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Biodegradable microparticles for the mucosal delivery of antibacterial and dietary antigens

Elias Fattal [a,*], Sophie Pecquet [b], Patrick Couvreur [a], Antoine Andremont [a]

[a] *School of Pharmacy, University of Paris Sud, UMR CNRS 8612, 5 rue Jean-Baptiste Clement, 92296, Châtenay-Malabry, France*

[b] *Food Immunology Group, Nestec S.A., Center de Recherche Nestlé CP 44, CH 1000 Lausanne, Switzerland*

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Abstract

Mucosal administration of antigen is known to be appropriate for vaccine purposes as well as tolerance induction. Biodegradable poly(DL-lactide-co-glycolide) (PLGA) microparticles were used to deliver both antibacterial phosphorylcholine (PC) and dietary antigen β lactoglobulin (BLG) by mucosal route. In a first study, the protective immunity elicited by intragastric vaccination with PC encapsulated in microparticles was evaluated in a mouse model against intestinal infection by *Salmonella typhimurium* and pulmonary infection *by Streptococcus pneumoniae*. A significant rise in anti-PC immunoglobulin A (IgA) titers, as measured by an enzyme-linked immunosorbent assay, was observed in the intestinal secretions after oral immunization with PC-loaded microparticles compared with the titers of mice immunized with free PC–thyr or blank microparticles. This antibody response correlated with a highly significant resistance to oral challenge by *S. typhimurium*. IgA in pulmonary secretion were not able to protect against *S. pneumoniae* infection. BALB/c mice were, therefore, immunized intranasally (i.n.). Immunization was followed by a rise in anti-PC IgA and IgG titers in serum and in pulmonary secretions by both free and encapsulated PC–Thyr. The survival rates were 91 and 76% in the two groups of mice, respectively. In a second study and in order to prevent allergy against milk by inducing oral tolerance, one of the major allergenic milk protein, BLG was entrapped into microparticles. Oral administration of microparticles containing BLG reduced significantly (by 10 000) the amount of protein necessary to decrease both specific anti BLG IgE and DTH response. These studies demonstrate the ability of microparticles to induce both mucosal immunity and oral tolerance.

Keywords: Microparticles; Poly(lactide-co-glycolide); Phosphorylcholine; Mucosal immunity; Oral tolerance

1. Introduction

The use of oral antigen delivery systems, made of biodegradable microparticles has allowed to protect orally administered antigens against enzymatic degradation, to increase antigens uptake by the M cells and the Peyer's patches (PP), to

* Corresponding author. Tel.: +33-1-46835568; fax: +33-1-46619334

E-mail address: elias.fattal@cep.u-psud.fr (E. Fattal).

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enhance mucosal immune response and finally to induce a long and slow antigen release (O'Hagan, 1996). The use of Poly (DL-lactide-co-glycolide) (PLGA) microparticles is particularly promising in that respect (Jenkins et al., 1995). However, number of properties were shown to be important to achieve such a targeting of the PP among which particle diameter (Andrianov and Payne, 1998). Following uptake of particles into the PP, Eldridge et al. (1990) found that particles smaller than 5 μm left the PP and migrated through the mesenteric lymph node within the phagocytic cells while particles of 5–10 μm remained in the PP. Those results were confirmed by Ebel (1990) who demonstrated that particle uptake depended on dose level, particle size and the fed state of the animal. The larger particles were not found in the spleen or the mesenteric lymph node indicating that these particles cannot penetrate further than the PP following uptake. Eldridge et al. (1990) have also shown the uptake of particles into PP and their subsequent transport into mesenteric lymph node in phagocytic cells. The fate of particles after uptake into PP was shown to be size dependent and particles of less than 5 μm were detectable to a high extent in the spleen 14 days post-administration. In these conditions, oral administration of vaccines, through their uptake by specialized epithelial cells and subsequent concentration in PP, is able to induce substantial increase in antigen specific antibodies, secretory IgA (sIgA), in all mucosal sites (gut lamina propia, respiratory tract, mammary glands, salivary glands, lachrymal glands and genito-urinary tract) (Mestecky, 1987).

