

Co-Delivery of Ovalbumin and CpG Motifs into Microparticles Protected Sensitized Mice from Anaphylaxis

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Key Words

Poly(lactide-co-glycolide) microparticles • CpG sequences • Immunotherapy • Anaphylactic shock

Abstract

Background: Two major drawbacks of current subcutaneous specific immunotherapy are the risk to induce severe anaphylactic reactions and the need of multiple injections of the allergen to reduce IgE-mediated hypersensitivity. The sustained release of allergens over time provided by poly(lactide-co-glycolide) microparticles (MP) could mimic the current therapeutic schedule and decrease their allergenicity. Moreover, MP could also co-deliver Th1 immunoadjuvants, such as CpG motifs. **Methods:** Ovalbumin (OVA)-sensitized BALB/c mice were treated intradermally with OVA or OVA plus CpG containing MP. OVA-specific IgG1 and IgG2a as well as IgE total levels and cytokine production were assessed throughout the experiment. The protection exerted by the MP against allergen challenge was estimated with body temperature changes, mortality and other symptoms. **Results:** Microparticulated treatments, irrespective of the presence of CpG motifs, elicited a lower IgE/IgG2a ratio than those induced by the allergen in solution (free or with adjuvants). However, after induction of the anaphylactic shock, only mice treated with MP co-encapsulating OVA plus CpG showed a significant lower decrease in body temperature

and were totally protected from death. Mice that were injected with OVA plus CpG in solution or with Alum displayed a marked fall of temperature accompanied by high mortality rates (70–100%). **Conclusion:** MP encapsulating both OVA, as an allergen model, and CpG sequences, as a pro-Th1 adjuvant, decreased the risk for OVA sensitization (IgE induction) and protected sensitized mice from anaphylactic shock after allergen provocation. Therefore, the combination of allergens and CpG sequences into MP could perform a safer alternative to current specific immunotherapy.

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Introduction

The WHO reported that immunotherapy is the only curative treatment for allergy [1]. However, the efficacy of specific immunotherapy (SIT) is currently hampered by the risk to induce severe anaphylactic reactions [2] and the need of long-term continuous injections. Although great expectations have been reported for the administration of the therapy by sublingual route [1], its limited efficacy and the need of high doses of allergen favor subcutaneous injection.

Since new administration routes do not ameliorate subcutaneous immunotherapy, the combination of novel adjuvants with allergens appears as an attractive alterna-

tive to bias the Th2 allergic response toward a more balanced specific immune profile [3–5]. Some of the most appropriate adjuvants in allergy seem to be CpG motifs, synthetic analogues of nonmethylated bacterial DNA, due to their intrinsic capability to enhance pro-inflammatory cytokines production via Toll-like receptor 9, and thus trigger the proliferation of Th1-type lymphocytes [6].

Previous work has established that the uptake of both the antigen and CpG motifs by the same antigen-presenting cell (APC) is a requisite for the induction of the most potent and antigen-specific Th1 immune response. To achieve this objective, current strategies include their co-administration [7], their covalent linkage [5] or their co-association with delivery systems, such as microparticles (MP) [8–13]. The co-delivery of antigen and CpG using polymeric devices represents an excellent strategy to improve both antigen and CpG phagocytosis by the same APC, by virtue of their particulate nature, and the protection exerted from degradation. The result is a potent antigen uptake, APC activation by CpG with a very clear Th1 bias and also stimulation of T regulatory cells, suitable for the restoration of immunological profile in pro-Th2 disorders [14]. Beside, the antigen-sustained release provided by polymeric carriers could reduce the number of injections of current SIT schedule, improving patient compliance and warranting the fulfillment of the treatment [12, 15, 16]. Finally, poly(lactide-co-glycolide) (PLGA) is a biodegradable and biocompatible material approved by the FDA for use in humans, which could perform an attractive substitute to the conventional pro-Th2 adjuvant Alum.

