



Research paper

A novel nanoparticulate adjuvant for immunotherapy with *Lolium perenne*

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ABSTRACT

Specific immunotherapy implies certain drawbacks which could be minimized by the use of appropriate adjuvants, capable of amplifying the right immune response with minimal side effects. In this context, previous studies of our group have demonstrated the adjuvant capacity of Gantrez[®] AN nanoparticles, which can effectively enhance the immune response. In this work, two types of nanoparticles (with and without LPS of *Brucella ovis* as immunomodulator) with encapsulated *Lolium perenne* extract are tested in a model of sensitized mice to this allergenic mixture. The results we obtained showed that *Lolium*–Gantrez[®] nanoparticles with LPS of *B. ovis* were able to induce significant Th1 responses, characterized by the IgG_{2a} isotype. Furthermore, in the challenge experiment of the sensitized mice, differences in the mortality rate and in the mMCP-1 levels were found between the treated groups and the control. Under the experimental conditions of this model of pre-sensitized mice to *L. perenne*, Gantrez[®] AN nanoparticles appeared to be a good strategy for immunotherapy.

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1. Introduction

During the last decades, the prevalence of allergic diseases has markedly increased in the developed areas of the world (Wills-Karp et al., 2001). Currently, the desensitization of allergic patients by the systemic injection of increasing doses of the allergen appears to be the most efficient therapy. However, although it has been shown to be effective, the risk of systemic reactions represents a major drawback for general application. Thus, it is of great importance to develop safer and more effective immunotherapies. Since IgE-allergic reactions are mediated by specific Th2 cells (Del Prete et al., 1993), a simple approach would consist in elicit a Th1 response after immunization and thereby downregulate the Th2 one (Sehara et al., 1998; Kumar et al., 1999; Toda et al., 2000).

Biodegradable particulate systems have demonstrated their efficacy as adjuvants to induce Th1 immune responses

(Gupta et al., 1998; Murillo et al., 2002). These systems, with a defined composition, size and surface, exhibit capacity to be efficiently captured by APCs (Gamvrellis et al., 2004). In the context of allergic immunotherapy, 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 (Scholl et al., 2004). In the same way, olive allergen encapsulated into PLGA microparticles induced a Th1 response which was not displayed after immunization with the non-encapsulated allergen (Batanero et al., 2003).

Grass pollen is the major outdoor seasonal source of airborne allergens in cool temperate climates. In addition, the allergenic role and importance of grass pollen in triggering hayfever and allergic asthma is well known and extensively documented (Knox and Suphioglu, 1996). Recent surveys have shown that 35% of young adults in a wide variety of countries have specific serum antibodies to grass pollen (Burney et al., 1997). In this context, the most clinically significant grass is rye-grass (*Lolium perenne*). Specifically, the major allergenic proteins of rye-grass pollen are Lol p1 and Lol

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p5, which together account for nearly all the IgE-binding reactivity of crude rye-grass pollen extract (Bond et al., 1993).

Previous studies of our group have demonstrated the adjuvant capacity of Gantrez® AN nanoparticles, which can effectively enhance the immune response (Salman et al., 2005, 2006, 2007; Gómez et al., 2006). Gantrez® AN is a copolymer of methyl vinyl ether and maleic anhydride that can easily react with amino groups, which makes easy to load or link different types of proteins, including allergens. Therefore, we describe here for the first time to our knowledge, the preparation, characterization and evaluation of Gantrez® AN nanoparticles with entrapped *L. perenne* protein extract (Lol), in order to be used in immunotherapy. In addition, in this study we examined the adjuvant capacity of these carriers with or without entrapped lipopolysaccharide (LPS) from *Brucella ovis*, as immunomodulator, in sensitized mice to *L. perenne*.

2. Materials and methods

2.1. Chemicals

Gantrez® AN 119 [poly(methyl vinyl ether-co-maleic anhydride); MW 200,000] was kindly gifted by ISP (Barcelona, Spain). 1,3-diaminopropane (DP), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and alum were purchased from Sigma-Aldrich Chemie (Germany). The peroxidase immunoconjugates (GAM/IgG₁/PO and GAM/IgG_{2a}/PO) were obtained from Nordic Immunology (The Netherlands). The IgE ELISA kit was purchased from BD Biosciences (Erembodegem, Belgium). All other chemicals used were of reagent grade and obtained from Merck (Spain).

