



Induction of antigen-specific immunity by pH-sensitive carbonate apatite as a potent vaccine carrier

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ABSTRACT

The ability of carbonate apatite (CO₃Ap) to enhance antigen-specific immunity was examined *in vitro* and *in vivo* to investigate its utility as a vaccine carrier. Murine bone marrow-derived dendritic cells took up ovalbumin (OVA) containing CO₃Ap more effectively than free OVA. Interestingly, mice immunized with OVA-containing CO₃Ap produced OVA-specific antibodies more effectively than mice immunized with free OVA. Furthermore, immunization of C57BL/6 mice with OVA-containing CO₃Ap induced the proliferation and antigen-specific production of IFN- γ by splenocytes more strongly than immunization with free OVA. Moreover, no significant differences were detected in the induction of delayed-type hypersensitivity responses, an immune reaction involving an antigen-specific, cell-mediated immune response between OVA-containing CO₃Ap and OVA-containing alumina salt (Alum), suggesting that CO₃Ap induced cell-mediated immune response to the same degree as Alum, which is commonly used for clinical applications. This study is the first to demonstrate the induction of antigen-specific immune responses *in vivo* by CO₃Ap.

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1. Introduction

The generation of robust and specific immune responses against infectious diseases is a primary goal of vaccination. Novel vaccine candidates under development are capable of inducing both cellular and humoral immune responses [1,2]. The development of new vaccination strategies depends on the discovery of novel adjuvants to enhance the potency and longevity of specific immune responses to antigens [3]. Adjuvants can be divided into two classes according to their mechanism of action: immunostimulants, such as toll-like receptor agonists, which directly enhance immune responses to vaccination, and vehicles that present antigens to the immune system through controlled release and delivery systems to increase the specific immune response to the antigen.

An efficient intercellular nucleic acid delivery system using pH-sensitive carbonate apatite (CO₃Ap) has been established as a carrier for applications in basic research, gene therapy and DNA vaccination [4–8]. Nucleic acids such as DNA, RNA and gene-silencing elements associated with CO₃Ap were effectively introduced into mammalian cells such as HeLa cells because the DNA

and RNA were adsorbed to CO₃Ap carriers and rapidly escaped from endolysosomes due to the high water solubility of the carbonate apatite in acidic pH environments, similar to endosomal pH [4–7]. In addition, the efficient delivery of proteins adsorbed to CO₃Ap into mammalian cells was recently confirmed [9]. As a model protein, fluorescent-labeled bovine serum albumin (BSA) was effectively delivered into nearly 100% of HeLa cells by the simple addition of protein/CO₃Ap complexes to the cells. Confocal microscopic imaging suggested the endosomal release of proteins delivered with CO₃Ap, probably due to the dissolution of CO₃Ap in acidic endosomal compartments and subsequent destabilization of endosomes. Additionally, intracellularly delivered β -galactosidase did not lose its enzymatic activity.

These results suggest that a simple intracellular protein delivery procedure using a CO₃Ap carrier may be a highly effective method for biological and clinical research. Moreover, apatite particles have been recognized as safe materials due to their biodegradability and resemblance to biological hard tissue components [10]. Thus, CO₃Ap may also be an effective carrier of antigenic proteins into antigen presenting cells (APC) such as dendritic cells (DC) for the development of novel vaccines. However, the ability of CO₃Ap to deliver proteins into APCs and the immunological responses of animals immunized with CO₃Ap-associated antigens have not yet been analyzed.

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In the present study, primary DCs freshly isolated from C57BL/6 mice demonstrated high CO₃Ap uptake. Furthermore, the stimulatory effects of CO₃Ap on antigen-specific immunity were examined in C57BL/6 mice immunized with CO₃Ap-associated ovalbumin (OVA). Finally, we compared the effect of the induction of OVA-specific delayed-type hypersensitivity (DTH) responses by CO₃Ap with that of alumina salt (Alum), which is used in many subunit vaccines as an adjuvant to enhance the body's response to immunogens.

2. Materials and methods

2.1. Mice

Six- to ten-week-old female C57BL/6NCrSlc mice were purchased from Japan SLC Inc. and maintained at the animal facilities of RIKEN BSI (Saitama, Japan) according to the guidelines of the institution. All experiments were approved by the RIKEN Animal Experiments Committee.

