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Influence of dose and immunization route on the serum Ig G antibody response to BSA loaded PLGA microspheres

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Abstract

BSA was entrapped into PLGA 50:50 microspheres and the in vitro release study was performed. Then 1 μg of microencapsulated antigen was subcutaneously administered to Balb/c mice and the serum Ig G response was compared to that obtained after the subcutaneous administration of the same amount of only free antigen or of free antigen emulsified 1:1 with Freund's complete adjuvant (FCA). The specific serum Ig G responses obtained from the microencapsulated antigen were higher than those obtained from the free antigen and similar to those obtained from the antigen emulsified with FCA. Therefore, the immune response obtained with the subcutaneous administration of 1 μg of microencapsulated antigen was used as a positive control to compare the immune response elicited by the administration of the spheres either by the oral or the intranasal route. There is a dose/response relationship in the serum Ig G response elicited after three consecutive oral administrations of microencapsulated antigen at a dosing range from 200 to 50 μg. However, this relationship does not seem to be clear for the intranasal administration of the spheres at the same dosing range. When comparing the serum Ig G responses at each dosing level for the different routes of administration, it can be observed that the intranasal route is a more powerful inducer of the production of specific Ig G antibody than the oral, which could be due to a greater permeability of the nasal epithelium and to qualitative differences between the mechanisms of induction of the immune response by each route. The serum Ig G2a versus Ig G1 ratio is not significantly different among all the groups that received antigen in microspheres.

Keywords: Microspheres; BSA; Immune response; Serum Ig G; Ig G1; Ig G2a; PLGA; Subcutaneous; Oral; Intranasal

1. Introduction

Vaccination has become one of the main goals of medical research [1]. Traditionally, vaccine research has mainly focused on the induction of systemic immunity through parenteral immunization, involving the intramuscular or the subcutaneous route, usually by means of an injection using a needle and a syringe [2,3]. However, most pathogens and allergens naturally access their hosts through the mucosal membranes [4]. Therefore, the development of new antigen delivery systems for mucosal vaccination would be an interesting goal, since mucosal vaccination has a number of advantages over the traditional parenteral immunization:

- mucosal vaccines are easier and less expensive to administer, not usually requiring the presence of trained personnel for vaccination;
- they are safer to use, avoiding the possibility of infections caused by inadequately sterilized needles or by a deficient hygiene;
- they might improve immunization efficiency, stimulating not only the systemic immunity, but also mucosal immunity at the portal of entry for most pathogens [5].

Of all the mucosal routes, the oral seems to be the most attractive, as it provides the easiest administration and requires a cheaper dosage form. However, the intranasal route could be preferable for a number of reasons:

- The environment of the nasal route is less harsh for the antigens since there is a less acidic pH and a lower level of proteolytic enzymes.
- The uptake of particles is less problematic in the nasal cavity than in the gut, since the intestinal lumen content makes the absorption of particulates through the intestinal wall difficult and more variable.
- The nasal mucosa is highly irrigated, which makes it more permeable when compared than other mucosal sites [5,6].
- Due to compartmentalization of the common mucosal immune system, intranasal immunization is the best route to induce a specific immune response in the respiratory system [7,8] as well as being able to induce a strong immune response at distant mucosal sites [9].

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Little is known about the intranasal administration of antigens encapsulated into biodegradable microspheres [10], and still less about its ability to elicit an immune response when compared with the oral or the subcutaneous route. Therefore, our research first investigated the dose dependence of the systemic immune response elicited either by the oral and the intranasal route, and then contrasted the immune response obtained by the oral with the intranasal route at each dosing level, also comparing these to that elicited by the subcutaneous route. Finally, we compared the Ig G2a versus Ig G1 ratio of the serum immune response elicited by the administration of microspheres by different routes and doses in order to determine if differences in total serum Ig G response could be related to differences in the activation of a specific Th $CD4^+$ lymphocyte subpopulation.

2. Materials and methods

2.1. Materials

The polymer poly(D,L - lactide - co - glycolide) (PLGA) (Resomer® RG 506) with a copolymer ratio of 50:50 (lactic/glycolic (%)) and an intrinsic viscosity of 0.8 dL/g in Cl_3CH was supplied by Boehringer Ingelheim K.G. (Ingelheim, Germany). Bovine serum albumin (BSA), polyvinylalcohol (PVA) (average molecular weight 30,000–70,000), affinity-purified goat anti mouse Ig G (peroxidase conjugate), Freund's complete adjuvant and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (diammonium salt) were supplied by Sigma (St. Louis, MO, USA). Affinity purified goat anti mouse Ig G1 and Ig G2a (peroxidase conjugate) were supplied from Southern Technology and the protein assay kit (micro BCA) was purchased from Pierce by Teknovas (Bilbao, Spain). All other chemicals were analytical grade and were supplied by Panreac S.A. (Barcelona, Spain).

2.2. Animals

Female Balb/c mice 6–8 weeks of age, weighing 15–18 g, were obtained from Criffa S.A. (Barcelona, Spain) and housed in groups of ten with free access to food and water throughout the study.

