Intradermal immunization with ovalbumin loaded poly-ε-caprolactone microparticles conferred protection in OVA-sensitized allergic mice

Beatriz San Roman[1], Socorro Espuelas[1], Sara Gómez[1], Carlos Gamazo[2], María L. Sanz[3], Marta Ferrer[3], Juan M. Irache[1]

Immunoadjuvant Unit, Departments of Pharmaceutical Technology[1] and Microbiology[2], University of Navarra, 31080 Pamplona, Spain.

[3] Department of Allergy and Clinical Immunology, Clínica Universitaria, 31080 Pamplona, Spain.

Corresponding autor:

Dr. Juan M. Irache

Department of Pharmaceutical Technology. University of Navarra

Ap.177

31080 Pamplona. Spain

Tel: +34 948 425600

Fax: +34 948 425649

e-mail: jmirache@unav.es

Abstract

Background Despite immunotherapy has been reported as the only treatment able to revert the Th2 response, its administration has some disadvantages such as the requirement of multiple doses, possible side effects provoked by conventional adjuvants and the risk of suffering an anaphylactic shock. For that reasons, drug delivery systems appear to be a promising strategy due to its ability to i) transport the allergens, ii) protect them from degradation, iii) decrease the number of administrations and iv) act as immuno-adjuvants.

Objective The aim of this work was to evaluate the properties of poly-ε-caprolactone (PCL) microparticles as adjuvants in immunotherapy using ovalbumin (OVA) as allergen model. For this purpose, the protection capacity of these microparticles (OVA PCL) against OVA allergy was studied in a murine model.

Methods The humoral and cellular induced immune response generated by OVA encapsulated into PCL microparticles was studied immunizing BALB/c mice intradermically. Beside, OVA-sensitized mice were treated with OVA PCL and OVA adsorbed to aluminium hydroxide (OVA-Alum). Fifteen days after therapy, animals were challenged with OVA and different signs of anaphylactic shock were evaluated.

Results One single shot by intradermal route with OVA PCL resulted in a Th2-type immune response. In OVA-sensitized mice, treatment with OVA PCL treatment elicited high OVA specific IgG but low levels of IgE. Furthermore, OVA PCL mice group displayed lower levels of serum histamine and higher survival rate in comparison with the positive control group.

Conclusion The anaphylactic shock suffered by OVA PCL treated mice was weaker than the one induced in the OVA-Alum group. Hence, the intradermal immunization with OVA PCL microparticles induced hyposensitization in OVA-allergic mice.

Keywords Microparticles, ovalbumin, immune response, adjuvant, allergy

Introduction

Mechanistic studies continue to support the idea that immunotherapy expands allergen-specific Th1 immunity and suppresses the Th2 responses resulting in clinical allergy [1,2]. Nowadays, subcutaneous immunotherapy has been considered by WHO [3] to be the only capable way of modifying the development of the allergic processes. The current guideline consists on repetitive administration of increasing doses of the allergen but the success is not always guaranteed. The need for qualified personnel and the difficulties to complain with the whole schedule of treatment limit its effectiveness. Furthermore, the adjuvants commonly used to enhance the strength and the length of the immune response (i.e. aluminium hydroxide or alum, calcium salts) can generate important side effects upon administration. Among them, sensitivity to alum [4], local granuloma formation [5] and anaphylactic shock after the injection [6] have been described. Taking into account the increasingly prevalence of the allergic diseases, and the cost for the healthcare public system [7], the design of a more successful immunotherapy remains a health priority.

During the last decades, drug delivery systems (such as nanoparticles, microparticles and liposomes) have arisen as a new strategy to administer drugs and biological molecules. These systems act like reservoirs to release the molecules in either a continuous or a pulsatile manner avoiding a multiple injection schedule and increasing the drugs half-life within the body [8]. Apart from these advantages, these carriers protect the antigen from pH conditions and enzymatic degradation requiring lower doses and decreasing toxicity [9]. Hence, the treatment would be more comfortable for the patient and the side effects would be minimized. As a consequence, the chance of suffering an anaphylactic shock and the cost of the therapy would decrease. Furthermore, another attractive property of these drug delivery systems is their ability to activate specific immune mechanisms after their uptake by the antigen presenting cells (APC) [8].

Considering all these aspects, the encapsulation of allergens into drug delivery systems seems to be an attractive alternative to the established specific immunotherapy. In fact, recently approaches have shown that encapsulated allergens are able to alter the allergic course in mice models. In this respect, recombinant birch pollen in poly(lactic-co-glycolic acid) (PLGA) nanoparticles has been described to be able to modulate an ongoing Th2 allergic situation after a single dose immunization by subcutaneous route [10]. Similarly, olive allergen into PLGA microparticles intraperitoneally administered induced a Th1 response which was not displayed after immunization with the non-encapsulated allergen [11].

Among synthetic materials, polyesters such as poly-ε-caprolactone (PCL) and PLGA have been widely used to fabricate microparticles because of their biocompatibility and biodegradability. PLGA, which is composed by monomers of poly glycolic and lactic acids, is approved by the FDA for its use in implants and reabsorbable sutures in humans. In contrast to PLGA, PCL is degraded more slowly [12] and without conferring an acidic medium [13] so it is an ideal candidate to design long-lasting degradable carriers without damaging the antigenicity of the encapsulated molecule. Moreover, *in vivo* administration of antigens into PCL microparticles has resulted in the encouragement of the Th1 immune response due to the fact that the hydrophobic behaviour of the polymer increase its interaction with the APC [14].

