



Ovalbumin encapsulation into liposomes results in distinct degrees of oral immunization in mice

A.C. Alves^{a,b}, G.A. Ramaltes^a, M.C. Oliveira^a, V.N. Cardoso^a, T.A. Mota-Santos^b,
A.M.C. Faria^b, C.M. Gontijo^{b,*}

^a Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^b Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, ICB – UFMG Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:

Received 13 March 2008

Accepted 8 July 2008

Available online 15 August 2008

Keywords:

Liposomes

Oral immunization

Oral tolerance

ABSTRACT

Oral administration of protein antigens, such as ovalbumin, may result in induction of either tolerance or immunization. To avoid oral tolerance, there are new strategies to protect the antigens from degradation within the gastrointestinal tract and to allow them to reach inductive immunological sites. One such strategy is the usage of liposomes. Different parameters may influence the stability of liposomes in the gastrointestinal tract. Herein, we studied the immunological consequences of oral administration of liposome-encapsulated ovalbumin in different strains of mice using different liposomes. Our data demonstrated that ovalbumin liposomes improved the induction of oral immunization and the degree of improvement depended on the liposome type and on the strain of mice used. The mechanism responsible for this differential effect of liposomes depended on the site of antigen release and absorption. Therefore, some liposomes might be suitable as adjuvants for oral immunization, others for oral tolerance induction.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

A variety of pathogenic microorganisms can enter the gastrointestinal tract causing local infectious diseases. The gastrointestinal route is also one of the most commonly way by which these microorganisms penetrate the body. On the other hand, oral tolerance is usually induced in animals when they contact non pathogenic antigens for the first time by the oral route. In this case, animals show a reduction in their ability to produce immune responses when they are subsequently challenged with the antigen, even in the presence of adjuvants [1–3]. However, immune responses such as systemic immunization and the production of secretory immunoglobulin A have also been observed in the gut after antigen feeding. For this reason, the oral route is being considered as an appropriate choice for immunization [4,5].

When used for oral vaccination, proteins were often unsuccessful in producing a detectable mucosal immune response because most of the antigens are degraded along the gastrointestinal tract. To avoid unresponsiveness, new strategies have been proposed to protect antigens from degradation within the gastrointestinal tract and help them reach the mucosa-associated lymphoid tissue. New

antigen delivery systems and mucosal adjuvants include poly(lactide-co-glycolide) microparticles, immune-stimulating complexes (ISCOMs), recombinant microorganisms, viral vectors, proteosomes, cholera toxin and lipidation of protein [6–9].

In order to develop oral vaccines, lipids have been widely studied. One of the lipid-based delivery systems is liposomes. Liposomes are lipid vesicles made mainly of phospholipids. Various parameters may influence the stability and kinetics of liposomes in the gastrointestinal tract, e.g. their lipid composition that forms the lipid bilayer or multilayer, surface charge, particle size, lamellar structure and localization and/or interaction of the antigen with their membrane [10,11]. Since one of the critical point for the oral use of liposomes for oral immunization is their stability in the gastrointestinal environment, several studies have been performed to develop stable liposomes. However, the correlation between these liposomal properties and the induction of immune responses has not yet been fully examined.

Opposing results have been observed when animals are orally treated with distinct forms of the antigen, such as in its particulate or soluble form [12–15]. These results suggest that the structure of the antigen may protect it from attack of digestive fluids and/or modify its absorption and distribution, leading to induction or suppression of immune responses. Therefore, further studies are needed to develop new delivery systems that can either induce immune responses (oral vaccines) or suppress them (oral tolerance).

* Corresponding author.

E-mail address: cgontijo@icb.ufmg.br (C. Gontijo).

Herein, we compared the immunological consequences of the oral administration of an antigen (ovalbumin-OVA) encapsulated into liposomes in different strains of mice, to evaluate the usefulness of liposomes in both oral vaccine and oral tolerance protocols.

2. Materials and methods

2.1. Animals

B6D2F1 mice, an abbreviation for (C57BL/6 × DBA2)F1 hybrids, C57BL/6 mice and BALB/c mice, were obtained from our breeding unit at the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil). Groups of seven female mice were first used when 8–9 weeks old.

2.2. Antigen

Chicken egg albumin (OVA) grades II and V was purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

2.3. Liposomes

Liposomes were composed of soy phosphatidylcholine (Lipoid GMBH, Germany), cholesterol (Sigma) and phosphatidylglycerol (Lipoid GMBH) in 7:2:1 molar ratio, distearoylphosphatidylcholine (Lipoid GMBH), cholesterol and phosphatidylglycerol in 7:2:1 molar ratio, soy phosphatidylcholine, cholesterol and phosphatidylserine (Sigma) in 7:2:1 molar ratio or distearoylphosphatidylcholine, cholesterol and phosphatidylserine in 7:2:1 molar ratio. Multilamellar vesicles were prepared according to the method of Bangham [16]. Large unilamellar vesicles were prepared as reported by Skoza and Papahadjopoulos [17] and small unilamellar vesicles were prepared by the dehydration–rehydration procedure described elsewhere [18]. All three types of liposome suspensions had a 30 mmol/L lipidic concentration and a final concentration of 40 mg/mL of OVA. The diameter of liposomes was determined by light scattering. The final concentration of OVA was measured by the modified Lowry method [19] and lipid concentration by the modified phosphorus assay method of Fiske and SubbaRow [20].

2.4. Oral tolerization and intraperitoneal immunization

Mice were intubated with a urethral polyvinyl catheter calibrated to reach the stomach, and given 20 mg of antigen (OVA solution or OVA into liposomes) in 0.5 mL of saline intragastrically (*ig*). Seven days after gavage, mice were injected *ip* with 10 µg of OVA plus 1 mg of Al(OH)₃ as adjuvant in 0.5 mL of saline. Two weeks later, mice were boosted with 10 µg of OVA (*ip*). One week after the booster, mice were bled and the intestinal mucosal secretion collected. Samples were stored at –20 °C. Some animals (immune control groups; seven mice per group) received saline as oral pre treatment.

2.5. Oral immunization

Mice were intubated with a urethral polyvinyl catheter calibrated to reach the stomach, and given 20 mg of antigen (OVA solution or OVA into liposomes) in 0.5 mL of saline intragastrically, once a week, during 4 weeks. One week after the last administration of OVA, mice were bled and the mucosal secretion collected. Samples were stored at –20 °C.

2.6. Induction of food allergy [21]

BALB/c mice were injected *ip* with 10 µg of OVA plus 1 mg of Al(OH)₃ as adjuvant in 0.5 mL of saline. Two weeks later, mice

were boosted with 10 µg of OVA (*ip*). One week after the booster, mice were given to drink a solution of ovalbumin (10 mg/animal/day) free or encapsulated, during 5 consecutive days. After 7 days, mice were bled and the intestinal secretion and mesenteric lymph nodes collected. Samples were stored at –20 °C. Some animals (immune control groups) received water instead of ovalbumin.

2.7. Analysis of cytokine production

Cell suspensions were prepared from mesenteric lymph nodes of BALB/c mice treated as previously described for induction of food allergy. Cells were placed in tissue culture plates (Nunc) and adjusted to a concentration of 5×10^6 cells/mL in RPMI Medium 1640 (Sigma) containing 10% of FCS, 2 mM L-glutamine, 50 µM 2-mercapto-ethanol, 100 U/mL penicillin, 100 µg/mL fungizone, 1 mM sodium pyruvate, 0.1 mM essential amino acids and 25 mM Hepes. They were stimulated with ovalbumin (100 µg/mL). After 48 and 72 h, supernatants were harvested and IFN-γ and IL-4 were measured by ELISA using antibodies to capture and detection according to manufacturer's protocol (PharMingen, USA).

2.8. Biodistribution analysis

Aliquots of ^{99m}Tc-Technetium-coupled(^{99m}Tc)-ovalbumin and ^{99m}Tc-ovalbumin encapsulated in liposomes were administered by gavage to mice. The animals were sacrificed at different times after treatment (30, 60, 120 and 180 min). Stomach, gut, Peyer's patches, mesenteric lymph nodes, spleen, and liver were excised from mice that were previously bled. The intestinal content was collected (intestinal wash) as well for analysis. All organs were washed with saline, dried and weighed. Radioactivity of the samples was measured by an automatic scintillation apparatus (ANSR-Abbot, USA). Data were expressed as percentage of the administered dose per gram of each organ's tissue.

Measurements of total anti-OVA antibodies by enzyme-linked immunosorbent assay (ELISA): Ninety-six-well polystyrene plates (Nunc, Copenhagen, Denmark) were coated overnight at 4 °C with 20 µg of OVA (grade V) diluted in 100 µL of coating buffer (NaHCO₃ buffer, 0.05 M pH 9.6) per well, washed with saline containing 0.05% Tween 20 (v/v), blocked with 0.25% casein in PBS for 1 h, washed again and then coated with serial dilutions of mouse serum samples starting at 1:20. After 1 h at 37 °C, plates were washed, incubated with goat peroxidase-conjugated anti-mouse Ig(H+L) (Southern Biotechnology Inc., Birmingham, USA) for 1 h at 37 °C, washed and developed by the addition of H₂O₂ and *ortho*-phenylene-diamine (Sigma). Reaction was interrupted after 20 min by the addition of 20 µL of H₂SO₄ 1 M and absorbance was read at 492 nm in a microplate reader (Bio-Rad, Hercules, USA). Production of anti-OVA antibody was reported as a score (ELISA*) which represents the mean ± standard deviation of the sum of absorbance values obtained from individual samples at the dilution range of 1:20–1:2560 as previously reported [22]. The highest absorbance values obtained at 1:20 dilution were in the linear response region of the curve, and the values obtained at the 1:2560 dilution always reached the blank level. Positive and negative control samples were run on every plate to standardize the assays.

