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Research paper

Association complexes between ovalbumin and cyclodextrins have no effect on the immunological properties of ovalbumin

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Abstract

Delivery systems are designed to deliver necessary amounts of drugs without modifying their biological features. Cyclodextrins (CD) are potential candidates for such a role due to their ability to alter physical and chemical properties of guest molecules by the formation of inclusion/association complexes. They have already been used to stabilize and solubilize peptides and proteins of biological importance. However, no systematic study has been reported on their immunological effects upon coupling to such proteins. Herein, we prepared and characterized the association complexes between α-, β- and hydroxypropyl-β-cyclodextrin and ovalbumin (Ova). Afterward we tested the effect of CD coupling in the Ova antigenicity and the immunological effects of CD coupling on Ova oral and subcutaneous administration. Our results clearly show that CD-coupled Ova elicits the same immunological activities as uncoupled Ova. Therefore, we conclude that CDs are immunologically safe for use as delivery systems in animals.

Keywords: Cyclodextrin; Ovalbumin; Immunization; Oral tolerance; Antigenicity

1. Introduction

The primary purpose of drug delivery systems is to deliver the necessary amount of drug to the target site, efficiently and precisely [1–3]. In addition, a fundamental aspect of these delivery systems is that they should work without modifying the desired biological features of the drug. Considerable efforts have been focused on developing new strategies for drug-controlled release systems based on biodegradable polymers [4], bioceramics [5], lipossomes [6], emulsions [7] and cyclodextrins (CD) [8,9].

CDs are potential candidates for such a role, due to their ability to alter physical, chemical and biological properties of guest molecules by the formation of inclusion and/or association complexes [10]. Recent advances in biotechnology (such as recombinant protein expression) have accelerated the economical, large-scale production of therapeutically active peptide and protein-based drugs. This rapid progress in molecular biology, however, has not been matched by the progress in the formulation, biological characterization and development of delivery systems for peptides and protein drugs.

CDs have been used to stabilize and solubilize peptides and proteins such as insulin, IL-2, TNF, monoclonal antibodies, aspartame and lactate dehydrogenase [11]. They can enhance drug absorption in the nasal mucosa and also protect it from chemical and enzymatic degradation [10,12]. These properties of CDs are very attractive especially for oral application. The oral route is the ideal route for pharmaceutical formulations, but it raises problems that had to be overcome. Proteins are unstable in the acid pH of the stomach, can be enzymatically degraded in the gastrointestinal tract, and have a poor absorption rate in the intestine which lowers their bioavailability. Hence coupling to CDs is a strategy to help overcome these problems.

An important aspect to remember when working with proteins is that they are antigenic molecules. Therefore,

Abbreviations: Ova, ovalbumin; CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; i.p., intraperitoneal; s.c., subcutaneous; CD, cyclodextrins; FTIR, Fourier transform infrared spectroscopy; TG, thermogravimetry curve; DTG, first derivative of thermogravimetry curve; DSC, differential scanning calorimetry; XRD, X-ray pattern diffraction.

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a systematic study on the immunological effects of these modified proteins is an obligatory requirement before their use in humans. It has been reported that even subtle modifications in a protein structure can completely change its antigenic properties leading to completely different reactivities in an organism [13–15].

It is well known that the administration route of a protein has a major influence on the immunological reactivity it evokes. Oral administration usually leads to oral tolerance, a state of systemic hyporesponsiveness to the antigen while oral immunization (systemic reactivity) occurs only in some exceptional situations [16]. Some strategies to achieve oral immunization and therefore enable the development of oral vaccines have been tried by coupling the antigen to adjuvants [17] or simply by modifying the protein's structure [13–15]. On the other hand, subcutaneous (s.c.) administration of an antigen usually leads to immunization but not without the help of adjuvants.

In the oral tolerance field, it is also well known that antigen modifications can alter its immunological properties. Results usually vary from enhancing [17,18] to impairing [19–22] oral tolerance induction.

