluid (pH 1.2). Chi-OVA released OVA very slowly in JP 14 second fluid, and ER-Chi-OVA suppressed the release of OVA from JP 14 first fluid and JP 14 second fluid (pH 6.8). Chi-OVA and ER-Chi-OVA tended to show a higher OVA-specific IgG level, though OVA solution overall exhibited the highest OVA-specific IgG level. On the other hand, the fecal OVA-specific IgA level was highest with ER-Chi-OVA, suggesting that ER-Chi-OVA should deliver Chi-OVA efficiently to Peyer's patches and activated GALT effectively. It is suggested that ER-Chi-OVA should display the better induction of OVA-specific IgA by achieving the delivery of Chi-OVA around Peyer's patches, the subsequent uptake by M-cells and the gradual release of OVA.

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