In this context, the aim of this work was to prepare and evaluate the ability of PCL microparticles as carriers for allergens using ovalbumin (OVA) as model. For this purpose, OVA loaded microparticles (OVA PCL) were fabricated by TROMS® method, a semi-industrial technique capable of encapsulation biological molecules such as adenoviruses [15], proteins or bacterial extracts [16] maintaining their native properties. Then, the immune response induced by administration of one single dose of OVA PCL by intradermal route and the protective capacity of

these microparticles in OVA sensitized BALB/c mice against an anaphylactic shock was evaluated.

Materials and methods

Materials

Poly-ε-caprolactone was purchased from Aldrich Chemical Company Inc. (USA). Ovalbumin (grade V) (OVA), Pluronic® F68, aluminium hydroxide (Alum), sorbitan monolaurate (Tween® 20), concanavalin A and 2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were supplied by Sigma-Aldrich Chemie (Germany). Methylene chloride (reagent grade) was obtained from Scharlau (Spain) and polyvinylalcohol (PVA), molecular weight 125,000, was obtained from Polysciences Inc. (USA). Microbicinchoninic acid (MicroBCA) protein assay kit was purchased from Pierce (USA). All other chemicals were of reagent grade and obtained from Merck & Co., Inc. (USA).

Microparticle preparation

Microparticles containing OVA (OVA PCL) were prepared by the solvent extraction/evaporation method using TROMS® to prepare the $W_1/O/W_2$ multiple emulsion [15,16]. For the preparation of the microparticles, the polymer solution (200 mg PCL in 5 ml of methylene chloride) was injected through a needle with an inner diameter of 0.17 mm (pumping flow of 50 ml/min) into a first vessel containing the inner aqueous phase (OVA dissolved in Pluronic® F68). Then, the previously formed inner emulsion (W_1/O) was forced to circulate through the system for 2 min under a turbulent regime (flow of 50 ml/min). After this step, the first emulsion was injected (maintaining the pumping flow constant) into a second vessel containing 30 ml of an aqueous phase 0.5% PVA (W_2 phase). The turbulent injection through the needle (inner diameter of 0.17 mm) resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was further homogenised by circulation into the system for 4 min. The resulting $W_1/O/W_2$ emulsion was stirred for at least 2 h under room conditions in order to eliminate the organic solvents by evaporation. The microparticles were purified by centrifugation at 9300 x *g* (12150-H, Sigma 3K30) and resuspended in distilled water a

total of three times. The washed microparticles were finally freeze-dried (Genesis 12EL, Virtis, USA) and stored at 4ºC.

In addition, empty microparticles (PCL) were also prepared in the same way as described above and used as a control formulation.

Characterization of Microparticles

Size and morphology

Microparticles were sized by laser diffractometry using a Mastersizer S laser sizer (Malvern Instruments, UK). The average particle size was expressed as the volume mean diameter (V_{md}) in micrometers (μm).

The shape and morphology of each formulation were examined by scanning electron microscopy (SEM). Freeze-dried microparticles were mounted on double-sided adhesive tape onto metal stubs, coated with gold to a thickness of 16 nm (Emitech K550 equipment). Further observations were made by SEM (Zeiss DSM 940 A) with a digital imaging capture system (DISS, Point Electronic GmBh)..

Determination of the OVA loading

To quantify the OVA content, approximately 5 mg of lyophilized microparticles were degraded with 1 ml of NaOH 0.1 N by shaking overnight on a magnetic stirrer at room temperature. The samples were centrifuged (27100 x *g*, 10 min) and the amount of protein in the supernatants was determined by the MicroBCA protein assay. The OVA loading was expressed as the amount (in micrograms) of antigen loaded per mg microparticles. The entrapment efficiency was determined by relating the OVA entrapped in the batch of microparticles to the initial weight of protein.

In vitro release of OVA

OVA loaded microparticles (10 mg) were dispersed in 1 ml of phosphate buffer saline (PBS, pH 7.4, 0.01 M). The sample tubes were incubated in a shaker at 37±1 ºC. At different time intervals, sample tubes were centrifuged (27100 x *g*, 20 min), the supernatants removed and the protein content was determined by MicroBCA assay and performed in a 96-well multiscaner autoreader (Labsystems iEMS Reader MF). The dissolution medium was replaced after the withdrawal of each aliquot. Unloaded microspheres were used as a control and subjected to the same procedure. Release profiles were expressed in terms of cumulative release and plotted versus time.

Structural integrity and antigenicity of the entrapped OVA

To evaluate the effects of the manufacturing process on the OVA structural integrity and antigenicity, 5 mg of OVA loaded microparticles were dissolved in methylene chloride. The organic solvent was evaporated and the residue was suspended in electrophoretic sample buffer (TRIS-HCl 62.5 mM (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.05% bromophenol blue). To eliminate polymeric residues, this suspension was centrifuged (2300 x *g*, 10 min). Then, the sample was boiled during 10 min to split up possible ovalbumin fragments. The protein profile was determined by SDS-PAGE and its antigenicity by immunoblotting.

For SDS-PAGE, samples were analysed by using 15% acrylamide slabs with the discontinuous buffer system of Laemmli [17] and gels stained with Coomasie Brilliant Blue R-250 [18] or alkaline-silver [19]. Immunoblotting was carried out as described previously [20] with immunoglobulin G against OVA from mouse and with horseradish conjugated rabbit anti-IgG. The substrate 4-chloro, 1-naphtol was used as chromogen.

Immunization studies

Mice immunization

Single dose immunization was assessed in BALB/c mice (Harlan Interfauna Ibérica, Barcelona, Spain) to analyze the effect of OVA PCL microparticles on the immune response. The experiments were performed in compliance with the regulations of the Ethical Committee of the University of Navarre in line with the European legislation on animal experiments (86/609/EU).

