



Gantrez[®] AN nanoparticles as an adjuvant for oral immunotherapy with allergens

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Abstract

The aim of this study was to investigate the adjuvant properties of oral-administered Gantrez[®] AN nanoparticles with ovalbumin (as allergen model) and, in some cases, lipopolysaccharide of *Brucella ovis* as immunomodulator. For this purpose, BALB/c mice were administered by oral gavage with OVA nanoparticles and both Th1 and Th2 markers (IgG2a and IgG1, respectively) were enhanced. On the other hand, these carriers administered by oral route were able to protect a model of sensitized mice to ovalbumin from anaphylactic shock. These results are highly suggestive for the valuable use of Gantrez[®] nanoparticles in oral immunotherapy with allergens.

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1. Introduction

The prevalence of atopic diseases such as asthma and allergic rhinitis has substantially increased over the past few decades. Whilst a number of pharmacological approaches can reduce the symptoms of allergic disease, only specific immunotherapy targets the underlying, Th2 cell-driven, disease process. Thus, many investigators have examined the effects of specific immunotherapies via the use of subcutaneous administrations of antigens to relieve the symptoms of the disease [1]. Although the effectiveness of subcutaneous immunotherapy has been established, it has also been pointed out that subcutaneous injections of antigen could potentially result in the occurrence of life-threatening anaphylactic reactions [2]. Therefore, safer routes of administration (noninjection or local routes) have been investigated and developed, but the efficacy of these routes still remains controversial. The only non-parenteral route that has been

supported by numerous controlled trials showing its efficacy in asthma and rhinitis, is the sublingual route (SLIT: sublingual immunotherapy), which is now accepted by the WHO as a valid alternative to the subcutaneous route for any type of patients including children [3]. However, some drawbacks remain including a relative difficulty in the administration of this type of formulations and the necessity of high doses of allergen [4,5]. In the last years, it has been also proposed the use of the oral route in order to improve the current immunotherapy treatments. The oral route has a lot of evident advantages, it is the most often used route because it is the most convenient and usually the safest and least expensive one. However, oral immunotherapy shows a lot of drawbacks related to the allergen degradation within the gut due to the gastric acidity or to the proteolytic enzymes [6]. Results of clinical trials obtained so far applying oral immunotherapy techniques with allergens were very poor [7–15]. Only three studies have shown a significant improvement of the allergic symptoms [8,10,11]. Moreover, these studies needed to administrate high doses of allergen to perform a successful treatment, and this means that, in order to decrease this dose of allergen, it would be necessary the use of a good adju-

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vant, capable of increase the immune response and protect the allergen from degradation in the gastrointestinal tract.

Nowadays, the only adjuvant which is approved for human use is alum, and as many studies have demonstrated, it does not have the capacity to elicit a sufficient mucosal immune response and it is not effective when administered by oral route [16,17]. On the other hand, several mucosal adjuvants have been described including monophosphoryl lipid A (MPL), CpG oligonucleotides or particulate mucosal delivery adjuvants such as immune-stimulating complexes (ISCOMs) [16,17]. Unfortunately, these mucosal adjuvants display a limited efficacy when administered by the oral route, and, in some cases, in order to enhance the immune response, it is necessary the use of parenteral prime-mucosal boost, or the combination of the adjuvant with liposomes [18–20].

Since many years, it has been proposed the use of controlled release delivery systems (micro or nanoparticles) as adjuvant, because they can offer: (i) protection of the loaded antigen from enzymatic degradation within the gastrointestinal tract, (ii) prolonged immune response, and (iii) enhancement of the delivery of the loaded antigen to the gut associated lymphoid tissue (GALT) [21–23]. In this context, our group has demonstrated the bioadhesive capacity of Gantrez[®] AN nanoparticles [24,25] and their high affinity toward normal mucosal tissue and Peyer's patches [26].

On the other hand, we demonstrated in previous studies that intradermal immunization with Gantrez[®] AN nanoparticles with LPS from *Brucella ovis* protected a model of sensitized mice from anaphylactic shock (unpublished data).

In this work, we studied the immunotherapeutic effect of these nanoparticulate systems administered by oral route to a model of sensitized mice.

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). Ovalbumin (OVA) (grade V), 1,3-diaminopropane (DP), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and alhydrogel 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). All other chemicals used were of reagent grade and obtained from Merck (Spain).

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

To prepare cells for extraction, tryptic soy broth (TSB) flask were inoculated with fresh cultures of *Brucella 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 [27,28].

2.3. Preparation of Gantrez[®] AN nanoparticles

Gantrez[®] AN nanoparticles were prepared by a solvent displacement method previously described [29]. The model protein was incorporated during the manufacture process (OVA-entrapped nanoparticles) or after the preparation of the adjuvant vectors (OVA-coated nanoparticles).