2.9. Measurements of specific IgG1 and IgG2a anti-OVA in the serum

The protocol used was the same to that described above for total anti-OVA antibodies, except that peroxidase-conjugated goat anti-mouse IgG1 (Southern Biotechnology Inc.) and goat anti-mouse IgG2a (Southern Biotechnology Inc.) were used for the detection step.

2.10. Measurements of specific IgA anti-OVA

The intestinal content from the entire gut was collected, mixed immediately with the same volume of saline at 4°C and centrifuged for 10 min at 3000 rpm. Supernatants were collected and frozen. The protocol we used was similar to that described for total anti-OVA antibodies with the following modifications: after serial dilutions (1:6–1:768) of the samples, plates were incubated for 1 h at 37°C and washed. The plates were then incubated with goat anti-mouse IgA (Southern Biotechnology Inc.) for 1 h at 37°C, washed and incubated with rabbit anti-goat IgG peroxidase-conjugated (Southern Biotechnology Inc.) for 1 h at 37°C. Reaction was developed and read as described previously.

2.11. Measurements of IgE anti-OVA antibodies by ELISA

Ninety-six-well polystyrene plates were coated overnight at 4°C with rat anti-mouse IgE UNLB diluted (1:250) in 50 µL of

coating buffer (NaHCO₃ buffer, 0.05 M pH 9.6) per well, washed with saline containing 0.05% Tween 20 (v/v), blocked with 0.25% casein in PBS for 1 h, washed again and then coated with mouse serum samples. After 2 h at room temperature, plates were washed, incubated with OVA-biotin 1 mg/mL (50 µL/well) for 1 h at room temperature, washed and then incubated for 45 min with streptavidin-peroxidase (Southern Biotechnology Inc., Birmingham, USA). After washing the plates, they were developed by the addition of H₂O₂ and *ortho*-phenylene-diamine (Sigma). Reaction was interrupted after 20 min by the addition of 20 µL of H₂SO₄ 1 M and absorbance was read at 492 nm in a microplate reader (Bio-Rad, Hercules, USA).

2.12. Statistical analysis

Data were analyzed statistically by ANOVA with the level of significance set at $P < 0.05$. All data represent results of at least three experiments and are expressed as the arithmetic mean

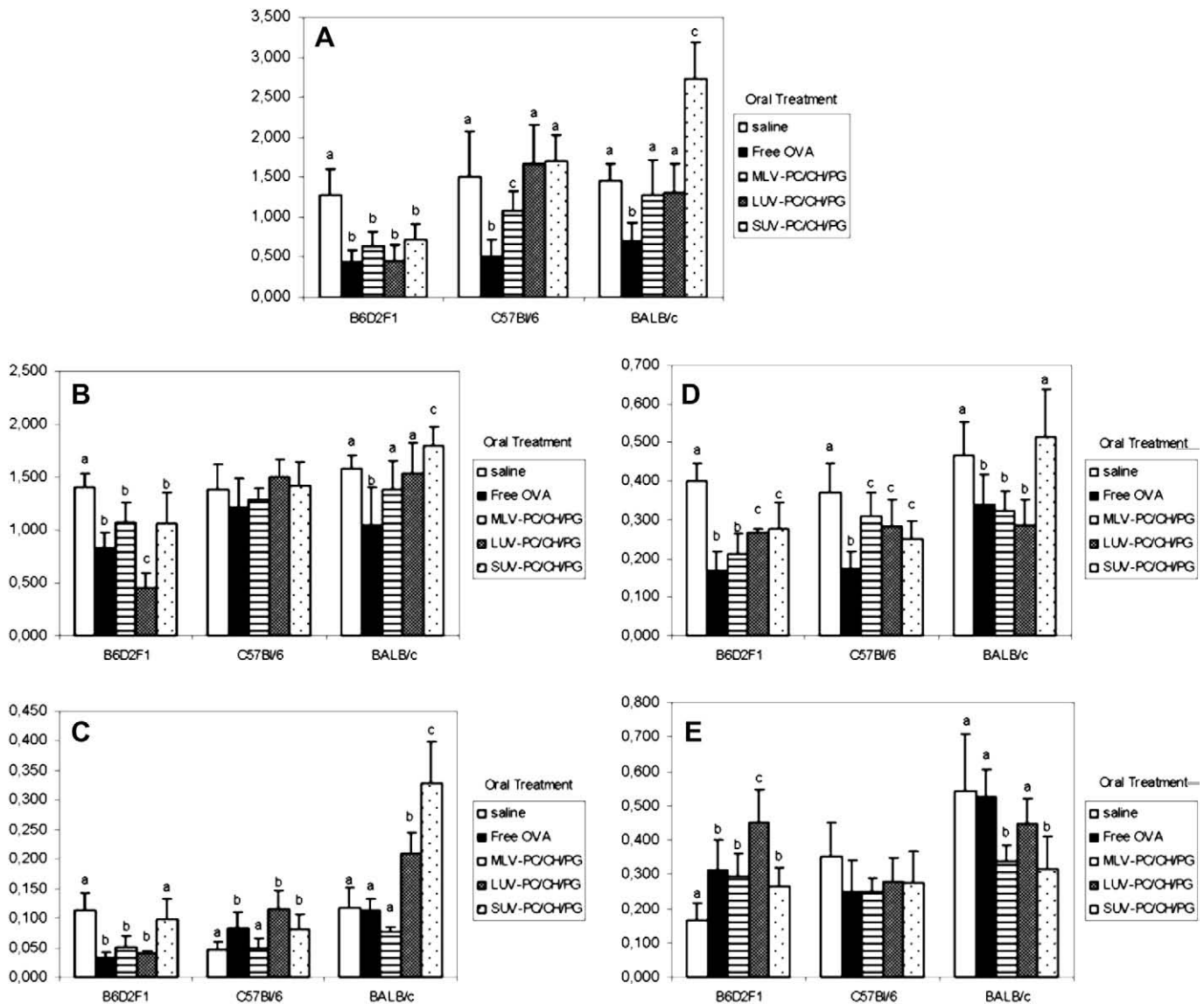


Fig. 1. Free or encapsulated ovalbumin was administered by gavage to B6D2F1, C57Bl/6 and BALB/c mice. After 1 week, mice were immunized *ip* with OVA in Al(OH)₃ and boosted 2 weeks later. Mice were bled after 1 week and specific anti-OVA antibodies were measured by ELISA. Total anti-OVA antibody production (A), anti-OVA IgG1 production (B), anti-OVA IgG2a production (C), anti-OVA IgA production (D) in mice's serum and anti-OVA IgA production (E) in the intestinal wash. Abbreviations: PC, phosphatidylcholine; Ch, cholesterol; PG, phosphatidylglycerol; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles. Data are expressed as means $\pm$ SD. Bars with the same letter (a, b or c) are statistically not different, although differ ($P < 0.05$) from bars that show a different letter.

with standard deviation of the mean (mean $\pm$ SD) for a sample size of seven.

3. Results

3.1. Effects of phosphatidylcholine liposomes

Several groups, including ours, have chosen (C57BL/6 $\times$ DBA2)F1 hybrids (B6D2F1 mice) orally treated with OVA, as one of the best models to study oral tolerance induction. Here, we have also used this, and two other strains, C57BL/6 and BALB/c, to test the effect of oral administration of such antigen under different conditions. Mice received 20 mg OVA administered by a single gavage 7 days before i.p. immunization with the same antigen in adjuvant. OVA was orally administered free or encapsulated into liposomes made of phosphatidylcholine (PC), cholesterol (CH) and phosphatidylglycerol (PG). Multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were used. Fig. 1 shows the serum levels total antibodies (Fig. 1A), IgG1 (Fig. 1B), IgG2a (Fig. 1C) and IgA (Fig. 1D), as well as secretory SIgA (Fig. 1E) that were produced by the different groups of mice and detected by ELISA.

All B6D2F1 mice that received OVA orally produced lower levels of total anti-OVA antibodies when compared to control immune mice (that received saline orally and then were i.p. immunized) (Fig. 1A). Oral tolerance induction can also be shown when different serum isotypes were tested (Fig. 1B–D), with only one single exception: the production of IgG2a in mice treated with OVA encapsulated into SUV. On the other hand, the production of secretory IgA was enhanced when B6D2F1 mice were previously treated with the antigen no matter the form it was administered. Interesting, among all encapsulated forms of OVA administration, the lowest values of IgG1, as well as the highest levels of SIgA production were obtained with LUV. Since most of the anti-OVA antibodies formed after OVA-alum immunization are IgG1, we concluded that OVA-LUV optimized oral tolerance induction. On the other hand, this liposome preparation had an opposite effect in anti-OVA SIgA (Fig. 1E).