Female BALB/c mice (n=5 per group, 8 weeks old) were immunized by the intradermal route with one of the following: i) free ovalbumin (10 μg per mouse) in 50 μl of PBS; ii) empty microparticles (PCL); iii) OVA PCL microparticles (10 μg of OVA per mouse); and iv) OVA in aluminium hydroxide (OVA-Alum). OVA-Alum was prepared by dissolving the protein in PBS (pH 7.4, 0.01 M) and subsequent mixing by sonication (XL2000 Ultrasonic Processor, Misonix Inc., USA) with aluminium hydroxide.

Blood samples were collected from the retro-orbital plexus at day 0, 7, 14, 28 and 35 after immunization. The samples were centrifuged and the resulting sera were pooled. Finally, the sera were diluted 1:10 in PBS and stored at –80ºC until assayed by ELISA.

Measurement of anti-OVA antibody levels in serum

An indirect ELISA was performed to determine the level of OVA-specific antibody isotypes in the serum [21]. The experiment was carried out as follows: 96-well microtitre plates (Thermo Labsystems, Finland) were coated overnight with 1 μg per well of ovalbumin in carbonate-bicarbonate buffer (pH 9.6) and maintained at 4ºC. After being washed in buffer (phosphate buffer saline containing 0.05% Tween® 20, PBS-T20), serum samples (100 μl) at different dilutions were added to wells and incubated during 4 h at 37ºC. Then, unbound antibody was washed prior to the addition of 100 μl of goat anti-mouse IgG1 or IgG2a horseradish peroxidase

conjugate (Nordic Immunology, Netherlands) diluted 1:1000 in PBS-T20 (37ºC, 1 h). After a final wash step, 100 µl of chromogen and substrate solution (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid and hydrogen peroxyde) was added. The plates were incubated under room temperature for 30 min, and, finally, the absorbance was measured at 405 nm by an iEMS Reader MF (Labsystems, Finland).

Cytokine assay

Naïve and immunized mice were sacrificed by cervical dislocation at day 35 after immunization and their spleens removed and placed in RPMI 1640 media (Gibco-BRL, UK) under sterile conditions. Each spleen was smashed and cells within experimental groups were pooled in one flask. The cellular suspension was centrifuged at 400 x *g* for 10 min, the supernatant discarded and the pellet washed twice with PBS. The splenocytes were suspended in lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA 0.1 mM) for 2 min to eliminate erythrocytes and refilled with RPMI 1640 to stop the reaction. This suspension was centrifuged (400 x *g*, 5 min) and the pellet was resuspended in RPMI 1640 medium supplemented 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 foetal bovine serum (all from Gibco-BRL, UK). The lymphocyte suspension was added to 96-well round bottom microtitre plates (Iwaki, Japan) (8×10^5 cells/well) along with test antigen (20, 80 and 160 µg OVA/ml in a final volume of 250 µl per well). Negative (wells without antigen) and positive (wells containing 1 µg/ml concanavalin A, used as mytogen) controls were used. The culture supernatants were collected for cytokine assay at 48 h after the stimulation. Then, the supernatants were kept frozen at -80ºC in a 96-well flat bottom microplate until testing. IFN-γ and IL-4 levels were determined using a commercial ELISA kit (Biosurce International, USA).

OVA sensitization, immunization and challenge

Female BALB/c mice (n=5 per group, 8 weeks old) were sensitized as described previously [22]. Fig. 1 shows the scheme of the sensitization and the immunization schedule. Mice were obtained and housed as described above and the experiments were conducted with Ethical Committee of the University of Navarre approved protocols in compliance with the European legislation (86/609/EU). To monitor serum antibody responses, blood samples were obtained from retro-orbital plexus along the sensitization and immunotherapy periods. Sera were collected and stored at –80ºC until their analysis.

Determination of total IgE antibody levels in serum

To determine IgE antibody level, microtitre plates (Nunc-Immuno™ Plate, Denmark) were coated overnight at 4ºC with anti-mouse IgE antibodies (pH 9.5) (BD OptEIA™ Set Mouse IgE, BD Biosciences, USA). Thereafter, plates were washed with PBS-Tween® 20 0.05% (PBS-T20) and blocked for 1 h at room temperature with PBS-FBS 10%, washed again and sera dilutions were incubated for 2 h at room temperature. After washing, plates were incubated for 1 h at room temperature with a mixture of anti-IgE antibodies marked with biotin and horseradish peroxidase-conjugated. Later, plates were washed in the same conditions and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in citric acid (pH 4.0) and hydrogen peroxide was added. The optical density was measured at 405 nm in a microplate autoreader (iEMS Reader MF, Labsystems, Finland).

Measurement of OVA specific IgG antibody levels in serum

Levels of specific IgG anti-OVA antibody in serum samples were determined by indirect ELISA. The assay was carried out as described above but in this case after washing the unbound

antibody samples were incubated for 1 h with goat anti-mouse IgG horseradish peroxidase conjugate, diluted 1:1000 in PBS-T20.

Serum histamine increase after OVA challenge

Histamine release test was performed on heparinized whole blood from the retro-orbital plexus obtained before and 30 min after the challenge. Samples were anticoagulated using EDTA (10 mM) to measure basal histamine level or lysed using perchloric acid (1.4% w/w) to determine whole blood histamine content. Also, blood samples were incubated with negative control (buffer only) and positive control (anti-IgE). Samples were centrifuged (10 min, 800 x *g*) and histamine production was assayed by a fluorometric method as previously described [23] using a Technicon II Analyzer (Technicon Instrument Corp., USA).

Body temperature decrease and hypersensitivity reactions analysis

The body temperature changes associated with anaphylactic shock were monitored by measuring the rectal temperature [24] without general anesthesia before and 10 min after the challenge. Anaphylactic symptoms (activity, bristly hair and cyanosis) were evaluated 30 min after the challenge using a scoring system modified from previous reports [25,26]. Reactions severity was classified in following categories depending on their gravity: i) (-) absent; ii) (+) weak; iii) (++) moderate; and iv) (+++) strong. Finally, the survival rates were recorded 24 h after intraperitoneal challenge.