The use of microparticles to induce mucosal immune response is applied here to phosphorylcholine (PC) which is an hapten present on different pathogenic bacteria including gram-positive bacteria such as *Streptococcus pneumoniae*, and gram-negative bacteria such as *Salmlonella typhimurium*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Proteus morganii*, and parasites, such as *Ascaris suum* and *Trichinella spiralis*, which colonize or invade host mucosa at different epithelial sites (for review see Trolle et al., 1998). We hypothesized that anti-PC mucosal immunity might confer protection against several diseases caused by these various pathogens including pneumonia, diarrhea and meningitis, and so fulfilling one of the WHO priority—namely 'multipurpose'—required for vaccine development. We describe here the formulation and the efficacy of PC-loaded PLGA microparticles to induce mucosal immune response against PC in a mouse model. Protection was evaluated in both *S. thyphimurium* and *S. pneumoniae* murine infections.

The advantages of biodegradable microparticles, first promoted for vaccine development, now appear attractive to improve the oral tolerance immune response, which has similar needs (Challacombe and Tomasi, 1980; Strobel, 1992). Oral administration of either self-antigens or food allergens has been demonstrated to provoke specific hyporesponsiveness of the immune system and is, thus, called oral tolerance. For the last decade, this strategy has been proposed as a promising treatment of auto-immune disorders and food allergies. To date, oral tolerance protocols in humans have mainly focused on auto-immune diseases, such as multiple sclerosis (Fukaura et al., 1996). Regarding food allergy, cow's milk allergy is a major issue when considering the high incidence of this allergy in the pediatric newborn population (up to 10%) (Kleinman, 1991). Several approaches are currently under evaluation. Using oral administration of β lactoglobulin (BLG), one of the major allergens in cow's milk, we have been able to demonstrate a capacity to induce specific oral tolerance in a mice model (Pecquet et al., 1999) at, however, very large doses. We describe here the ability of reducing the tolerogenic dose of BLG by using PLGA microparticles.

2. Material and methods

2.1. Preparation and characterization of PC loaded microparticles

Microparticles of D,L-lactide-co-glycolide (PLGA) (ratio: 75/25, MW:128 000 Da, Birmingham Polymers, Birmingham, USA) were prepared by multiple emulsion solvent evaporation method, as described (Allaoui-Attarki et al., 1998). They were loaded as described (Allaoui-Attarki et al.,

1998) with PC–thyr using a w/w PC–thyr-to-polymer ratio of 1:8. Blank microparticles were prepared identically in the absence of PC–thyr. Microparticles were collected by 5 min of centrifugation at $700 \times g$, washed three times with distilled water and freeze-dried in 0.1% polyvinylalcohol (PVA). Diameters, measured as described (Allaoui-Attarki et al., 1998), were ≤ 10 μm for 94 and 97% of the microparticles, respectively, loaded with PC–thyr or unloaded. The amount of encapsulated thyroglobulin was assayed, after release of the conjugate from 30 mg microparticles incubated in 3 ml 1 N NaOH overnight with stirring. The encapsulation efficiency of the PC–Thyr conjugate was 88% The PC-to-protein molar ratios of the conjugates were estimated at 108 by a microphosphate assay as described (Bartlett, 1959). The 475 nm specific absorbance spectrum of the PC–thyr conjugate was identical before and after encapsulation, as was its PC-specific ELISA reactivity.

2.2. Oral immunization procedures

Female, 8 weeks old, BALB/c mice (IFFA-CREDO, Saint-Germain sur l'Arbresle, France) were randomly assigned to one of four immunization groups (six mice per group). The first two groups were immunized on 3 consecutive days by intra-gastric administration of 500 μl of saline containing 75 mg of PLGA microparticles, either loaded with PC–thyr (280 μg of PC), or unloaded. A third group of mice received free PC–thyr suspended in 500 μl of saline under similar conditions. Three identical booster doses were, respectively, administered 29, 30, and 31 days later, as described (Allaoui-Attarki et al., 1998). The anti-PC IgG, IgA, and IgM responses were assayed 45 days after the first immunization dose. Blood samples were collected by retro-orbital puncture. Sera were diluted 1:40 in bicarbonate buffer contents were collected immediately after death, as described (Allaoui-Attarki et al., 1998).