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

B. ovis REO 198 strain was grown on tryptic soy broth (TSB) supplemented with 0.5% of yeast extract (DIFCO). To prepare cells for extraction, TSB flask were inoculated with fresh cultures of *B. ovis*, and incubated at 37 °C for 3 days in air, under constant shaking. Rough lipopolysaccharide was obtained from complete cells as described previously (Gómez et al., 2006) by the phenol-chloroform-petroleum ether extraction method (Galanos et al., 1969). The final product had a protein content of less than 1%.

2.3. Preparation of *L. perenne* extract

Pollen sample of *Lolium perenne* was purchased from Biopol (USA). Crude protein extract was obtained by stirring 5 g of pollen in 100 mL of PBS for 12 h at 4 °C.

After centrifugation (2×30 min, at 14,000 g at 4 °C), supernatant was collected and dialyzed versus distilled water during 24 h. Then, samples were filtered through 0.2 µm filter and stored in aliquots at −80 °C. Finally they were freeze-dried for further preservation.

2.4. Preparation of *Lolium*-entrapped Gantrez nanoparticles (Lol-NP) and *Lolium* nanoparticles containing rough lipopolysaccharide of *B. ovis* (LPS-Lol-NP)

Lol-NP was prepared by a solvent displacement method previously described (Gómez et al., 2006) with minor

modifications. Briefly, 16 mg of the freeze-dried *Lolium* extract were dissolved in 1 mL of water and then 5 mL of acetone were added in order to precipitate the proteins. This suspension was left during one night at −20 °C. Then it was centrifuged at 4000 rpm, during 20 min at 4 °C. The pellet was dispersed in 4 mL acetone by ultrasonication (Microson™) for 1 min under cooling. In some batches (LPS-Lol-NP), 4 mg LPS were also dispersed in 4 mL acetone by ultrasonication (Microson™) for 1 min under cooling. The protein dispersion, and also the LPS suspension in LPS-Lol-NP, were then added to 16 mL acetone containing 400 mg Gantrez AN and the mixture was stirred for 30 min at room temperature. Then, the polymer was desolvated by the addition of 80 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 20 µg 1,3-diaminopropane/mg copolymer for 5 min under magnetic stirring at room temperature. The resulting carriers were purified by centrifugation at 27,000×g for 20 min. The supernatants were removed and the pellets resuspended in water. The purification procedure was repeated twice and finally, the formulations were freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5%) as cryoprotector.

Control nanoparticles (NP) were prepared in the same way, in the absence of *Lolium*.

2.5. Characterisation of nanoparticles

2.5.1. Size, zeta potential and morphology of nanoparticles

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

2.5.2. Yield

The yield of the nanoparticles preparation process was determined by gravimetry as described previously (Arbos et al., 2002). Briefly, Gantrez® AN nanoparticles, freshly prepared, were freeze-dried. Then, the yield was calculated as the difference between the initial amount of the polymer used to prepare nanoparticles and the weight of the freeze-dried carriers.

2.5.3. Protein content

The quantification of the amount of protein extract associated to nanoparticles was determined by HPLC. The analysis was performed in a HPLC model 1050 series LC, Agilent (Waldborn, 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 Na₂HPO₄) pH 7, methanol and water (40/10/50v/v/v). The flow rate was set to 1 mL/min and effluent

was monitored with fluorescence detection ($\lambda_{\text{exc}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$).

For HPLC analysis, nanoparticles were 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. Then, 10 μL aliquot was injected onto HPLC column. Calibration curves from 1 to 100 $\mu\text{g}/\text{mL}$ of protein ($r^2 > 0.999$) were performed. Each sample was assayed in triplicate and results were expressed as the amount of protein (in μg) per mg nanoparticles. Similarly, the encapsulation efficiency (E.E.) was calculated as follows:

$$E.E.(\%) = (Q_{\text{associated}} / Q_{\text{initial}}) \times 100 \quad (1)$$

where Q_{initial} is the initial amount of protein extract added per mg of polymer that form the NP and $Q_{\text{associated}}$ is the amount of entrapped protein per mg of NP, which was calculated by HPLC.