In this study, we demonstrated that the microencapsulation of ovalbumin (OVA) or the antigen plus CpG motifs into PLGA MP is an available strategy to improve the safety of conventional allergen SIT (OVA administered with Alum), as indicated by the decrease in the levels of IgE during the treatment and the protection exerted against the allergen provocation. The benefits of CpG co-delivery are discussed.

Materials and Methods

Materials

The co-polymer formed by lactic and glycolic acid, Resomer® RG 502 (PLGA, MW 12,000) was supplied by Boehringer Ingelheim. OVA (grade V) was purchased from Sigma-Aldrich and phosphorothioates CpG oligonucleotide [No. 1826, sequence (5'–3'): tccatgacgttcctgacgtt] was obtained from Coley Pharmaceutical Group. DOTAP was obtained from Avanti Polar Lipids Inc. Oligreen® ssDNA Quantitation kit (Molecular Probes) and mi-

crobicinchoninic acid protein assay (Micro BCA; Pierce) were used to quantify the oligonucleotide and the OVA content, respectively. Other chemical compounds were of reagent grade and obtained from Sigma-Aldrich.

MP Fabrication

MP were fabricated by the emulsion and solvent evaporation technique using TROMS (Total Recirculation One Machine System) [17, 18]. Briefly, the first emulsion was obtained by injection and mixing of the polymer solution (50 mg PLGA and 5 mg DOTAP in 1.25 ml methylene chloride) onto Pluronic F68® (250 µl, 6% w/v) containing OVA (3.125 mg) or OVA plus CpG sequences (300 nmol, approximately), depending on the formulation. Afterwards, this W1/O emulsion was injected onto polyvinyl alcohol (7.5 ml, 0.5% w/v) to obtain a double emulsion, which was magnetically stirred to allow organic solvent evaporation and MP formation. Finally, MP were centrifuged, washed and lyophilized for their storage.

DOTAP [N/P 1 (relation between positive charge of amino groups of the lipid and the negative charge of phosphoric acid of the oligonucleotide)] was added to the organic phase of the formulations, to allow the formation of a complex with CpG sequences and to increase its encapsulation.

MP Characterization

The average size was determined with a Mastersizer S laser sizer and the zeta potential using a Zetasizer Nano ZS, both from Malvern Instruments.

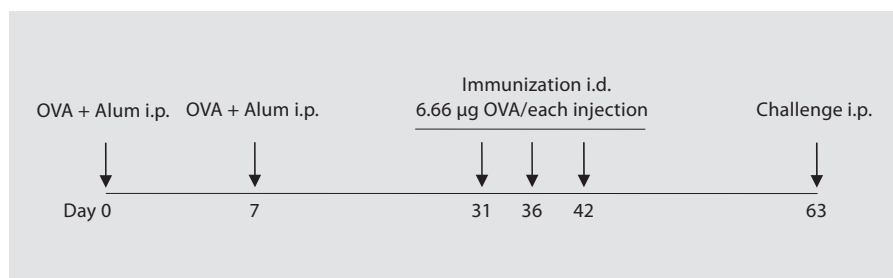
The OVA content in the MP was determined using the Micro BCA protein assay, according to the manufacturer's specifications. To analyze the amount of CpG associated to MP, digested MP were incubated with Oligreen® ssDNA Quantitation reagent and their fluorescence was measured. Protein and CpG encapsulation was expressed as microgram per milligram of MP.

Therapeutic Mouse Model

The design of this experiment was modified from previous reports [19, 20]. Briefly, BALB/c mice (n = 7–8) were sensitized twice intraperitoneally with 50 µg OVA adsorbed in 2 mg of Alum on days 0 and 7 (fig. 1). Once the allergic state was confirmed serologically with IgE total determination (4,799 ng/ml for sensitized mice vs. 1,945 ng/ml for naïve animals), mice were immunized with 20 µg OVA and 25 µg CpG motifs divided in 3 intradermal injections at days 31, 36 and 42 in the following combinations: (1) phosphate-buffered saline (PBS), (2) OVA in solution, (3) mixture of OVA and CpG in solution (OVA + CpG), (4) MP containing OVA alone (OVA MP), (5) OVA and CpG sequences loaded MP (OVA CpG MP) and (6) OVA adsorbed in aluminum hydroxide (OVA + Alum). At day 63 after the first sensitization, anaphylactic shock was provoked by the intraperitoneal injection of 1 mg OVA. At this moment, different symptoms and parameters were evaluated in blind experimental conditions: body temperature, piloerection, mobility, cyanosis, mouse mast cell protease 1 (MMCP-1) level and survival rate. Throughout all experiments, blood samples were obtained from the retro-orbital plexus to determine antibody levels.