Different profiles were observed with C57BL/6 mice: serum levels of total anti-OVA antibodies in mice that received OVA into LUV and SUV liposomes were comparable with levels of control immune animals. Although mice treated with MLV did not exhibit the same suppression of antibody production when compared to animals treated with non-encapsulated OVA, they can be considered partially tolerant since they produced intermediary levels of specific antibodies that were significantly different from intraperitoneal immunized animals (Fig. 1A). There was no difference among the groups when the levels of serum anti-OVA IgG1 (Fig. 1B) or anti-OVA SIgA (Fig. 1E) were detected. When the production of anti-OVA IgA in mice sera was analyzed, a profile similar to that showed by the total anti-OVA antibodies was observed (Fig. 1D). In this case, mice orally treated with OVA into every liposome tested became partially tolerant when compared to those that received free OVA or were immunized i.p.

The profiles shown by BALB/c mice were even more different. Although mice treated with free OVA showed impaired production of total antibodies and IgG1 against OVA, but not of IgG2a or secretory IgA, this was an exception when mice were previously treated with encapsulated OVA. In fact, tolerance was only noted in the levels of serum IgA when mice were treated with OVA into MLV and LUV liposomes, as well as secretory IgA when MLV and SUV liposomes were used. Usually, mice respond equally well when compared to immunized controls. Under some circumstances, animals produce even higher amounts of serum antibodies, particularly in those that received the antigen into SUV liposomes. This was noticed for total Ig (Fig. 1A), IgG1 (Fig. 1B), and, interesting, IgG2a, in spite of the effect of aluminum salt (Fig. 1C).

3.2. Effect of distearoylphosphatidylcholine liposomes

Although every strain became tolerant after oral pre-treatment with free OVA, the data obtained with PC/CH/PG liposomes in Fig. 1 has shown interesting features: first, B6D2F1 mice is, among all the strains tested, the one in which oral tolerance is easier to be observed, while the opposite seems to be noticed in BALB/c mice and an intermediary profile could be noticed in C57BL/6 mice; second, the production of SIgA seems to run in an opposite direction when compared with serum antibodies profiles and, finally in third, among the liposomes tested, SUV (the smallest one) seems to exhibit greater differences when compared to the administration of free OVA, while the largest liposomes (MLV) induce quite similar data.

Taken these considerations together, we decided to further investigate SUV liposomes under conditions that make them even more stable. It is important for effective oral administration of OVA into liposomes that they maintain integrity in the gastrointestinal environment until they reach the GALT. Changes in the phospholipid composition of liposomes can increase their stability. In previous studies, distearoylphosphatidylcholine (DSPC)-based liposomes showed to be relatively stable in the gastrointestinal tract [23–25] since liposomes composed of lipids with higher transition temperatures had more rigid membranes. Therefore, SUV liposomes with different composition (DSPC or PC/CH/PG) were tested in B6D2F1, C57BL/6 and BALB/c mice that were given 20 mg of OVA by gavage (free or encapsulated). After one week of the oral treatment, mice were immunized as described before.

Fig. 2 compares results obtained with OVA encapsulated into SUV-PC and SUV-DSPC. B6D2F1 mice treated with SUV-DSPC produced higher levels of all anti-OVA antibodies isotypes analyzed than SUV-PC treated and free OVA-treated animals. Quite the same was observed in C57BL/6 mice: higher levels of total and IgA anti-OVA antibodies was observed in the serum when they received SUV-DSPC liposomes (Fig. 2A and D). No difference was observed in IgG1 anti-OVA production among all groups tested. Higher levels of total anti-OVA antibodies were also found when C57BL/6 mice were orally treated with OVA-DSPC-liposomes, regardless of which liposome size was used (MLV, LUV or SUV) (data not shown). On the other hand, BALB/c mice showed lower levels of anti-OVA antibodies when treated with SUV-DSPC liposomes than with SUV-PC liposomes.

Anti-Ova SIgA production was also evaluated for DSPC/CH/PG liposomes (Fig. 2E). The lowest concentration of anti-OVA SIgA was observed in C57BL/6 mice treated with OVA incorporated into SUV-DSPC. Nevertheless, B6D2F1 mice showed an increase in specific SIgA production when treated with OVA incorporated into SUV-DSPC.

3.3. Effect of incorporation of phosphatidylserine into liposomes

An interesting feature that can be obtained from the data shown in Fig. 2: DSPC induced an immunogenic effect in B6D2F1 mice that, from all strains we have use, is the one where oral tolerance is easier to be induced. This effect was also noticed in C57BL/6 mice, a strain that, from the data of Fig. 1, has shown to be not so affected by the administration of OVA in PC-based liposomes. Now, we decided to test whether the reverse, oral tolerance induction, could be achieved.

Some studies [26,27] have shown that incorporation of phosphatidylserine (PS) in liposomes reduces their immune adjuvant activity. To verify this inhibitory effect and to evaluate the influence of negatively charged PS liposomes in the establishment of oral tolerance, we tested their effect on anti-OVA immune responses. C57BL/6 mice were chosen for this purpose since this strain of mice was found to have a profile between B6D2F1

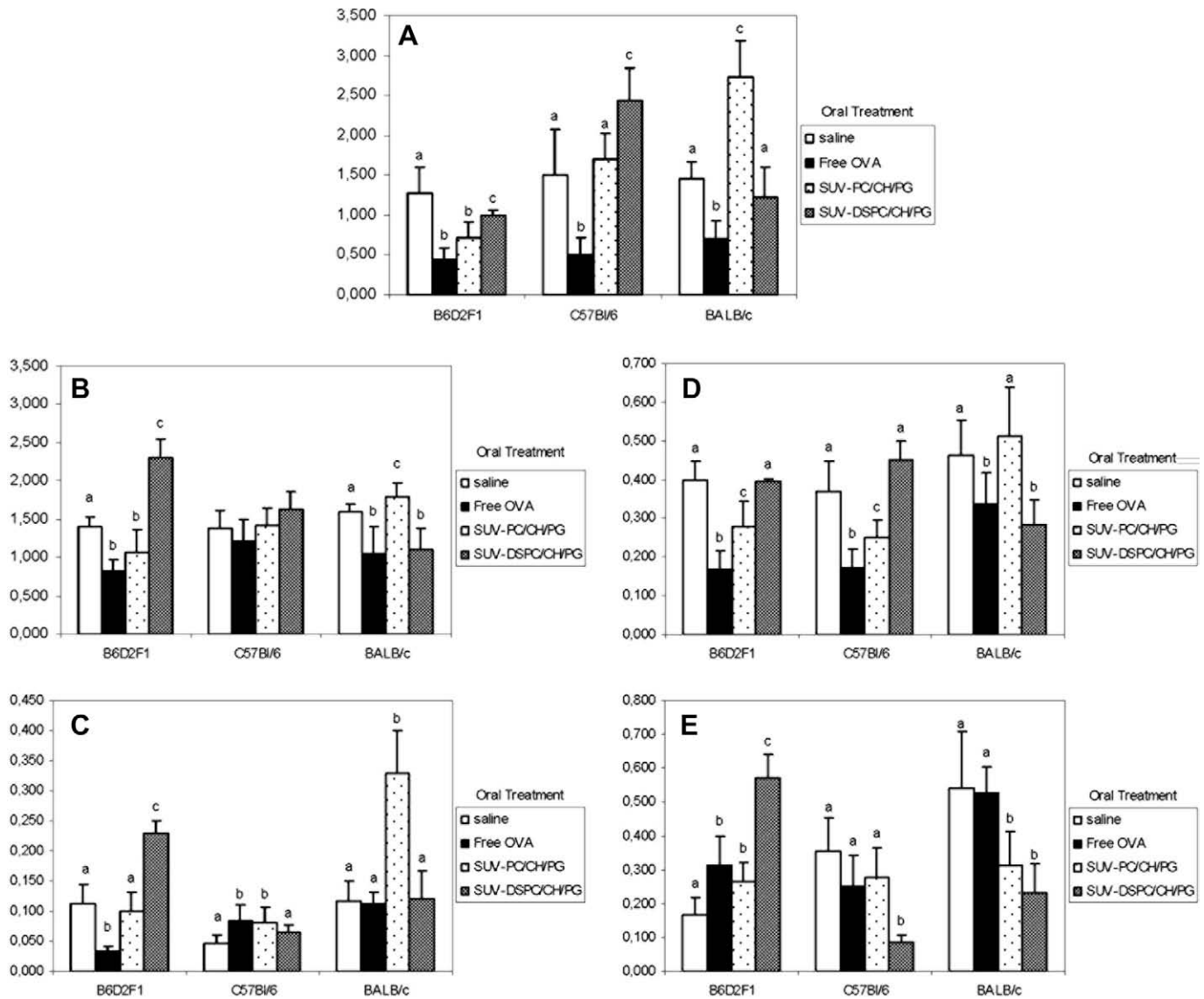


Fig. 2. Free or SUV encapsulated ovalbumin was administered by gavage to B6D2F1 mice, C57BL/6 mice or BALB/c mice. After 1 week, mice were immunized *ip* with OVA in Al(OH)₃ and boosted 2 weeks later. Mice were bled after 1 week and specific anti-OVA antibodies were measured by ELISA. Total anti-OVA antibody production (A), anti-OVA IgG1 production (B), anti-OVA IgG2a production (C), anti-OVA IgA production (D) in mice's serum and anti-OVA SIgA production (E) in the intestinal wash. Abbreviations: SUV, small unilamellar vesicles; PC, phosphatidylcholine; DSPC, distearoylphosphatidylcholine; CH, cholesterol; PG, phosphatidylglycerol. Data are expressed as means $\pm$ SD. Bars with the same letter (a, b or c) are statistically not different, although differ ($P < 0.05$) from bars that show a different letter.