2.2. Preparation of carbonate apatite (CO₃Ap) and FITC-OVA/CO₃Ap complexes

CO₃Ap was prepared as previously reported [9]. Briefly, 100 µg OVA or fluorescein isothiocyanate (FITC) labeled-OVA (FITC-OVA) was added to 1 ml bicarbonate-buffered DMEM (Gibco, Invitrogen). Then, 6 µl of 1 M CaCl₂ solution was added to the protein solution and immediately mixed slowly with a vortex. The mixed solution was incubated at 4 or 37 °C for 30 min to obtain CO₃Ap particles containing OVA or FITC-OVA.

2.3. Uptake by bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated according to a previously reported protocol with slight modifications [11]. Briefly, bone marrow cells were collected by flushing the femurs and tibias of 6- to 10-week-old female C57BL/6 mice. Cells were then cultured at 1×10^6 cells/ml in 6-well plates in RPMI 1640 medium (GIBCO) supplemented with L-glutamine, 10% fetal bovine serum (FBS; Sigma–Aldrich), 5 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; Prospec-Tany Technogene Ltd.), and 100 µg/ml penicillin, streptomycin and gentamicin (GIBCO). On days 3 and 5 of culture, plates were swirled gently before two-thirds of the conditioned medium was removed along with the non-adherent cells, and fresh GM-CSF-containing culture medium was added to the cells. On day 7 or 8, the non-adherent cells were harvested after vigorous pipetting of DC clusters.

For the detection of OVA-containing CO₃Ap uptake, BMDCs (1×10^5 in 100 µl RPMI 1640 medium) were incubated for 1 h at 4 or 37 °C with 1 µg/ml free FITC-labeled OVA or CO₃Ap containing FITC-labeled OVA. After incubation, the cells were washed three times with 5 mM ethylenediaminetetraacetic acid (EDTA) solution to remove residual complexes, and the percentage of FITC-positive cells was analyzed on a FACSCalibur flow cytometer (Becton–Dickinson).

2.4. Immunization and isolation of blood samples and spleens

Six-week-old female C57BL/6 mice were administered 3.3 mg/kg free OVA or OVA-containing CO₃Ap diluted with PBS on days 0 and 14. Blood samples were collected from the tail vein once per week for antibody titration. On day 28, whole blood samples were collected by heart puncture under deep anesthesia with diethyl ether and spleens were harvested.

Spleens of mice were individually prepared as single-cell suspensions in 3 ml RPMI 1640 supplemented with L-glutamine, 10% FBS, and 100 µg/ml penicillin, streptomycin and gentamycin. The red blood cells were removed by suspension in a lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA₂Na). Splenocytes were washed with complete RPMI and counted using the trypan blue exclusion method.

2.5. In vitro cytokine release measurement by enzyme-linked immunosorbent assay (ELISA)

Splenocytes (2×10^5 in 100 µl RPMI 1640 medium) were incubated for 72 h with 1, 5 or 25 µg/ml free OVA or OVA-containing CO₃Ap. After incubation, the concentration of IFN-γ was analyzed by ELISA. NUNC MaxiSorp 96-well ELISA plates (Nalge Nunc International) were coated with purified rat anti-mouse IFN-γ (eBioscience; 100 µl, 1 µg/ml) in 0.1 M NaHCO₃, incubated overnight at 4 °C, and washed with $1 \times$ phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). The plates were blocked with 3% BSA in PBS for 1 h at room temperature and washed with PBS-T. Standard recombinant IFN-γ (100 µl) diluted in PBS containing 1% BSA was added to the wells, and the plates were incubated for 1 h at room temperature and then washed with PBS-T. A biotin-labeled rat anti-mouse IFN-γ mAb (eBioscience; 100 µl, 1 µg/ml) was added to the wells, after which the plates were incubated for 1 h at room temperature and then washed with PBS-T. Horseradish-peroxidase (HRP)-conjugated avidin (eBioscience; 100 µl, 1 µg/ml) was added to the wells, and the plates were incubated for 1 h at room temperature and then washed with PBS-T. Finally, colorimetric signals were generated using 1-Step Ultra TMB-ELISA solution (Thermo Scientific Pierce, Rockford, IL). After 5 min, the reaction was stopped with 1 N H₂SO₄, and the absorbance at 450 nm was measured using a Wallac ARVO plate reader (Perkin–Elmer). The concentration of IFN-γ was interpolated on the basis of standard curves.