The experiments were performed in compliance with the regulations of the Ethical Committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU).

Fig. 1. Experimental design. BALB/c mice received 50 µg OVA adsorbed onto 2 mg Alum at days 0 and 7. After that, mice were immunized with OVA and OVA CpG MP and OVA + Alum at days 31, 36 and 42 (6.66 µg OVA per immunization). For induction of systemic anaphylactic shock mice were intraperitoneally challenged with 1 mg OVA at day 63. i.p. = Intraperitoneally; i.d. = intradermally.



Measurement of Anti-OVA IgG Antibody Levels in Serum

OVA (1 µg/well in carbonate-bicarbonate buffer)-coated microplates (Thermo Labsystems) were maintained overnight at 4°C. After being washed, serum samples were incubated at 37°C for 4 h. The unbound antibody was washed before the addition of goat anti-mouse IgG1 or IgG2a horseradish peroxidase conjugate (Nordic Immunology) diluted 1:1,000 in PBS-Tween 20 0.05% (PBS-T20; 37°C, 1 h). Afterwards, plates were washed and a solution containing 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and hydrogen peroxide was incubated under room temperature for 30 min. Finally, the colorimetric reaction was determined at 405 nm (iEMS Reader MF; Labsystems).

Measurement of IgE Antibody Levels in Serum

The IgE antibody level was assessed by a sandwich ELISA. For this purpose, anti-mouse IgE (BD OptEIA™ Set Mouse IgE; BD Biosciences) coated microtiter plates (Nunc-Immuno™ Plate) were maintained overnight at 4°C. After washing the microplates (PBS-T20), these were blocked with PBS containing 10% fetal bovine serum for 1 h at room temperature. Thereafter, microplates were washed again and the samples were incubated for 2 h at room temperature. After washing, plates were incubated for 1 h with a solution of anti-IgE antibodies labeled with biotin and horseradish peroxidase conjugate. Later, plates were washed in the same conditions and tetramethylbenzidine was added to develop a colorimetric reaction. The absorbance was measured at 405 nm in an iEMS Reader MF (Labsystems).

Cytokine Assay

At day 62, mice (n = 2–3) were sacrificed and their spleens and axillary lymph nodes were removed and pooled in RPMI 1640 media (Gibco-BRL) under sterile conditions. The cellular suspensions were purified and the erythrocytes were eliminated (NH₄Cl 0.15 M, KHCO₃ 10 mM, EDTA 0.1 mM). The resulting cells were centrifuged and resuspended in complete medium (RPMI 1640 with 0.1% β-mercaptoethanol 50 mM, 0.5% sodium pyruvate 100 mM, 1 IU/ml penicillin, 1 µg/ml streptomycin and 10% v/v fetal bovine serum). The suspensions (8 × 10⁵ cells/well) were incubated in microtiter plates (Iwaki) with OVA (80 µg/ml), complete medium (negative control) and concanavalin A (positive control) for 48 h. IFN-γ and IL-4 level in the supernatants were measured using commercial ELISA kits (Pharmingen).

Evaluation of the Anaphylactic Shock

Anaphylactic symptomatology (lack of mobility, cyanosis and piloerection) was evaluated for 30 min using a scoring system described in previous reports [21] and modified for this study. Reac-

tions severity was classified in the following categories depending on their gravity: + = weak; ++ = moderate; +++ = strong.