2.3. Preparation of the microspheres

PLGA microspheres were prepared by a modification of the solvent extraction technique using a W/O/W double emulsion system previously described [11]. Briefly, the microparticles were prepared dissolving the appropriate amount of the antigen in 250 μl water and emulsifying with 5 ml of a 5% (w/v) polymer solution in methylene chloride by sonication at 50W (Branson Sonifier® 250). The primary emulsion was then added to 25 ml of distilled water containing polyvinyl alcohol (8% w/v) and mixed at high speed to obtain a W/O/W emulsion with an Ultraturrax T-25 homogenizer. Finally, an aqueous solution of isopropanol (2% v/v) was added and the system was mechanically stirred for 2 h. The resulting microspheres were separated by centrifugation, washed three times in distilled water and freeze-dried.

2.4. Characterization of the spheres

Sphere morphology and surface appearance were examined by scanning electron microscopy (SEM; Jeol® JSM-35 CF). Size was determined by laser diffractometry using a Coulter Counter® LS130 particle size analyser. Zeta potential of a suspension of microspheres in KCl 1 mM was determined in triplicate using a Malvern® Zetasizer 3000.

2.5. BSA loading assessment

Total loaded protein was estimated using the Lowry assay after disruption of the microspheres with 0.2 M NaOH solution. The surface-associated protein was assessed by suspending the particles in 20 mM phosphate buffered saline pH 7.4 and incubating at 37 °C under continuous orbital rotation for 30 min. The spheres were separated by centrifugation and the supernatant was analyzed for BSA by the micro-BCA assay. Surface-associated protein represents the percentage of total protein loaded that is present on the microsphere surface.

2.6. In vitro release studies

The release profile of BSA from PLGA microspheres was determined by incubating 1.5 mg of encapsulated antigen in a test tube containing 2 ml of 0.02 M phosphate-buffered saline PBS (pH 7.4) and shaking with a rotatory shaker at 25 rpm at 37 ± 0.5 °C. At appropriate intervals, the release medium was removed by centrifugation and replaced with 2 ml of fresh PBS. The amount of protein released was determined using the micro BCA method. Protein release profiles were generated for each microsphere formulation in terms of cumulative protein release versus time. All the release tests were performed in triplicate and results reported as means of these triplicate assays.

2.7. Immunization protocol

2.7.1. Experiment 1

One group of ten mice was subcutaneously immunized at day 0 with 1 μg of microencapsulated BSA. Immediately before administration, the required dose of freeze dried microparticles was weighed and resuspended in PBS 0.02 M. Two more groups of mice received the same quantity of antigen in solution (PBS 0.02 M) or emulsified 1:1 in FCA (1 μg of free antigen in 50 μl PBS 0.02 M emulsified with 50 μl of FCA). The volume of administration was 0.1 ml.

2.7.2. Experiment 2

Four groups of ten mice were orally immunized at days 0–2 with an appropriate quantity of microencapsulated BSA, according to the following protocol:

- one group received 500 μg BSA per dose;
- the second group received 200 μg BSA per dose;
- the third group received 100 μg BSA per dose;
- the fourth group received 50 μg BSA per dose.

Immediately before administration, the required dose of freeze-dried microparticles was weighed and resuspended in $NaHCO_3$ 0.3% in water. The volume of administration was 0.4 ml. The animals were fasted overnight before the immunization.

2.7.3. Experiment 3

Four groups of ten mice were intranasally immunized at days 0–2 with an appropriate quantity of microencapsulated BSA, according to the following protocol:

- one group received 200 μg BSA per dose;
- the second group received 100 μg BSA per dose;
- the third group received 50 μg BSA per dose;
- the fourth group received 10 μg BSA per dose.

Immediately before administration, the required dose of freeze-dried microparticles was weighed and resuspended in saline. The volume of administration was 50 μl in concordance with the results obtained by Eyles et al. [8].

A control group of ten mice was not immunized. Blood samples were collected from the retroorbital plexus of the mice, under anaesthesia with ether, at weeks 3, 5, 7, 9 and 12. These samples were centrifuged and serum was collected and stored frozen at −30 °C until assayed by ELISA (anti-BSA total Ig G, Ig G1 and Ig G2a).

2.8. Anti-BSA antibody detection

Anti-BSA antibodies were determined by a conventional ELISA. Briefly, microtitre plates (Nunc-Immuno Plate® FP 96 Maxisorp. NUNC) were coated with 100 μl per well of a 10 μg/ml BSA in a 0.05% PBS-Tween 20 (v/v)-dry milk 2% solution (pH 7.4) (PBSTM) and incubated overnight at 4 °C. The plates were then washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked for 1 h with 200 μl PBST. Serum samples were serially diluted 1:2 starting from 1:100 dilution in PBS, and 100 μl of each sample was added to each well of the coated ELISA plates. The plates were incubated for 1 h at 37 °C and washed three times with PBST. An amount of 100 μl of horseradish peroxidase-labelled goat anti-mouse IgG, IgG1 or IgG2a diluted 1 in 1000 in PBSTM was added to each well. The plates were covered and after incubation at 37 °C for 1 h, washing was repeated. 100 μl of a 0.1 mg/ml solution of the ABTS in 50 mM citrate buffer plus hydrogen peroxide (1 in 10,000), was added to each well and incubated at 37 °C for 15 min for colour development. The end-point titres were expressed as the $\log_2$ of the last dilution reciprocal, which gave an OD_{405} above the mean OD_{405} of negative controls.