Statistical analysis

Sensitization data were compared using ANOVA followed by Tukey test to assess statistical significance. Previously, Kolmogorov-Smirnov test was applied to evaluate if data (n=5) followed

a normal distribution. Results were considered statistically significant if $p<0.05$. All calculations were performed using SPSS® statistical software program (SPSS® 10, Microsoft, USA).

Results

Microparticle characterization

Microparticles obtained by TROMS® displayed a homogeneous mean size of about 1.5 µm when measured using laser diffractometry. The protein loading was of 55.4±6.0 µg/mg, which corresponded with an encapsulation efficiency of 77.3±4.6% (Table 1). In addition, the analysis of the morphology by SEM (Fig. 2) indicated that the particles were spherical, well shaped and with uniform size, confirming the size distribution obtained by laser diffractometry.

The *in vitro* release profile of ovalbumin from OVA PCL was assessed in PBS at 37ºC±1. Microparticles released the protein in a biphasic way, characterised by an initial and short release period of 6% in the first 24 h, followed by a long sustained release for at least 35 days. At the end of the experiment the quantity of ovalbumin detectable in the supernatant was approximately 20% (data not shown).

The effect of the preparative process on both the structural conformation and the antigenicity of OVA were studied by SDS-PAGE and immunoblotting analysis, respectively (Fig. 3). In comparison with the free OVA, the electrophoretic profile suggested that the structural integrity of the entrapped ovalbumin was not affected by TROMS®. Furthermore, it is interesting to note that the OVA released from the microparticles maintained its electrophoretic profile as well.

Antibody and cellular immune response

The serum levels of OVA-specific IgG1 elicited in BALB/c mice after a single dose of 10 µg of OVA (free OVA, OVA mixed with Alum or OVA encapsulated in MP) are depicted in Fig. 4. None of the treatments was capable of inducing detectable OVA-specific IgG2a levels using this immunization schedule (data not shown). One single intradermal immunization with free OVA or empty microparticles was not able to induce an specific antibody response. In contrast, mice

immunized either with OVA-Alum or OVA PCL elicited a Th2 response (high levels of IgG1 and no detectable specific IgG2a antibodies).

These data were confirmed by studying the cellular immune response induced. Thus, at day 35 after intradermal immunization OVA PCL treated mice presented a Th2 cytokine profile, with higher splenic levels than mice immunized with free OVA (574.0 pg/ml versus 443.3 pg/ml for IFN-γ and 103.2 pg/ml versus 37.1 pg/ml for IL-4).

Mice sensitization and induction of active systemic anaphylaxis (ASA)

BALB/c mice were sensitized with 50 μg OVA adsorbed in aluminium hydroxide (OVA-Alum) by intraperitoneal route at days 0 and 7. Once confirmed the allergic status to OVA, mice were immunized intradermically at days 13, 16 and 19 with either OVA PCL or OVA-Alum (as positive control) (3.33 μg OVA per immunization). Finally, at day 34 mice were challenged with 1 mg of OVA (i.p.) to provoke a systemic anaphylaxis.

The IgE levels were found to be higher for the animals immunized with OVA-Alum than those with OVA PCL (Fig. 5a). At day 35, the amount of IgE for the untreated control group was two times higher than the one produced by mice immunized with OVA PCL (20.08 μg/ml versus 11.27 μg/ml). On the other hand, the levels of specific IgG to OVA were similar along the experiment with any treatment (Fig. 5b).

In order to estimate the IgE-mediated mast cell degranulation, the serum histamine level from the immunized animals was quantified 30 min after the intraperitoneal challenge. Fig. 6 summarizes these results. The histamine secretion in mice treated with OVA PCL microparticles was reduced with respect to the conventional formulation OVA-Alum (310.8 ng/ml versus 699.1 ng/ml; $P<0.05$). Likewise, the decrease of the body temperature was less strong in animals treated with OVA PCL than with OVA-Alum (2.1ºC±0.5, $P<0.01$, and 3.6ºC±1.0, $P<0.001$, comparing with basal body

temperature) (Table 2). The severity of the anaphylactic shock induced in the animals by both formulations was evaluated measuring cyanosis, activity and bristly hair. OVA PCL treated mice displayed a normal activity and motion with slight cyanosis while mice treated with OVA-Alum exhibited a total lack of mobility and a high degree of cyanosis. So, animals treated with OVA PCL displayed less intense symptoms than the control group. Finally, the dead rate was also lower for the immunized animals with microparticles than those treated with OVA-Alum (60% versus 80%, respectively).

Discussion

Nowadays, immunotherapy is the only way to diverse the allergic march and to improve the quality of life of these patients. However, there is a real need to look for safer vaccine systems. Particulate delivery systems have been demonstrated to be a good chance as immunomodulator adjuvants to generate prolonged and effective immune responses for a large number of antigens [27-29].

When working with biological molecules such as proteins, an essential aspect is the maintenance of their properties during the fabrication process. Thus, in this work TROMS® technology has been confirmed to act as a guarantor for the microencapsulation of ovalbumin without altering the antigenic and immunogenic properties of the protein. This fact is supported on the avoidance of aggressive shearing forces used in some traditional fabrication processes such as sonication. Besides, other advantages have been described for this innovative system like the possibility of semi-industrial manufacturing and the high reproducible batches [15].

Regarding the physico-chemical characterization, the microparticles fabricated in this work showed more homogeneous size distribution with higher encapsulation efficacy comparing with other authors [30,31]. Additionally, OVA PCL displayed no burst effect (only 6% was released during the first 24 h) combined with a constant release until the end of the experiment (20% of OVA release in 35 days). These results suggest that the largest amount of protein was in the OVA PCL polymeric matrix. The distribution of the antigen along the microparticle could determine the release profile. In this case, the OVA distribution might be induced by the fabrication method, in which the emulsification was carried out by turbulent injection of the phases.