MMCP-1, a well-defined indicator of allergic episodes, was measured with an MMCP-1 ELISA kit purchased from Moredun Animal Health Ltd. Briefly, blood samples (obtained 1 h after the challenge) were incubated for 1.5 h in microplates coated with sheep anti-MMCP-1 capture antibody. After being washed, the horseradish peroxidase conjugate diluted (1:800) with 4% BSA in PBS-T20 was incubated for 1 h. Then, another washing step was carried out and tetramethylbenzidine was added. Ten minutes later, the reaction was stopped with 0.25 M sulphuric acid and the absorbance was measured at 450 nm in a plate autoreader (iEMS Reader MF; Labsystems).

Another parameter classically used to estimate the gravity of anaphylaxis after allergen provocation is body temperature change [22]. Therefore, the rectal temperature was monitored before and 1 h after the OVA challenge.

Besides, the severity of the allergic reaction could lead the animals to an extreme status provoking their death. Hence, the survival rate was assessed 1 h after the intraperitoneal challenge.

Data Analysis and Statistics

Since data did not follow a normal distribution, the tested treatments were compared using nonparametric Mann-Whitney U analysis. Results were considered statistically significant if p < 0.05. All calculations were performed using the SPSS® statistical software program (SPSS 11; SPSS Inc.).

Results

Characterization of MP

The main physicochemical characteristics of MP are summarized in table 1. All formulations displayed a similar size (1–1.5 µm) and their zeta potential moved from +8 mV of OVA containing MP toward slightly negative values when co-encapsulating CpG plus OVA. This modification in the zeta potential suggests a different location of the cation within the polymeric MP in the presence of CpG motifs: within the inner aqueous phase complexed with the oligonucleotides or more external and near the particle surface in their absence. The current hypothesis about inner structure and location could also explain

Table 1. Physicochemical characterization of PLGA MP containing OVA and OVA and CpG sequences and prepared using DOTAP to form a complex with the oligonucleotide

Formulation	Size μm	Zeta potential, mV	OVA loading $\mu\text{g}/\text{mg}$ MP	CpG content $\mu\text{g}/\text{mg}$ MP
OVA MP	1.0 ± 0.1	8.7 ± 0.7	22.1 ± 1.3	–
OVA CpG MP	1.3 ± 0.1	-12.4 ± 6.1	42.0 ± 3.9	45.2 ± 6.5

Data are expressed as means $\pm$ SD (n = 3).

Table 2. Cytokine production (IFN- γ and IL-4) by lymphocytes

Treatment	IFN- γ , pg/ml	IL-4, pg/ml	IFN- γ /IL-4
PBS	$1,241 \pm 779$	12 ± 17	103
OVA	$1,170 \pm 761$	44 ± 17	26
OVA + CpG	$1,837 \pm 157$	65 ± 53	28
OVA MP	$2,163 \pm 184$	44 ± 11	49
OVA CpG MP	$2,020 \pm 166$	75 ± 70	27
OVA + Alum	$2,252 \pm 106$	139 ± 27	16

Lymphocytes were obtained from spleens and axillary lymph nodes of sensitized BALB/c mice (n = 2–3) 61 days after intradermal immunization with 20 μg OVA and/or 25 μg CpG motifs (divided in 3 injections at days 31, 36 and 42) in the following combinations: (1) PBS, (2) OVA in solution, (3) mixture of OVA and CpG in solution (OVA + CpG), (4) MP containing OVA alone (OVA MP), (5) OVA and CpG sequences loaded MP (OVA CpG MP) and (6) OVA emulsified with aluminum hydroxide (OVA + Alum). The resulting cellular suspensions were recalled in vitro with 80 μg OVA/ml for 48 h to analyze IFN- γ and IL-4 production.

well the influence of the cation DOTAP in the OVA loading. Both OVA and DOTAP are amphiphilic molecules and could compete for the interphase between the inner aqueous and the organic phase of the MP. Hence, OVA could be forced to diffuse to the external aqueous phase resulting in lower antigen loading efficiencies. In the presence of CpG sequences, DOTAP-oligonucleotides complexes would be entrapped within the inner aqueous phase and OVA could retrieve its position in the interphase.