There are considerable hurdles to be overcome before practical use of therapeutic peptides and proteins can be achieved, mainly due to chemical and enzymatic instability, poor absorption through biological membranes and tissues, rapid plasma clearance, peculiar dose–response curves, and unknown immunological effects. Many strategies have been attempted to circumvent these problems with proteins and peptides by their chemical modification or usage of delivery systems [11]. The main purpose of this work is to characterize the physical–chemical nature of the interaction between ovalbumin (Ova; an antigenic protein largely employed in immunological studies) and α-, β- and hydroxypropyl-β-CD (modern delivery systems). We also present a systematic study of the immunological effects of the coupling process on the antigenicity and immunogenicity of Ova.

2. Materials and methods

2.1. Materials

The α-, and β-CD were donated by Wacker Corporation (Eddyville, IA, USA). The hydroxypropyl-β-CD was purchased from Amaizo® (Hammond, IN, USA) and used without further purification. Ova, molar weight 43,000, grade V, approx. 99% (agarose eletrophoresis) was purchased from Sigma (St Louis, MO, USA).

2.2. Methods

2.2.1. Preparation of association complexes with ovalbumin and α-, β- and hydroxypropyl-β-cyclodextrin

Three types of association compounds were prepared: Ova:α-CD (ovalbumin coupled to α-cyclodextrin), Ova:β-CD (ovalbumin coupled to β-cyclodextrin) and Ova:HP-:β-CD (ovalbumin coupled to hydroxypropyl-β-cyclodextrin). In brief, the association compounds were prepared by the mixture of 1.0 g (2.3×10^{-5} mol) of Ova and 1.0 g of CDs, i.e. (1.0×10^{-3} mol α-CD, 8.0×10^{-4} mol β-CD and 7.0×10^{-4} HP-:β-CD) by the freeze-drying method, using Labconco Freezone model 177, for a period of approximately 48 h. Physical mixtures of Ova and the different CDs were also prepared in the same molar ratio of the association compounds for comparison. Characterization of the association compounds was achieved by X-ray pattern diffraction (XRD) powder pattern diffraction, thermogravimetry curve (TG) and differential scanning calorimetry (DSC) thermal analysis and Fourier transform infrared (FTIR) spectroscopy. The XRD was obtained using a RIGAKU diffractometer filtered with an LiF monochromator and employing the Cu Kα (1.5405 nm) radiation as a source (15 kV, 15 mA). The TG and first derivative of thermogravimetry curve (DTG) were performed on a Shimadzu TGA-50 thermogravimetric analyzer, using a dynamic N_2 atmosphere and a heating rate of 10 °C/min. DSC curves were obtained from a Shimadzu DSC-50 apparatus with the same heating rate and atmosphere. Finally, FTIR spectra were recorded in a MATTSON FTIR-3500, in KBr pellets, in the range of 4000–500 cm^{-1}.

2.2.2. Animals used for biological tests

We used 8–10-week-old B6D2F1 (C57BL6 × DBA/2J) mice that were bred and maintained in our animal facility at Instituto de Ciências Biológicas, UFMG. The animal experiments were developed following standard procedures approved by UFMG ethical committee.

2.2.3. Oral administration of the antigen

Animals received a gavage (intragastric administration) of 10 mg of Ova, alone or coupled to CDs as described, diluted in sterile saline.

2.2.4. Immunizations with ovalbumin

Seven days after the oral treatments, mice were s.c. immunized in their tail base, with 100 μg Ova emulsified in 20 μl of complete Freund's adjuvant (CFA, Sigma, St Louis, MO, USA). For antibody assays they were bled 28 days post-immunization.

2.2.5. Immunizations with cyclodextrin-coupled Ova

Mice were s.c. immunized at the base of the tail with 100 μg Ova (alone or coupled to CDs) diluted in 40 μl of sterile saline. They were boosted 14 days later with an i.p. immunization of 10 μg Ova.