2.3. Intranasal immunization procedures

Groups of female, 8 weeks old, BALB/c mice (IFFA-CREDO, Saint-Germain sur l'Arbresle, France) were immunized intranasally (i.n.) under light anesthesia with sodium pentobarbital. Then on 3 consecutive days, a dose comprising 7.5 mg of either blank or PC–Thyr-loaded microparticles, or of 15 μg of PC in free PC–Thyr in 50 μl sterile saline, was deposited in the nostrils (Trolle et al., 1999, 2000). Mice were boosted with identical doses 29, 30 and 31 days later. The anti-PC IgA and IgG responses were assessed by ELISA, 45 days after the first immunization dose (Trolle et al., 1999, 2000). Blood samples were collected by retro-orbital puncture. Sera were diluted 1:40 in bicarbonate buffer. Lung washes were collected immediately after death, as described (Trolle et al., 2000).

2.4. Antibody assays

Two-fold dilutions of samples were assayed in duplicate for anti-PC IgM, IgG, and IgA by ELISA using PC-BSA-coated plates and goat anti-mouse IgA, IgG, or IgM conjugated to alkaline phosphatase (Sigma, St. Louis, MO). Titers of test samples were determined by calculating the dilution of the sample which gave a 405 nm absorbance equivalent to that of negative controls consisting either of pooled sera or of pooled intestinal samples or pulmonary secretions from 40 non-immunized mice, and which were included in each assay. Titers were expressed as the $\log_{10}$ of the reciprocal of the dilution, and compared between groups by Student's t-test.

2.5. Oral challenge by Salmonella typhimurium

Oral infection with *S. typhimurium* strain C5 (kindly provided by M.Y. Popoff, Institut Pasteur, Paris, France) was performed, as described (Allaoui-Attarki et al., 1997), at the peak of the IgA anti-PC response (45 days after primer immunization). Briefly, mice were water-deprived overnight and then given free access for 18 h to a drinking bottle containing 100 ml of the inoculum (10^5 bacteria per ml). Afterwards, the inoculum was replaced by acidic sterile water (pH 3). The number of animals that died was recorded daily for 4 weeks post challenge and the between-groups differences in death rates were then compared using the log-rank test.

2.6. Pulmonary challenge by S. pneumoniae

Animals were infected 45 days after primer immunization. *S. pneumoniae* strain P4142, serotype 3 (kindly provided by J.J. Pocidalo, INSERM U13, Paris, France) was used. Ether-anaesthetized mice were given 50 ml of the bacterial inoculum i.n. (10^6 cfu per mouse). Preliminary experiments (not shown) indicated that 3 h after challenge, the total inoculum was present in the lung, and that after inoculation, total lung bacterial counts gradually increased, reaching 10^9 cfu per lung at the time of death. All mice developed bacteremia, and when death occurred, it took place between the sixth and the tenth days. The number of animals that died was recorded daily for 3 weeks after challenge (Trolle et al., 1999, 2000).

2.7. Preparation and characterization of β lactoglobulin loaded microparticles

Microparticles of D,L-lactide-co-glycolide (D,L-PLGA) (ratio 75/25, MW: 19 500, Birmingham Polymers, Birmingham, AL, USA) were prepared using the double emulsion (w/o/w) solvent evaporation technique as previously described (Rojas et al., 1999). Microparticles were loaded with BLG, or they remained unloaded for control purposes. The microparticles were isolated by centrifugation (4000 rpm for 10 min), washed three times with double-distilled water and freeze dried. Microparticles diameter was determined using a coulter Multisizer II (Coultronics, France). The zeta potential of the microparticles was measured by a Zetasizer 4 apparatus (Malvern, France). The level of BLG encapsulated was determined as follows: weighed amounts of freeze-dried microparticles were dispersed in 0.1M NaOH containing 1% (w/v) SDS, to give a concentration of 5.0 mg per 1 ml. The suspension was shaken at room temperature for 24 h. Samples were centrifuged (4000 rpm for 10 min) and the BLG concentration was determined in the supernatant by a Bio-Rad DC protein microassay against a series of BLG standards prepared in 1 ml 0.1 N NaOH, SDS 1%. Protein assay sensitivity was 10 mg/ml. The encapsulation efficiency was expressed by the ratio between the actual and the theoretical loading (actual BLG loading/theoretical BLG loading × 100). All samples and standards were assayed in triplicate.