One main advantage claimed in the use of drug delivery systems is that the continuous antigen release may validate the efficacy of one single shot instead of the more inconvenient multiple

dosage [31,32]. In this work, single immunization with OVA PCL resulted in an immune response biased towards a Th2 profile.

However, when the administration schedule was modified from single to three injections mice treated with OVA PCL did not show an allergic immune response but a moderated anaphylactic shock in comparison with OVA-Alum treated animals. This modification in the immunotherapeutical schedule might induce a reversion from high levels of IgG1 to a more balance situation between IgG1 and IgG2a. In fact, current treatments for human specific immunotherapy which have confirmed their benefits in medium-term periods [33,34] are based on the repetitive administration of increasing doses of the allergen. The high specific IgG levels induced by OVA PCL and OVA-Alum would be able to counteract the production of IgE. Nevertheless, the balance between both antibodies would be in favor of OVA PCL because of the lower IgE level. Thus, IgG antibodies would block the allergen avoiding its interaction with IgE antibodies [35]. In this way, IgG competes with IgE for allergen binding and inhibit IgE-mediated granular release of mast cells and basophils. This partial deviation of the Th2 environment induced by OVA PCL would be responsible for the improvement in the symptoms associated to anaphylactic shock, such as a less increase of histamine level in serum, a lower decrease of the body temperature and a lower mortality rate than OVA-Alum treated mice. In summary, we believe that the administration of the microparticles is a less aggressive system to induce hyposensitization.

Consequently, OVA PCL could be the starting point to fabricate in semi-industrial scale polymeric carriers for allergens. These vehicles could be associated to different immunomodulators that could strengthen a Th1 response. New experiments with natural allergens are in course in order to test PCL microparticles as safe and efficacious adjuvants against allergy.

Acknowledgements

The authors would like to thank Esther Luquin for excellent technical assistance. This work was financially supported by “Gobierno de Navarra”, “Fundación Universitaria de Navarra” and “Ministerio de Ciencia y Tecnología” (SAF 2001-0690-C0301) from Spain.

References

1 Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. J Exp Med 1993;178:2123-2130.

2 Bousquet J, Becker WM, Hejjaoui A, Chanal I, Lebel B, Dhivert H, Michel FB. Differences in clinical and immunologic reactivity of patients allergic to grass pollens and to multiple-pollen species. II. Efficacy of a double-blind, placebo-controlled, specific immunotherapy with standardized extracts. J Allergy Clin Immunol 1991;88:43-53.

3 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102:558-562.

4 Clemmensen O, Knudsen HE. Contact sensitivity to aluminium in a patient hyposensitized with aluminium precipitated grass pollen. Contact Dermatitis 1980;6:305-308.

5 Vogelbruch M, Nuss B, Korner M, Kapp A, Kiehl P, Bohm W. Aluminium-induced granulomas after inaccurate intradermal hyposensitization injections of aluminium-adsorbed depot preparations. Allergy 2000;55:883-887.

6 Williams AP, Krishna MT, Frew AJ. The safety of immunotherapy. Clin Exp Allergy 2004;34:513-514.

7 Edwards PE. Asthma management at the source. Case Manager 2004;15:59-61.

8 Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. J Control Release 2003;90:261-280.

9 Reddy LH. Drug delivery to tumours: recent strategies. J Pharm Pharmacol 2005;57:1231-1242.

10 Scholl I, Weissenbock A, Forster-Waldl E, Untersmayr E, Walter F, Willheim M, Boltz-Nitulescu G, Scheiner O, Gabor F, Jensen-Jarolim E. Allergen-loaded biodegradable poly(D,L-

lactic-co-glycolic) acid nanoparticles down-regulate an ongoing Th2 response in the BALB/c mouse model. Clin Exp Allergy 2004;34:315-321.

11 Batanero E, Barral P, Villalba M, Rodriguez R. Encapsulation of Ole e 1 in biodegradable microparticles induces Th1 response in mice: a potential vaccine for allergy. J Control Release 2003;92:395-398.

12 Lemoine D, Francois C, Kedzierewicz F, Preat V, Hoffman M, Maincent P. Stability study of nanoparticles of poly(epsilon-caprolactone), poly(D,L-lactide) and poly(D,L-lactide-co-glycolide). Biomaterials 1996;17:2191-2197.

13 Jameela SR, Suma N, Jayakrishnan A. Protein release from poly(epsilon-caprolactone) microspheres prepared by melt encapsulation and solvent evaporation techniques: a comparative study. J Biomater Sci Polym Ed 1997;8:457-466.

14 Murillo M, Grillo MJ, Rene J, Marin CM, Barberan M, Goni MM, Blasco JM, Irache JM, Gamazo C. A Brucella ovis antigenic complex bearing poly-epsilon-caprolactone microparticles confer protection against experimental brucellosis in mice. Vaccine 2001;19:4099-4106.

15 del Barrio GG, Novo FJ, Irache JM. Loading of plasmid DNA into PLGA microparticles using TROMS (Total Recirculation One-Machine System): evaluation of its integrity and controlled release properties. J Control Release 2003;86:123-130.

16 Estevan M, Gamazo C, Gonzalez-Gaitano G, Irache JM. Optimization of the entrapment of bacterial cell envelope extracts into microparticles for vaccine delivery. J Microencapsul 2006;23:169-181.

17 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.