mice in which we can easily induce tolerance and BALB/c mice that are more resistant to oral tolerance induction when OVA-PG-liposomes were used for oral treatment. We also chose LUV liposomes since they appeared not to be as immunogenic as SUV liposomes. Thus, C57BL/6 mice were given, by gavage, 20 mg of free or encapsulated OVA using LUV made of phosphatidylcholine (PC), cholesterol (CH) and phosphatidylserine (PS) or distearoylphosphatidylcholine (DSPC), cholesterol (CH) and phosphatidylserine (PS). After 1 week of the oral treatment, mice were immunized as described in Methods.

Fig. 3 shows that LUV-PC/CH/PS and LUV-DSPC/CH/PS were both able to induce suppression of total anti-OVA antibodies and anti-OVA IgG1 antibodies in C57BL/6 (Fig. 3B), a fact that was not observed when PG was used. This suggests that the incorporation of PS into the liposomes rendered tolerant mice that were previously resistant when LUV-PG liposomes were used. The effect triggered by PS in C57BL/6 was similar to the one observed in B6D2F1 when PG was used (Fig. 1). Animals treated with LUV-PS liposomes

showed similar levels of anti-OVA IgG2a to animals treated with OVA alone (Fig. 3C). Serum levels of anti-OVA IgA were even lower in animals orally treated with LUV-DSPC/CH/PS than animals that received OVA alone, OVA encapsulated in PG-liposomes or OVA encapsulated in LUV-PC/CH/PS (Fig. 3D). On the other hand, C57BL/6 treated with LUV-DSPC/CH/PS showed the highest concentration of anti-OVA SIgA antibodies (Fig. 3E).

3.4. Effect of liposomes in immunization by the oral route

After we had evaluated the effect of oral pre-treatment with encapsulated OVA in the establishment of oral tolerance, we analyzed whether free or liposome-encapsulated OVA were capable of orally immunizing mice without the use of any parenteral immunization. To perform such experiment, we have chosen C57BL/6 mice for the same reason we have mentioned above. Mice were immunized with the antigen given weekly exclusively by the oral route, as described in Methods.

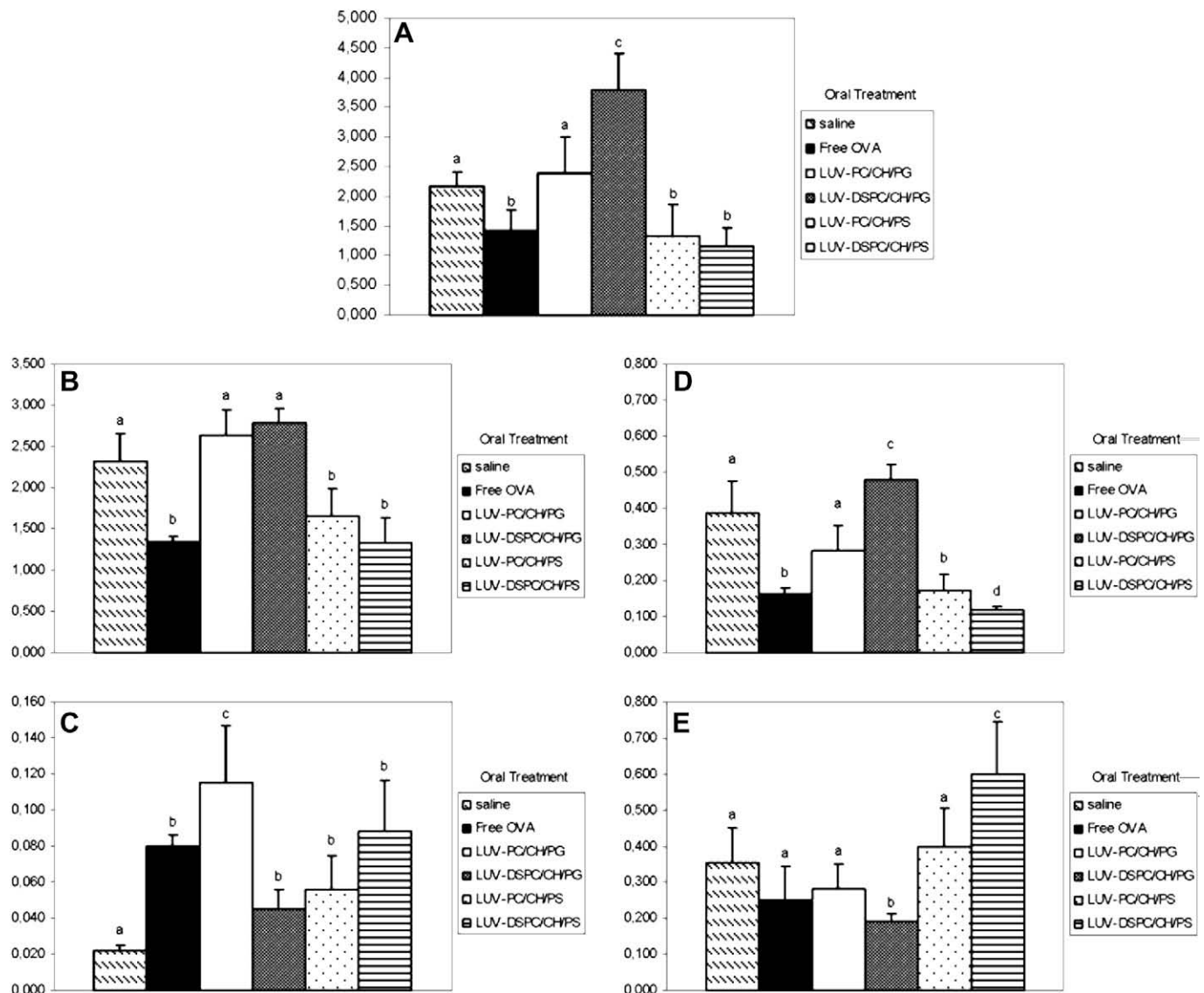


Fig. 3. Free or LUV encapsulated ovalbumin was administered by gavage to C57BL/6 mice. After 1 week, mice were immunized *ip* with OVA in Al(OH)₃ and boosted 2 weeks later. Mice were bled after 1 week and specific anti-OVA antibodies were measured by ELISA. Total anti-OVA antibody production (A), anti-OVA IgG1 production (B), anti-OVA IgG2a production (C), anti-OVA IgA production (D) in mice's serum and anti-OVA SIgA production (E) in the intestinal wash. Abbreviations: PC, phosphatidylcholine; CH, cholesterol; DSPC, distearoylphosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; LUV, large unilamellar vesicles. Data are expressed as means $\pm$ SD. Bars with the same letter (a, b, c or d) are statistically not different, although differ ($P < 0.05$) from bars that show a different letter.

Fig. 4 shows specific anti-OVA antibodies found in mice sera and mucosal secretions after the fourth oral administration of OVA (free or encapsulated). We decided to use SUV made of phosphatidylcholine (PC), cholesterol (CH) and phosphatidylglycerol (PG) or distearoylphosphatidylcholine (DSPC), cholesterol (CH) and phosphatidylglycerol (PG) since these liposomes appeared to be more effective for systemic immunization than the others. An orally induced immune response was obtained since the production of total anti-OVA antibodies (Fig. 4A), anti-OVA IgG1 (Fig. 4B) and anti-OVA IgG2a (Fig. 4C) increased when compared with mice that received free OVA. SUV-DSPC was shown to be the most effective for oral immunization. Actually, the levels of antibodies obtained were even higher for IgG2a when compared with levels of such isotype obtained in mice immunized with OVA with adjuvant *i.p.* Liposomes were able to induce a Th1 and Th2 response and they might be a good choice to obtain both responses. This was not observed when animals were immunized *i.p.* since we used a Th2 inducer adjuvant. Although oral treatment with liposomes has not increased serum levels of IgA, a local response was observed

(Fig. 4D and 4E). Moreover, SUV-DSPC treated mice produced higher levels of SIgA than mice treated with SUV-PC or with free OVA.