2.6. Splenocyte proliferation assay

Splenocytes (4×10^6 in 2 ml RPMI 1640 medium) were incubated for 96 h with 1, 5 or 25 µg/ml free OVA or OVA-containing CO₃Ap. Splenocyte proliferation was determined using the FITC BrdU Flow Kit (Becton–Dickinson) according to the manufacturers' instructions. Briefly, 5-bromo-2-deoxyuridine (BrdU; 5 µM) was added for the last 2 h of incubation. After washing, cells were fixed, permeabilized and treated with DNase I, as described in the assay kit. Cells were washed and analyzed on a FACSCalibur flow cytometer (Becton–Dickinson) using CellQuest software (Becton–Dickinson).

2.7. Antibody titration

Specific antibody titers against OVA were determined by performing an endpoint ELISA. NUNC MaxiSorp 96-well ELISA plates were coated with 5 µg/ml OVA in 0.1 M NaHCO₃, incubated overnight at 4 °C and washed with PBS-T. The plates were blocked with 3% BSA in PBS for 1 h at room temperature and washed with PBS-T. After serial dilution (beginning at 40-fold) in PBS containing 1% BSA, serum samples (100 µl) were added to the wells and the plates were incubated for 1 h at room temperature. The plates were washed with PBS-T and 100 µl peroxidase-conjugated goat anti-mouse IgG (heavy chain- and light chain-specific, diluted 32,000-fold with 1% BSA in PBS; Jackson Immuno Research Laboratory Inc.) was added to each well. The plates were incubated for 1 h at room temperature and then washed with PBS-T. Finally, colorimetric signals were generated using 1-Step Ultra TMB-ELISA solution. The reaction was stopped with 1 N H₂SO₄ after 5 min, and the

absorbance at 450 nm was measured using a Wallac ARVO plate reader (Perkin–Elmer). The antibody titer was determined as the highest dilution below the cut-off value indicated by the mean OD value of the background $\pm$ 2 SD.

2.8. Induction and measurement of DTH responses

Six-week-old B57BL/6 mice were immunized by subcutaneous injection of 100 μ g OVA in 100 μ l of PBS, Alum (aluminum hydroxide hydrate gel suspension LG-6000, COSMO BIO CO., Ltd., Tokyo, Japan), or CO₃Ap. One week after immunization, mice were challenged by subcutaneous injection of 50 μ g OVA in 50 μ l of PBS, Alum or CO₃Ap into the left footpad and PBS only into the right footpad. Footpad swelling was measured 48 h later. DTH response was calculated as the difference in thickness between right footpad and left footpad. The mean swelling of three mice was calculated, and the average difference was compared by Student's *t* test.

2.9. Statistical analysis

All data are expressed as mean $\pm$ SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with post hoc analysis followed by Bonferroni's test.

3. Results and discussion

3.1. Association of CO₃Ap containing OVA with DCs

To examine the ability of CO₃Ap to deliver antigenic proteins into the cytoplasm of primary DCs, BMDCs freshly isolated from C57BL/6 mice were incubated with FITC-labeled free OVA (FITC-OVA) or CO₃Ap containing FITC-labeled OVA (FITC-OVA/CO₃Ap) for 1 h at 4 or 37 $^{\circ}$ C. After the incubation, the fluorescence intensity of FITC-labeled OVA in BMDCs was analyzed by flow cytometry (Fig. 1). Compared to cells treated with free OVA, a larger population of BMDCs displayed more intense fluorescence following treatment with the FITC-OVA/CO₃Ap complex. The population of FITC-positive BMDCs incubated with FITC-OVA/CO₃Ap complexes ($55.10 \pm 6.05\%$ positive cells) was approximately 3.5-fold larger than that of BMDCs incubated with OVA ($15.40 \pm 2.15\%$ positive cells) at 37 $^{\circ}$ C ($P < 0.01$). By contrast, no uptake of FITC-OVA/CO₃Ap complexes occurred in BMDCs incubated at 4 $^{\circ}$ C ($4.21 \pm 0.68\%$