CpG loading was very high (80% encapsulation efficiency), whereas the antigen encapsulation increased when both were associated into the same MP formulation. OVA encapsulation efficiency increased from 20 to 50% for OVA-loaded MP or CpG and OVA co-encapsulated MP, respectively.

Antibody Response

The OVA-specific IgG1 and IgG2a antibodies were determined in sensitized BALB/c mice treated with OVA in solution (alone or combined with CpG motifs or Alum as adjuvants) or microencapsulated OVA (fig. 2). The combination of CpG sequences with OVA resulted in an enhancement in the IgG2a/IgG1 ratio (0.43 for OVA + CpG) compared with the injection of the allergen alone in solution (IgG2a/IgG1 = 0.33) or PBS (IgG2a/IgG1 = 0.27) and improved the ratio induced by the conventional treatment, OVA plus Alum (IgG2a/IgG1 = 0.40). The encapsulation of the allergen into MP led to a more IgG2a-biased response (IgG2a/IgG1 = 0.50–0.54).

Regarding the IgE level (fig. 2b), all microparticulated treatments, either loading OVA alone or OVA plus CpG, produced a lower increase in this antibody compared with the groups of mice receiving OVA in solution, combined with CpG sequences or adsorbed onto Alum. In fact, at day 51 after first sensitization, the level of IgE detected in these groups was in the range of 18,000–22,000 ng/ml, whereas the production of this antibody decreased to 11,000–14,000 ng/ml in mice receiving MP. The IgE level detected in sensitized and untreated mice was approximately 7,500 pg/ml. This behavior was maintained along IgE determinations, although at the end of the experiment (before the challenge), the differences between microencapsulated or soluble antigens were not so marked.

Analyzing the IgE/IgG2a ratio (fig. 2c), the microencapsulation of the allergen resulted in a more prominent Th1 tendency (IgE/IgG2a close to 1,600 at the end of the experiment) compared with the administration of the allergen in solution or physically mixed with adjuvants (IgE/IgG2a approximately 2,800). Mice receiving CpG sequences, microparticulated or physically mixed with OVA, did not show any benefit in humoral response in comparison with those immunized with the allergen, alone in MP or in solution.

Cellular Immune Response

The determination of IFN- γ and IL-4 (table 2) indicated which subset of Th cell population was predominant in sensitized mice treated with free or encapsulated allergen by intradermal route. All treatments increased IL-4 production compared with PBS group (12 ± 17 pg/ml). Therein, the particulate allergen induced IL-4 levels (45–75 pg/ml, approximately) similar to those obtained with immunization with OVA in solution (44 ± 17 pg/ml) or OVA plus CpG sequences (65 ± 53 pg/ml).

Regarding the IFN- γ level, the combination of CpG sequences with the allergen and MP treatments resulted

Fig. 2. OVA-specific IgG1 (black bars) and IgG2a (hatched bars) (expressed in titers; **a**) and total IgE antibodies (in ng/ml; **b**) response and IgE/IgG2a ratio (**c**) measured by ELISA on sera from BALB/c mice sensitized twice (at days 0 and 7) by intraperitoneal route (50 μ g OVA in 2 mg of Alum) and treated 1 month later with 20 μ g OVA and 25 μ g CpG motifs (divided in 3 intradermal injections) in the following combinations: (1) PBS, (2) OVA in solution, (3) mixture of OVA and CpG in solution (OVA + CpG), (4) MP containing OVA alone (OVA MP), (5) OVA and CpG sequences loaded MP (OVA CpG MP) and (6) OVA emulsified with aluminum hydroxide (OVA + Alum). The antibody titer was determined as the reciprocal of the highest serially diluted serum [starting from dilution 1:40 (titer 1) to 1:655,360 (titre 15)], whose optical density (405 nm) was equal or above 0.2 that of blank samples.