18 Fairbanks G, Steck TL, Wallach DF. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 1971;10:2606-2617.

19 Oakley BR, Kirsch DR, Morris NR. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 1980;105:361-363.

20 Gamazo C, Winter AJ, Moriyon I, Riezu-Boj JI, Blasco JM, Diaz R. Comparative analyses of proteins extracted by hot saline or released spontaneously into outer membrane blebs from field strains of Brucella ovis and Brucella melitensis. Infect Immun 1989;57:1419-1426.

21 Faquim-Mauro EL, Macedo MS. Induction of IL-4-dependent, anaphylactic-type and IL-4-independent, non-anaphylactic-type IgG1 antibodies is modulated by adjuvants. Int Immunol 2000;12:1733-1740.

22 Steerenberg PA, van Dalen WJ, Withagen CE, Dormans JA, van Loveren H. Optimization of route of administration for coexposure to ovalbumin and particle matter to induce adjuvant activity in respiratory allergy in the mouse. Inhal Toxicol 2003;15:1309-1325.

23 Castillo JG, Gamboa PM, Oehling A, Wong E, de la Cuesta CG. Variations in antigen-specific histamine release related with immunotherapeutic treatment. Allergol Immunopathol (Madr) 1989;17:149-153.

24 Watanabe N, Matsuda E, Masuda A, Nariai K, Shibasaki T. The effects of fexofenadine on eosinophilia and systemic anaphylaxis in mice infected with Trichinella spiralis. Int Immunopharmacol 2004;4:367-375.

25 Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999;103:206-214.

26 Poulsen OM, Hau J, Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clin Allergy 1987;17:449-458.

27 Jones DH, McBride BW, Jeffery H, O'Hagan DT, Robinson A, Farrar GH. Protection of mice from Bordetella pertussis respiratory infection using microencapsulated pertussis fimbriae. Vaccine 1995;13:675-681.

28 Maloy KJ, Donachie AM, O'Hagan DT, Mowat AM. Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles. Immunology 1994;81:661-667.

29 Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan--DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 1999;5:387-391.

30 Benoit MA, Baras B, Gillard J. Preparation and characterization of protein-loaded poly(epsilon-caprolactone) microparticles for oral vaccine delivery. Int J Pharm 1999;184:73-84.

31 Slobbe L, Medlicott N, Lockhart E, Davies N, Tucker I, Razzak M, Buchan G. A prolonged immune response to antigen delivered in poly (epsilon-caprolactone) microparticles. Immunol Cell Biol 2003;81:185-191.

32 Uchida T, Martin S, Foster TP, Wardley RC, Grimm S. Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin. Pharm Res 1994;11:1009-1015.

33 Malling HJ, Dreborg S, Weeke B. Diagnosis and immunotherapy of mould allergy. V. Clinical efficacy and side effects of immunotherapy with Cladosporium herbarum. Allergy 1986;41:507-519.

34 Kagi MK, Wuthrich B. Different methods of local allergen-specific immunotherapy. Allergy 2002;57:379-388.

35 Vrtala S, Ball T, Spitzauer S, Pandjaitan B, Suphioglu C, Knox B, Sperr WR, Valent P, Kraft D, Valenta R. Immunization with purified natural and recombinant allergens induces mouse

IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. J Immunol 1998;160:6137-6144.

Table 1: Physico-chemical characteristics of microparticles prepared by TROMS®. PCL: empty microparticles; OVA PCL: ovalbumin loaded poly-ε-caprolactone microparticles. Data are expressed as mean ± SD (n = 3).

	Size (µm)	**OVA loading (µg/mg MP)**	**Encapsulation efficiency (%)**
PCL	1.61±0.37	-	-
OVA PCL	1.72±0.29	55.43±6.03	77.30±4.63

Table 2: Systemic anaphylactic reactions and body temperature measurement 30 min and 10 min after intraperitoneal challenge, respectively, and death rate 24 h later. Score system: (-) absent; (+) weak; (++) moderate; (+++) strong. Data are expressed as mean ± s.d. (n=5); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (ANOVA followed by Tukey`s test) compared with the basal body temperature (38.4±0.2ºC) in BALB/c mice before challenge.

	Body temperature (ºC)	Activity	Cyanosis	Bristly hair	Death rate (%)
OVA-Alum	34.0±1.5 ***	-	+ + +	+ +	80
OVA PCL	35.7±0.9 **	+ +	+	+ +	60

Figure legends

Fig. 1: Experimental design. BALB/c mice received 50 µg OVA adsorbed onto 1 mg Alum at days 0 and 7. After that, mice were immunized with OVA PCL and OVA-Alum at days 13, 16 and 19 (3.33 µg OVA per immunization). For induction of systemic anaphylactic shock mice were intraperitoneally challenged with 1 mg OVA.

Fig. 2: Scanning electron microscopy of lyophilized OVA-loaded microparticles prepared by TROMS®, showing homogeneous sized and spherical shaped particles.

Fig. 3: Study of the structure and the antigenicity of OVA. Lane 1 and 2: Coomasie Brilliant Blue R-250 stain of free OVA and OVA extracted with methylene chloride from OVA PCL, respectively. Lane 3: SDS-PAGE for OVA released from the particles after 35 days of incubation in PBS at 37ºC. Alkaline-silver stain for proteins was employed. Lane 4: Western-blot analysis of OVA from OVA PCL microparticles using a commercial serum anti-OVA from mouse. Load was the equivalent to 6 µg OVA/lane.

Fig. 4: Serum antibody response to ovalbumin measured by indirect ELISA on sera from BALB/c mice intradermically immunized (10 µg OVA) with the following: OVA in solution (OVA) (■), OVA adsorbed in aluminium hydroxide (OVA-Alum) (●) and ovalbumin loaded microparticles (OVA PCL) (▲). The antibody titre is defined as the reciprocal of a serum dilution whose optical density was equal or above 0.2 than blank samples reading the absorbance at 405 nm, starting from sample dilution 1:40.

