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Research paper

Allergen immunotherapy with nanoparticles containing lipopolysaccharide from *Brucella ovis*

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ABSTRACT

The adjuvant and protective capacity against anaphylactic shock of the association between rough lipopolysaccharide of *Brucella ovis* (LPS) coencapsulated with ovalbumin (OVA), as a model allergen, in Gantrez® AN nanoparticles was investigated. Several strategies were performed in order to study the adjuvant effect of the LPS either encapsulated or coating the nanoparticles. OVA, as well as LPS, was incorporated either during the manufacturing process (OVA-encapsulated or LPS-encapsulated nanoparticles, respectively) or after the preparation (OVA-coated or LPS-coated nanoparticles, respectively). After the administration of 10 μg of OVA incorporated in the different formulations, all the nanoparticles, with or without LPS, were capable of amplifying the immune response (IgG_1 and IgG_{2a}). However, in a model of sensitized mice to OVA, the formulation with OVA and LPS-entrapped inside the nanoparticles administered intradermally in three doses of 3 μg of OVA each was the only treatment that totally protected the mice from death after a challenge with an intraperitoneal injection of OVA. In contrast, the control group administered with OVA adsorbed onto a commercial alhydrogel adjuvant showed 80% mortality. These results are highly suggestive for the valuable use of Gantrez® nanoparticles combined with rough LPS of *B. ovis* in immunotherapy.

1. Introduction

Allergy, or hypersensitivity type I, applies to an abnormal reaction against innocuous environmental compounds (allergens) and involves complex interactions between exogenous and genetically determined factors. The development of allergen-specific IgE is closely linked to allergen-specific T helper cell responses, characterised by a predominant production of type-2 cytokine, as it is seen at sites of acute allergic inflammations [1]. The prevalence of type I allergies has constantly increased within recent years. Concretely, the European Academy of Allergology and Clinical Immunology organisation affirms that, in EU countries, allergic disease is the most common chronic illness of childhood, affecting more than one child in four in some countries [2]. On the other hand, Arbes et al. found that 54.3% of the United States population, aged 6–59 years, had a positive allergy skin test to at least one of 10 common allergens [3]. Nowadays, the only effective "treatment" to cure allergy is specific immunotherapy (SIT).

SIT involves repeated administrations of the sensitizing allergen, usually by subcutaneous injection or, more recently, by sublingual application. SIT has been shown to be a robust and clinically effective approach [4,5], improving the quality of life of the treated individuals, through the reduction of symptoms and medication usage [6]. However, historically, variability in safety and clinical efficacy has limited the widespread application of SIT [7]. Many strategies have been proposed in an attempt to solve these drawbacks, including the use of recombinant allergens and allergen derivatives [8], peptides containing aminoacid sequences of allergen T-cell epitopes [9], low molecular weight fractions of allergen extracts [10], mimotopes [11] and DNA vaccines [12].

The SIT drawbacks could also be overcome by the use of appropriate adjuvants in order to decrease the administered allergen dose and to promote the adequate immune response [13]. In this context, we have demonstrated in previous studies the adjuvant capacity of Gantrez® nanoparticles, which can effectively enhance the immune response when administered by intradermal route [14]. However, this effect may be improved and or modulated by the incorporation of a PAMP (pathogen-associated molecular pattern) to nanoparticles.

The dendritic cells (DCs) are antigen presenting cells (APCs) involved in initiating, directing, and controlling both innate and

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adaptive immune responses [15,16]. The recognition of the antigens by the DCs is mediated by their toll-like receptors (TLRs) located on their surface and within endosomal compartments. These TLRs are one class of PAMP recognizing receptors, and some examples of PAMPs are lipopolysaccharide from Gram-negative bacteria [17], unmethylated bacterial CpG DNA sequences [18] or peptidoglycans from Gram-positive bacteria among others [19,20]. For all these reasons, the addition of PAMPs to nanoparticles could be a good strategy to induce activation of TLR-mediated signalling pathways in DCs, maturation of DCs and, consecutively, strong activation of antigen-specific T lymphocytes. In this context some PAMPs, such as lipopolysaccharide or lipid A (which is known to be the region of the lipopolysaccharide responsible for its adjuvant capacity [21]) have been incorporated into some vehicles such as microparticles [22,23] or nanoparticles [24,25] in order to enhance their adjuvant effect. However, a main drawback when using LPS is its intrinsic toxicity [26,27]. Therefore, in this investigation a rough lipopolysaccharide from *Brucella ovis,* which is known to be low-endotoxic [28] was selected. On the other hand, OVA was chosen as the model allergen because mice rendered allergic to OVA represent a suitable animal model for human allergen vaccination.