2.3.1. Preparation of OVA-entrapped Gantrez[®] AN nanoparticles (OVAⁱⁿ-NP)

OVAⁱⁿ-NP was prepared as described previously [30]. Briefly, 5 mg OVA were dispersed in 1 mL acetone by ultrasonication (MicrosonTM) for 1 min under cooling. The OVA dispersion was then added to 4 mL acetone containing 100 mg Gantrez[®] AN 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 1,3-diaminopropane/mg Gantrez[®] AN for 5 min under magnetic stirring at room temperature. Eventually, the cross-linked nanoparticles were fluorescently-labelled by incubation with 1.25 mg of Rhodamine B isothiocyanate (RBITC) (Sigma, Madrid, Spain) for 5 min. Nanoparticles were purified by centrifugation and freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5%) as cryoprotector.

2.3.2. Preparation of OVA and LPS-entrapped Gantrez[®] AN nanoparticles (OVAⁱⁿ-LPSⁱⁿ-NP)

Briefly, 5 mg OVA was dispersed in 1 mL acetone by ultrasonication (MicrosonTM) for 1 min under cooling; similarly, 1 mg LPS was also dispersed in 1 mL acetone by ultrasonication (MicrosonTM) for 1 min under cooling. The OVA and the LPS dispersions were added to 3 mL acetone containing 100 mg Gantrez[®] AN 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 1,3-diaminopropane/mg Gantrez[®] AN for 5 min under magnetic stirring at room temperature. Eventually, the cross-linked nanoparticles were fluorescently-labelled by incubation with 1.25 mg of rhodamine B isothiocyanate (RBITC) (Sigma, Madrid, Spain) for 5 min. The formulation was purified by centrifugation and lyophilised as described above.

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

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). 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.

2.4. Characterization of the 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 deionised water and measured at room temperature with a scattering angle of 90°. All measurements were performed in triplicate.

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 (Waldoborn, 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 (40/10/50, 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 0.1N NaOH 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, 2-keto-3-deoxy-octulosonic acid (KDO), by the thiobarbituric acid method [31]. For this purpose, a solution containing digested nanoparticles (NaOH 0.1N, 24 h, 4 °C) was added to five 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 results were expressed as the amount of LPS (in µg) per mg nanoparticles.

The yield of the nanoparticles preparation process was determined by gravimetry from freeze-dried samples as described previously [29].

2.5. In vitro release study of ovalbumin from nanoparticles

OVA-loaded nanoparticles (10 mg) were dispersed in eppendorf tubes by vortexing in 1 mL phosphate buffer saline (PBS, pH 7.4). Release study was conducted at 37 ± 1 °C under agitation in a VorTemp 56™ Shaking Incubator (Labnet International, Inc.) during 7 days. At defined times, the sample tubes were centrifuged at 26,500 × g for 20 min and the protein content was determined in the pellet by the HPLC method described above. The pellets were digested by incubation for 24 h at 37 °C in 75 µL of 1N NaOH. Empty nanoparticles were used as control and subjected to the same procedure. Release profiles were expressed in terms of cumulative release, and plotted versus time.

2.6. 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, 8 weeks old females (supplied by Harlan Interfauna Ibérica, Spain), were randomized into groups of five mice. Animals were immunised by oral gavage with 25 µg OVA incorporated in one of the following formulations: (i) OVA-entrapped nanoparticles (OVA^{in} –NP) or (ii) OVA and LPS-entrapped nanoparticles (OVA^{in} – LPS^{in} –NP) and (iii) free OVA dissolved in sterile buffered saline solution. Blank Gantrez® AN nanoparticles (NP) were also administered as control (All the formulations were dispersed in a total volume of 250 µL of sterile buffered saline solution).

Blood samples from the retroorbital 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.

2.6.1. Quantification of anti-OVA antibodies in serum

Specific antibodies against OVA (IgG₁ and IgG_{2a}) were determined in the pooled sera by indirect ELISA. Briefly, microtiter wells (cliniplate EB, Labsystems, Finland) were coated with 1 µg OVA in 100 µL sodium carbonate–bicarbonate buffer (0.05 M; pH 9.6) at 4 °C for 15 h. The plates were washed with PBS-Tween 20 (1%) and serum samples were added in two-fold serial dilutions in PBS-Tween 20 (1%) starting with 1:40, and incubated at 37 °C for 4 h. After washing again with PBS-Tween 20 (1%), 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 (1%). The plates were washed and, finally, incubated with the substrate chromogen solution (H₂O₂–ABTS). The optical density (OD) was determined at λ_{max} 405 nm (iEMS Reader MF de Labsystems, Finlandia). 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. Sensitization, vaccination and challenge studies

BALB/c mice, 8 weeks old females (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.