3.5. Effect of liposomes in the biodistribution of ovalbumin

Since different liposomes could elicit variable responses in mice, we performed a biodistribution experiment to better understand the differences found. Our working hypothesis was that the different effects observed could be due to the distinct sites of antigen delivery by distinct liposomes preparations. Aliquots of free ^{99m}Tc-ovalbumin and ^{99m}Tc-ovalbumin encapsulated in LUV or SUV liposomes were administrated by gavage to C57BL/6 mice to verify if the antigen was equally absorbed and distributed in tissues. As seen in Table 1, absorption and tissue distribution of OVA were dependent on the size of liposomes and on the way that the antigen was administered to the animals (soluble or in particulate form). The measured diameter for the SUV-PC/CH/PG liposome was 108 nm (SD $\pm$ 5) and for the LUV-PC/CH/PG liposome was

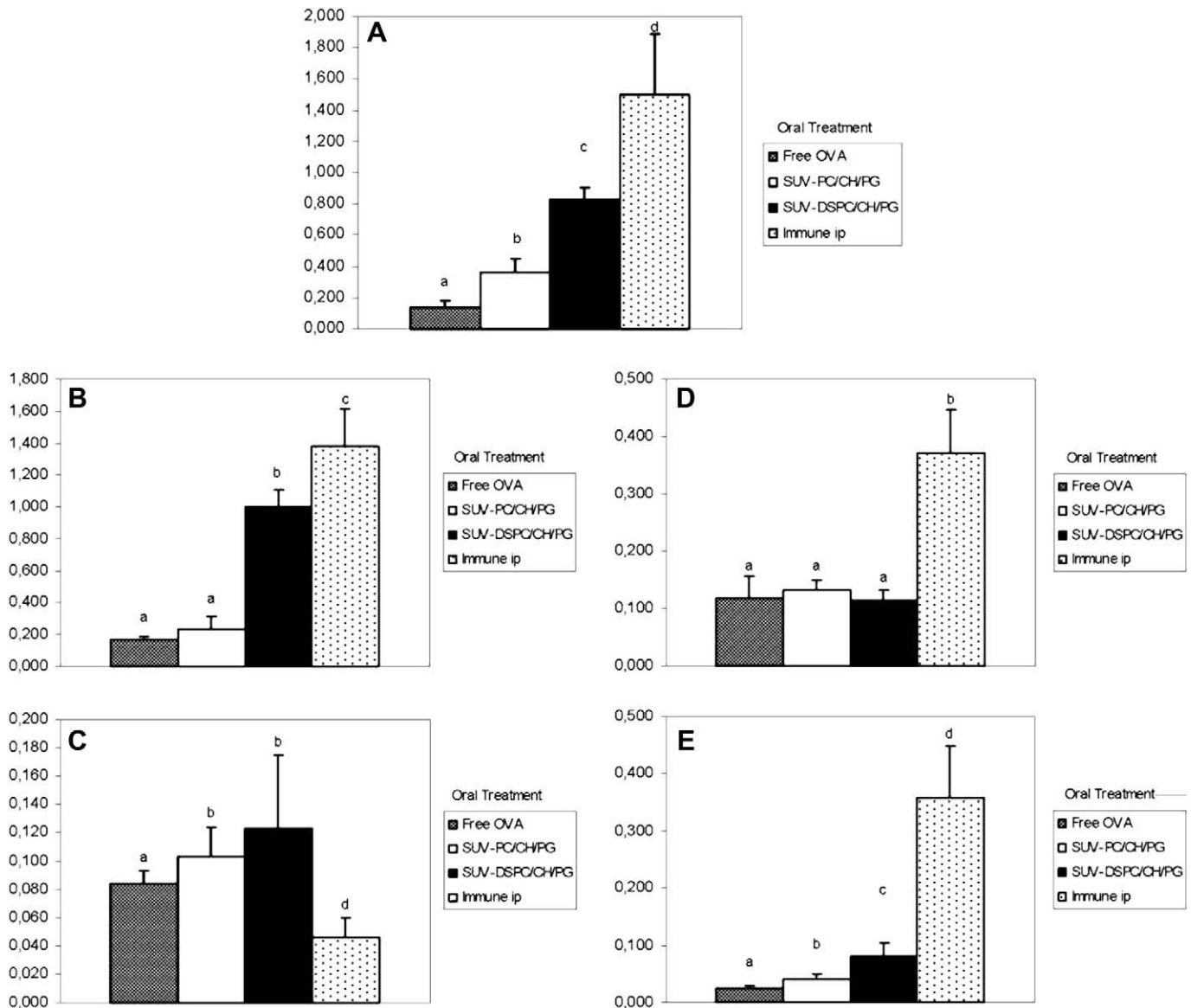


Fig. 4. C57Bl/6 mice were orally immunized with free or SUV encapsulated ovalbumin. Total anti-OVA antibody production (A), anti-OVA IgG1 production (B), anti-OVA IgG2a production (C), anti-OVA IgA production (D) in mice's serum and anti-OVA SIgA production (E) after the last gavage. Abbreviations: PC, phosphatidylcholine; CH, cholesterol; PG, phosphatidylglycerol; DSPC, distearoylphosphatidylcholine; SUV, small unilamellar vesicles. Data are expressed as means $\pm$ SD. Bars with the same letter (a, b, c or d) are statistically not different, although differ ($P < 0.05$) from bars that show a different letter.

642 nm ($SD \pm 75$). The majority of the antigen was not absorbed and remained inside the gut, independently of the way in which ^{99m}Tc -OVA was administered. Nevertheless, the percentage that was absorbed was greater for ^{99m}Tc -OVA-SUV than ^{99m}Tc -OVA-LUV or free ^{99m}Tc -OVA. Besides, ^{99m}Tc -OVA-SUV showed a controlled release of the antigen in Peyer's patches, spleen and blood. ^{99m}Tc -OVA was found earlier and in higher concentration in the blood when administered encapsulated in SUV liposomes. ^{99m}Tc -OVA-LUV was found mostly in the small intestine and scarcely in other tissues and blood. The absorption of ^{99m}Tc -OVA-LUV was even lower than free ^{99m}Tc -OVA.

3.6. Putative effect of liposomes in induction of food allergy

A major concern regarding protocols for oral immunization is the development of undesirable effects such as hypersensitivity reactions. To test whether our liposome formulations would increase the susceptibility to allergic reactions, we assessed anti-OVA IgE production in mice orally treated with free OVA as com-

pared to OVA encapsulated into liposomes and found no detectable levels of specific IgE (data not shown). However, this would not be enough to ensure the safety of liposomes since atopic individuals or animals may have a special susceptibility to hypersensitivity reactions in the gut when exposed to liposome preparations. Therefore, we used a modified experimental model of food allergy in an allergy-prone strain of mice (BALB/c) to verify whether our liposomal formulation could increase the susceptibility to an induced hypersensitivity reaction. After induction of experimental food allergy, levels of total serum anti-OVA antibodies present in BALB/c that received OVA into LUV and SUV liposomes were comparable with those that received free OVA (Fig. 5A). Moreover, anti-OVA IgE production in OVA-SUV treated animals was also comparable to mice that received free OVA (Fig. 5B). BALB/c mice treated with OVA-LUV showed the same levels of anti-OVA IgE as animals that did not received OVA orally. Production of IL-4 and IFN- γ was also similar among all groups tested (Fig. 5C and D). Our results suggest that the liposome-antigen formulations used in this study do not elicit nor increase the susceptibility to hypersensitivity reactions.

Table 1
Distribution of ^{99m}Tc -OVA orally administered to C57BL/6 mice

Tissue	Delivery system	% Dose			
		30"	60"	120"	180"
Stomach	Free OVA	56.31±9.79	47.78±8.78	22.56±4.70	4.86±1.05
	OVA LUV-PC/CH/PG	29.92±3.56	28.90±5.63	21.80±6.35	3.01±0.81
	OVA SUV-PC/CH/PG	41.23±9.04	33.76±10.57	16.05±2.62	14.22±3.54
Small intestine	Free OVA	13.49±2.56	12.54±2.64	2.40±0.30	1.40±0.21
	OVA LUV-PC/CH/PG	23.04±4.58	14.41±4.35	6.85±2.43	2.31±0.67
	OVA SUV-PC/CH/PG	12.43±2.39	10.24±1.57	2.05±0.73	1.35±0.33
Small intestine wash	Free OVA	27.01±6.97	38.26±7.98	39.24±9.40	5.34±0.64
	OVA LUV-PC/CH/PG	37.82±9.58	51.17±10.65	33.24±10.08	4.31±1.13
	OVA SUV-PC/CH/PG	60.75±7.27	70.43±3.03	11.72±3.02	7.20±1.24
Large intestine	Free OVA	0.62±0.18	0.38±0.08	0.43±0.17	0.46±0.15
	OVA LUV-PC/CH/PG	0.11±0.03	0.08±0.02	0.23±0.06	8.58±2.19
	OVA SUV-PC/CH/PG	0.24±0.07	0.34±0.10	0.49±0.12	1.19±0.34
Large intestine wash	Free OVA	0.50±0.13	0.93±0.26	1.06±0.08	24.13±7.90
	OVA LUV-PC/CH/PG	0.20±0.05	0.57±0.08	23.67±8.59	31.75±5.89
	OVA SUV-PC/CH/PG	0.61±0.04	0.50±0.07	4.28±0.98	22.78±5.41
Peyer's patches	Free OVA	2.07±0.62	0.90±0.280	0.86±0.260	0.03±0.010
	OVA LUV-PC/CH/PG	0.07±0.02	0.02±0.007	0.05±0.006	0.01±0.001
	OVA SUV-PC/CH/PG	2.68±0.83	2.04±0.491	0.66±0.091	0.56±0.113
Mesenteric lymphnodes	Free OVA	0.34±0.12	0.17±0.05	0.41±0.12	0.06±0.01
	OVA LUV-PC/CH/PG	0.10±0.03	0.03±0.01	0.02±0.01	0.29±0.06
	OVA SUV-PC/CH/PG	0.22±0.03	0.23±0.07	0.16±0.02	0.15±0.04
Spleen	Free OVA	0.01±0.004	0.05±0.011	0.67±0.162	0.04±0.009
	OVA LUV-PC/CH/PG	0.02±0.008	0.01±0.002	0.01±0.001	0.01±0.002
	OVA SUV-PC/CH/PG	0.19±0.027	0.18±0.040	0.35±0.091	0.16±0.040
Liver	Free OVA	0.09±0.02	0.14±0.04	1.98±0.48	0.08±0.02
	OVA LUV-PC/CH/PG	0.10±0.04	0.19±0.02	0.23±0.02	0.06±0.02
	OVA SUV-PC/CH/PG	0.43±0.08	0.40±0.08	1.02±0.29	0.45±0.11
Blood	Free OVA	0.03±0.010	0.07±0.02	0.50±0.15	0.05±0.01
	OVA LUV-PC/CH/PG	0.02±0.007	0.08±0.02	0.03±0.01	0.06±0.02
	OVA SUV-PC/CH/PG	0.59±0.110	0.58±0.08	1.28±0.30	0.38±0.03