2.8. Induction of oral tolerance and immunization procedures

Female BALB/c mice were obtained from IFFA-Credo (L'Abresle, France) and experiments commenced at 3 weeks of age. The mice were bred and raised on a cow's milk free diet. On day 1, mice were orally administered, by gastric feeding, saline water, various amounts of D,L-PLGA microparticles loaded with BLG (3.8 mg BLG/100 mg of polymer), soluble BLG or unloaded microparticles. Gavage products were suspended in 300 ml of saline water. All mice were immunized on day 5 by intra-peritoneal administration of 0.08 mg of BLG and 0.08 mg of OVA, in 0.04 ml sterile saline mixed with 0.16 ml 2% $Al(OH)_3$ (Superfos Biosecotr A/S, Denmark). On day 28, immune response was evaluated in the different groups according to the method developed by Pecquet et al. (1999). Briefly, intestinal secretions and blood were collected and IgE antibodies anti-BLG and anti-OVA were determined by ELISA according to the method developed by Pecquet et al. (1999). Delayed type hypersensitivity (DTH) evaluation was done by duplicate thickness measurements of the left rear footpad prior to and following intradermal immunization with 0.1 mg of BLG diluted in 0.05 ml of sterile saline, using a dial gauge microcaliper (Mitutoyo, MFG, Japan). DTH, serum and intestinal IgE were compared using ANOVA test.

3. Results and discussion

3.1. Efficacy of PC-loaded microparticles as antibacterial vaccine

PC was chosen as an antigen because it was found to elicit an immune response after intestinal exposure of mice to PC-bearing *S. typhimurium* (Pecquet et al., 1992), and because anti-PC immunity protects mice against *S. pneumoniae*, another

PC-bearing microorganism (Briles et al., 1981b). This hapten is also present on different microorganisms that invade or colonize different mucosa (Trolle et al., 1998). The anti-PC IgG, IgA and IgM titers measured after oral immunization procedures show that IgA rose in serum and intestinal secretions, after intragastric administration of PC–thyr-loaded microparticles (Fig. 1). No rise of IgG, IgA, and IgM was observed in serum and intestinal secretion after intragastric administration of free PC–thyr (Fig. 1). All serum and intestinal samples from the mice given blank microparticles were negative (Fig. 1). The ability observed here of PLGA microparticles to elicit a mucosal immune response is due to protection of orally administered antigen and to the increase of particle uptake by intestinal lymphoid tissue. However, in our findings, the immune response was both of mucosal and systemic type. This effect might be due to the presence of small

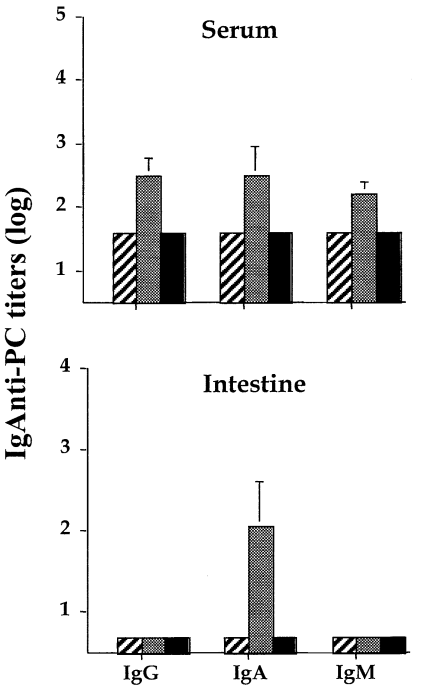

Fig. 1. Antiphosphorylcholine IgG, IgA, and IgM antibody titers in serum and intestinal secretions of mice immunized intragastrically with free PC–thyr (▨), PC–thyr-loaded microparticles (▩) or blank microparticles (■). Each value is the mean ± S.E. for six mice.