Once the animals were sensitized to OVA, they were divided into several groups (five mice per group), depending on the formulation administered.

On days 14, 17, 20 and 23, the animals received oral gavages of 12.5 µg of OVA each. The formulations tested were: OVAⁱⁿ-NP and OVAⁱⁿ-LPSⁱⁿ-NP (dispersed in a total volume of 250 µL of sterile buffered saline solution), and as control we administered OVA dissolved in PBS. Finally, on day 35 the animals were challenged by an injection of 1 mg of OVA by intraperitoneal route.

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 [32] using a Technicon II Analyzer (Technicon Instrument Corp., USA).

The body temperature changes associated with anaphylactic shock were monitored by measuring the rectal temperature [33] 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 [34,35]. Reactions severity was classified in 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 rate was recorded 24 h after intraperitoneal challenge.

2.8. Bioadhesion studies

The bioadhesion study was carried out using a protocol described previously [25], in compliance with the regulations of the responsible committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU). Briefly, an aqueous suspension containing 10 mg of the nanoparticles loaded with RBITC (approximately 45 mg particles/kg body weight) was administered perorally to male Wistar rats fasted overnight (average weight 225 g; Harlan, Spain). The animals were sacrificed by cervical dislocation at 0.5, 1, 3 and 8 h post-administration. The abdominal cavity was opened and the gastrointestinal tract was removed. Then, the gut was divided into six anatomical regions: stomach (Sto), intestine (I1, I2, I3 and I4) and

caecum (Ce). Each mucosa segment was opened lengthwise, rinsed with PBS, digested with NaOH 3 M for 24 h. RBITC was extracted from the digested samples by addition of 2 mL methanol, vortexed for 1 min and centrifuged at 2000 × g for 10 min. Aliquots (1 mL) of the obtained supernatants were diluted with water (3 mL) and assayed for RBITC content by spectrofluorimetry at λ_{ex} 540 nm and λ_{em} 580 nm (GENios, TECAN, Groedig, Austria) to estimate the fraction of adhered nanoparticles to the mucosa. The standard curves of the bioadhesion study were prepared by addition of RBITC-solutions in 3N NaOH (0.5–10 µg/mL) to control tissue segments following the same steps of extraction ($r > 0.996$).

In addition, the total adhered fraction in the whole gastrointestinal tract was plotted versus time and, from these curves, the kinetic parameters of bioadhesion (Q_{max} , AUC_{adh} , K_{adh} and MRT_{adh}) were estimated using the WinNonlin 1.5 software as described previously [25], with the only difference that these parameters were estimated from 0 to 8 h.

2.9. Statistical analysis

The bioadhesion data and the physico-chemical characteristics were compared using the nonparametric Mann–Whitney *U*-test and Student *t*-Test, respectively. *P* values of <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[®] AN nanoparticles

The main physico-chemical characteristics of Gantrez[®] AN formulations are summarised in Table 1. The yield of the process was always high and close to 80% of the polymer transformed into nanoparticles. The size of OVA nanoparticles was 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 resulting carriers.

On the other hand, the amount of OVA loaded in nanoparticles ranged from 24 to 30 µg protein/mg, whereas the LPS content (for OVAⁱⁿ-LPS^{out}-NP and OVAⁱⁿ-LPSⁱⁿ-NP) was calculated to be about 15 µg/mg.

Fig. 1 shows the *in vitro* release of OVA from nanoparticle formulations in PBS buffer (pH 7.4). All the formulations displayed a similar profile characterised by a burst effect followed by a sustained period of slow release. However, the coencapsulation of OVA and LPS dramatically influ-

Table 1

Physico-chemical characteristics of Gantrez® AN nanoparticles. Data are represented by mean $\pm$ S.D. ($n = 10$)

Treatment	LPS content ($\mu\text{g}/\text{mg}$)	Ova content ($\mu\text{g}/\text{mg}$)	Encapsulation efficiency (%)	Size (nm)	Zeta potential (mV)	Yield (%)
NP	–	–	–	158 ± 3	-45.1 ± 0.5	85.3 ± 0.8
OVA ⁱⁿ -NP	–	30.1 ± 4.5	42.1 ± 6.3	239 ± 4	-50.8 ± 2.9	78.2 ± 1.1
OVA ⁱⁿ -LPS ^{out} -NP	15.2 ± 0.5	24.1 ± 5.4	33.9 ± 7.6	231 ± 3	-46.1 ± 3.1	79.1 ± 0.9
OVA ⁱⁿ -LPS ⁱⁿ -NP	13.8 ± 3.0	26.5 ± 0.3	37.3 ± 0.4	227 ± 4	-34.1 ± 3.4	78.5 ± 1.4

NP: empty nanoparticles; OVAⁱⁿ-NP: OVA-entrapped nanoparticles; OVAⁱⁿ-LPS^{out}-NP: OVA-entrapped and LPS-coated nanoparticles; OVAⁱⁿ-LPSⁱⁿ-NP: OVA and LPS-entrapped nanoparticles.