4. Discussion

Oral tolerance is usually induced in experimental animals when they first meet antigens by the oral route. The phenomenon of oral tolerance is an essential event to prevent induction of active immunity against harmless antigens, such as food proteins and commensal bacterial. Thus, the development of oral vaccines is not an easy task, because many protein antigens are either degraded in the gastrointestinal tract before they reach the mucosa-associated lymphoid tissue, or even they induce immune tolerance if they reach immune inductive sites. In order to develop oral vaccines, new anti-

gen delivery systems have been tested. An ideal mucosal antigen delivery vehicle should be stable and capable of binding to M cell, as well as to induce a mucosal immune response. Some of those amphiphilic molecules based systems are immune-stimulating complexes (ISCOMS). ISCOMS are made of lipids and Quil A that have adjuvant activity. They have been successfully used in oral immunization but they are tested mainly for hydrophobic antigens [28–30].

Another delivery system used is liposomes. Liposomes have been used for delivering antigens in mucosal sites since they can carry hydrophobic and hydrophilic molecules [11,31]. Liposomes vary in resistance to acids, bile and enzymes [32]. In this study, we intended to vary the phospholipid composition, size and number of bilayers of the liposomes to analyze their influence in the ability of liposome to induce a systemic and/or mucosal immune response in different strains of mice.

It is well known that oral administration of a soluble protein antigen quite often induces oral tolerance, characterized by a marked reduction in specific immune responses when these animals are subsequently immunized with the same antigen. Induction of oral tolerance depends on many factors such as structure and dose of the administered antigen, as well as age and genetic background of the animal [1,3,9,33–35]. It has been also shown that antigen presentation is a crucial event for the induction of oral tolerance [36,37]. Previous studies using Flt3L, a growth factor for dendritic cells, have shown that intestinal dendritic cells (DCs) are the main APCs involved in oral tolerance induction to protein antigens [36]. The way by which antigens are processed and presented to the immune system seems to be critical for the induction of either tolerance or active immunity. DCs are converted from tolerogenic into immunogenic APCs, for instance, when cholera toxin or IL-1 are administered to previously Flt3L-treated mice, and this effect is due to augmentation of costimulatory molecules CD80 and CD86 induced by these inflammatory agents [37]. Modulation of DC function in the gut is however complex and still poorly understood. Some pro-inflammatory cytokines, such as IL-18, have been shown to downregulate the immunogenic function of DCs and to support the generation of antigen specific regulatory T cells (Th3 and Tr1) in Peyer's patches [38].

In our studies, we have showed initially that the mouse strain is particularly important on the effect of liposomes over two opposite phenomena: immunization or oral tolerance induction. As expected, B6D2F1 hybrids have shown to be the most suitable strain for inducing oral tolerance. With the exception of secretory IgA, the levels of every sera isotype specific to OVA became lower, no matter the type of PC/CH/PG liposome used for pre-treating mice with the antigen (Fig. 1). On the other hand, C57BL/6 and BALB/c mice responded differently, although all strains became tolerant when we used free OVA (soluble protein) to treat mice before immunization. Nevertheless, we could not induce tolerance in Swiss mice with free or encapsulated OVA. Actually, to establish tolerance in Swiss mice we had to modify the experimental protocol and use a 3 times higher amount of antigen (data not shown). When liposomes were used, absence of oral tolerance induction could be detected, in some cases. In fact, even stronger responses were noted specially when SUV, the smallest liposome, was used to pre-treat the animals. Interestingly, Swiss mice exhibited a strong anti-OVA response when pre-treated with the antigen in SUV-liposomes (data not shown).

The production of SIgA antibodies by B6D2F1, C57BL/6 and BALB/c strains has shown an opposite profile when compared to sera immunoglobulins (Fig. 1E). Differences between strains seem to be particularly more evident with BALB/c mice. Actually, this is even more observed when we have focused our study with SUV-DSPC/CH/PG liposomes which make them more stable [23–25]. The production of every isotype in BALB/c mice became

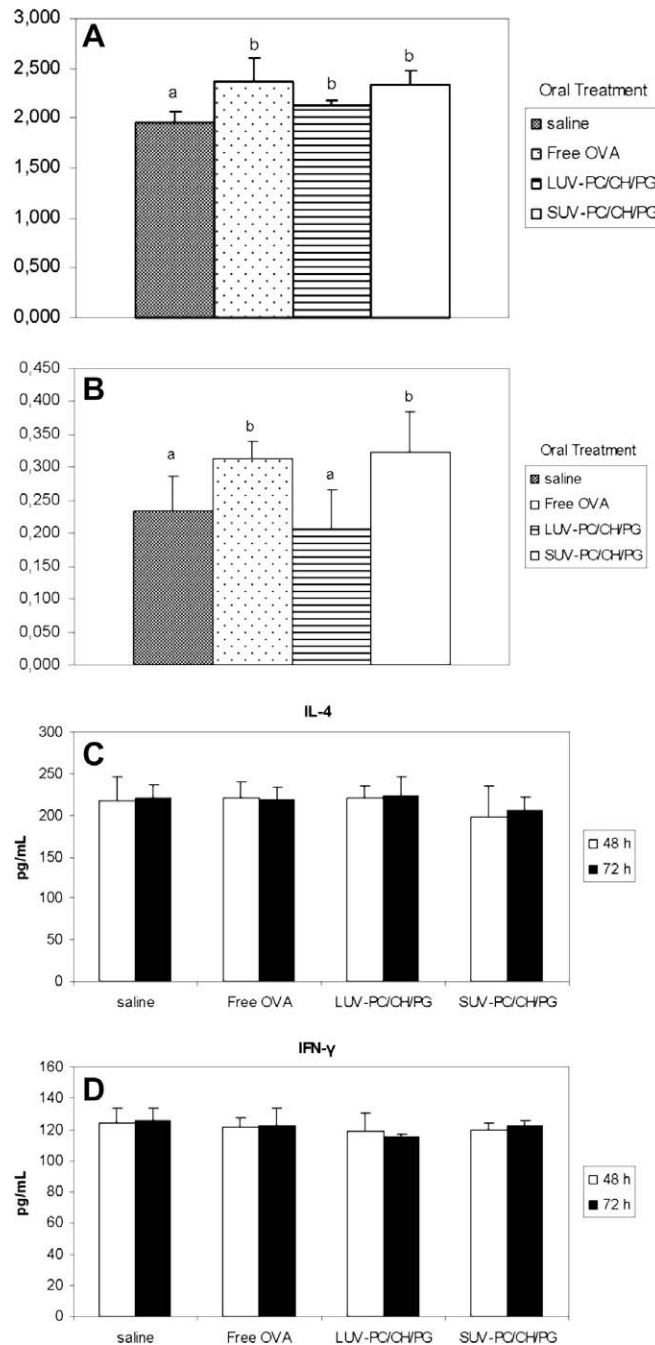


Fig. 5. After been immunized i.p. with ovalbumin, free or encapsulated ovalbumin was orally administered to BALB/c mice, for 5 consecutive days. Total anti-OVA antibody production (A) and anti-OVA IgE production (B) in mice's serum. IL-4 production (C) and INF- γ production (D) in mesenteric lymph nodes. Abbreviations: PC, phosphatidylcholine; CH, cholesterol; PG, phosphatidylglycerol; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles. Data are expressed as means $\pm$ SD. Bars with the same letter (a or b) are statistically not different, although differ ($P < 0.05$) from bars that show a different letter.

lower when SUV-DSPC base-liposomes were used instead of SUV-PC vesicles (Fig. 2). Except for the production of SIgA in C57BL/6, in general the opposite could be noted with both B6D2F1 and C57BL/6 mice.