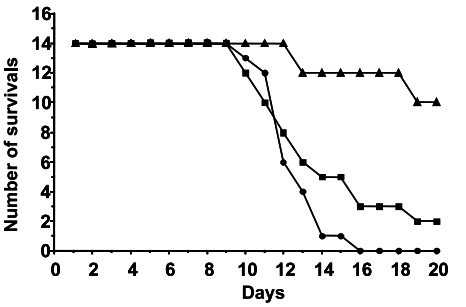

Fig. 2. Cumulative mortality rates after oral challenge by *S. typhimurium* C5 of mice immunized intragastrically with free PC–thyr (■), PC–thyr-loaded microparticles (▲), or blank microparticles (●).

particles that were shown to leave PP after uptake and to be transported to the mesenteric lymph nodes and spleen where they elicit a systemic anti-PC response (Eldridge et al., 1991).

Protection was first evaluated after oral challenge by *S. typhimurium* strain C5. Death rates were recorded in the various immunization groups after oral challenge by *S. typhimurium* strain C5. The results show that the mice orally immunized with PC–thyr-loaded microparticles were significantly better protected against this challenge than those which were intragastrically immunized with unloaded microparticles or with free PC–thyr (Fig. 2). The combined increases in both the IgA response and the protective immunity observed after intragastric immunization with PC–Thyr-loaded microparticles were strikingly larger than those observed after oral immunization with free PC–Thyr. This confirms PLGA microparticles uptake by PP. Since no protection was observed after oral administration of blank microparticles (Fig. 2), protective immunity observed with PC–Thyr-loaded microparticles appeared to be due to the anti PC immune response.

It was also shown that both free PC–Thyr and PC–Thyr-loaded microparticles administered i.n. also confer a strong mucosal and systemic anti-PC immune response (Table 1). This immunity was protective against an intranasal lethal challenge by *S. pneumoniae* serotype 3 (Table 2). The survival rates were 91 and 76% in mice immunized with free and entrapped PC–Thyr, respectively (Table 2). Protection following oral immunization

with the same preparations was significantly lower although oral administration of PC–Thyr-loaded microparticles induced significant increases of pulmonary IgA (Trolle et al., 2000). The same was observed in a previously reported comparison of the relative efficacy of immunization by intranasal and oral routes with tetanus toxoid encapsulated in PLGA microparticles. In that experiment, intranasal administration induced better systemic and local immune responses than oral immunization (Almeida and Alpar, 1996). The mechanisms responsible for such differences may result from that IgA-secreting cells induced in gastrointestinal tract may have a greater propensity to relocate in compartments other than the upper respiratory tract.

The immune protection acquired against pneumococcal pneumonia, as assessed by both pulmonary clearance and survival after the lethal challenge with *S. pneumoniae* strain 4142, correlated with the anti-PC immune response observed in the immunized mice. The high serum anti-PC IgG titers obtained after either i.n. immunization was a key factor in conferring protection against mortality. Indeed, it was previously shown in mice that an increase in serum IgG antibodies correlated with the degree of protection against an intravenous challenge with *S. pneumoniae* (Briles et al., 1981a; McDaniel et al., 1984; Watson et al., 1995). It was not surprising that high IgG levels were found in the serum of the mice immunized i.n., because the presence of high levels of serum IgG against soluble or particulate antigens has repeatedly been reported after immunization via the respiratory tract (Shahin et al., 1992, 1995; Cahill et al., 1995; Roberts et al., 1993; Russell et al., 1996). The high levels of anti-PC IgG found in the lungs of the immunized mice may be explained, at least in part, by transudation from serum. Indeed, it has been previously suggested that serum IgG can reach the lungs (Toews et al., 1985). Our results showed that the protection provided against *S. pneumoniae* by i.n. immunization with PC–Thyr-loaded microparticles was not significantly different than that observed after i.n. immunization with free PC–Thyr. Free PC–Thyr is a soluble antigen which might, therefore, freely penetrate the respiratory tract epithelium and reach the immune system (Kuper et al., 1992). Thus, in our model, the advantages of using a particulate carrier for nasal immunization of protein-linked PC can only be hypothetical, for instance to stabilize the immunogen, induce long-lasting local immunity by interacting with mucosal surfaces or generate a mucosal memory response (McGhee and Kiyono, 1993). Lastly, important questions have been raised about the safety of PC immunization, it has been postulated that cells secreting anti-ds DNA antibodies may be routinely activated in the course of the response to parenteral immunization with PC coupled to a protein (Limpanasithikul et al., 1995). In mice, some of these cross-reactive antibodies can deposit in the kidneys, in a pattern similar to that seen in auto-immune diseases (Ray et al., 1996). However, attempts to observe such phenomena in non-auto-immune mice have been frustrating, as ds-DNA binding cells have been generated from PC-immunized mice only rarely, and only after interfering with the regulatory