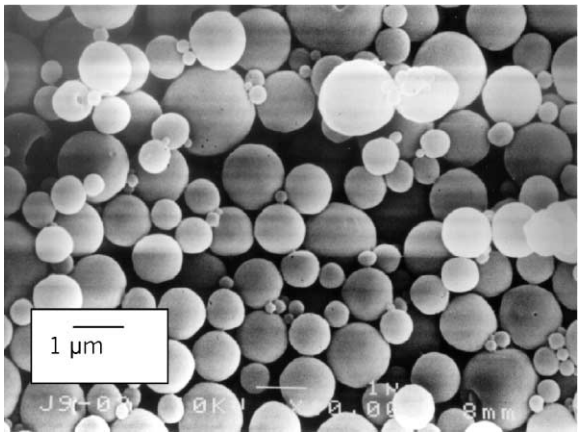

Fig. 1. Scanning electron photograph of the microspheres.

2.9. Statistical analysis

The results were expressed as mean ± S.D. for each group of mice. Statistical analysis was made with SPSS 10.1 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by means of Kolmogorov-Smirnov trial. Tahmane's T2 test was performed to assess statistical significance. Results were considered statistically significant if $P < 0.05$.

3. Results and discussion

3.1. Characterization of the microspheres

When observed under scanning electron microscopy (SEM), the microspheres appeared spherical with a smooth uniform surface, as shown in Fig. 1. The mean particle size was about 1 μm and the total loaded protein and surface associated protein were 9.09 and 29.85% respectively. The mean zeta potential was −8.52 mV.

3.2. In vitro release studies

Release profile of BSA from the spheres is shown in Fig. 2. As can be seen in Fig. 2, 1 μm particles show a biphasic release profile, with an initial period of fast release and a second period in which the spheres release the protein in a sustained way, which is in agreement with previously reported studies [12,13]. Approximately, 45% of BSA remained inside the spheres after 34 days (816 h) of incubation. This may be due to low diffusion caused by the electrical interaction between basic amino acids of protein and the acidic carboxyl groups of the polymer [14].

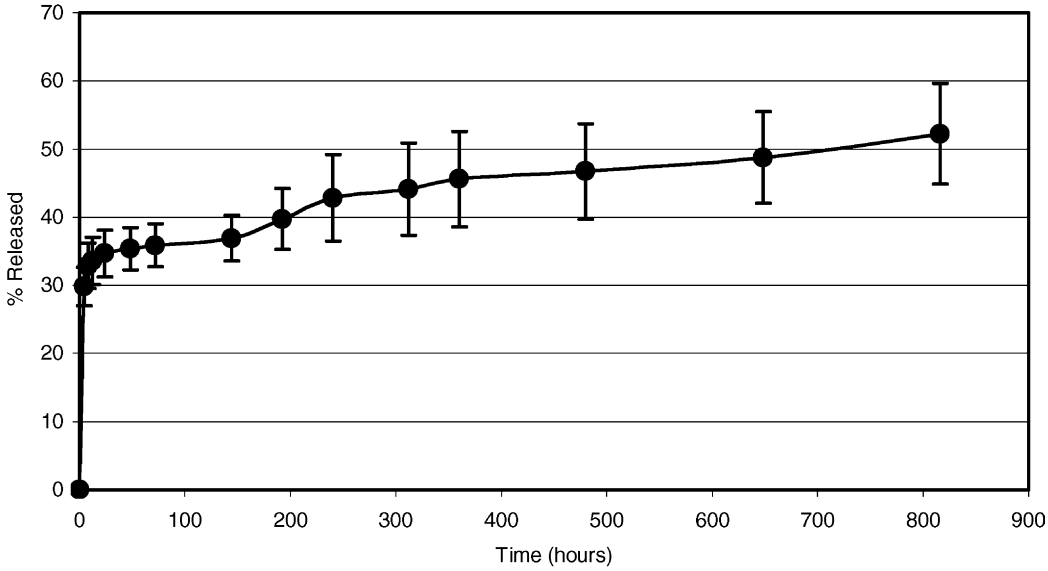

Fig. 2. Release profile of BSA from PLGA microspheres.

3.3. *In vivo antibody response in mice*

The level of anti-BSA antibodies (Ig G) was determined for all experimental groups up to week 12. Fig. 3 shows serum antibody response obtained after a single subcutaneous administration of 1 μg BSA in different vehicles.

As can be seen in this Fig. 3, the subcutaneous administration of 1 μm particles elicited higher serum Ig G response to that obtained with the administration of free antigen and a similar immune response (no statistically significant differences) to that obtained with Freund's complete adjuvant, employed as a positive control. This difference between free and encapsulated antigen was statistically significant at all time points ($P < 0.05$). The level of anti-BSA antibody induced by microspheres reached its maximal value around week 5 and remained high over the entire time period studied. The administration of free antigen elicited a nearly undetectable immune response.