2.6. In vitro histamine release test with basophils

The allergenicity of the *Lolium*-Gantrez[®] nanoparticles was probed by an in vitro histamine release test from basophils.

The blood was obtained from patients recruited from those coming to the Department of Allergy. Inclusion criteria were having nasal, ocular or bronchial symptoms during pollen season. In addition, having positive skin prick test to Phleum and specific IgE levels against Phleum allergen and total IgE measured by CAP Phadia (Uppsala-Sweden) technique.

In order to quantify the in vitro release of histamine from the basophils, 45 μL of blood from the *Lolium*-allergic patients and healthy donors were incubated with 10 μg of *Lolium* protein extract incorporated in LPS-Lol-NP and Lol-NP during 15 min at 37 °C. As controls *Lolium* extract and blank Gantrez[®] nanoparticles (NP) were used. After the incubation of the formulations with the blood, 850 μL of EDTA solution was added and the resulting suspension was centrifuged (10 min, 1200 $\times$ g). The histamine released was quantified in the supernatant by a fluorometric method previously described (Castillo et al., 1989) using a Technicon II Analyzer (Technicon Instrument Corp., USA). In order to quantify the total histamine 45 μL of blood was added to 850 μL of perchloric acid 2%. The resulting suspension was centrifuged (10 min, 1200 $\times$ g) and the total histamine was quantified in the supernatant as described above. The percentage of histamine released from the basophils was calculated as follows:

$$\text{Histamine released}(\%) = (\text{Histamine released} / \text{Total histamine}) \times 100 \quad (2)$$

2.7. Sensitization, immunotherapy and challenge 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 sensitized by receiving two intraperitoneal administrations of 50 μg *Lolium* protein

extract emulsified in 2 mg alum (alhydrogel) adjuvant (Sigma-Aldrich Chemie, Germany) in a total volume of 150 μL , on days 1st and 8th.

Once sensitized, animals were divided into 5 groups of 5 animals each, and on days 20th, 23rd and 26th, the animals received intradermal injections with 50 μg of *Lolium* protein extract each incorporated in either Lol-NP or LPS-Lol-NP. As controls, *Lolium* extract dispersed in Alum (Lol-Alum), blank nanoparticles (NP) and PBS were used. Finally on day 41st the animals were challenged in order to provoke them an anaphylactic shock by an injection of 4 mg of *Lolium* extract by intraperitoneal route. Along the experiment, blood samples were obtained from retro-orbital plexus on days 0, 7th, 13th, 20th, 27th, 34th and 40th.

2.7.1. Quantification of antibodies in serum

In order to quantify antibody responses, sera were collected and stored at -80°C until their analysis. To determine IgE antibody level, microtitre plates (Nunc-Immuno[™] Plate, Denmark) were coated overnight at 4 °C 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% and blocked for 1 h at room temperature with 10% foetal bovine serum in PBS (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. The plates were washed and, finally, incubated with the substrate chromogen solution (TMB). The color reaction was stopped with H_2SO_4 2 N and the optical density (OD) was determined at 450 nm (iEMS Reader MF de Labsystems, Finland).

Levels of specific antibodies against Lol (IgG₁ and IgG_{2a}) were determined by indirect ELISA. Briefly, microtiter wells (cliniplate EB, Labsystems, Finland) were coated overnight with 1 μg *Lolium* extract in 100 μL sodium carbonate-bicarbonate buffer (0.05 M; pH 9.6) at 4 °C. The plates were washed with PBS-Tween 20 (0.05%) and serum samples were added in ten-fold serial dilutions in PBS-Tween 20 (0.05%) starting with 1:10, and incubated at 37 °C for 4 h. After washing again with PBS-Tween 20 (0.05%), the plates were incubated, at 37 °C for 2 h, with anti-mouse IgG₁ or IgG_{2a} peroxidase conjugates diluted 1:1000 in PBS-Tween 20 (0.05%). The plates were washed and, finally, incubated with the substrate chromogen solution (ABTS and hydrogen peroxide). The optical density (OD) was determined at 405 nm (iEMS Reader MF de Labsystems, Finland). Measurements were performed by triplicate and data were expressed as the reciprocal of a serum dilution whose optical density was 0.2 above blank samples.