In this work, Gantrez® nanoparticles containing LPS of *B. ovis* were prepared. The effect of LPS location (coating the surface or encapsulated into the matrix) in the carriers was evaluated in immunization studies as well as in sensitization experiments and further challenge against OVA.

2. Methods

2.1. Chemicals

Gantrez® AN 119 [poly(methyl vinyl ether-co-maleic anhydride); MW 200,000] was kindly gifted by ISP (Barcelona, Spain). Ovalbumin (OVA) (grade V), 1,3-diaminopropane (DP), 2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) and alum were purchased from Sigma–Aldrich Chemie (Germany). The peroxidase immunoconjugates (GAM/IgG_1/PO and GAM/IgG_{2a}/PO) and the fluorescein–isothiocyanate-conjugated goat anti-rabbit IgG were obtained from Nordic Immunology (The Netherlands). The IL-10 ELISA kit was purchased from Biosource International (California, USA). All other chemicals used were of reagent grade and obtained from Merck (Spain).

2.2. Rough lipopolysaccharide (LPS) of B. ovis extraction

To prepare cells for extraction, tryptic soy broth (TSB) flask were inoculated with fresh cultures of *B. ovis* REO 198 strain, and incubated at 37 °C for 3 days in air, under constant shaking. The rough lipopolysaccharide fraction was obtained from complete cells as described previously by the phenol–chloroform–petroleum ether extraction method [29,30].

2.3. Preparation of nanoparticles

Gantrez® nanoparticles were prepared by a solvent displacement method previously described [31].

2.3.1. Preparation of OVA-entrapped nanoparticles (OVA^{in}–NP)

OVA^{in}–NP was prepared as described elsewhere [14]. Briefly, 5 mg OVA were dispersed in 1 mL acetone by ultrasonication (Microson™) for 1 min under cooling. The OVA dispersion was then added to 4 mL acetone containing 100 mg Gantrez® and the mixture was stirred for 30 min at room temperature. Then, the polymer was desolvated by the addition of 20 mL ethanol: water phase (1:1 by volume). The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland) and the resulting nanoparticles dispersed in the aqueous media were cross-linked by incubation with 5 μg DP/mg copolymer for 5 min under magnetic stirring at room temperature. Nanoparticles were purified by centrifugation and lyophilised using sucrose 5% as cryoprotectant.

2.3.2. Preparation of LPS-coated/OVA-entrapped nanoparticles (OVA^{in}–LPS^{out}–NP)

Five milligrams of OVA were dispersed in 1 mL acetone by ultrasonication (Microson™) for 1 min under cooling. The OVA dispersion was then added to 4 mL acetone containing 100 mg Gantrez® and the mixture was stirred for 30 min at room temperature. Then, the polymer was desolvated by the addition of 20 mL ethanol: water phase (1:1 by volume). The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland). The prepared nanoparticles were then incubated with 1 mg LPS in 1 mL of water for 1 h at room temperature under magnetic stirring. Nanoparticles were purified by centrifugation and lyophilised as described above.

2.3.3. Preparation of OVA and LPS-entrapped in nanoparticles (OVA^{in}–LPS^{in}–NP)

Five milligrams of OVA was dispersed in 1 mL acetone by ultrasonication (Microson™) for 1 min under cooling, similarly, 1 mg LPS was also dispersed in 1 mL acetone by ultrasonication (Microson™) for 1 min under cooling. The OVA and the LPS dispersions were added to 3 mL acetone containing 100 mg Gantrez® and stirred for 30 min at room temperature. Then, the desolvation of the polymer was induced by the addition of 20 mL ethanol: water phase (1:1 by volume). The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland). The resulting nanoparticles dispersed in the aqueous media were cross-linked by incubation with 5 μg DP/mg copolymer for 5 min under magnetic stirring at room temperature. The formulation was purified by centrifugation and lyophilised as described above.

2.4. Characterisation of nanoparticles

The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy and electrophoretic laser doppler anemometry, respectively, using a Zetamaster analyser system (Malvern Instruments, UK). The samples were diluted with deionized water and measured at room temperature with a scattering angle of 90°. All measurements were performed in triplicate.