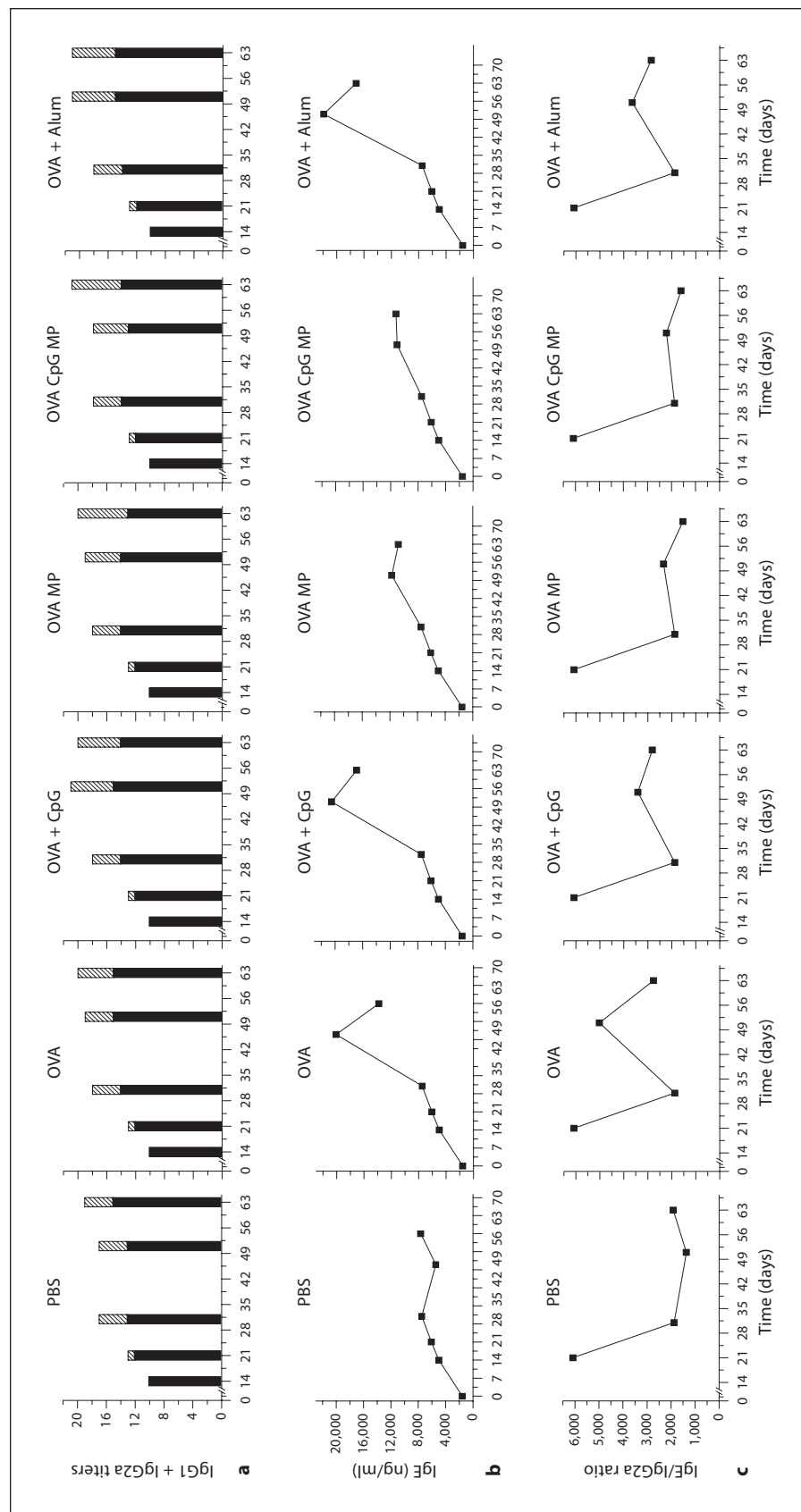


Table 3. Systemic anaphylactic reactions

Treatment	Mobility	Cyanosis	Piloerection
PBS	++	+++	++
OVA	+++	++	++
OVA + CpG	+	+++	++
OVA MP	++	+	+
OVA CpG MP	++	+	+
OVA + Alum	++	++	++