2.7.2. 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 $\times$ g) and histamine production was assayed by a fluorometric method as previously described (Castillo et al., 1989) using a Technicon II Analyzer (Technicon Instrument Corp., USA).

2.7.3. Mouse Mast Cell Protease 1 (mMCP-1) quantification

Levels of mMCP-1 in serum were measured with an mMCP-1 enzyme-linked immunosorbent assay kit purchased from Moredun Animal Health Ltd. (Penicuik, United Kingdom), by using a technique described previously (Huntley et al., 1990).

2.7.4. Evaluation of anaphylaxis

The body temperature changes associated with anaphylactic shock were monitored by measuring the rectal temperature (Watanabe et al., 2004) 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 (Poulsen et al., 1987; Stampfli et al., 1999). 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 1 h after intraperitoneal challenge.

2.8. Statistical analysis

The physico-chemical characteristics were compared using the Student's *t*-test. *p* values < 0.05 were 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. *p* < 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

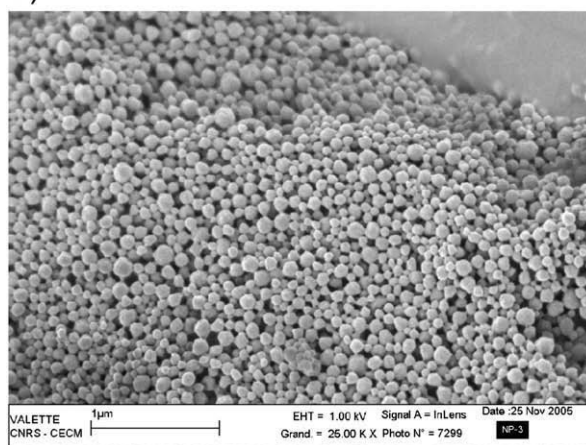
The main physico-chemical characteristics of Gantrez® formulations are summarised in Table 1. The size of *Lolium*-loaded nanoparticles was significantly higher than the size of empty nanoparticles (NP) (*p* < 0.05). Furthermore, the size of *Lol*-encapsulated nanoparticles slightly increased with the presence of LPS. Overall, nanoparticle batches were found to be homogeneous spheres (Fig. 1). In addition, no important difference was visualized when comparing SEM photographs of nanoparticles containing only *Lolium* (Lol-NP, Fig. 1a) with nanoparticles containing also LPS (Lol-LPS-NP, Fig. 1b).

Table 1

Physico-chemical characteristics of Gantrez® AN nanoparticles. Data were represented by mean ± SD (*n* = 10). NP: empty nanoparticles; Lol-NP: *Lolium* extract-entrapped nanoparticles; LPS-Lol-NP: *Lolium* extract and LPS-entrapped nanoparticles.

	Size (nm)	Zeta potential	Protein (µg/mg)	Encapsulation efficiency (%)	Yield (%)
NP	158 ± 2	−45.1 ± 0.5	–	–	85.3 ± 0.8
Lol-NP	294 ± 11	−36.6 ± 1.1	39.4 ± 5.4	66.9 ± 9.1	68.2 ± 1.3
LPS-Lol-NP	341 ± 14	−39.4 ± 1.6	36.8 ± 3.5	65.1 ± 6.1	70.7 ± 0.9

a)



b)