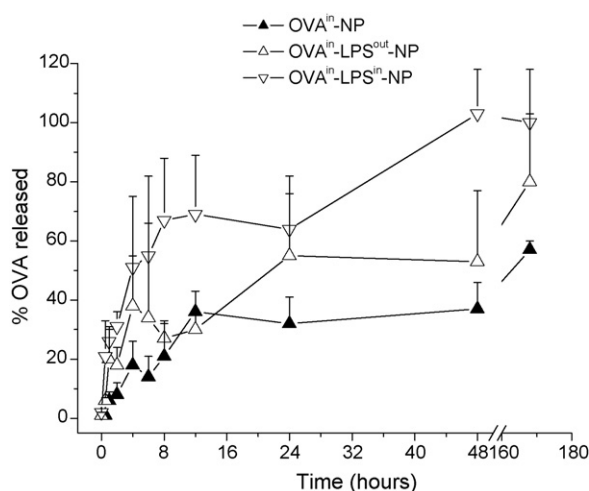


Fig. 1. *In vitro* release of OVA from Gantrez® AN formulations in PBS. (▲) OVAⁱⁿ-NP: OVA-entrapped nanoparticles; (△) OVAⁱⁿ-LPS^{out}-NP: OVA-entrapped and LPS-coated nanoparticles; (▽) OVAⁱⁿ-LPSⁱⁿ-NP: OVA and LPS-entrapped nanoparticles. Data express the mean of the cumulative amount of OVA released vs. time (mean $\pm$ S.D., $n = 3$).

enced the profile of the protein release. Thus, the burst effect for OVAⁱⁿ-LPSⁱⁿ-NP was about 60% of the loaded protein, whereas for the LPS-coated formulation and OVAⁱⁿ-NP, this value ranged from 25% to 30%. Similarly, for OVAⁱⁿ-LPSⁱⁿ-NP, the protein was totally released after 48 h, whereas for OVAⁱⁿ-LPS^{out}-NP and OVAⁱⁿ-NP only about 50% and 35% was released in the same period of time.

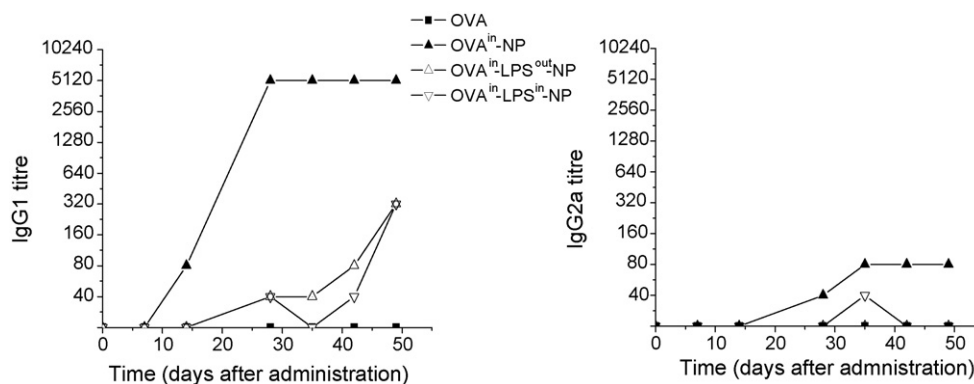


Fig. 2. Anti-OVA IgG₁ and IgG_{2a} titres in sera after oral immunization with: (■) OVA solution (OVA), (▲) OVA-entrapped nanoparticles (OVAⁱⁿ-NP), (△) OVA-entrapped and LPS-coated nanoparticles (OVAⁱⁿ-LPS^{out}-NP), and (▽) OVA and LPS entrapped nanoparticles (OVAⁱⁿ-LPSⁱⁿ-NP). The antibody titre is defined as the reciprocal dilution giving an optical density (OD) reading at 405 nm of ≥ 0.2 .