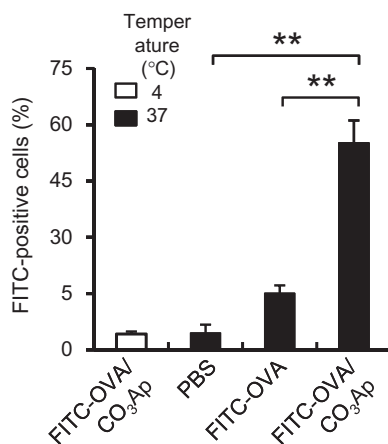


Fig. 1. Uptake by dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were incubated with 10 μ g/ml FITC-labeled free OVA or OVA-containing CO₃Ap for 1 h at 4 $^{\circ}$ C ($\square$) or 37 $^{\circ}$ C ($\blacksquare$). After incubation, the uptake of FITC-labeled OVA was detected by flow cytometry. The percentage of FITC-positive cells is shown. The results are expressed as the mean $\pm$ SEM of three independent experiments. Significant differences between the groups are indicated by $^{**}P < 0.01$.

positive cells). This experiment demonstrated high CO₃Ap uptake by primary DCs for the first time.

3.2. OVA-specific antibody production by CO₃Ap containing OVA

To investigate the induction of humoral immune responses by OVA/CO₃Ap complex in mice, serum samples were collected once per week from C57BL/6 mice intraperitoneally immunized with OVA or OVA/CO₃Ap complex, and OVA-specific antibody titers were measured using an endpoint ELISA. On day 28, the OVA-specific antibody titer in mice immunized with the OVA/CO₃Ap complex was more than 320,000-fold higher than that of mice immunized with free OVA (Fig. 2A). The trend for high antibody production in mice immunized with the OVA/CO₃Ap complex was observed as early as 7 days after the first immunization and continued until day 28 (Fig. 2B).

3.3. Induction of cellular immunity by CO₃Ap containing OVA

To investigate the induction of OVA-specific cell-mediated immunity by OVA/CO₃Ap complex, C57BL/6 mice were intraperitoneally immunized with OVA or OVA/CO₃Ap complex, and splenocytes from the immunized mice were collected and cultured for 72 or 96 h in the absence or presence of 1, 5 or 25 μ g/ml OVA. Splenocytes from mice treated with OVA or OVA/CO₃Ap complexes were successfully immunized and secreted IFN- γ after *in vitro* stimulation with OVA; however, no IFN- γ was secreted by splenocytes from mice treated with PBS as a negative control (Fig. 3A). IFN- γ secretion in response to OVA stimulation increased in a dose-dependent manner, and at all OVA concentrations, splenocytes from OVA/CO₃Ap-immunized mice secreted significantly

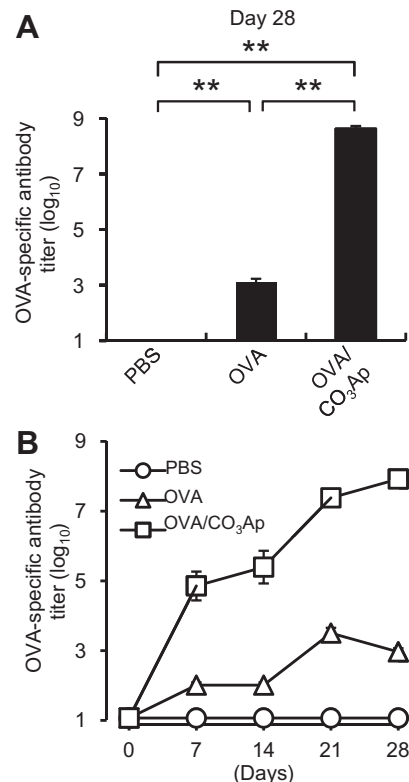


Fig. 2. OVA-specific antibody production. OVA-specific antibody titers in the serum of C57BL/6 mice immunized with free OVA or OVA-containing CO₃Ap as determined by an endpoint ELISA on day 28 (A) and throughout the entire experimental period (B). The results are expressed as the mean $\pm$ SEM of at least three mice. Significant differences between groups are indicated by $^{**}P < 0.01$.