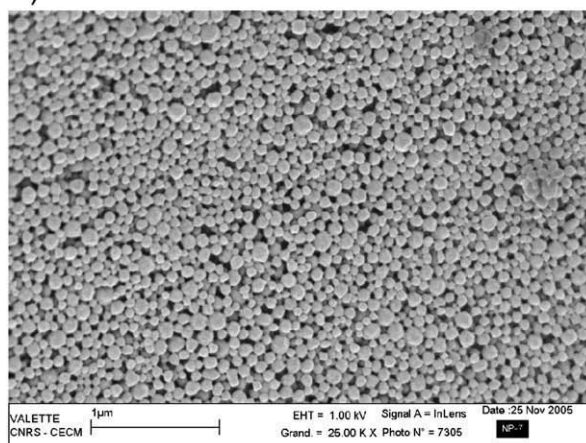


Fig. 1. SEM photographs of: a) *Lolium* nanoparticles (Lol-NP) and b) *Lolium* and LPS-entrapped nanoparticles (LPS-Lol-NP).

However, the encapsulation of the *Lolium* extract decreased to some extent the negative charge of conventional nanoparticles.

Concerning the protein content, it is interesting to note that, the presence of LPS did not affect the protein content. Finally, the nanoparticles yield decreased with the encapsulation of the *Lolium* protein extract (*p* < 0.05).

3.2. In vitro histamine release test with basophils

In order to probe the in vitro allergenicity of the *Lolium*-Gantrez® nanoparticles, LPS-Lol-NP and Lol-NP were incubated with blood from both allergic patients and healthy donors, and histamine released from activated basophils was quantified. As controls, *Lolium* extract and blank Gantrez® nanoparticles were used. No significant differences were found between the *Lolium*-entrapped nanoparticles (LPS-Lol-NP and Lol-NP) and the *Lolium* extract (*p* < 0.05), although LPS-Lol-NP appeared to slightly increase the release of histamine from basophils (Table 2).

Table 2

Percentage of histamine released upon in vitro stimulation of whole blood of allergic donors and control patients. The formulations tested were Lol (*Lolium* extract); Lol-NP (*Lolium*-entrapped nanoparticles); LPS-Lol-NP (LPS and *Lolium*-entrapped nanoparticles); NP (blank nanoparticles).

	Lol	Lol-NP	LPS-Lol-NP	NP
Allergic donors	31.7 ± 14.5	48.3 ± 19.3	56.6 ± 20.8	5.7 ± 4.8
Control patients	2.1 ± 3.4	0.9 ± 2.1	2.2 ± 2.3	1.1 ± 2.8

Data are expressed as mean ± SD (n = 10).

3.3. Immunotherapeutic schedule

3.3.1. Antibody response

BALB/c mice were sensitized with 50 µg *Lolium* protein extract adsorbed in aluminium hydroxide by intraperitoneal route on days 1st and 8th. Once the animals were sensitized to *Lolium*, they received intradermal injections on days 20th,