Similar results have been obtained by other authors [15,16], and confirm the ability of microspheres to elicit a considerable serum immune response after a single subcutaneous administration. Bearing these results in mind we used this subcutaneous administration of 1 μg BSA in microspheres as standard to compare the immune response elicited by the oral and intranasal administration of the spheres.

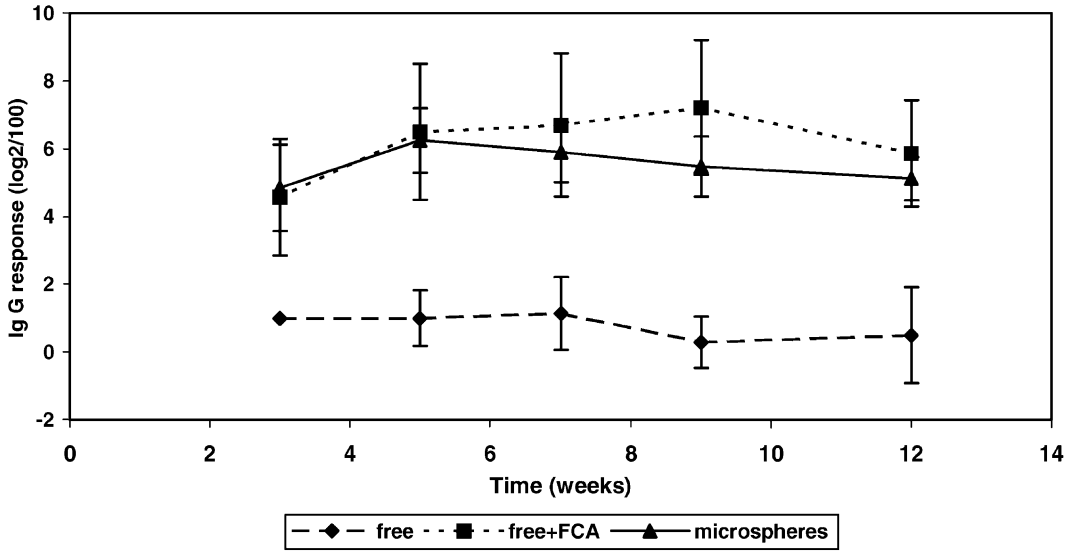

Fig. 3. Serum Ig G antibodies after subcutaneous immunization with 1 μg of BSA in 1 μm sized spheres or with free BSA (either alone or emulsified 1:1 with FCA).

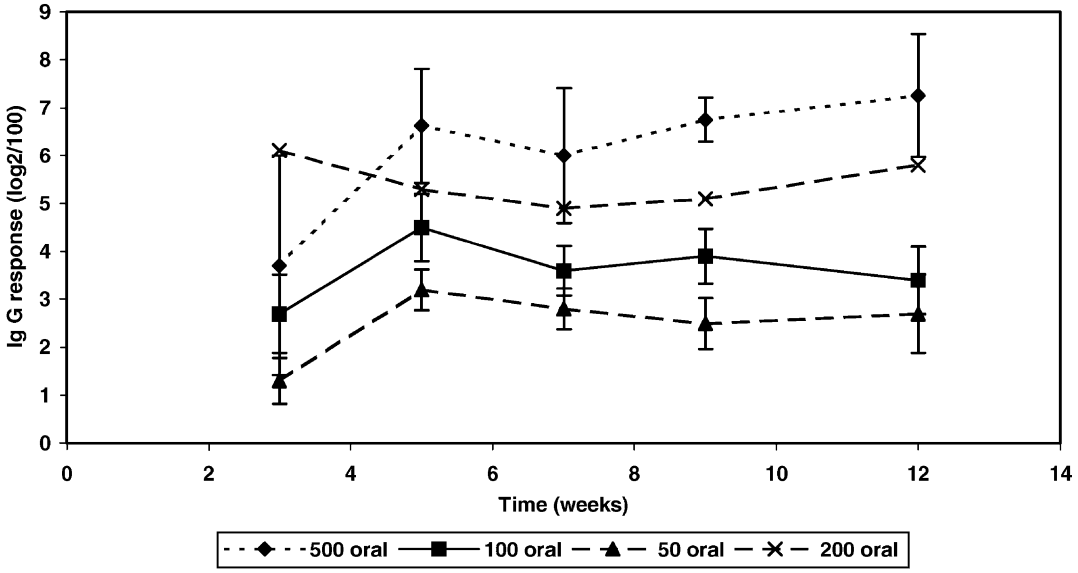

Fig. 4. Serum Ig G antibodies after three consecutive oral doses of 500, 200, 100 or 50 μg of BSA per dose in 1 μm sized spheres.