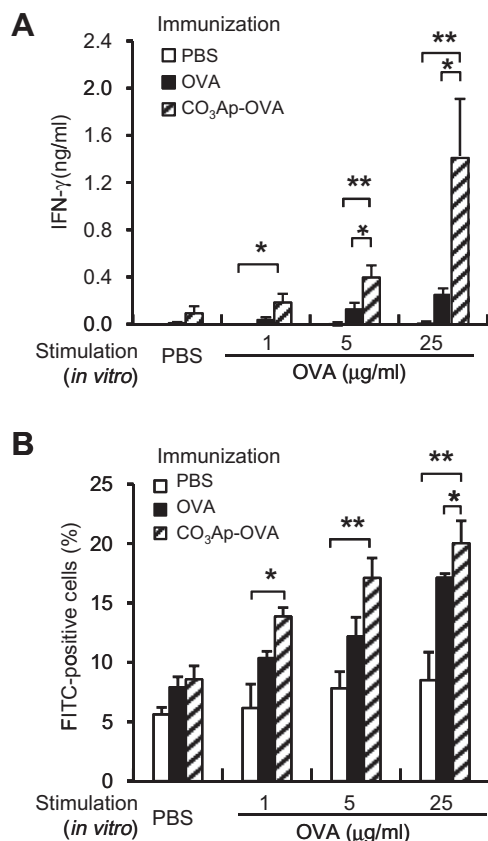


Fig. 3. OVA stimulation of immunized splenocytes *in vitro*. Splenocytes isolated from C57BL/6 mice immunized with free OVA or with CO₃Ap were incubated with 1, 5 or 25 μg/ml OVA for 72 h (A) or 96 h (B). Interferon (IFN)-γ secretion was detected by ELISA (A), and cell proliferation was detected by 5-bromo-2-deoxyuridine (BrdU) uptake analysis (B). The results are expressed as the mean ± SEM of at least three independent experiments. Significant differences between the groups are indicated by **P* < 0.05 and ***P* < 0.01.

more IFN-γ than splenocytes from mice that had been immunized with free OVA. This result indicated that OVA/CO₃Ap complexes were able to efficiently induce an immune response in mice. Furthermore, the proliferative response of primary splenocytes was examined by BrdU-uptake analysis (Fig. 3B). Splenocyte proliferation in response to *in vitro* stimulation with OVA was observed in splenocytes from mice that had been immunized with OVA or the OVA/CO₃Ap complex, but not in splenocytes from mice administered PBS.

3.4. The induction of DTH by CO₃Ap containing OVA

To investigate whether the CO₃Ap-OVA complex induces an OVA-specific cellular immune response in mice, mouse footpad assay for DTH reactions in mice was used, with the footpad thickness measured by micrometer. The CO₃Ap-OVA group of C57BL/6 mice sensitized and challenged with CO₃Ap-OVA significantly differed from the PBS-sensitized group (*P* = 0.00844) and from the OVA-sensitized and challenged group (*P* = 0.0001258; Fig. 4). This result clearly demonstrated that CO₃Ap-OVA induced OVA-specific cell-mediated immune responses in mice. Moreover, we compared the induction of DTH reactions by CO₃Ap with that of Alum, which is commonly used for clinical applications. No significant differences were detected between the CO₃Ap-OVA group and Alum-OVA group, suggesting that CO₃Ap induced a cell-mediated immune response to the same degree as Alum.

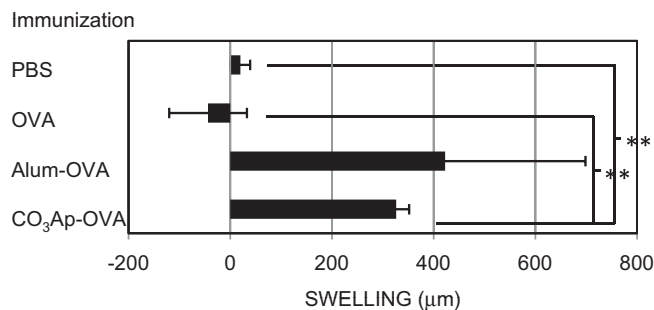


Fig. 4. Comparison of delayed-type hypersensitivity response in mice that were sensitized and challenged with CO₃Ap-OVA or Alum-OVA. For sensitization, 100 μg OVA was applied with PBS, Alum, or CO₃Ap to the dorsal skin of C57BL/6 mice by subcutaneous injection in experimental groups. The control experiment was performed with PBS alone. Seven days after the inoculation, 50 μg of OVA applied with the same compounds used for sensitization was injected into the left footpad of mice, and PBS was injected alone to the right footpad. Footpad thickness was measured before and 48 h after challenge, and swelling was computed. Each symbol and bar of swelling percentage represent the mean ± SEM (*n* = 3). ***P* < 0.01 for CO₃Ap-OVA stimulated groups and control groups. No significantly differences were observed between Alum-OVA stimulated group and control groups. PBS: phosphate-buffered saline, OVA: ovalbumin, Alum: aluminum hydroxide gel, CO₃Ap: pH-sensitive synthesized carbonate apatite.