3.2. Antibody response in BALB/c mice

Fig. 2 shows the anti-OVA IgG₁ and IgG_{2a} titres (Th2 and Th1 markers, respectively) in sera after oral immunization of mice with the different formulations. All nanoparticle formulations induced higher levels of both IgG₁ and IgG_{2a} than the control (OVA), however, the enhancement of the immune response was considerably higher for the group treated with OVA-entrapped nanoparticles without LPS (OVAⁱⁿ-NP). For this group of mice, the IgG₁ levels displayed a profile characterised by a short lag-time, of about 1 week, followed by an intense period of anti-OVA IgG₁ secretion. At the end of this period (day 28th), a plateau of antibody levels was reached and maintained till the end of the experiment (day 49th). On the other hand, LPS-containing nanoparticles displayed a delayed induction of the secretion of anti-OVA IgG₁ antibodies. In fact, the IgG₁ titres were quite low for both OVAⁱⁿ-LPSⁱⁿ-NP and OVAⁱⁿ-LPS^{out}-NP till day 35 of the experiment. Then, these levels significantly increased. The levels of anti-OVA IgG_{2a} antibodies were low for all the formulations tested; although the induced titres were at least four times higher for OVAⁱⁿ-NP than for LPS containing nanoparticles.

3.3. Sensitization studies

The induced OVA-allergic mice received the immunotherapeutic schedule, and were challenged with OVA on day 35. In order to analyse the intensity of the anaphylactic shock,

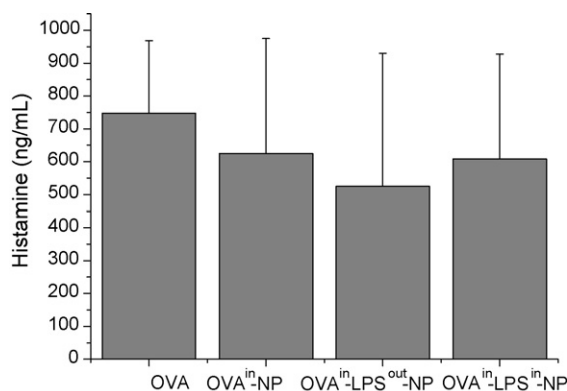


Fig. 3. Increase of the histamine blood level after the challenge with 1 mg of OVA i.p. After sensitization to OVA, the different groups of animals were treated orally (days 14th, 17th, 20th and 23rd) with OVA-entrapped nanoparticles (OVAⁱⁿ-NP), OVA-entrapped and LPS-coated nanoparticles (OVAⁱⁿ-LPS^{out}-NP) and OVA and LPS entrapped nanoparticles (OVAⁱⁿ-LPSⁱⁿ-NP). OVA solution in water was used as control. Data were expressed as mean $\pm$ S.D. ($n = 10$).

several parameters were determined. Fig. 3 shows the difference of histamine blood levels before the challenge and 30 min later. No significant differences were found between the different groups.

Table 2 shows the overall anaphylactic symptoms score including the mortality rate. OVAⁱⁿ-LPS^{out}-NP and OVAⁱⁿ-LPSⁱⁿ-NP groups showed a lower decrease on the body temperature than the control (OVA), although the differences were not significant ($P < 0.05$). The degree of piloerection and cyanosis seemed to be lower for animals treated with OVAⁱⁿ-NP and OVAⁱⁿ-LPSⁱⁿ-NP than with the control or OVAⁱⁿ-LPS^{out}-NP. Similarly, the mobility of the animals treated with OVAⁱⁿ-NP and OVAⁱⁿ-LPSⁱⁿ-NP appeared to be normal; while for the other groups, the animals were found to be static. However, the most determinant data was the mortality rate. In this context, the challenge of animals with OVA induced a 60% of mortality within the control group. On the contrary, in the group of animals treated with OVAⁱⁿ-LPSⁱⁿ-NP, the mortality rate was 20%, whereas all the animals treated with OVAⁱⁿ-NP survived to the challenge (Table 2).

3.4. Bioadhesion study

RBITC loaded nanoparticles dispersed in water (10 mg, approximately 45 mg particles/kg body weight) were per-

orally administered to male Wistar rats fasted overnight. The animals were sacrificed at 0.5, 1, 3 and 8 h post-administration and the fluorescence was quantified in the different parts of the gut.

To ensure that the fluorescence intensity determined in the gastrointestinal parts was due to the RBITC-associated nanoparticles, *in vitro* release of RBITC was firstly examined. The percentage of RBITC released after 30 min incubation in simulated gastric fluid and 3 h in simulated intestinal fluid was similar in all nanoparticles formulations, and represented about 10% in gastric and 12% in intestinal fluids from the initial loaded amount of RBITC in the nanoparticles. The amount of free RBITC found adhered to the gut 3 h post-administration was less than 2%. Therefore, it could be assumed that the fluorescence found in the gut of the animals would be due to the RBITC-associated to the nanoparticles.