Fig. 5: Evolution of total IgE (a) and specific anti-OVA IgG (b) in sera from BALB/c mice during

the sensitization and challenge study. Mice were sensitized to OVA (50 μg OVA by intraperitoneal route, days 0 and 7) and immunized (10 μg OVA, divided in three doses of 3.33 μg; see arrows (↓) in the figures) with OVA-Alum (●) and OVA PCL (▲).The antibody titre is defined as the reciprocal of a serum dilution whose optical density was equal or above 0.2 than blank samples reading the absorbance at 405 nm.

Fig. 6: Plasma histamine increase (ng/mL) 30 minutes after the intraperitoneal challenge (day 34) with 1 mg OVA in mice sensitized with OVA (50 μg OVA at days 0 and 7) and immunized with 3.33 μg OVA (adsorbed in Alum or encapsulated in PCL microparticles) at days 13, 16 and 19. Basal histamine level: 9.8±4.5 ng/mL. Data are reported as mean ± s. d. * $P < 0.05$ comparing both treatments with ANOVA followed by Tukey`s test.

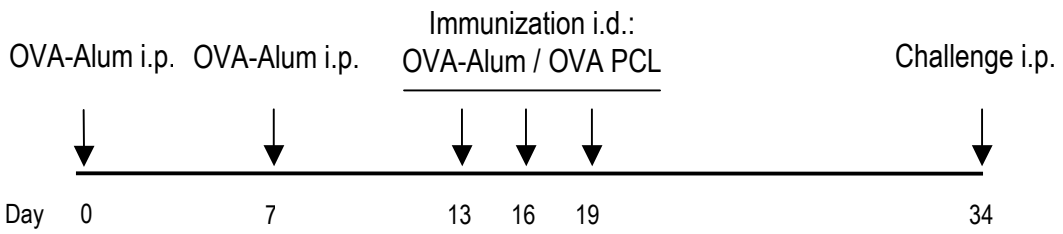

OVA-Alum i.p.
OVA-Alum i.p.
Immunization i.d.:
OVA-Alum / OVA PCL
Challenge i.p.
Day
0
7
13
16
19
34