The morphological characteristics of the nanoparticles were observed by scanning electron microscopy (LEO Electron Microscopy Inc., Thornwood, NY) operating at 3 kV with a filament current of about 0.5 mA. Prior to observation, the nanoparticles were coated with a platinum layer of about 2 nm using a Cressington sputter-coated 208HR with a rotatory–planetary-tilt stage, equipped with a MTM-20 thickness controller.

The quantification of the amount of OVA associated to nanoparticles was determined using HPLC. The analysis was performed in a HPLC model 1050 series LC, Agilent (Waldbornn, Germany) coupled with fluorescence detector. Data were analyzed by Hewlett-Packard computer using the Chem-Station G2171 program. The separation was carried out at 25 °C on a reversed-phase Zorbax GF-25 column (4.6 mm × 250 mm; particles size 4 μm) obtained from Agilent Technologies (California, USA). The mobile phase composition was phosphate buffer (130 mM NaOH, 20 mM KCl, 50 mM Na_2HPO_4) pH 7, methanol and water (40:10:50 v/v/v). The flow rate was set to 1 mL/min and effluent was monitored with fluorescence detection (λ_{exc} = 280 nm, λ_{em} = 340 nm).

For HPLC analysis, nanoparticles were previously digested with NaOH 0.1 N for 24 h at 4 °C. Then, the samples were transferred to auto-sampler vials, capped and placed in the HPLC auto-sampler.

The amount of associated LPS to nanoparticles was indirectly estimated by determining one of its exclusive markers, KDO, by the thiobarbiturate acid method [32]. For this purpose, a solution containing digested nanoparticles (NaOH 0.1 N, 24 h, 4 °C) was added to 5 volumes of a solution of methanol and 1% methanol saturated with sodium acetate to precipitate the LPS content. The pellet obtained was then resuspended in 0.2% SDS solution and used in the KDO assay. Each sample was assayed in triplicate and the results were expressed as the amount of LPS (in μg) per mg nanoparticles.

In order to corroborate the presence of LPS at the surface of nanoparticles, an indirect immunofluorescence assay was carried out. For this purpose, slides containing air-dried fixed samples of nanoparticles (10 μL of 3 mg/mL stock) were incubated with serum from hyperimmunized rabbits against LPS (diluted 1:50) in a humid chamber for 30 min at room temperature and washed five times with PBS (0.01 M, pH 7.2). Bound anti-LPS antibodies were detected by fluorescein–isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1:100). After incubation (30 min in dark, humid chamber at room temperature) slides were washed with PBS and counterstained with 4% Evans Blue for 10 min. The slides were finally observed for specific fluorescent staining by epifluorescence microscopy (Zeiss Axioscop). Negative control consisted of slide incubated with nanoparticles and no serum, and then conjugated as above. Positive control consisted in whole *B. ovis* bacteria incubated with serum and conjugated as above.

2.5. Immunization studies

Animal protocols 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).

BALB/c mice, females of 8 weeks old (supplied by Harlan Interfauna Ibérica, Spain), were randomized into six groups of five mice. Animals were intradermally immunised with 10 μg of OVA incorporated in one of the following formulations: (i) OVA-entrapped nanoparticles (OVA^{in}–NP); (ii) OVA-entrapped and LPS-coated nanoparticles (OVA^{in}–LPS^{out}–NP); (iii) OVA and LPS-entrapped nanoparticles (OVA^{in}–LPS^{in}–NP); (iv) OVA adsorbed in alhydrogel (OVA–alum) and (v) free OVA dissolved in sterile PBS.

Blood samples from the retro-orbital plexus were collected on days 0, 7, 14, 28, 35, 42 and 49 post-immunization. The samples were centrifuged (3000*g*, 10 min) and the resulting sera were pooled. Finally, each pool was diluted 1:10 in PBS and stored at −80 °C until analysis.