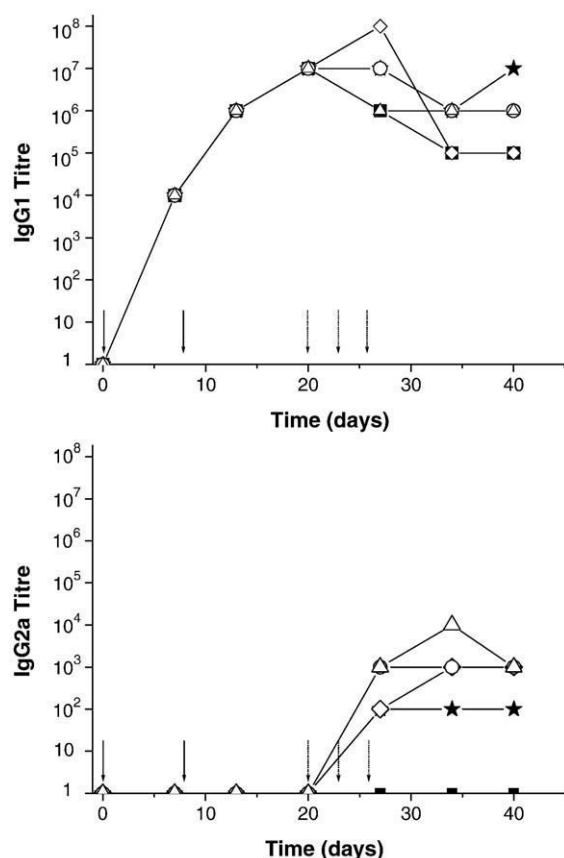


Fig. 2. Immunogenicity in BALB/c mice. Anti-Lol IgG₁ and IgG_{2a} levels during the sensitization and immunotherapy periods. Mice were sensitized with two intraperitoneal administrations of 50 µg Lol extract adsorbed in 2 mg Al(OH)₃ (↓). Once sensitized, the animals were intradermally administered on days 20th, 23rd and 26th (↓) with 50 µg Lol incorporated in: *Lolium*-entrapped nanoparticles (Lol-NP) (○), *Lolium* and LPS-entrapped nanoparticles (LPS-Lol-NP) (Δ). As controls, the mice were administered with: PBS (■), *Lolium* adsorbed in alhydrogel (Lol-Alum) (★) and blank nanoparticles (NP) (◇). The antibody titre is defined as the reciprocal dilution giving an optical density (OD) reading at 405 nm of ≥0.2.

23rd and 26th, with 50 µg of *Lolium* protein extract each incorporated in either Lol-NP or LPS-Lol-NP. As controls, *Lolium* extract dispersed in Alum (Lol-Alum), blank nanoparticles (NP) and PBS were used. Finally, on day 41st the animals were challenged with an injection of 4 mg of *Lolium* extract (i.p.) to provoke systemic anaphylaxis.

Fig. 2 shows the specific anti-*Lolium* IgG₁ and IgG_{2a} titres (Th2 and Th1 markers, respectively) in sera along the immunizations with the different formulations.

Both Lol-NP and LPS-Lol-NP formulations were able to decrease the Th2 response. On day 40th of the experiment Lol-NP and LPS-Lol-NP showed a 10-times lower titre than the Lol-Alum formulation.

Furthermore, *Lolium*-Gantrez® nanoparticles were able to induce significant Th1 responses. Both LPS-Lol-NP and Lol-NP induced 10-times higher IgG_{2a} levels than the control group Lol-Alum. Surprisingly, NP also induced higher IgG_{2a} titres than Lol-Alum, showing the same IgG_{2a} titre on day 40th than LPS-Lol-NP and Lol-NP. This fact could be due to an unspecific effect of control nanoparticles (NP). The PBS control group displayed undetectable levels of IgG_{2a} during all the experiment.

Fig. 3 shows the total IgE concentration found along the experiment. All the animals displayed an increment of the IgE level due to the sensitization status, and the group of animals that displayed the highest levels of IgE after immunizations (on days 20th, 23rd and 26th) was found to be Lol-Alum. On the other hand, LPS-Lol-NP also displayed higher titres of IgE than the Lol-NP group, which slightly increased the concentration along the experiment.

3.3.2. Challenge studies

The Lol-sensitized mice received the immunotherapeutic schedule as shown above, and on day 41st they were challenged with Lol i.p. In order to analyse the intensity of the anaphylactic shock, several parameters were evaluated.

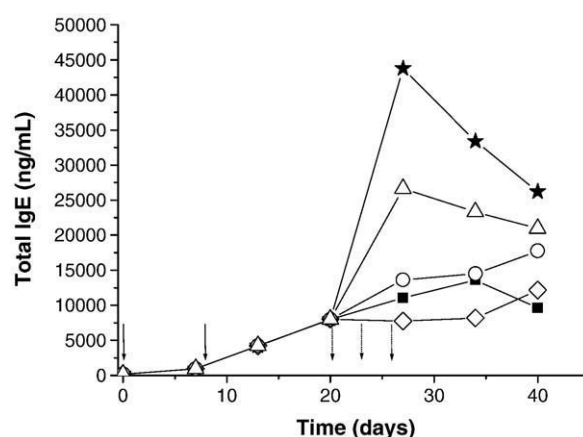


Fig. 3. Total IgE levels during the sensitization and immunotherapy periods. Mice were sensitized with two intraperitoneal administrations of 50 µg Lol extract adsorbed in 2 mg Al(OH)₃ (↓). Once sensitized, the animals were intradermally administered on days 20th, 23rd and 26th (↓) with 50 µg Lol incorporated in: *Lolium*-entrapped nanoparticles (Lol-NP) (○), *Lolium* and LPS-entrapped nanoparticles (LPS-Lol-NP) (Δ). As controls, the mice were administered with: PBS (■), *Lolium* adsorbed in alhydrogel (Lol-Alum) (★) and blank nanoparticles (NP) (◇).