Table 1
Anti-PC antibodies in sera and secretions of immunized mice

Immunization group	Route	Anti-PC titer in indicated fluid			
		IgA		IgG	
		Serum	Broncho-alveolar fluid	Serum	Broncho-alveolar fluid
PC–Thyr in microparticles	i.n.	3.6 ± 1.1	5.1 ± 2.0	9.1 ± 1.3	8.5 ± 2.3
Free PC–Thyr	i.n.	2.5 ± 0.9	6.3 ± 4.7	9.2 ± 1.2	6.1 ± 0.4
Unloaded microparticles	i.n.	0.2 ± 0.6	0	0.5 ± 0.5	0

Means $\pm$ S.E.M. of the log endpoint dilution are reported.

Table 2
Survival rates of immunized BALB/c mice after intranasal challenge with 106.5 CFU of *S. pneumoniae* strain 4241 (serotype 3)

Immunization group	Inoculation route	Survivals/challenged	Percent survival
PC ± Thyr in microparticles	i.n.	22/29	76
Thyr in microparticles	i.n.	0/12	0
Blank microparticles	i.n.	4/18	22
Free PC ± Thyr	i.n.	29/32	91
Free Thyr	i.n.	2/12	17
Saline	i.n.	2/11	18

i.n., Intranasal.

mechanisms of the immune system (Ray et al., 1996; Limpanasithikul et al., 1995). In our work, we could not evidence any anti ds-DNA antibodies, or glomerular immunoglobulin deposition, either in the mice immunized i.n. with free or loaded microparticles (Data not shown).

3.2. Oral tolerance induced by β lactoglobulin-loaded microparticles

BLG microparticles were prepared using a concentration of BLG of 50 mg/ml and different concentrations of Tween 20 (molar ratio protein:surfactant from 0 to 8). Mean diameter, encapsulation and loading efficiency, burst release effect and zeta potential were determined (Table 3). Microparticles have a diameter that vary from 6.2 to 7.2 μm. The presence of Tween 20 does not modify particle diameter. Nevertheless, the introduction of Tween 20 within the first emulsion influences the encapsulation and loading efficiency. Indeed, from a protein/Tween 20 ratio of 1:0.018, an increase of the encapulation efficiency reaching 91% for a protein/Tween 20 ratio of 1/8 was observed (Table 3). Release studies have shown that microparticles prepared without Tween 20 induced a burst release of BLG whereas microparticles prepared with Tween 20 (molar ratio: BLG/Tween 20 of 1:8), displayed only 1% of protein release in the first minutes following incubation in the receiving medium (Table 3). Zeta potential measurements have shown that Tween 20 was able to migrate to the external surface of particles and displace BLG from the surface. Indeed, unloaded particles has a potential of −24.8 mV and BLG loaded microparticles prepared without Tween 20 a potential of −8.9 mV. BLG, provides to microparticles positive charges, neutralizing microparticles zeta potential (Table 3). Nevertheless, when the amount of Tween 20 increases, microparticles zeta potential decreases until reaching value close to unloaded microparticles (Table 1). This study has allowed to design a microparticulate system able to encapsulate large quantities of BLG and to control its release. The use of a non-ionic surfactant Tween 20 in the formulation was considered as crucial to optimize both parameters. Indeed, in one study, it was shown that encapsulation of BLG was due to a strong absorption of the protein onto particle surface (Leo et al., 1998). This absorption induces a burst release of the protein. This release was controlled by the presence of Tween 20 in the internal phase of the first emulsion. It was shown that in the presence of Tween at the ratio used in our study, BLG was displaced from oil/water interfaces (Courthadon et al., 1991). Observation using laser scanning confocal microscopy has evidenced a modification of fluorescent BLG in the particles when Tween 20 was present, characterized by a disappearance of the protein from the outer surface of the microparticles (Rojas et al., 1999). Scanning electron microscopy observations has allowed to show that Tween 20 induces a reduction of pores and channels in the internal structure of the microparticles (Rojas et al., 1999). All these observations allow to explain the role of Tween 20 in increasing encapsulation efficiency and a better controlled release of BLG from microparticles.