2.2.6. Enzyme-linked immunosorbent assay (ELISA) for serum antibody measurements

Serum levels of anti-Ova antibodies were determined by standard ELISA assay. In short, 96-well microtiter assay plates were coated overnight at 4 °C with 2 μg Ova in

sodium carbonate buffer (pH 9.6). Plates were washed with saline containing 0.05% Tween 20, blocked with 0.25% casein in PBS, washed and incubated with diluted serum samples for 1 h. Plates were then washed again and incubated with goat anti-mouse IgG1 antibodies (Southern Biotechnology, Birmingham, AL, USA), washed and incubated with rabbit anti-goat IgG HRP (Southern Biotechnology, Birmingham, AL, USA). They were then washed and incubated with H_2O_2 in the presence of orthophenylene diamine (OPD) in sodium citrate buffer (pH 5.0) for 15 min. Reaction was interrupted by the addition of 20 μl of 2N H_2SO_4. Optical densities were obtained using an automatic ELISA reader (Bio-Rad Model 450 Microplate Reader) at 492 nm.

2.2.7. ELISA for antigenicity tests

To access the stability of Ova epitopes upon coupling to CDs, we carried out a modified ELISA. In short, 96-well microtiter assay plates were coated overnight at 4 °C with 2 μg of Ova or Ova coupled to α-, β- and hydroxypropyl-β-CD in sodium carbonate buffer (pH 9.6). The ELISA was then carried out as described above with serum samples from Ova-immunized mice.

2.2.8. Antigen-specific T-cell proliferation for oral tolerance study

Eight days after immunization with Ova in CFA, mice inguinal lymph nodes were aseptically removed and single-cell suspensions were prepared in RPMI 1640 medium containing 10% fetal calf serum (FSC), L-glutamine, penicillin, streptomycin and gentamycin (Gibco BRL). Cells were cultured with 5 mg/ml Ova for 72 h at 37 °C in a moist atmosphere of 5% CO_2. During the last 7 h of incubation, cells were pulsed with 1 μCi of tritiated thymidine (^{3}[H]thymidine) and then harvested. The amount of radioactivity incorporated by cell DNA was determined by scintillation counting in a counter for beta radiation. Cells cultured without antigen served as negative controls and cells cultured with concanavalin A as positive controls.

2.2.9. In vitro proliferation for immunogenicity tests

Spleen cells from Ova-immunized mice were harvested 14 days post-immunization. Red blood cell lysis was carried out and single cell suspensions were obtained and cultured with 0.5 mg/ml Ova alone or coupled CDs. The proliferation was accessed as described above.

2.3. Statistical analysis

Comparisons between groups were performed using analysis of variance (ANOVA) followed by Tukey test (family error of 5%).

3. Results and discussion

3.1. Ova and cyclodextrins form association compounds

Although FTIR infrared spectra normally is not a good probe to monitor the host–guest interaction using CDs, since the strong CD's vibrational modes are underneath the guest's vibrational ones, there is some infrared modification. After Ova:α-CD interaction we notice a narrowing of the symmetric and asymmetric OH, NH stretching, at 3500–3200 cm^{-1}, when compared to the FTIR spectra of the physical mixture and of the Ova (Fig. 1). This result suggests that breakdown and formation of new hydrogen bonding after the association with the protein are taking place. A higher protein crystallinity of the Ova:α-CD is also observed when compared to the physical mixture and to the Ova spectra at lower frequencies (below 1000 cm^{-1}). This result could be due to changes in the Ova partition coefficient upon CD interaction. The same vibrational behavior is also observed for Ova:β-CD and Ova:β-CD compounds (results not shown).

The association compounds TG/DTG curves in all cases do not reveal major evidence of the protein–CD interaction. But the DSC curves for the Ova:CD compounds show some evidence of host–guest interaction. The α-CD DSC curve shows four endothermic peaks at 50, 75, 141 and 280 °C and one exothermic peak at 320 °C. Thus, the two initial thermal