2.6. Quantification of anti-OVA antibodies in serum

Specific antibodies against OVA (IgG_1 and IgG_{2a}) were determined in the pooled sera by indirect ELISA. Briefly, microtiter wells (Cliniplatte EB, Labsystems, Finland) were coated with OVA (1 μg/well) at 4 °C overnight. Serum samples were added in twofold serial dilutions in PBS–Tween 20 (1%) starting with 1:40, and incubated at 37 °C for 4 h. Anti-mouse IgG_1 or IgG_{2a} peroxidase conjugates diluted 1:1000 in PBS–Tween 20 (1%) were added followed by the substrate chromogen solution (H_2O_2–ABTS). Optical density (OD) was determined at λ_{max} 405 nm (iEMS Reader MF de Labsystems, Finland). Measurements were performed in triplicate and data were expressed as the reciprocal of a serum dilution whose optical density was 0.2 above blank samples.

2.7. Quantification of IL-10

The IL-10 in the pooled sera of immunized mice was quantified by a commercial ELISA kit of Biosource International (California, USA). Measurements were performed by triplicate and data were expressed as pg/mL of IL-10 in sera.

2.8. Sensitization, vaccination and challenge studies

BALB/c mice, females of 8 weeks old (supplied by Harlan Interfauna Ibérica, Spain), were sensitized by intraperitoneal injection of 50 μg of OVA emulsified in 1 mg alum (alhydrogel) adjuvant (Sigma–Aldrich Chemie, Germany) in a total volume of 150 μL on days 1 and 8. On days 14, 17 and 20, the animals (5 mice per group) received intradermal injections with 3 μg of OVA each incorporated in either OVA^{in}–NP, OVA^{in}–LPS^{out}–NP or OVA^{in}–LPS^{in}–NP. As controls, OVA dispersed in alum (Ova–alum) and PBS were used. Finally, on day 35 the animals were challenged by an injection of 1 mg of OVA by intraperitoneal route.

2.8.1. Histamine quantification

Histamine release test was performed on heparinized whole blood from the retro-orbital plexus obtained before and 30 min after the challenge. Samples were lysed using perchloric acid (1.4% w/w) to determine whole blood histamine content. The resulting suspensions were centrifuged (10 min, 800*g*) and histamine production was assayed by a fluorometric method as previously described [33] using a Technicon II Analyzer (Technicon Instrument Corp., USA).

2.8.2. Evaluation of anaphylaxis

The body temperature changes associated with anaphylactic shock were monitored by measuring the rectal temperature [34] 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 the previous reports [35,36]. Reactions severity was classified in the following categories depending on their gravity: (i) (−) absent; (ii) (+) weak; (iii) (++) moderate; and (iv) (+++) strong, and the mobility was classified in (i) low or (ii) normal, depending on the activity of the animals. Finally, the mortality rates were recorded 24 h after intraperitoneal challenge.

2.9. Statistical analysis

The physico-chemical characteristics were compared using the Student's *t-test. A* p-value <0.05 was considered significant. For the evaluation of the histamine increase and temperature decrease, statistical comparisons were performed using the one-way analysis of variance test (ANOVA) and Tukey HSD test. A p-value <0.05 was considered as a statistically significant difference. All calculations were performed using SPSS® statistical software program (SPSS® 10, Microsoft, USA).

3. Results

3.1. Characterisation of Gantrez® nanoparticles

The main physico-chemical characteristics of Gantrez® formulations are summarised in Table 1. The size of OVA nanoparticles were significantly higher than empty nanoparticles (NP) ($p < 0.05$); however, the presence of LPS in the OVA formulations did not affect the size of the nanoparticles. Overall, nanoparticle batches were found to be homogeneous spheres (Fig. 1). In addition, no important differences were visualized when compared

Table 1
Physico-chemical characteristics of Gantrez® nanoparticles

Formulations[a]	LPS content (μg/mg)	OVA content (μg/mg)	Encapsulation efficiency (%)	Size (nm)	Zeta potential (mV)
NP	–	–	–	158 ± 3	−45.1 ± 0.5
OVA^{in}–NP	–	30.1 ± 4.5	42.1 ± 6.3	239 ± 4	−50.8 ± 2.9
OVA^{in}–LPS^{out}–NP	15.2 ± 0.5	24.1 ± 5.4	33.9 ± 7.6	231 ± 3	−46.1 ± 3.1
OVA^{in}–LPS^{in}–NP	13.8 ± 3.0	26.5 ± 0.3	37.3 ± 0.4	227 ± 4	−34.1 ± 3.4

Data were represented by means ± SD (n = 10).
[a] *Formulations:* NP, empty nanoparticles; OVA^{in}–LPS^{out}–NP, OVA-coated and LPS-entrapped nanoparticles; OVA^{in}–NP, OVA-entrapped nanoparticles; OVA^{in}–LPS^{out}–NP, OVA-entrapped and LPS-coated nanoparticles; OVA^{in}–LPS^{in}–NP, OVA and LPS-entrapped nanoparticles.