In Table 4 are reported humoral and mucosal IgE titers obtained after oral administration of

Table 3
Characterization of microparticles containing BLG and influence of Tween 20 on the average diameter, encapsulation, burst release and zeta potential

BLG:Tween 20 (molar ratio)	Mean diameter (μm)	Encapsulation efficiency (mg BLG/100 mg MS)	Entrapment yield (%)	Amount of BLG released after 2 min incubation (%)	Zeta potential (mV)
1:0	6.6	2.4	32	23	−8.9
1:0.01	6.8	2.5	33	N.D.	−1..1
1:0.018	6.7	3.4	46	N.D.	N.D.
1:0.1	7.0	3.4	46	N.D.	N.D.
1:0.5	6.9	3.7	50	N.D.	N.D.
1:1	7.0	4.1	55	N.D.	−15.5
1:4	7.2	5.9	80	16	−14.5
1:8	6.2	6.7	91	1	−22.3
0:8	6.3	–	–	–	−25.6
0:0	6.4	–	–	–	−24.8

N.D., not determined.

soluble and encapsulated BLG. It appears that BLG entrapped into microparticles (0.5μg/g of mouse) induces a reduction of IgE production in serum and intestinal secretion. This dose was considered as optimal after comparing different doses of BLG entrapped with microparticles (data not shown). In order to obtain a similar effect with soluble BLG, a dose of 5 mg/g of mouse was given (Table 4). The dose of 0.5 μg/g of mouse of soluble BLG does not produce any reduction of antibody secretion (Table 4). It was also shown that this response was BLG-specific since there was no effect on the production of anti-Ovalbumin IgE (data not shown) and that unloaded microparticles did not modify the immune response. The DTH response was evaluated. On both mice groups that received soluble BLG at a dose of 5 mg/g of mice or microparticles (0.5 μg/g of mouse) a reduction of DTH response was observed as compared with other treatments. If this type of immune response is the first to be observed with biodegradable microparticles, a similar effect was observed by Elson et al. (1996) using emulsions containing albumin. On the other hand, the study of Flaganan et al. (1996) should be taken into account, which showed that ovalbumin encapsulated into microparticles broke oral tolerance in mice and initiated a powerful immune response, resulting in Th2 cell activation (Flaganan et al., 1996). This fact reintroduces the crucial role of the oral dose in the oral tolerance phenomenon. Oral tolerance dose dependency is known to be essential (Friedman and Weiner, 1994) and indeed to observe a significant decrease of specific antibodies, as well as DTH suppression, a high oral dose of soluble BLG is required in our mouse model. Such a drastic decrease in the quantity of the required oral dose is a real benefit in this system of tolerogen vectorisation. The greatest potential of these adjuvants may be their use in oral tolerance induction of precious antigens.

Table 4
Humoral and mucosal anti BLG IgE titers(log10) ± S.E.M. in BALB/c mice after administration of BLG entrapped in Poly(-lactide co-glycolide) microparticles or soluble BLG

	Humoral IgE	Mucosal IgE
Sodium chloride	3.69 ± 0.25	2.32 ± 0.16
BLG-loaded (500 μg) microparticles per gram of mouse	3.58 ± 0.21	2.08 ± 0.34
BLG-loaded (0.5 μg) microparticles per gram of mouse	2.39 ± 0.27	1.48 ± 0.26
BLG (5 mg) free per gram of mouse	2.24 ± 0.32	1.24 ± 0.25
BLG (0.5 μg) free per gram of mouse	3.58 ± 0.21	2.18 ± 0.34
Unloaded microparticles	3.68 ± 0.1	2.38 ± 0.16

Results are geometric means from ten mice per group.

4. Conclusion

Taken together, our results highlight the potential of antigen encapsulation in DL-PLGA microparticles for eliciting protective immunity against invasive intestinal bacterial diseases and the induction of oral tolerance against a dietary antigen. If the ability of PLGA microparticles to stimulate mucosal immunity was already demonstrated, this is the first time this concept is applied to a multipurpose vaccine. In addition, our results concerning oral tolerance broadens the application of biodegradable microparticles in the field of immunology.

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