Table 3

Mortality rate, histamine levels and symptoms scored after the challenge with *Lolium* extract i.p. Treatments: PBS: Phosphate buffered saline; Lol–Alum: *Lolium* extract adsorbed to aluminium hydroxide; Lol–NP: *Lolium*-entrapped nanoparticles; LPS–Lol–NP: *Lolium* and LPS-entrapped nanoparticles.

Treatment	Temperature decrease (°C)	Piloerection	Mobility	Cianosis	Histamine (ng/mL)	Mortality rate (%)
PBS	5.8 ± 0.4	+	Low	+	228.6 ± 0.4	25
Lol–Alum	4.0 ± 1.2	+	Low	+	158.2 ± 86.5	0
NP	5.6 ± 0.7	+	Low	+	223.1 ± 6.1	40
Lol–NP	5.1 ± 0.3	++	Low	+	220.2 ± 5.3	0
LPS–Lol–NP	5.2 ± 0.3	+	Very Low	+	231.8 ± 12.4	0

Table 3 shows the overall anaphylactic symptoms score including the mortality rate. No remarkable differences were found in the anaphylactic symptoms between the different groups. The only data in which some differences were found was the mortality rate. In this context, the groups Lol–Alum, Lol–NP and LPS–Lol–NP showed a mortality rate of 0% 1 h after the challenge, while in PBS and NP groups, 25% and 40% of the animals respectively, have died 1 h post-challenge.

The difference of histamine blood levels before and 30 min after the challenge is also shown in Table 3. All the treated groups showed an increase of the histamine levels of around 200 ng/mL of histamine, and no significant differences were found between the groups.

Finally, mMCP-1 (mouse mast cell protease-1) concentration was quantified in blood. This protease is highly elevated in anaphylactic process and it is considered to be a good parameter to quantify the intensity of the phenomenon. Fig. 4 shows the mMCP-1 blood levels 1 h after the challenge with Lol i.p. The group of animals treated with LPS–Lol–NP showed a significant ($p < 0.05$) lower mMCP-1 concentration after the challenge than the control group PBS.

4. Discussion

Particulate delivery systems have been demonstrated to be a good choice as immunomodulator adjuvants to generate prolonged and effective immune responses for a large number of allergens and antigens (Maloy et al., 1994; Roy et al., 1999; Batanero et al., 2003; Scholl et al., 2004; Balenga et al., 2006). On the other hand, previous studies of our group have demonstrated the immunotherapeutic capability of Gantrez® AN nanoparticles, which can effectively protect

from anaphylactic death to a model of pre-sensitized mice to ovalbumin (Gómez et al., 2008). Thus, the aim of this study was to perform a model of pre-sensitized mice to *L. perenne* and then, evaluate the protective effect of *Lolium*–Gantrez® nanoparticles on the animals after a challenge.

For this purpose, we firstly prepared Gantrez® nanoparticles with *Lolium* protein extract by a reproducible method of solvent displacement previously described for ovalbumin as a model protein (Gómez et al., 2006). In some batches, LPS of *B. ovis* was added within the matrix of the nanoparticles in order to act as immunomodulator. In this context, this LPS has demonstrated in previous studies that, when it is entrapped in the nanoparticles, it is able to improve the adjuvant capacity of the Gantrez® nanoparticles (Gómez et al., 2008). Then, the resulting formulations were: *Lolium*-entrapped nanoparticles (Lol–NP) and LPS and *Lolium*-entrapped nanoparticles (LPS–Lol–NP).