Fig. 4 describes the distribution of the adhered amounts of nanoparticles in the gut mucosa as a function of time. Overall, all the formulations tested were able to develop adhesive interactions within the gut, although, OVA-nanoparticle formulations appeared to interact with the mucosa in a larger extent than control nanoparticles (NP). OVAⁱⁿ-NP displayed a maximum of adhesion in the stomach, where, about 19% of the given dose was found adhered 1 h post-administration. Interestingly, about 20% of the loaded dose of these nanoparticles was also found adhered to the small intestine (mainly duodenum and jejunum, portions I1–I3 in the figure) during at least the first 3 h post-administration. On the other hand, the presence of LPS modified the distribution of OVA nanoparticles within the gut. Thus, OVAⁱⁿ-LPSⁱⁿ-NP displayed two maximums of adhesion: in the stomach (about 12% of the given dose 1 h after administration) and in the duodenum (11% of the given dose 3 h post-administration). In this context, 3 h post-administration, OVAⁱⁿ-LPSⁱⁿ-NP showed twice higher adhered amount in I1 than OVAⁱⁿ-NP. In summary, OVAⁱⁿ-LPSⁱⁿ-NP displayed higher affinity by the proximal parts of the intestine than the same formulation without LPS (OVAⁱⁿ-NP).

Fig. 5 shows the curves of bioadhesion obtained by representing the total amount of the adhered particles to the whole gastrointestinal tract over time. Both OVA formulations displayed a higher bioadhesive profile than control nanoparticles (NP). OVAⁱⁿ-NP showed a peak of maximum adhesion 1 h after administration. On the other hand, OVAⁱⁿ-LPSⁱⁿ-NP

Table 2
Symptoms score after the challenge with OVA i.p.

Treatment	Temperature decrease (°C)	Piloerection	Mobility	Cyanosis	Survival rate (%)
OVA	7.2 $\pm$ 1.1	+	Low	+++	40
OVA ⁱⁿ -NP	7.3 $\pm$ 0.2	+	Normal	++	100
OVA ⁱⁿ -LPS ^{out} -NP	4.5 $\pm$ 3.6	++	Low	++	20
OVA ⁱⁿ -LPS ⁱⁿ -NP	6.1 $\pm$ 2.2	+	Normal	++	80

Severity of the symptoms: (–) absent; (+) weak; (++) moderate and (+++) strong. Treatments: OVA: OVA dissolved in phosphate buffer saline; OVAⁱⁿ-NP: OVA-entrapped nanoparticles; OVAⁱⁿ-LPS^{out}-NP: OVA-entrapped and LPS-coated nanoparticles; OVAⁱⁿ-LPSⁱⁿ-NP: OVA and LPS-entrapped nanoparticles ($n = 10$).