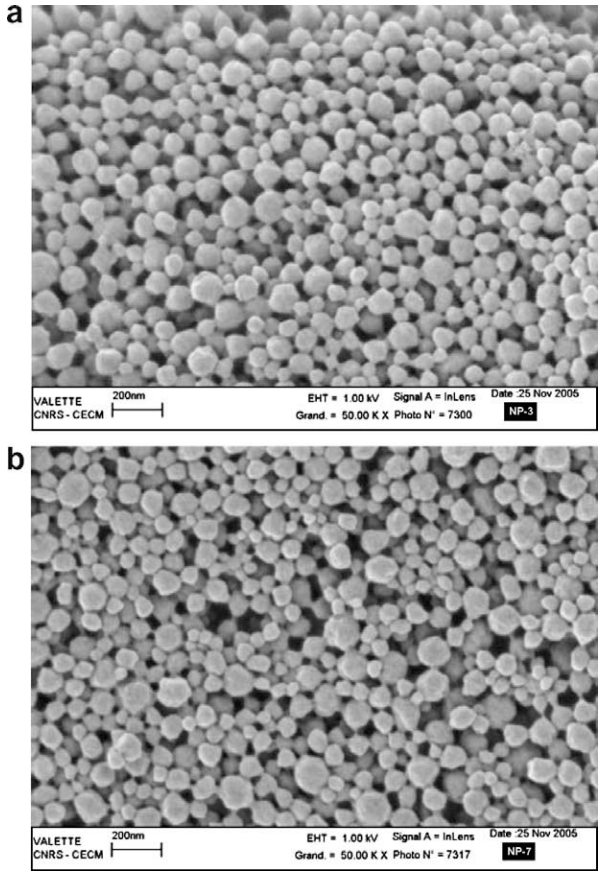

Fig. 1. SEM photographs of (a) OVA^{in}–NP (OVA-entrapped nanoparticles) and (b) OVA^{in}–LPS^{in}–NP (OVA and LPS-entrapped nanoparticles).

SEM photographs of nanoparticles containing only OVA (OVA^{in}–NP, Fig. 1a) with nanoparticles also containing LPS (OVA^{in}–LPS^{in}–NP, Fig. 1b). Concerning the OVA content, it is interesting to note that the addition of LPS slightly decreased the OVA content and the encapsulation efficiency (see Table 1).

Confirmation of the presence of LPS on the surface of OVA^{in}–LPS^{out}–NP was demonstrated by indirect immunofluorescence by using specific sera against LPS. OVA^{in}–LPS^{in}–NP showed a positive but lower fluorescence signal image. On the contrary, OVA^{in}–NP and NP had negligible or minimal background fluorescence (photographs not shown).

3.2. Antibody response in BALB/c mice after intradermal administration of the OVA-nanoparticle formulations

Fig. 2 shows the anti-OVA IgG_1 and IgG_{2a} titres (Th2 and Th1 markers, respectively) in sera after intradermal immunization of mice with the different formulations. All nanoparticle formulations induced a similar profile characterised by a short lag-time, of about 1 week, followed by a rapid increase of anti-OVA IgG_1 levels for at least 3 weeks. At the end of this period (day 28), a plateau of antibody levels was reached and maintained till the end of the experiment (day 49). The levels of anti-OVA IgG_1 antibodies were always higher for nanoparticle formulations than those induced by the control OVA–alum. On the other hand, the presence of LPS on the surface of the nanoparticles (OVA^{in}–LPS^{out}–NP) increased the level of IgG_{2a} antibodies against OVA (Th1 response). Free OVA titres were close to the basal line.

3.3. IL-10 quantification

Fig. 3 shows the IL-10 concentration in sera after intradermal immunization of mice with the different nanoparticles formulations. OVA^{in}–NP appeared to be the most effective formulation in inducing IL-10 production. On the other hand, the association of LPS to the nanoparticles decreased the seric levels of IL-10. Significantly, a peak of IL-10 concentration was found on day 14th for all OVA-entrapped nanoparticles.