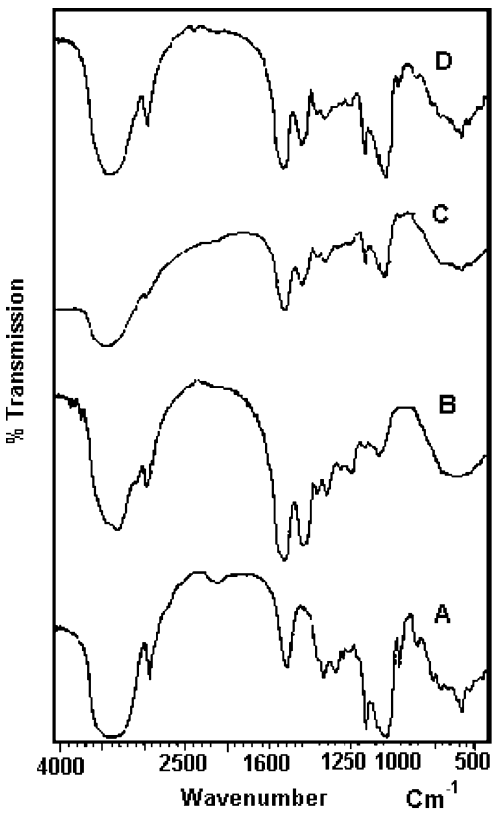

Fig. 1. FTIR spectra of α-cyclodextrin (A), ovalbumin (B), physical mixture (C) and association complex (D).

phenomena could be associated to two types of loss of water, the third peak with CD phase transition, the fourth one to melting point and the exothermic peak to the thermodecomposition process. Interestingly the Ova:α-CD DSC curve shows a different thermal profile in comparison to the Ova or α-CD alone or their physical mixture, since the CD phase transition peak (141 °C) and the Ova phase transition peak (228 °C) were not observed. In contrast,