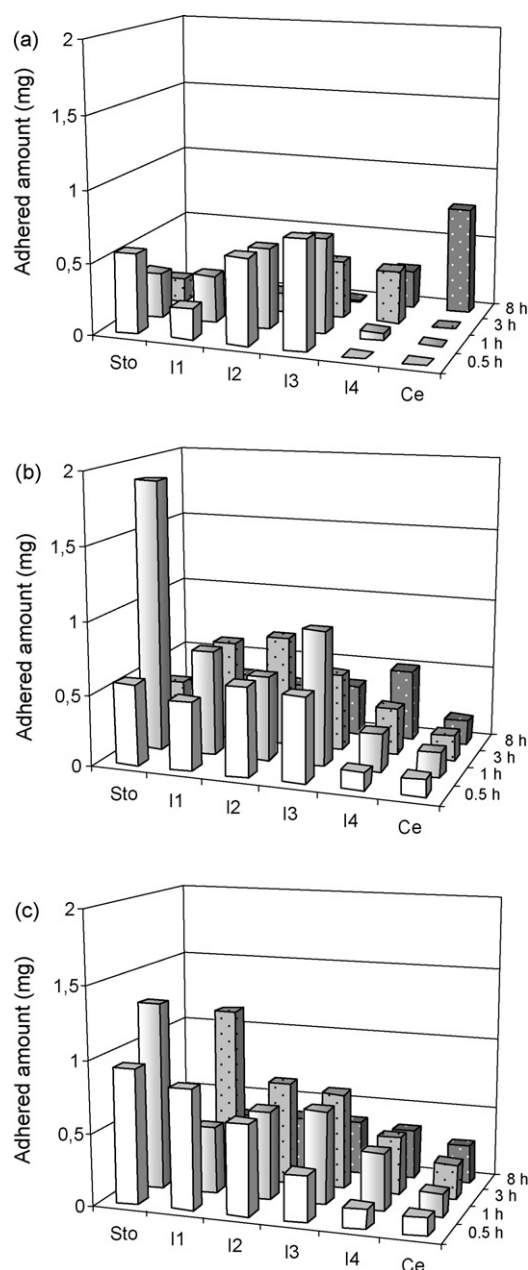


Fig. 4. Distribution of nanoparticles formulation in the gastrointestinal tract of rats. (a) control nanoparticles (NP), (b) OVA-entrapped nanoparticles ($\text{OVA}^{\text{in}}\text{-NP}$), and (c) OVA and LPS entrapped nanoparticles ($\text{OVA}^{\text{in}}\text{-LPS}^{\text{in}}\text{-NP}$) after the oral gavage of 10 mg RBITC-loaded nanoparticles. The (x-axis) represents the different gut segments; [Stomach: Sto; intestine portions: I1, I2, I3, I4; caecum: Ce], z-axis represents the adhered fraction of the nanoparticle in the mucosa (mg); (y-axis) represents the time after administration (0.5, 1, 3 and 8 h). Each value was represented by the mean ($n=4$; S.D. was less than 20% of the mean).

did not show this peak, this formulation displayed a plateau of adhesion between 1 and 3 h post-administration.

Table 3 shows the parameters of bioadhesion. The capacity of the nanoparticles to develop adhesive interactions within the gut (expressed as Q_{max}) and the intensity of the phenomena (expressed as AUC_{adh}) were found to be around twice higher for OVA loaded nanoparticles than for control ones

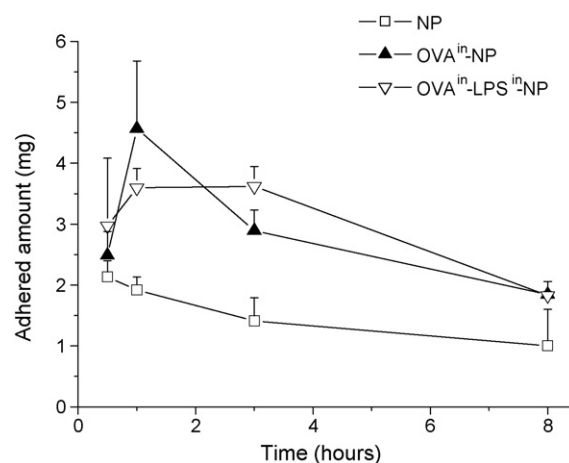


Fig. 5. Curves of bioadhesion represented by the adhered fraction of nanoparticles in the whole gastrointestinal tract over time. Dose: 10 mg nanoparticles. ($\square$) Control nanoparticles (NP), ($\blacktriangle$) OVA-entrapped nanoparticles ($\text{OVA}^{\text{in}}\text{-NP}$), and (∇) OVA and LPS entrapped nanoparticles ($\text{OVA}^{\text{in}}\text{-LPS}^{\text{in}}\text{-NP}$). The data were expressed as mean $\pm$ S.D. ($n=4$ for each time of the experiment).

(NP) ($P<0.01$). On the contrary, for all formulations, the fraction of adhered nanoparticles appeared to be eliminated from the gut mucosa at a similar rate (K_{adh}). Thus, the mean residence time of the adhered fraction of nanoparticles was similar for all the formulations and close to 3.5 h.

4. Discussion

In this study we provide evidence that oral immunotherapy is possible by using the appropriate immunoadjuvant. Gantrez[®] AN nanoparticles were demonstrated to be good adjuvants for immunotherapy when administering intradermally (unpublished data), nevertheless oral immunotherapy was a great challenge because of reasons related to the degradation of the allergens in the gastrointestinal tract and instability, or the insufficient enhancement of the immune response. Until now, just three published clinical trials showed a significant improvement of the allergic symptoms [8,10,11] when using oral immunotherapy, and all of them needed high doses of allergen to succeed in the treatment. This means that, with the use of a good adjuvant, the oral immunotherapy could be more efficient and safe, and thus, it would allow the use of lower doses of allergen. In this context, nanoparticles of biodegradable polymers could allow the design of a more efficient oral immunotherapy.

Gantrez[®] AN nanoparticles were prepared by a method previously described [25], and OVA, as a model allergen, was incorporated during the manufacturing process (OVA-entrapped nanoparticles) as described previously [30]. Furthermore, LPS of *Brucella ovis*, which is known to show a low endotoxic effect [36] and to enhance Th1-like responses [37], was incorporated in some batches in order to study its influence on the efficacy of the resulting adjuvant. When the

Table 3
Parameters of bioadhesion

Treatment	$Q_{\max}^a$ (mg)	AUC_{adh}^b (mg h)	K_{adh}^c (h^{-1})	MRT^d (h)
NP	2.13 ± 0.18	10.95 ± 0.14	0.13 ± 0.08	3.45 ± 0.32
OVA ⁱⁿ -NP	$4.56 \pm 1.68^{**}$	$21.68 \pm 5.43^{**}$	0.11 ± 0.02	3.41 ± 0.15
OVA ⁱⁿ -LPS ⁱⁿ -NP	$3.62 \pm 1.53^{**}$	$23.22 \pm 8.98^{**}$	0.09 ± 0.03	3.48 ± 0.20

^a Maximal amount of nanoparticles adhered to the gut surface.