3.5. CO₃Ap as a potent and novel carrier of antigenic protein

Our previous study suggested that an intracellular protein delivery system using a CO₃Ap carrier and a very simple procedure may be a highly effective method for biological and clinical research [9]. The present study has now confirmed the induction of antigen-specific immune responses *in vivo* by CO₃Ap. CO₃Ap containing OVA was taken up by primary BMDCs more efficiently than free OVA (Fig. 1), and the uptake of CO₃Ap by DCs resulted in the efficient induction of antigen-specific immune responses. In fact, our present results demonstrate that CO₃Ap containing OVA induced cellular immunological responses *in vivo*, such as enhanced IFN-γ production and proliferation response in primary splenocytes from OVA/CO₃Ap-immunized mice (Fig. 3). DCs present antigen to antigen-specific T cells via surface MHC molecules, which results in cytokine production and clonal expansion of T cells.

The increased IFN-γ production and proliferation of splenocytes in response to antigen stimulation suggested efficient induction of antigen-specific immune responses by DCs. IFN-γ is critical for the induction of cellular immunity such as protection against parasites [12], in part by enhancing the cytotoxicity of cytotoxic T lymphocytes (CTLs) [13] and natural killer (NK) cells [14]. Thus, enhanced IFN-γ production by splenocytes in response to the presentation of antigens delivered to DCs by CO₃Ap indicates that CO₃Ap can induce effective cellular immunity against antigen-containing CO₃Ap. Moreover, an increase in OVA-specific antibody production was observed in OVA/CO₃Ap-immunized mice, indicating the enhancement of antigen-specific humoral immunity (Fig. 2). Thus, our results demonstrate that CO₃Ap is not only a promising method for various fields including molecular biology, cellular biology and tissue engineering, but also a potent antigen carrier for the development of effective vaccines.

Several attempts have been made to identify strategies that induce potent immunostimulatory activity [15–19]. The aluminum salt has been widely approved for clinical use at the present time. The enhancing effects of antigen co-delivery with CO₃Ap on antigen-specific IgG production seem to be almost equivalent or slightly higher than those of Complete Freund's adjuvant, MPL or CD40 mAb and clearly higher than those of Alum in OVA-immunized C57BL/6 mice [19–22]. In addition, our results demonstrated that CO₃Ap/OVA-immunized mice expressed a DTH response sim-

ilar to that of Alum/OVA-immunized mice. Comparing splenocyte IFN- γ production between CO₃Ap/OVA-immunized mice and previously reported Alum/OVA-immunized mice, splenocytes from CO₃Ap/OVA-immunized mice produced IFN- γ (around 1.3 ng/ml, Fig. 3A) at similar levels to Alum/OVA-immunized mice (around 1.5 ng/ml) and at lower levels than Complete Freund's adjuvant-immunized mice (around 4 ng/ml) [22]. In summary, CO₃Ap-conjugated antigen strongly induced humoral immune response but weakly induced cellular immune response.

CO₃Ap is characterized by its sensitivity to pH. Although CO₃Ap is highly stable at neutral pH, it degrades quickly in acidic environments [9]. Antigens encapsulated within CO₃Ap may escape effectively from the acidic endosome into the cytosol of DCs as well as in HeLa cells [6,8] to result in improved antigen presentation by DCs to cytotoxic T lymphocytes via MHC class I. These changes in DC phenotype in response to antigens may be related to the immune-enhancing effects observed in mice immunized with OVA associated with CO₃Ap in this study; however, further investigations are still required to understand this mechanism.

In conclusion, this study demonstrates that antigen-specific immunity can be enhanced *in vivo* by complex formation between antigen and CO₃Ap. Our results suggest that potent immune responses could be generated by CO₃Ap carrying not only OVA but also other peptides or proteins. Thus, these results suggest that CO₃Ap is a promising antigen carrier for the development of novel effective vaccines.

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