Fig. 4 shows the dose dependant immune response after the triple administration of 1 μm-sized particles by the oral route. As could be expected, there is a direct dose/response relationship, decreasing the immune response as the dose decreases (except for 200 μg dose at week 3). For almost all groups, there is an initial rise of the antibody titter, up to week 5. However, whilst the immune response diminishes or is maintained for those groups that received 100 or 50 μg of antigen per dose, it increases up to week 12 for the other two groups. This could be related to a better stimulation of the immune system at higher dosing levels, mainly due to the poor absorption of the microspheres by the intestinal wall, which can be as low as 1% of the administered dose [9,17–22]. The serum Ig G responses obtained with doses of 500 and 200 μg was similar (no statistically significant difference), and significantly higher ($P < 0.05$) at weeks 3, 7, 9 and 12 to that obtained with 100 μg and at all time points when compared to that obtained with 50 μg. No statistically significant differences were found between these last two groups.

Three doses of 200 μg by the intranasal route elicited a very strong serum Ig G response, as can be seen in Fig. 5. This figure shows that the response reached its maximal value at week 5, and then began to decrease slightly until

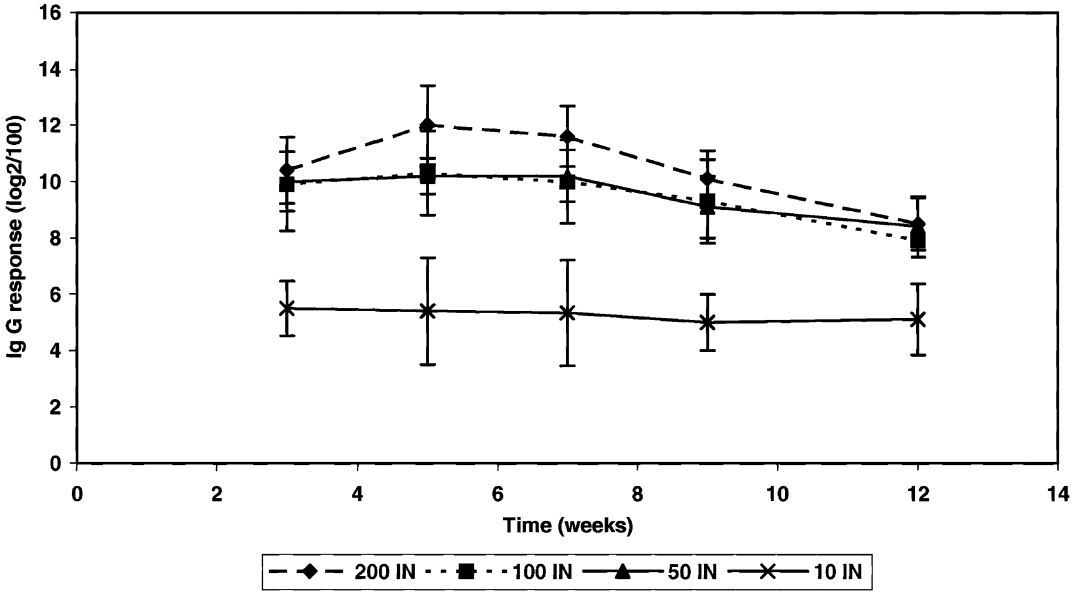

Fig. 5. Serum Ig G antibodies after three consecutive intranasal doses of 200, 100, 50 or 10 μg BSA per dose in 1 μm sized spheres.

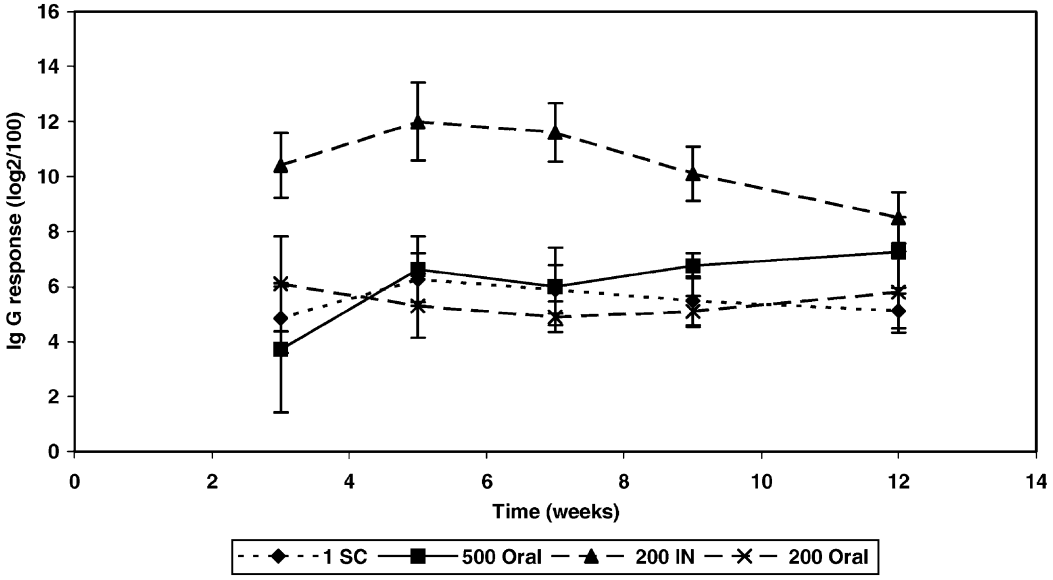

Fig. 6. Serum Ig G antibodies after three consecutive oral doses of 500 or 200 μg, three consecutive intranasal doses of 200 μg BSA or a single subcutaneous dose of 1 μg BSA in 1 μm sized spheres.