When the different formulations were characterized, we could observe that both formulations Lol–NP and LPS–Lol–NP displayed higher size than the blank nanoparticles (NP) ($p < 0.05$). The presence of LPS slightly increased the size of the resulting carriers (see Table 1) although it did not affect the *Lolium* encapsulation.

In order to analyse the allergenicity preservation of the *Lolium* extract after the nanoparticles preparation, the basophils activation experiment was performed and it was demonstrated that our manufacture process did not affect the immunogenic properties of the extract.

After corroborating this fact, the immunotherapeutic capacity of these formulations was evaluated in a model of pre-sensitized mice to *Lolium*. This sensitization model was designed following the studies described in the literature by other authors (Scholl et al., 2004; Charng et al., 2006; Hisbergues et al., 2007). Once the animals were sensitized, the nanoparticles were administered to the mice and afterwards they were challenged with *Lolium* extract i.p., and the intensity of anaphylaxis was observed.

The antibody response data on day 20th showed that the animals were successfully sensitized to *Lolium*, with high levels of total IgE in serum. However, after immunization, animals tested with LPS–Lol–NP showed a 100-times higher titre of IgG_{2a} (Th1) than the controls or Lol–Alum tested mice. This result would indicate the rise of Th1 response, which was further confirmed by other biological markers. Thus, mMCP-1 (mouse mast cell protease 1) was reduced in LPS–Lol–NP treated animals, which is a clear evidence that the mast cell degranulation in this group had significantly decreased (Faulkner et al., 1997; Knoops et al., 2005). Furthermore, the success of the immunotherapy with LPS–Lol–NP is also correlated with the increase of the level of IgE just after the

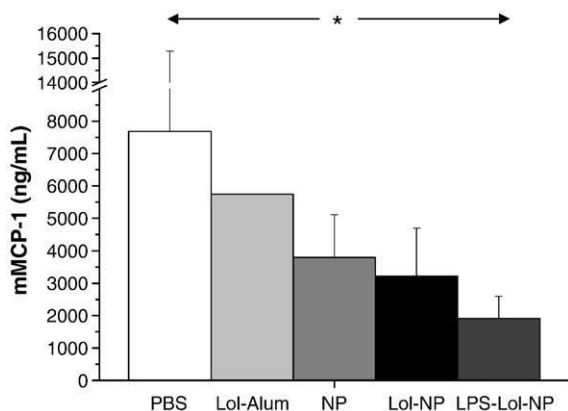


Fig. 4. Mouse mast cell protease 1 (mMCP-1) blood level after the challenge with 4 mg of *Lolium* extract i.p.

immunization. In this context, it is well described that just after immunization, an early increase on IgE levels is observed followed by a late decrease of the antibody (Gleich et al., 1982; Van Ree et al., 1997; Akdis and Blaser, 2000). This finding corroborates the results obtained in our previous studies with ovalbumin as model allergen. In those studies, the same formulation but with ovalbumin (LPS–OVA–NP) was tested and it could be appreciated that the presence of LPS in the nanoparticles was able to improve the adjuvant capacity of the particles (Gomez et al., 2008).

However, the anaphylactic symptoms, including histamine levels, temperature, cyanosis, piloerection and lack of mobility, did not appear to change from one formulation to other. The only result that was improved with the administration of *Lolium* nanoparticles, was the mortality rate: 1 h after the challenge 25% of PBS group have died, while in *Lolium*-nanoparticles groups all the animals survived. This lack of significant differences in the anaphylactic symptoms among the groups seemed to be due to the low intensity of the anaphylaxis compared to the same experiment performed with ovalbumin instead of *Lolium* extract (Gomez et al., 2008). In fact, this low intensity of the anaphylaxis may be related with the sensitization protocol used, since *Lolium* allergens are aeroallergens, and thus, the natural presentation is through the inhalatory route (Del Pozo et al., 1992; Weller et al., 1993; Shi et al., 2000). Therefore, it is possible that the sensitization and challenge of the animals by intraperitoneal route did not implicate the right biological mechanisms. Nevertheless this work suggests that, under the tested experimental conditions, LPS–Lol–NP appears to be a promising formulation to be used in immunotherapy against *L. perenne*.

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