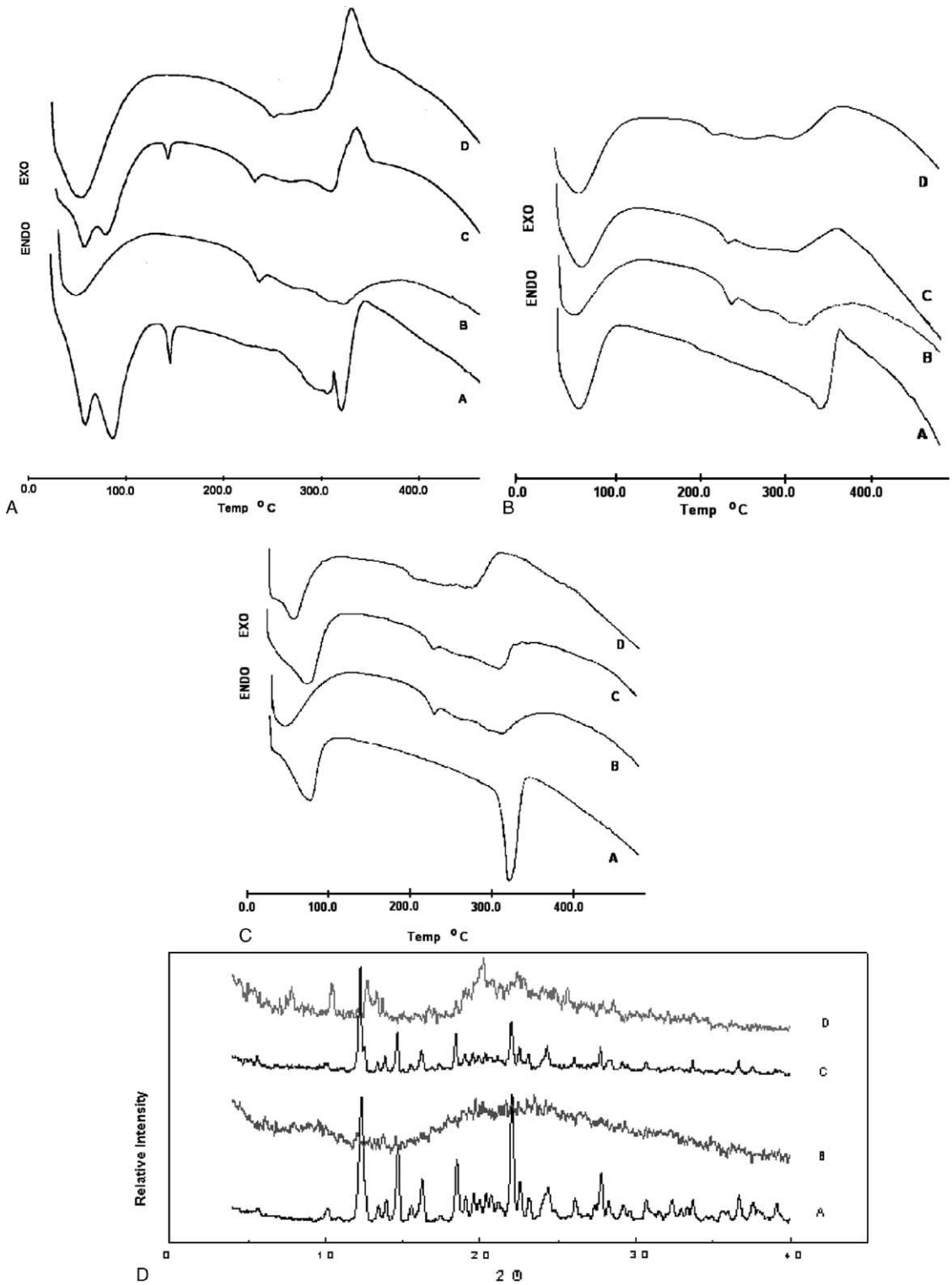

Fig. 2. (A) DSC curves of α-cyclodextrin (A), ovalbumin (B), physical mixture (C) and association complex (D). (B). DSC curves of HP-β-cyclodextrin (A), ovalbumin (B), physical mixture (C) and association complex (D). (C). DSC curves of β-cyclodextrin (A), ovalbumin (B), physical mixture (C) and association complex (D). (D). XRD pattern diffraction of α-cyclodextrin (A), ovalbumin (B), physical mixture (C) and association complex (D).

the physical mixture DSC curve shows four endothermic peaks (Fig. 2A). These results suggest the Ova:α-CD interaction.

The DSC curves of the Ova:HP-β-CD and Ova:β-CD reveal a different thermal behavior when compared to the curves from Ova, HP-β-CD, β-CD alone or their respective physical mixture (Fig. 2B and C). The most important evidence of host–guest interaction in these cases is the absence of Ova phase transition peak or lower this temperature (228 °C) in the respective association complex.

Finally the Ova:α-CD XRD diffraction pattern revealed formation of new crystalline phases that are not observed in the physical mixture. Moreover higher crystallinity was also observed when compared to the amorphous Ova XRD pattern, reinforcing the FTIR results discussed above (Fig. 2D). The same increase in crystallinity was observed in Ova:HP-β-CD and Ova:β-CD compounds (results not shown).

3.2. Antigenicity of ovalbumin is not modified by coupling to cyclodextrins

The first biological parameter analyzed was a putative change in the antigenicity of Ova after coupling to CDs. To access this possibility we stimulated spleen cells from Ova-immunized mice in vitro with Ova, Ova:α-CD, Ova:β-CD or Ova:HP-β-CD in concentrations of 0.5 mg/ml. As we can see in Fig. 3, cells are equally stimulated by Ova or Ova coupled to the CDs indicating that the antigenicity of Ova is not affected by coupling to any of the CDs. This profile only changes when we raise the concentration to 5 mg/ml, when in vitro stimulation with Ova:α-CD and Ova:β-CD leads to cell death (data not shown), probably due to their toxicity at high concentrations as already reported by others in

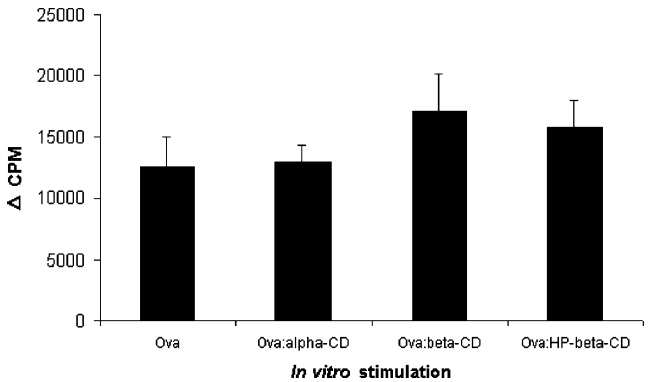

Fig. 3. Proliferative responses of spleen cells from Ova primed mice stimulated in vitro with either 0.5 mg/ml of Ova or of cyclodextrin-coupled Ova. Mice were immunized (s.c.) with Ova adsorbed in CFA. Fourteen days later, cells were isolated from their spleens and stimulated in vitro for 72 h with 0.5 mg/ml of the antigens as described. In the last 8 h of culture, cells were pulsed with [^{3}H] thymidine and the radioactivity determined in a Beta-counter. Bars represents values (Δ mean ± SEM) of a group ($n = 4$). $P = 0.49$.