week 12. The serum Ig G profile was similar for 100 and 50 μg dosage levels, and slightly smaller than that obtained with 200 μg. However, there were no statistically significant differences between the three groups, which could indicate a better stimulation of the immune system by this route, mainly due to a better absorption of the particles by the nasal associated lymphoid tissue (NALT), so a bigger percentage of the administered dose could have reached the immune system, minimizing the effect of the dose. Moreover, the bronchus associated lymphoid tissue (BALT) could also be implicated, since the administered volume makes the microspheres suitable to reach the bronchoalveolar tissue [8]. Only the administration of three doses of 10 μg of antigen elicits a considerable smaller immune response (statistically significant at all time points compared to the other three groups).

When we compare the immune response elicited by the oral versus the intranasal route, the intranasal administration of BSA encapsulated into microspheres stimulates a stronger systemic immune response at all dosing levels, this difference being bigger when the administered dose is smaller. At higher dosing levels (Fig. 6) it can be seen that three

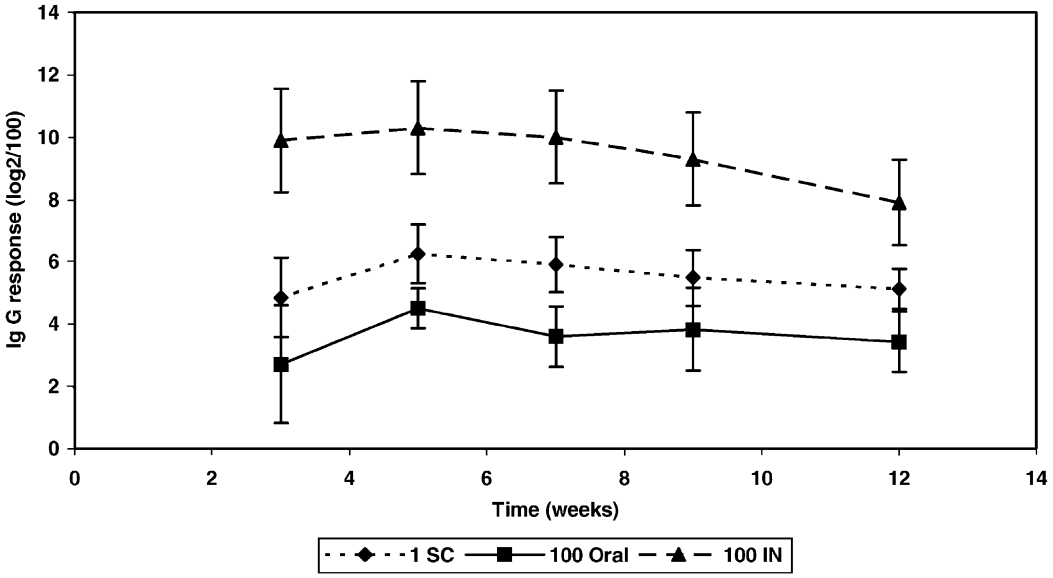

Fig. 7. Serum Ig G antibodies after three consecutive oral or intranasal doses of 100 μg BSA, or a single subcutaneous dose of 1 μg BSA in 1 μm sized spheres.