Systemic anaphylactic reactions were determined 30 min after intraperitoneal challenge with 1 mg OVA in OVA-sensitized and immunized mice. Score system: + = weak; ++ = moderate; +++ = strong. Data are expressed as the means of the parameters for each group (n = 5–6)

Table 4. Body temperature decrease, MMCP-1 level and death rate after intraperitoneal OVA challenge

Treatment	Temperature °C	MMCP-1 ng/ml	Survival rate, %
PBS	8.5 ± 3.1	4,563 ± 3,079	40
OVA	8.6 ± 1.3	9,009 ± 4,685	0
OVA + CpG	10.3 ± 0.6	12,267 ± 799	0
OVA MP	9.2 ± 1.8	4,637 ± 1,207	50
OVA CpG MP	6.1 ± 4.4 ^a	3,169 ± 1,190 ^b	100
OVA + Alum	10.1 ± 0.4	3,345 ± 738 ^b	33

Measurements were taken after intraperitoneal OVA challenge (1 mg OVA/animal) in BALB/c mice sensitized (50 µg OVA in 2 mg Alum at days 0 and 7) and immunized with 20 µg OVA and 25 µg CpG motifs (divided in 3 intradermal injections at days 31, 36 and 42) in the following combinations: (1) PBS, (2) OVA in solution, (3) mixture of OVA and CpG in solution (OVA + CpG), (4) MP containing OVA alone (OVA MP), (5) OVA and CpG sequences loaded MP (OVA CpG MP) and (6) OVA emulsified with aluminum hydroxide (OVA + Alum). Data are expressed as means ± SD of the parameters for each group (n = 5–6 for each treatment) ^a p < 0.05 compared with OVA + CpG and OVA + Alum; ^b p < 0.01 compared with OVA and OVA + CpG.

in an increase in the production (around 200 pg/ml), compared with the administration of OVA in solution or PBS (around 1,100 pg/ml).

However, all treatments decreased the IFN-γ/IL-4 ratio, from 100 for PBS group to IFN-γ/IL-4 ratios of 26–28 or 16 for OVA + Alum-treated mice. Only the treatment of sensitized mice with OVA microencapsulated alone (without CpG motifs) induced a higher ratio between

IFN-γ- and IL-4-secreting cells of around 49. In any case, these differences in the IFN-γ or IL-4 levels were not statistically significant.

Anaphylactic Shock Evaluation

The anaphylactic shock induced by the administration of OVA by intraperitoneal route was estimated with the analysis of different factors such as allergic promoters (MMCP-1), physiological parameters (temperature) and systemic reactions (piloerection, mobility and cyanosis in mouth, ears and feet).

Animals that received any microparticulated treatment displayed less intense anaphylactic systemic reactions than the control groups (PBS, OVA, OVA mixed with CpG sequences and OVA adsorbed onto Alum), particularly as far as cyanosis and piloerection are concerned (table 3).

Moreover, the MMCP-1 level (table 4), associated with allergic situations, showed that the co-encapsulation of both OVA and CpG oligonucleotide into MP was able to reduce the production of this enzyme (significant differences in comparison with OVA and OVA + CpG) reaching the same levels induced by OVA + Alum treatment (3,500 ng/ml).

The results of the decrease in body temperature, which could lead to death in extreme situations, are shown in table 4. The temperature decrease measured 1 h after challenge in most groups was higher than 7°C. With OVA CpG MP, mice displayed lower decrease in temperature (6.1 ± 4.4°C) than the respective control groups, OVA plus CpG (10.3 ± 0.6°C, p < 0.05) and the conventional treatment, OVA combined with Alum (10.1 ± 0.4°C, p < 0.05).