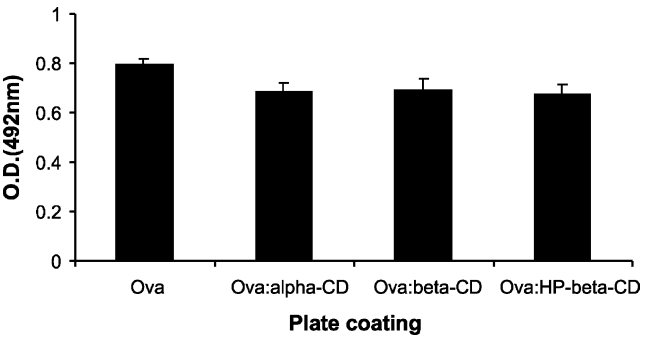

Fig. 4. Ova epitopes remain unchanged upon coupling to cyclodextrins. Microplates were coated with Ova, Ova:α-CD, Ova:β-CD or Ova:HP-β-CD and their binding affinities to anti-Ova antibodies were accessed using serum from Ova-immunized mice. The bars represent the mean values (± error deviation) of a group of at least five mice. Serum dilution, 1:2000. $P = 0.152$.

previous studies [23,24]. This stimulation is Ova-specific since spleen cells from Ova-immunized mice stimulated in vitro with culture medium only have a mean proliferation accessed as 3523 c.p.m. and spleen cells from unimmunized mice have a mean proliferation accessed as 1500 c.p.m. after all stimuli tested.

We also evaluated whether coupling of CDs to Ova would change the epitopes usually recognized by anti-Ova immunoglobulins. To access this effect, we coated 96 well plates with Ova or Ova coupled to CDs and then added to the plates sera from mice immunized with Ova in CFA. As we can observe in Fig. 4, there is no significant difference between plates coated with Ova or with CD-coupled Ova. Thus coupling CDs to Ova does not change its available epitopes. The binding is specific since no Ova coating or coating with normal mice sera mixed with Ova or with Ova:CD gives an absorbance below 0.1.

3.3. Cyclodextrin coupling to ovalbumin does not affect tolerance induction by oral route

Oral treatment of mice either with Ova alone or with Ova coupled to CDs before immunization with Ova suppresses their subsequent antigen-specific proliferative response in vitro (Fig. 5A). No differences were found between either Ova or Ova coupled to CDs in its ability to induce suppression of anti-Ova antibodies (Fig. 5B). Therefore, we can conclude that CDs coupling to Ova does not affect the tolerogenic capacity of Ova when administered by the oral route.

3.4. Cyclodextrins coupling to Ova have no adjuvant effect in subcutaneous immunization

We analyzed the ability of CDs to enhance an immune response, that is their ability to act as adjuvants, since there has been an incredible demand for new adjuvants for immunization. CFA is too toxic for human use and $Al[OH]_3$