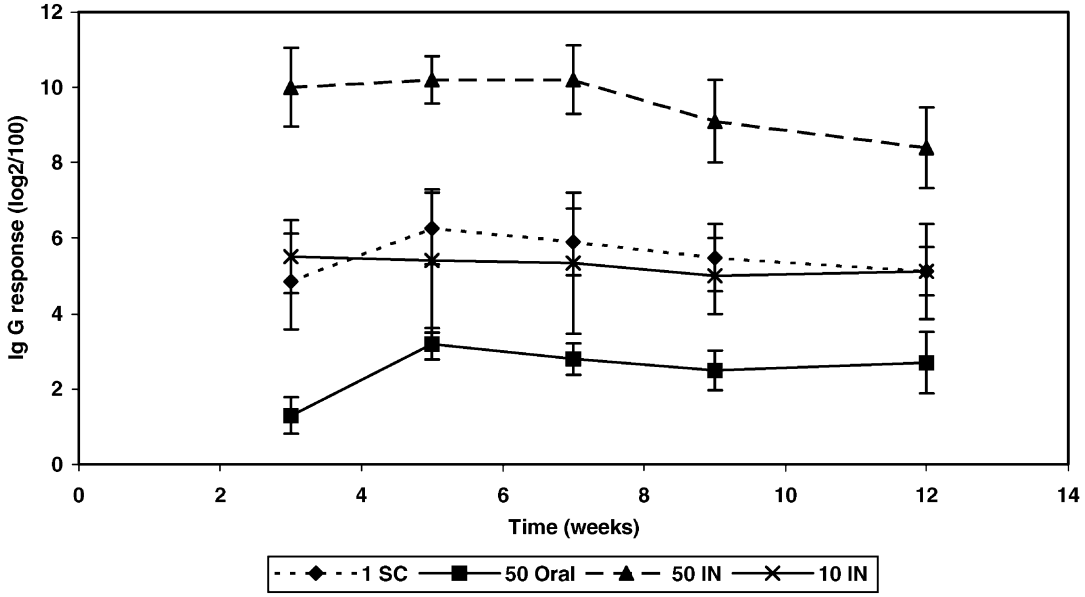

Fig. 8. Serum Ig G antibodies after three consecutive oral or intranasal doses of 50 μg BSA, or a single subcutaneous dose of 1 μg BSA in 1 μm sized spheres.

oral administrations of 200 or 500 μg of encapsulated BSA elicited a similar (no statistically significant difference) immune response to that obtained with a single subcutaneous administration of 1 μg of encapsulated antigen (employed as a standard group), and a lower response than that elicited by three administrations of 200 μg of encapsulated protein by the intranasal route, with statistically significant difference at all time points for three oral administrations of 200 μg and at weeks 5 and 7 for three oral doses of 500 μg ($P < 0.05$). To the extent that the administered dose is lower, the difference between the immune response elicited by each route increases. At a 100 μg dosing level (Fig. 7) the intranasal route continues eliciting a bigger immune response, which is statistically significant at all time points compared to the oral route ($P < 0.01$). At the smallest dosing level (Fig. 8), the difference between the oral and the intranasal route is greatest ($P < 0.01$ for a 50 μg dosing level), the immune response elicited by three oral administrations of 500 μg of antigen being lower than that obtained with ten times lower amount of antigen administered by the intranasal route and similar to that obtained with 50 times lower (10 μg) intranasal doses.

The difference in the ability of each route to induce an immune response could be due to various reasons. The nasal mucosa is a more permeable epithelium than the intestinal mucosa [23]. Moreover, microspheres have to be transported over a very small distance, remaining for only 15 min in the nasal cavity, and are less exposed to luminal contents by the intranasal route than by the oral route [24], which means that a bigger amount of antigen can be systemically available employing similar doses, and the absorption is less irregular than by the oral route. Other authors have also reported high antibody titters after the intranasal administration of particulated antigens [25–27]. Sjölander et al. [27] have recently reported a similar serum Ig G response by the intranasal route employing only a 15 times higher dose compared to the subcutaneous route. However, this bigger availability of antigen by the intranasal route may not explain by itself the different capacity of each route to induce an immune response. We had observed in preliminary studies that the administration of three oral doses of 1 mg of BSA in microspheres did not improve the serum Ig G titter obtained with three oral doses of 500 μg of microencapsulated BSA (data not shown). This would indicate that we have reached the maximum serum Ig G response obtainable with our microspheres by the oral route. However, by the intranasal route the serum Ig G titter elicited is clearly higher, which suggests that the induction mechanisms of the immune responses by each route could be qualitatively different. It has been reported that the nature of the antigen-presenting cell (APC), which presents the antigen to the specific naïve T cells, will favor the isotype and magnitude of the B cell antibody response [28–31]. There are three main antigen presenting cell populations in the mouse: B-lymphocytes, macrophages and dendritic cells, which have a different body distribution [28,32–39]. Neutra et al. [32] reported the different body distribution of dendritic cells on the epithelial barriers at different sites in the body. These cells are well described as the initiators and modulators of the immune response [40,41]. Therefore, similar quantities of antigen could be differently presented, depending on the immunization route, eliciting higher antibody titters by the intranasal route compared to oral, being the differences mostly due to a different tissue distribution of APC, which could even result in differences