Our results are mostly in concert with previous studies on the influence of the genetic background in oral tolerance induction [39–41]. It is likely that most formulations increase or decrease susceptibility to either oral tolerance or oral immunization only to the extent of the genetically established possibilities of the mouse strain. Some formulations such as PS, however, seem to be less suitable for oral immunization (and may be suitable for oral tolerance) since they overcome this genetic barrier (Fig. 3).

Oral tolerance is a result of many factors such as genetic background and delivery system. Induction of oral tolerance in B6D2F1 mice was more dependent on the strain characteristics, whereas in C57BL/6 and BALB/c mice both factors were important. When C57BL/6 mice were orally immunized with OVA-SUV we obtained higher levels of total anti-OVA antibodies than with free OVA (Fig. 4), in other words, the particulated nature of OVA-liposome led to a different response.

To exert their effect, liposomes should retain their integrity after oral administration. Cholesterol was used in our formulation because this component is known to enhance membrane integrity [10]. Negatively charged phosphatidylglycerol was also

incorporated to augment vesicle stability and increase liposome uptake by Peyer's patches [11]. Different phospholipids were used as well: phosphatidylcholine or distearoylphosphatidylcholine, with transition temperatures below and above 37 °C to assess which one would be more appropriated to elicit an immune response. In the present study, we found that SUV made of DSPC/CH/PG were more suitable for systemic immunization in C57BL/6 mice (Fig. 4). On the other hand, the local immune response elicited, measured by the production of anti-OVA SIgA, was considerably lower than the one observed in animals that received OVA *ip* (Fig. 4E). These findings are in agreement with previous studies showing that liposomes made of DSPC are more stable in presence of lipases and bile salts and could be good choices for a mucosal antigen delivery vehicle [11,25,41]. On the contrary, BALB/c mice produced higher levels of anti-OVA antibodies when orally treated with PC/CH/PG liposomes, particularly SUV liposomes. A mucosal immune response was obtained when we used LUV-PC/CH/PG, although the level of anti-OVA SIgA was higher for free OVA. BALB/c mice also produced more IgA and IgG1 in serum when treated with antigen in PC/CH/PG liposomes (Fig. 2). Surprisingly, BALB/c mice orally treated with ovalbumin in LUV and SUV PC/CH/PG liposomes showed high levels of anti-OVA IgG2a antibodies even after an immunization protocol using alum as adjuvant. The highest amount was noticed upon administration of ovalbumin in SUV-PC/CH/PG (Fig. 1C). Although PC liposomes are not as stable as DSPC liposomes, they were able to elicit an immune response. Perhaps, they may form mixed micelles or complexes with lipids and cholates that can protect the ovalbumin from degradation and deliver it into Peyer's patches.

We showed that SUV was more suitable for systemic immunization against OVA while LUV induced a local immune response and have a profile more similar to free OVA. This may be related to the different sizes of the liposomes. Previously [42], we reported that Ovalbumin encapsulated in SUV liposomes was more absorbed than when free OVA was orally administered to Swiss mice. As can be seen in Table 1, OVA in SUV were captured by Peyer's patches and could be found in blood, but OVA delivered by LUV remained for a longer period in the stomach and gut. When LUV was used, OVA could hardly be found in the blood. Data obtained to C57BL/6 mice were similar to those of Swiss mice. As pointed out earlier, Swiss mice orally treated with soluble antigen did not become tolerant while C57BL/6 mice were. Thus, it seems that the higher efficiency of SUV to be absorbed favored immunogenicity against OVA, whereas the ability of LUV to be retained in the gut enhances local immune responses such as oral tolerance and SIgA production.

The tolerogenic effect of LUV may be due to its greater contact with distinct mucosal DC subsets that lead to local immune responses and systemic hyporesponsiveness. These subsets have been described in Peyer patches and *lamina propria* as being CD11c⁺CD11b⁺CD8 α [−] [43]. Other authors suggested that B220⁺pDC may be the most tolerogenic DC subsets and this is still a matter of debate [44]. On the other hand, OVA-SUV may be captured and presented either by activated DC or by other subsets of DC on a different immunological environment that elicit immune responses [43,45].

One major concern when oral route is used for immunization purposes is the possibility of eliciting food allergy reactions. However, we clearly showed using an experimental model of food allergy in a Th2-prone strain of mice (BALB/c) that none of our liposome formulations have any boosting effect in the allergy reaction induced (Fig. 5).

Oral tolerance can be a desired effect when the abrogation of hypersensitivity reactions and autoimmunity are the main goal. Immune suppression induced by oral tolerance may involve inhi-

bition of lymphocytes and other immune cells by different anti-inflammatory mechanisms [3]. Apoptosis is of vital importance as a regulator of cell homeostasis and it is known to elicit the release of anti-inflammatory mediators such as TGF- β and PGE-2 by macrophages [46]. Apoptotic events in the gut are related in many ways to tolerance induction. Lymphocytes from orally tolerized mice [47] as well as activated human *lamina propria* T cells [48] display an enhanced susceptibility to apoptosis. In addition to the secretion of anti-inflammatory mediators as a result of clearance of apoptotic cells by macrophages [46], the apoptotic T cells themselves release TGF- β in its latent and active form [49]. Phosphatidylserine is a phospholipid that has inhibitory properties over macrophages due exactly to its recognition by macrophage during apoptosis [50,51]. Therefore, we tested the effect in immune responses and oral tolerance induction of its incorporation into liposome-antigen formulation. Indeed, PS liposomes were able to induce oral tolerance in C57BL/6 mice, a strain that had shown poor tolerance results when free OVA or PG liposomes were used as oral antigens. Our results suggest that PS liposomes may be an alternative to improve the induction of oral tolerance (Fig. 3).

In summary, some liposomes might be suitable for oral immunization inducing systemic as well as local antibody production with different profiles of antibody isotypes. Other liposome formulations, specially the ones including PS, might be used to improve oral tolerance induction. These different effects seem to be related to the site of antigen delivery and absorption and they can be used to manipulate the immune responses to oral administered soluble antigens.

Acknowledgments

We are thankful to Ilda Marçal de Souza for her excellent assistance in taking care of the animals. This study was financially supported by research fellowships (A.C.A. and A.M.C.F.) from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

References

- [1] M.J.C. Rios, M.A.C. Pereira, L.M. Lopes, C. M. Gontijo, E.B. Castanheira, N.M. Vaz, Tolerance and priming initiated by mucosal contact with protein antigen in inbred strains of mice, *Braz. J. Med. Biol. Res.* 21 (1988) 825–836.
- [2] A. McI Mowat, A.M.C. Faria, H.L. Weiner, Oral tolerance: physiological basis and clinical applications, in: J. Mestecky, M.W. Lamm, W. Strober, J. Bienenstock, J.R. McGhee, L. Mayer (Eds.), *Handbook of Mucosal Immunology*, third ed., Elsevier Academic Press, San Diego, pp. 487–538.
- [3] A.M. C Faria, H. Weiner, Oral tolerance, *Immunol. Rev.* 206 (2005) 232–259.
- [4] A.W. Cripps, J.M. Kyd, A.R. Foxwell, Vaccines and mucosal immunization, *Vaccine* 19 (2001) 2513–2515.
- [5] J. Mestecky, Z. Moldoveanu, C.O. Elson, Immune response versus mucosal tolerance to mucosally administered antigens, *Vaccine* 23 (2005) 1800–1803.
- [6] H. Chen, Recent advances in mucosal vaccine development, *J. Control. Release* 67 (2000) 117–128.
- [7] J. Holmgren, J. Adamsson, F. Anjuère, Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin B subunit and CpG DNA, *Immunol. Lett.* 97 (2005) 181–188.
- [8] D.J. Brayden, A.W. Baird, Microparticle vaccine approaches to stimulate mucosal immunization, *Microbes Infect.* 3 (2001) 867–876.
- [9] F.M. Oliveira, E.M. Santos, T.A. Mota-Santos, V. Ruiz-de-Souza, C.M. Gontijo, Covalent coupling of palmitate to ovalbumin inhibits and blocks the induction of oral tolerance, *Scand. J. Immunol.* 55 (2002) 570–576.
- [10] G. Gregoriadis, Engineering liposomes for drug delivery: progress and problems, *Trends Biotechnol.* 13 (1995) 527–537.
- [11] F. Zhou, M.R. Neutra, Antigen delivery to mucosa-associated lymphoid tissues using liposomes as a carrier, *Biosci. Rep.* 22 (2002) 355–369.
- [12] F.M. Oliveira, A.F. Silva-Neto, C.A. Silva, C.M. Gontijo, Coupling of palmitate residues to ovalbumin affects the induction of oral tolerance against the antigen, *Braz. J. Med. Biol. Res.* 31 (1998) 1421–1424.
- [13] C.J. Clarke, C.R. Stokes, The intestinal and serum humoral immune response of mice to systemically and orally administered antigens in liposomes. I. The response to liposome-entrapped soluble proteins, *Vet. Immunol. Immunopathol.* 32 (1992) 125–138.
- [14] Y. Fujii, Y. Aramaki, T. Hara, K. Yachi, H. Kikuchi, S. Tsuchiya, Enhancement of systemic and mucosal immune response following oral administration of liposomes, *Immunol. Lett.* 36 (1993) 65–70.