^b Area under the curve of bioadhesion.

^c Terminal elimination rate of the adhered fraction in the gastrointestinal mucosa.

^d Mean residence time of the adhered fraction of nanoparticles in the mucosa.

^{**} $P < 0.01$ vs. control nanoparticles NP (Man-Whitney U -test).

different formulations were characterized, we could observe that all the nanoparticles displayed a similar and homogeneous size. The presence of LPS did not affect the size of the resulting carriers, although rendered more electropositive particles (see Table 1). Concerning OVA loading, the presence of LPS did not affect it and, likewise, the LPS content was similar for all formulations.

When these formulations were orally administered to mice, the only formulation that really enhanced the immune response was OVAⁱⁿ-NP. Surprisingly, the presence of the LPS on the NP (OVAⁱⁿ-LPSⁱⁿ-NP) decreased both OVA-specific IgG_{2a} (Th1 marker) and IgG₁ (Th2 marker) levels (Fig. 2). This fact did not correlate with the data previously obtained by intradermal route (unpublished data).

On the other hand, in order to evaluate the protective effect of these formulations with or without LPS on a model of OVA-sensitized mice, the animals were treated with the different types of OVA-entrapped nanoparticles (with or without LPS) by oral route and finally they were challenged and the different anaphylactic symptoms were observed. We found that although the histamine levels or the temperature data did not vary too much between some formulations and others, the anaphylactic symptoms and mortality rate were really determinant. In this context, the ultimate parameter to test the efficacy of the immunization was the protection against mortality. OVAⁱⁿ-NP was found to be the best formulation. In fact, OVAⁱⁿ-NP protected all the mice from death, in contrast to the control group administered orally with OVA solution. OVAⁱⁿ-LPSⁱⁿ-NP also diminished the mortality rate against the control, but it did not protect all the mice. However, when the LPS was coating the nanoparticles (OVAⁱⁿ-LPS^{out}-NP), the mice were not protected, and the mortality rate was similar to that observed for the control group. In summary, the presence of LPS in the nanoparticles did not improve at all its adjuvant capacity when administered by oral route.

Trying to explain this phenomenon, OVAⁱⁿ-NP and OVAⁱⁿ-LPSⁱⁿ-NP were administered to rats and some bioadhesion studies were performed. We could observe that effectively both formulations showed higher bioadhesive capacity than the control nanoparticles (NP), but each formulation showed a different profile. OVAⁱⁿ-LPSⁱⁿ-NP displayed a higher affinity by the proximal parts of the intestine (Fig. 4) than OVAⁱⁿ-NP. This fact could explain the efficacy of OVAⁱⁿ-NP protection in sensitized mice, because the upper parts of the intestine are supposed to be more aseptic and thus they

promote Th2-like responses, while distal parts, with a great number of bacteria, are related with Th1-like profiles [38]. So, as OVAⁱⁿ-LPSⁱⁿ-NP is preferably adhered to the proximal parts, this fact could drive to a high Th2 response and thus worsening of the allergic symptoms.

On the other hand, the *in vitro* release of OVA from the nanoparticles (Fig. 1) showed that the presence of LPS induced a faster release of the loaded protein. In fact, for OVAⁱⁿ-LPSⁱⁿ-NP, after 48 h, all the loaded protein was released from the nanoparticles. On the contrary, for OVAⁱⁿ-NP, only 35% of the protein was released in a similar period of time. These observations may be the reason of the success of OVAⁱⁿ-NP in the challenge studies. This formulation without LPS was able to release the OVA in a more sustained way, and thus to protect the protein from the degradation of the gut.

Moreover, Otte et al. have recently proposed that the continuous presence of specific bacterial components in the gastrointestinal tract, including LPS, would induce a status of hyporesponsiveness and down-regulation of the Toll-like receptors (TLR) cell surface expression [39]. This phenomenon could be another reason because of which the association of LPS with the nanoparticles did not affect the efficacy of the formulations.

Finally, as a conclusion of this work, we have demonstrated that Gantrez[®] AN nanoparticles seemed to be a good alternative for oral immunotherapy. In this context, OVAⁱⁿ-NP were able to protect sensitized mice from the death by anaphylactic shock. It is remarkable that protection from anaphylactic death is not usually described in the bibliography [40–43], therefore, the effect of Gantrez[®] AN nanoparticles is a significant finding for its application in oral immunotherapy.

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