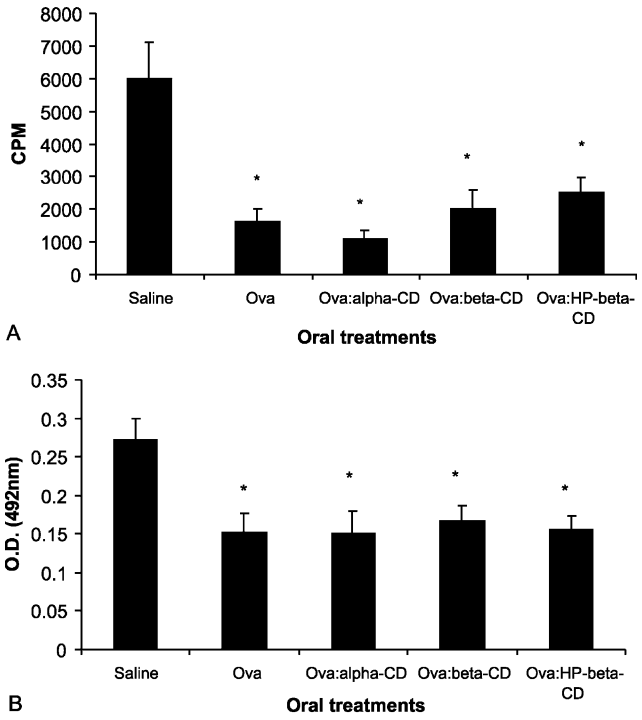

Fig. 5. Oral tolerance induction and immune responses of mice treated by oral route with cyclodextrin-coupled Ova. Mice received oral treatments (10 mg of antigen as described above) and 7 days later they were subcutaneously (s.c.) immunized with Ova adsorbed in CFA. (A) For proliferative responses, lymph node cells suspensions were obtained 8 days after immunization and cultured in vitro with Ova (5 mg/ml) for 72 h. In the last 8 h cultures were pulsed with [^{3}H] thymidine and the radioactivity determined in a Beta-counter. Bars represents values (Δ mean $\pm$ SEM) of a group ($n = 5$). $P < 0.01$. (B) For anti-Ova IgG1 production, mice were bled 28 days after the s.c. immunization with Ova + CFA and anti-Ova serum immunoglobulin concentration was determined by ELISA. Bars represents values (mean $\pm$ SEM) of a group ($n = 5$). Serum dilution 1:16000. $P < 0.05$.

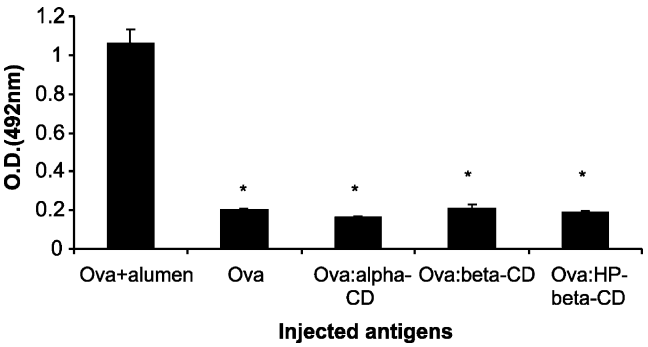

Fig. 6. Anti-Ova IgG1 production in mice that were immunized with either Ova alone, cyclodextrin-coupled Ova or Ova adsorbed in alumen. Mice were immunized (s.c.) with Ova adsorbed or not as described above and received an intraperitoneal (i.p.) booster 14 days later with soluble Ova. They were bled 7 days later and anti-Ova serum immunoglobulin concentration was determined by ELISA. Bars represents values (mean $\pm$ SEM) of a group ($n = 5$). Serum dilution 1:100. $P < 0.05$.

(another common adjuvant used in experimental studies) is not the ideal adjuvant because it is not effective in triggering Th1 patterns of reactivity (cellular reactivity). Therefore, we injected mice with Ova coupled to the CDs and analyzed its ability to induce serum anti-Ova antibodies. Since no serum-specific antibody could be measured by a standard ELISA after this treatment, we boosted the animals with soluble Ova by intraperitoneal injection. Seven days after the booster we could find some anti-Ova antibodies in the mice sera but their levels were much lower than the ones achieved by immunization with Ova plus $Al[OH]_3$ (Fig. 6). Thus Ova coupled to α-, β- or HP-β-CD elicits equivalent low amount of anti-Ova antibodies suggesting that CDs have poor adjuvant capacity when administered by parenteral route.

Hence, we can conclude that α-, β- and HP-β-CD are immunologically safe materials to be used either by oral route or by parenteral route. Our study clearly shows that they do not alter the antigenicity, tolerogenicity or immunogenicity of proteins coupled to them since they have no effect on the oral administration of proteins (they do not enhance oral tolerance induction or lead to oral immunization) and neither have any promising effects as adjuvants. These results strongly suggest that CDs are promising compounds to be used as excipients or as host molecules in inclusion compounds in the pharmaceutical area.

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