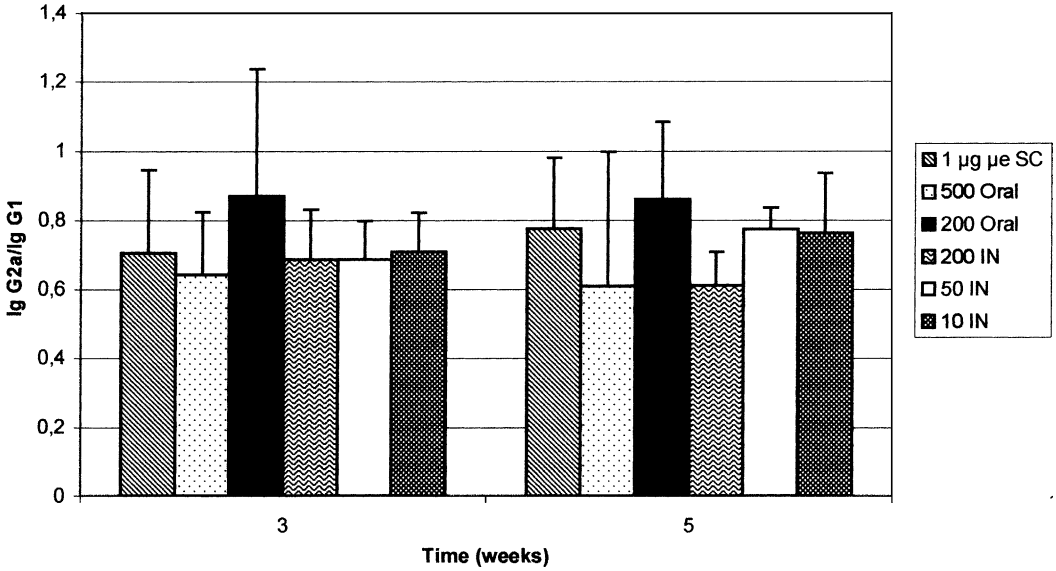

Fig. 9. Serum Ig G2a vs. Ig G1 ratio elicited after the administration of a single subcutaneous dose of 1 μg or three consecutive oral doses of 500 or 200 μg or intranasal doses of 200, 50 or 10 μg BSA in 1 μm sized spheres.

in their ability to stimulate the specific Th $CD4^+$ lymphocyte subpopulation.

In order to determine if this theoretically different antigen presentation originates differences in the ability of the microspheres to stimulate a specific Th cell subset, we determined the Ig G2a/Ig G1 ratio at weeks 3 and 5 for the highest oral and intranasal doses and lowest intranasal doses. Results are expressed as mean + S.D. of individual Ig G2a/Ig G1 ratios in each group in Fig. 9. This Figure shows that microspheres administered either by the subcutaneous, oral or intranasal route are capable to elicit a combined serum Ig G2a/Ig G1 responses, with a predominance of the Ig G1 response. Previously reported articles have demonstrated that soluble antigens usually elicit high levels of Ig G1 antibody isotype and absent or very low levels of Ig G2a [8]. Ig G1 is an antibody associated to inflammatory and mast-cell or eosinophil responses, while Ig G2a is able to act as an opsonin, activates the complement and binds to macrophages FcγRI enhancing the phagocytic response [42]. It has been determined that switching to Ig G2a is induced by IL-12 and IFN-γ (secreted by Th1 $CD4^+$ cell subset) and inhibited or diminished by IL-4, IL-5 and IL-10 (secreted by Th2 $CD4^+$ cell subset) [31]. Our results are consistent with those previously reported by Banchereau et al. [41]. According to this author, particulated antigens can be processed and presented either by major histocompatibility complex (MHC) classes I and II by dendritic cells and macrophages, which stimulates Th1 and Th2 lymphocyte subpopulations, while soluble antigens are exclusively presented by class II MHC, stimulating the Th2 response. Therefore, the apparition of Ig G2a isotype in this study by the oral and nasal route confirms that Th1 cell subset must have been activated. Some authors have also observed this pattern of response for particulated antigens by the subcutaneous [43], the oral [44–46] and the intranasal route [47,48] although variations in the immune response obtained can be observed, mostly due to different polymer compositions, release profiles and particle sizes. In our case, the Ig G2a versus Ig G1 ratio seems to be bigger at lower doses (independently of the route of administration) and, at a 200 μg dosing level, bigger for the oral route. The influence of the dose on the Th1/Th2 response relationship would be in agreement with results obtained by Morokata et al. [30], who reported that high availability of antigen would result in a favored Th2 response in Balb/c mice. Constant et al. [31] also report that Th1 clones are more susceptible than Th2 clones to activation-induced cell death triggered by high doses of antigen, so the net result could be the outgrowth of Th2 lymphocytes because the negative cross-regulation usually conferred by the Th1 cells has been reduced. However, there are no statistically significant differences between the groups tested in our study, and lacking of statistical analysis for calculated ratio on previously reported articles makes comparison difficult. Therefore, we cannot conclude that, in this case, a bigger serum Ig G response results in a different Ig G2a or Ig G1 production pattern. More studies concerning T lymphocyte proliferative assays and cytokine production should be necessary to completely characterize the different immune response elicited by each route.

4. Conclusions

This study shows that there is a dose/response relationship after the oral administration of BSA encapsulated into 1 μm sized PLGA microspheres at a dose range from 200 to 50 μg.

However this relationship is not as clear when using intranasal administration at the range of doses employed in the oral route and only employing doses of 10 μg the immune response is significantly lower. The intranasal route is capable of eliciting a higher serum Ig G response than the oral route after the administration of the antigen in microspheres at each dosage level. Moreover, it is possible to obtain a higher serum Ig G response after the administration of three intranasal doses of 50 μg than that obtained after the oral administration of three doses of 500 μg, and a similar response employing three intranasal doses of 10 μg. This suggests a better induction of the serum Ig G response by using the intranasal route, which could be due to the greater availability of the antigen by the nasal route. The administration of microspheres by the subcutaneous, the oral or the intranasal route elicits a combined serum Ig G2a/Ig G1 response where Ig G1 antibody levels are slightly predominant. No statistically significant differences have been found on Ig G2a versus Ig G1 ratio between routes of adminitration or between antigen doses, suggesting that in this case a different degree of stimulation of the immune system does not result in an altered Ig G2a versus Ig G1 pattern response.

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