- [15] U. Chandrasekhar, S. Sinha, H.R. Bhagat, V.B. Sinha, B.S. Srivastava, Comparative efficacy of biodegradable liposomes and microspheres as carriers for delivery of *Vibrio cholerae* antigens in the intestine, *Vaccine* 12 (1994) 1934–1988.
- [16] A.D. Bangham, M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, *J. Mol. Biol.* 13 (1965) 238–252.
- [17] F.C. Skoza, D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation, *Proc. Natl. Acad. Sci. USA* 75 (1978) 4194–4198.
- [18] B. Zadi, G. Gregoriadis, A novel method for high-yield entrapment of solutes into small liposomes, *J. Liposome Res.* 10 (2000) 73–80.
- [19] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, *Anal. Biochem.* 83 (1977) 346–356.
- [20] G.R. Bartlett, Phosphorus assay in column chromatography, *J. Biol. Chem.* 234 (3) (1959) 466–468.
- [21] J.C.S. Saldanha, D.L. Gargiulo, S.S. Silva, F.H. Carmo-Pinto, M.C. Andrade, J.I. Alvarez-Leite, M.M. Teixeira, D.C. Cara, A model of chronic IgE-mediated food allergy in ovalbumin-sensitized mice, *Braz. J. Med. Biol. Res.* 37 (2004) 809–816.
- [22] B.A. Verdolin, S.M. Ficker, A.M.C. Faria, N.M. Vaz, C.R. Carvalho, Stabilization of serum antibody responses triggered by initial mucosal contact with the antigen independently of oral tolerance induction, *Braz. J. Med. Biol. Res.* 34 (2001) 211–219.
- [23] Y. Aramaki, H. Tomizawa, T. Hara, K. Yachi, H. Kikuchi, S. Tsuchiya, Stability of liposomes in vitro and their uptake by rat Peyer's patches following oral administration, *Pharm. Res.* 10 (8) (1993) 1228–1231.
- [24] G.A. Ramaltes, J.R. Deverre, J.M. Grognet, F. Puisieux, E. Fattal, Use of an enzyme immunoassay for the evaluation of entrapment efficiency and in vitro stability in intestinal fluids of liposomal bovine serum albumin, *Int. J. Pharm.* 143 (1996) 1–11.
- [25] M. Kokkono, P. Kallinteri, D. Fatouros, S.G. Antimisari, Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition, *Eur. J. Pharm. Sci.* 9 (2000) 245–252.
- [26] N.C. Phillips, L. Gagne, N. Ivanoff, G. Riveau, Influence of phospholipid composition on antibody responses to liposome encapsulated protein and peptide antigens, *Vaccine* 14 (9) (1996) 898–904.
- [27] M. Otsuka, K. Goto, S. Tsuchiya, Y. Aramaki, Phosphatidylserine-specific receptor contributes to TGF- β production in macrophages through a MAP kinase ERK, *Biol. Pharm. Bull.* 28 (9) (2005) 1707–1710.
- [28] A.Mcl. Mowat, Dendritic cells and immune responses to orally administered antigens, *Vaccine* 23 (2005) 1797–1799.
- [29] A. Mcl Mowat, A.M. Donachie, G. Reid, O. Jarrett, Immune-stimulating complexes containing Quil-A and protein antigen prime class I MHC-restricted T lymphocytes in vivo and are immunogenic by the oral route, *Immunology* 72 (3) (1991) 317–322.
- [30] E. Medina, C.A. Guzmán, Modulation of immune responses following antigen administration by mucosal route, *Immunol. Med. Microbiol.* 27 (2000) 305–311.
- [31] M.T. Magistris, Mucosal delivery of vaccine antigens and its advantages in pediatrics, *Adv. Drug Deliv. Rev.* 58 (2006) 52–67.
- [32] G.F.A. Kersten, D.J.A. Crommelin, Liposomes and ISCOMs, *Vaccine* 21 (2003) 915–920.
- [33] A.G. Lamont, A. Mcl Mowat, M.J. Browning, D.M.V. Parrot, Genetic control of oral tolerance to ovalbumin in mice, *Immunology* 63 (1987) 737–739.
- [34] K.S. Barone, M.R. Reilly, M.P. Flanagan, J.G. Michael, Abrogation of oral tolerance by feeding encapsulated antigen, *Cell. Immunol.* 199 (2000) 65–72.
- [35] T.A. Kraus, L. Toy Chan, J. Childs, A. Cheifetz, L. Mayer, Failure to induce oral tolerance in Crohn's and ulcerative colitis patients: possible genetic risk, *Ann. NY Acad. Sci.* 1029 (2004) 225–238.
- [36] J.L. Viney, A. Mcl Mowat, J.M.O. O'Malley, E. Williamson, N.A. Fanger, Expanding dendritic cells in vivo enhances the induction of oral tolerance, *J. Immunol.* 160 (12) (1998) 5815–5825.
- [37] E. Williamson, G.M. Westrich, J.L. Viney, Modulating dendritic cells to optimize mucosal immunization protocols, *J. Immunol.* 163 (7) (1999) 3668–3675.
- [38] N.M. Tsuji, B. Nowak, IL-18 and antigen-specific CD4(+) regulatory T cells in Peyer's patches, *Ann. NY Acad. Sci.* 1029 (2004) 413–415.
- [39] N.M. Vaz, M.J. Rios, L.M. Lopes, C.M. Gontijo, E.B. Castanheira, F. Jacquemart, L.A. Andrade, Genetics of susceptibility to oral tolerance to ovalbumin, *Braz. J. Med. Biol. Res.* 20 (6) (1987) 785–790.
- [40] A.G. Lamont, A.M. Mowat, M.J. Browning, D.M. Parrott, Genetic control of oral tolerance to ovalbumin in mice, *Immunology* 63 (4) (1988) 737–739.
- [41] K. Masuda, K. Horie, R. Suzuki, T. Yoshikawa, K. Hirano, Oral delivery of antigens in liposomes with some lipid compositions modulates oral tolerance to the antigens, *Microbiol. Immunol.* 1 (2002) 55–58.
- [42] A.C. Alves, C.M. Gontijo, M.C. Oliveira, S.O.F. Diniz, F.M. Oliveira, V.N. Cardoso, G.A. Ramaltes, Biodistribution of free ^{99m}Tc -ovalbumin and ^{99m}Tc -ovalbumin encapsulated in liposomes, *Braz. Arch. Biol. Technol.* 48 (2005) 235–242.
- [43] B.L. Kelsall, F. Leon, Involvement of intestinal dendritic cells in oral tolerance immunity to pathogens and inflammatory bowel disease, *Immunol. Rev.* 206 (2005) 132–148.
- [44] J. Bilsborough, T.C. George, A. Norment, J.L. Viney, Mucosal CD8 α + DC with a plasmacytoid phenotype induce differentiation and support function of T cells with regulatory properties, *Immunology* 108 (2003) 481–492.
- [45] A.Mcl. Mowat, Anatomical basis of tolerance and immunity to intestinal antigens, *Nat. Rev. Immunol.* 3 (4) (2003) 331–341.
- [46] V.A. Fadok, D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, P.M. Henson, Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β PGE2 and PAF, *J. Clin. Invest.* 101 (4) (1998) 890–898.
- [47] P. Garside, M. Steel, E.A. Worthey, P.J. Kewin, S.E. Howie, D.J. Harrison, D. Bishop, A.Mcl. Mowat, Lymphocytes from orally tolerized mice display enhanced susceptibility to death by apoptosis when cultured in the absence of antigen in vitro, *Am. J. Pathol.* 149 (6) (1996) 1971–1979.
- [48] M. Boirivant, R. Pica, R. DeMaria, R. Testi, F. Pallone, W. Strober, Stimulated human lamina propria T cells manifest enhanced Fas-mediated apoptosis, *J. Clin. Invest.* 98 (11) (1996) 2616–2622.
- [49] W. Chen, W. Jin, H. Tian, P. Sicurello, M. Frank, J.M. Orenstein, S.M. Wahl, Requirement for transforming growth factor beta1 in controlling T cell apoptosis, *J. Exp. Med.* 194 (4) (2001) 439–453.
- [50] M.N. Huynh, V.A. Fadok, P.M. Henson, Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF- β 1 secretion and the resolution of inflammation, *J. Clin. Invest.* 109 (2002) 41–50.
- [51] V.E. Kagan, G.G. Borisenko, B.F. Serinkan, Y.Y. Tyurina, V.A. Tyurin, J. Jiang, S.X. Liu, A.A. Shvedova, J.P. Fabisiak, W. Uthaisang, B. Fadeel, Appetizing rancidity of apoptotic cells for macrophages: oxidation externalization and recognition of phosphatidylserine, *Am. J. Physiol. Lung* 285 (2003) 1–17.