Figure 1

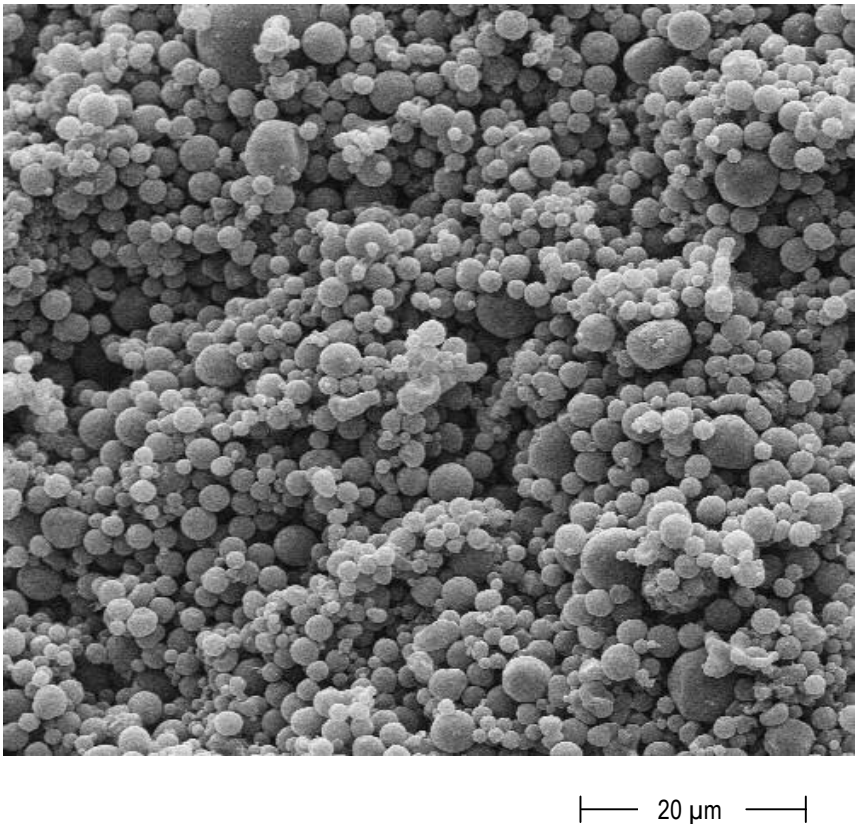


Figure 2

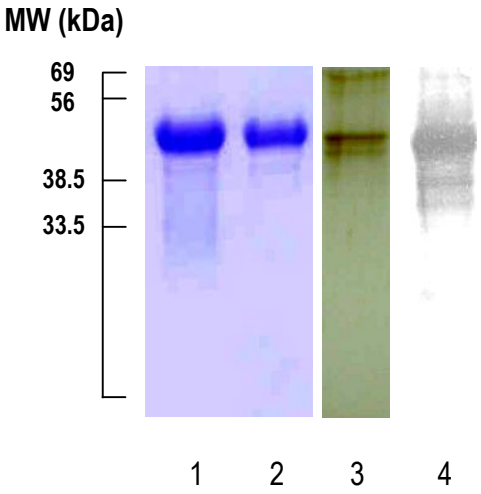


Figure 3

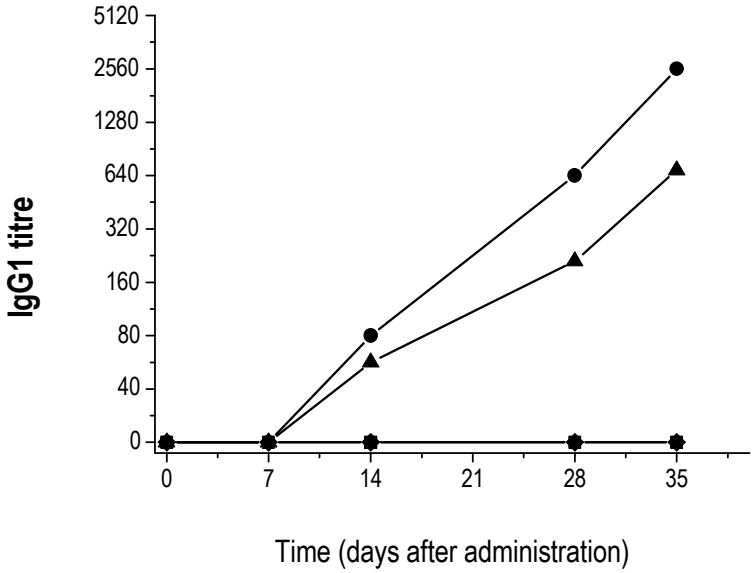
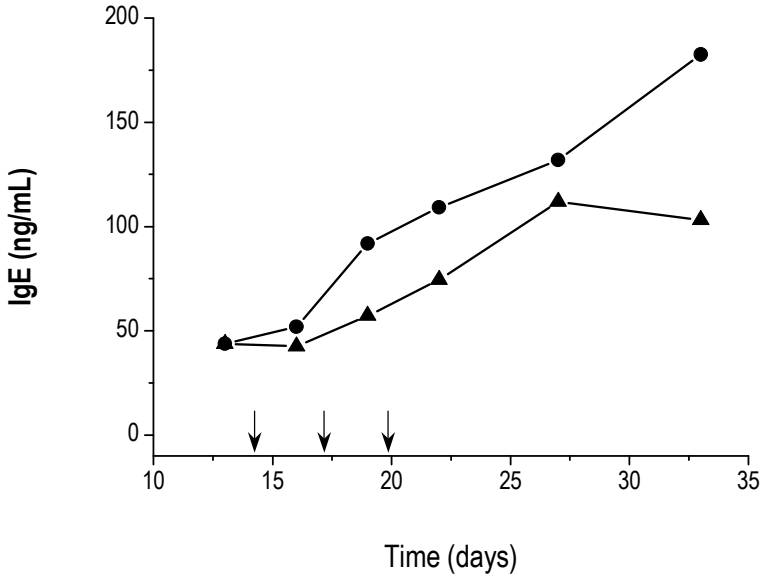

5120
2560
1280
640
320
160
80
40
0
IgG1 titre
0
7
14
21
28
35
Time (days after administration)

Figure 4

a)


IgE (ng/mL)
200
150
100
50
0
10
15
20
25
30
35
Time (days)

b)

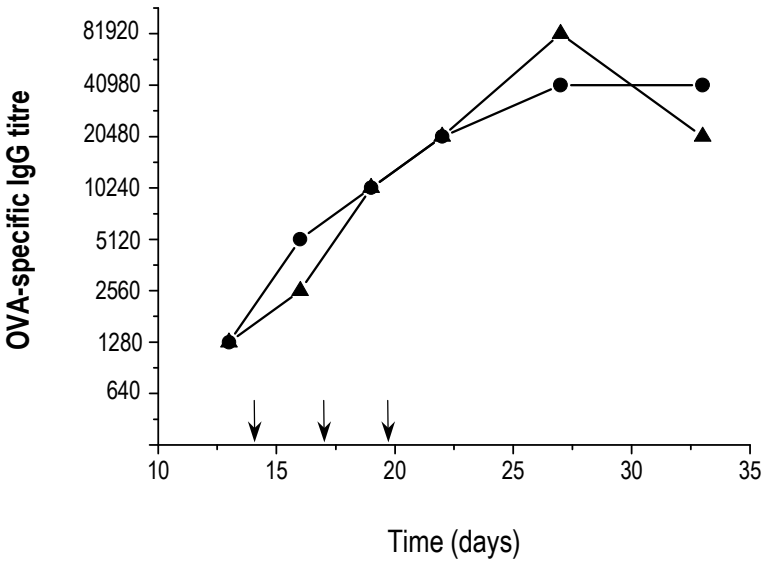

OVA-specific IgG titre
81920
40980
20480
10240
5120
2560
1280
640
10
15
20
25
30
35
Time (days)

Figure 5

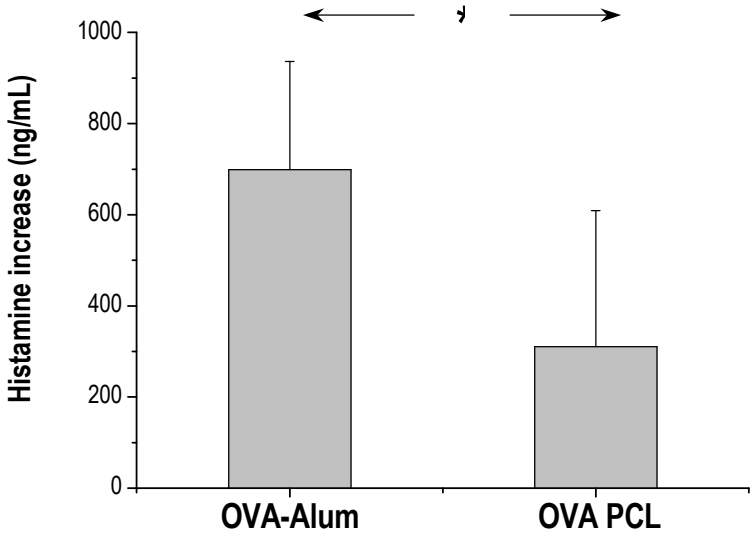

Histamine increase (ng/mL)
1000
800
600
400
200
0
OVA-Alum
OVA PCL

Figure 6