Analyzing the survival rate 1 h after the provoked challenge (table 4), the treatments based on MP achieved a survival rate higher than 50%, whereas mice treated with the free allergen or with the allergen combined with CpG sequences did not survive (0% survival rate for both treatments). Beside, only 33 and 40% of animals receiving OVA plus Alum and PBS, respectively, survived. Finally, it is noteworthy emphasizing the capacity of OVA CpG MP to avoid death in an anaphylactic episode (100% survival rate).

Discussion

Although the administration of allergens with CpG oligodeoxynucleotides has been shown to be effective for the prevention of Th2 bias and the reversion of estab-

lished allergic responses in mouse models [4, 5], the strategy does not tackle 2 major problems of SIT: the allergen can still cause anaphylactic reactions through interaction with IgE, and multiple injections are necessary to achieve a sufficient immune response. In this study, we analyzed the allergenicity and efficacy of CpG and allergen (OVA) co-encapsulated PLGA MP in OVA-sensitized BALB/c mice. The system could associate the depot properties of MP with Th1 and T regulatory-triggering properties of CpG oligonucleotides. Allergen-sustained release from MP could prevent the side effects through interaction with IgE.

SIT has been associated with the increase in the ratio between allergen-specific IgG2a and IgE antibodies. In the current study, we observed that the administration of microencapsulated allergen in OVA-sensitized mice diminished the IgE/IgG2a ratio in comparison with OVA, OVA + Alum and mixtures OVA + CpG (IgE/IgG2a: 1,540–1,600 for the allergen in MP and 2,770–2,810 for the allergen in solution, with CpG or with Alum; fig. 2c). However, the IgE production observed in mice treated with PLGA-entrapped OVA was similar to sensitized and untreated groups. These results indicated that the encapsulation of the allergen can decrease the risk of sensitization observed during the initial stages of conventional SIT (related to transient IgE increase), resulting in enhanced security [23]. The contribution of CpG co-encapsulation (the OVA/CpG ratio was 1:1) was rather modest (fig. 2b). In fact, higher levels of allergen-CpG conjugation (1:3 or superior) have been previously reported as critical to show therapeutic benefits [4, 5], although similar studies for MP are still lacking.

The modification in antibody levels observed during the SIT is currently mediated by the generation of allergen-specific T regulatory cell and IL-10 production [24] and/or eventually allergen-specific T cell reduction with deviation of Th2 cells in favor of Th1 responses [25]. IFN- γ and IL-4 levels after allergen-specific stimulation of splenocytes indicated that none of the treatments evaluated in our study, when compared with sensitized and untreated mice (PBS group), induced suppression of allergen-specific T cells or significant shifting from Th2 to Th1 response, although IL-4 production induced with microencapsulated antigen was lower than observed in mice treated with OVA + Alum. These results, in agreement with IgE/IgG2a evolution, seem to indicate that the ongoing Th2 response was not downregulated with our schedule of administration.

One of the early benefits of allergen SIT (that even preceded IgE downregulation and restoration of Th2/Th1

balance) is a decrease in mast cell and basophil activity and systemic anaphylaxis in response to allergen provocation. Our results have shown the co-encapsulation of antigen and CpG decreased the risk for allergen sensitization throughout SIT as indicated by the gravity of symptoms as cyanosis or piloerection, lower body temperature change and MMCP-1 levels and the mortality provoked by the allergen challenge (tables 3 and 4). Similarly, Martinez Gomez et al. [12] published that CpG co-encapsulation with the major allergen of bee venom into PLGA MP was a requisite for the induction of IgG2a antibodies and protection against allergen challenge (evaluated as body temperature change) in sensitized mice.

To conclude, MP encapsulating both OVA, as an allergen model, and CpG sequences, as a pro-Th1 adjuvant, decreased the risk for OVA sensitization and IgE induction observed in sensitized mice during SIT. Although our results did not evidence the regulatory mechanisms in which these physiological benefits are based on, PLGA MP could be proposed as a safer alternative to current administration of allergens with Alum.

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