3.4. Immunotherapeutic schedule

The induced OVA-allergic mice received the immunotherapeutic schedule previously described, and on day 35 they were chal-

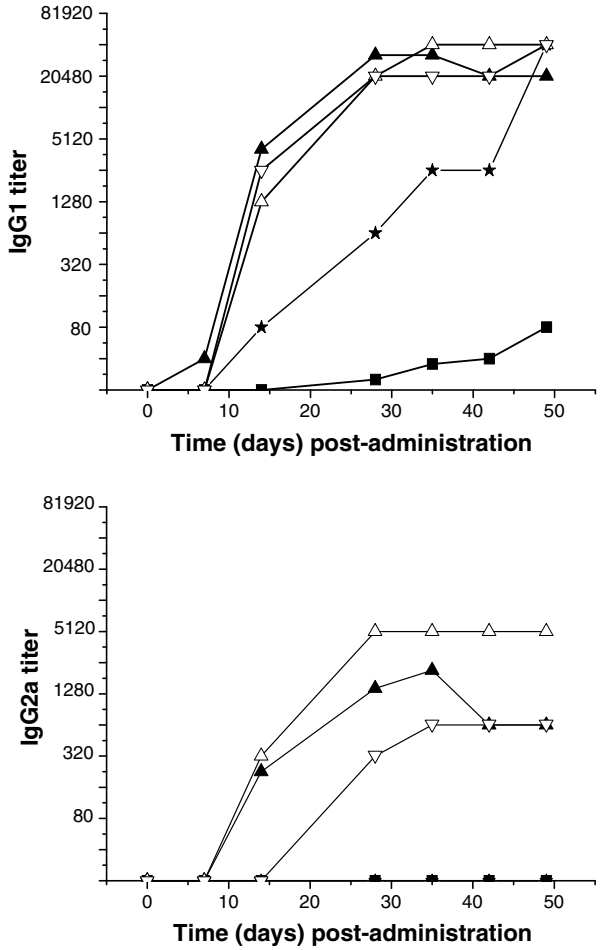

Fig. 2. Anti-OVA IgG_1 and IgG_{2a} titres in sera after intradermal immunization with OVA solution (OVA) (■), OVA adsorbed in alhydrogel (OVA–alum) (★), OVA-entrapped nanoparticles (OVA^{in}–NP) (▲), OVA-entrapped and LPS-coated nanoparticles (OVA^{in}–LPS^{out}–NP) (△), and OVA and LPS-entrapped nanoparticles (OVA^{in}–LPS^{in}–NP) (▽)). The antibody titre is defined as the reciprocal dilution giving an optical density.

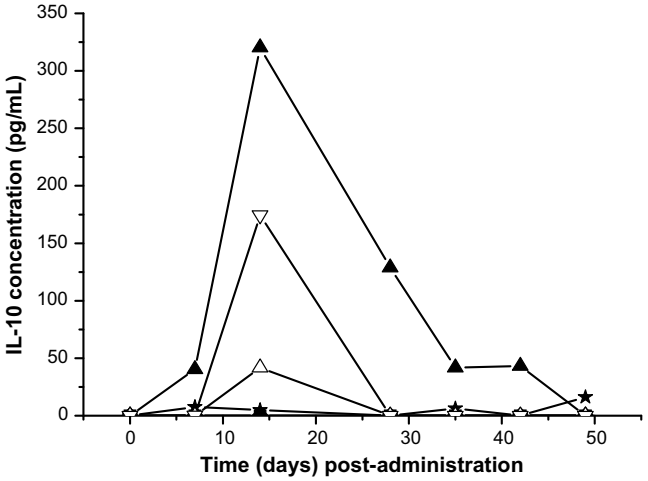

Fig. 3. IL-10 concentration (pg/mL) in sera after intradermal immunization with OVA solution (OVA) (■), OVA adsorbed in alhydrogel (OVA–alum) (★), blank nanoparticles (NP) (♦), OVA-entrapped nanoparticles (OVA^{in}–NP) (▲), OVA-entrapped and LPS-coated nanoparticles (OVA^{in}–LPS^{out}–NP) (Δ) and OVA and LPS-entrapped nanoparticles (OVA^{in}–LPS^{in}–NP) (▽)).

lenged with OVA. In order to analyse the intensity of the anaphylactic shock, several parameters were determined. Fig. 4 shows the difference of histamine blood levels before the challenge and 30 min later. Groups treated with either OVA^{in}–LPS^{in}–NP or OVA^{in}–NP showed a significant lower increase of the histamine levels in comparison with the control groups (OVA–alum and PBS). In contrast, the group of animals treated with OVA^{in}–LPS^{out}–NP did not show a significant difference with the controls.

Table 2 shows the overall anaphylactic symptoms score including the mortality rate. OVA^{in}–NP, OVA^{in}–LPS^{out}–NP and OVA^{in}–LPS^{in}–NP groups showed a slightly lower decrease in the body temperature than the controls (OVA–alum and PBS), although, the differences were not significant ($p < 0.05$). The piloerection and cyanosis seemed to be lower for OVA^{in}–NP and OVA^{in}–LPS^{in}–NP than for the other formulations. Furthermore, the mobility seemed

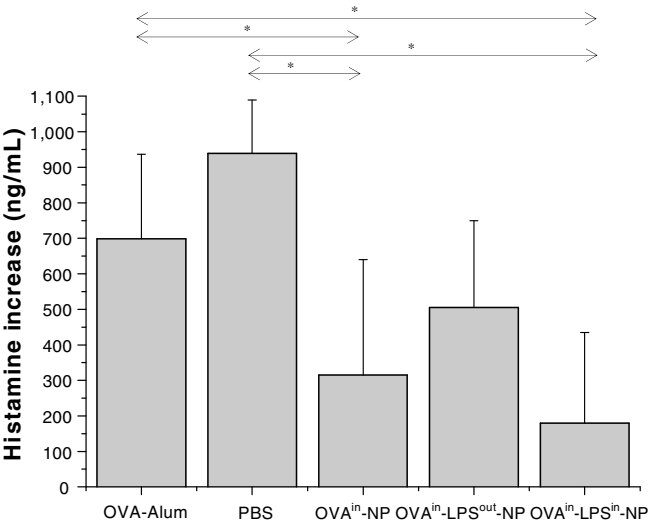

Fig. 4. Increase of the histamine blood level after the challenge with 1 mg of OVA i.p. After sensitization to OVA, the different groups of animals were treated (days 14, 17 and 20) with OVA-entrapped nanoparticles (OVA^{in}–NP), OVA-entrapped and LPS-coated nanoparticles (OVA^{in}–LPS^{out}–NP) and OVA and LPS-entrapped nanoparticles (OVA^{in}–LPS^{in}–NP). OVA adsorbed in alhydrogel (OVA–alum) and PBS were used as controls.

Table 2
Anaphylactic symptoms in the treated vs. non-treated OVA-allergic mice

Immunotherapeutic treatment[a]	Temperature decrease (°C)	Piloerection	Mobility	Cyanosis	Mortality rate (%)
OVA–alum	3.62 ± 1.03	+ +	Low	+ +	80
PBS	3.54 ± 3.14	+ + +	Low	+ + +	60
OVA^{in}–NP	2.96 ± 1.42	+	Low	+	40
OVA^{in}–LPS^{out}–NP	2.83 ± 1.27	+ + +	Low	+ + +	60
OVA^{in}–LPS^{in}–NP	1.19 ± 1.42	+	Normal	+ +	0

Severity of the symptoms: (+) weak; (++) moderate and (+++) strong.
[a] *Treatments:* OVA–alum, OVA adsorbed to aluminium hydroxide; PBS, phosphate buffer saline; OVA^{in}–NP, OVA-entrapped nanoparticles; OVA^{in}–LPS^{out}–NP, OVA-entrapped and LPS-coated nanoparticles; OVA^{in}–LPS^{in}–NP, OVA and LPS-entrapped nanoparticles.

not to be affected in the animals treated with OVA^{in}–LPS^{in}–NP, while for the other groups the animals were found to be static with a high difficulty to coordinate any simple movement. Finally, the OVA^{in}–LPS^{in}–NP group protected the animals from death, while, for the other groups tested, the mortality rate was between 40% and 80%.

4. Discussion

The physico-chemical and biodegradation properties of some nanoparticle formulations may be worthy as useful immunoadjuvants [37–39]. In the context of the immunotherapy of allergic processes, potent adjuvants are required. Thus, and based on the previous studies with nanoparticles of the polymer Gantrez® AN [14], the aim of the present work was to study the effect of the incorporation of an immunostimulant, such as the low-endotoxic LPS from *B. ovis* [40], on the adjuvant capacity of these nanoparticles. The special chemical nature of Gantrez® nanoparticles may afford the binding on their exposed chemical reactive groups of microbial markers, that may interact with scavenger ligands on the immune cells involved in signal transduction and the secretion of cytokines [40]. For this purpose, Gantrez® nanoparticles containing ovalbumin (OVA) were prepared. This protein was incorporated during the manufacture process (OVA-entrapped nanoparticles) as described previously [14], and the LPS was incorporated either during the nanoparticle manufacture (LPS^{in}, entrapped) or after the preparation of nanoparticles (LPS^{out}, coating).

When these formulations were intradermally administered to mice, the presence of the LPS on the NP (OVA^{in}–LPS^{out}–NP) increased the OVA-specific IgG_{2a} levels (Th1 marker). On the contrary, the levels of IgG_1 (Th2 marker) were not affected by the presence of the LPS. These results are in agreement with our previous observations related with the ability of *B. ovis* LPS to promote the production of Th1 cytokines [41]. Thus, OVA^{in}–LPS^{out}–NP (LPS coating the surface of nanoparticles) is "mimicking" the Gram-negative bacteria structure with the LPS on the surface, activating the Th1 pathway. Other authors have used smooth *Brucella* spp. lipopolysaccharides in assisting as a potential carrier for vaccine development in situations requiring a strong Th1-like response for protection against even xeno-infections [42–46], but this is the first report describing the capability of *B. ovis* rough LPS as an immunostimulant.

From the immunological point of view, an allergic episode is characterised by the presence of IgE and IgG_1, produced by the polarization of Th naïve lymphocyte into a Th2 subset population [47]. As it is well known, immunotherapy in humans should be based on the decrease of the Th2 by enhancing the Th1, and this balance is achieved by increasing the activity of the T_{reg} lymphocytes [48]. T_{reg} cytokines (IL-10, TNF-α) play a key role in preventing IgE and Th2 expansion [49,50]. Thus, we have demonstrated

that LPS-nanoparticles do exert an effect on the T_{reg} lymphocytes, since IL-10 could be detected after immunization. Moreover, only when OVA was associated to Gantrez® nanoparticles, IL-10 could be detected in sera (see Fig. 3).

In order to evaluate the protective effect of these formulations with or without LPS on a model of OVA-sensitized mice, the mice were treated with the OVA-entrapped nanoparticles with or without LPS by intradermal route and finally were challenged and the different anaphylactic symptoms were observed. Results indicated that histamine levels, one of the most dangerous substance released during anaphylaxis, was much lower ($p < 0.005$) for LPS-entrapped nanoparticles (OVA^{in}–LPS^{in}–NP) than for control groups (Fig. 4). These results correlated well with the temperature decrease after the challenge (Table 2). However, the ultimate parameter to test the efficacy of the immunization was the protection against mortality. Again, OVA^{in}–LPS^{in}–NP was found to be the best formulation. In fact, OVA^{in}–LPS^{in}–NP protected all the mice from death, in contrast to the control groups immunized with OVA–alum or PBS. However, when the LPS was coating the nanoparticles (OVA^{in}–LPS^{out}–NP), the mice were not protected, and the mortality rate was similar than that observed for the untreated group. The appropriate co-stimulation of the dendritic TLR-4 with the LPS at the time of allergen (OVA) exposure would develop a protective Th1 immune response. Confirmation of the presence of some LPS molecules on the surface of the LPS-entrapped nanoparticles was demonstrated by indirect immunofluorescence by using specific sera against LPS. Finally, when the LPS was encapsulated in the nanoparticles with the OVA (OVA^{in}–LPS^{in}–NP), both LPS and OVA are supposed to be released at the same time, the OVA presentation being more effective. However, when the OVA is entrapped in the nanoparticles and the LPS is coating the surface (OVA^{in}–LPS^{out}–NP), the LPS would be released first, followed by the OVA.

We have demonstrated the ability of combined use of the innocuous rough LPS from *B. ovis* and Gantrez® nanoparticles to induce high levels of IL-10 thus showing highly interesting adjuvant properties for immunotherapy. However, its most remarkable effect is the capability of these particles to protect from anaphylactic shock. It is even more interesting when considering that protection from anaphylactic death is not usually described in the bibliography [51–54], therefore, this effect is a significant finding for its application in immunotherapy.

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