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Encapsulation of $A\beta_{1-15}$ in PLGA microparticles enhances serum antibody response in mice immunized by subcutaneous and intranasal routes

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ABSTRACT

The aim of the present work was to develop an easy, safe and effective vaccine in Balb/c mice using the $A\beta_{1-15}$ peptide as immunogen entrapped in PLGA microparticles to reduce the risk of an adverse T cell-mediated response. $A\beta_{1-15,}$ which contains the N-terminal antibody epitope of the full $A\beta_{1-42}$ peptide was encapsulated in PLGA by a modified solvent evaporation/extraction technique using a double emulsion system. Microparticles were characterized in terms of size distribution (1.22 ± 0.28 μm), encapsulation efficiency (75.05 ± 4.17%), surface associated peptide (59.81 ± 0.96%) and "*in vitro*" release profile. Balb/c mice were immunized by subcutaneous and intranasal routes with three 30 μg doses of the peptide microencapsulated in PLGA. A solution of the peptide alone and an emulsion in the Freund's adjuvant were administered subcutaneously as control groups. Antibody levels elicited against the toxic $A\beta_{1-40}$ fraction in the serum of PLGA microparticles treated groups were higher than that of the peptide alone groups. Our initial results indicate that immunotherapy with $A\beta_{1-15}$ loaded PLGA microparticles could be a promising approach for the future development of a safe vaccine against Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is the most common cause of mental deterioration in elderly people. Although AD was described almost a century ago, there is currently no effective treatment or cure. However, new therapeutic approaches, including amyloid beta (Aβ) immunotherapy are currently being investigated (Fu et al., 2010).

From an anatomic point of view, AD is characterized by an atrophy of the cerebral cortex and by a massive loss of excitatory cholinergic and glutamatergic projections made by the nucleus basal is towards the cortex (Francis et al., 1999). Histopatologically, the extracellular accumulation of Aβ in the form of plaques and small aggregates (oligomers) is the main pathological hall-mark of the disease (Hardy and Selkoe, 2002). Aβ peptide is considered a cleavage product of neuronal amyloid precursor protein (APP) by β-and γ-secretases (Selkoe, 1994). Cleavage can yield either $A\beta_{1-40}$ or $A\beta_{1-42}$. An abnormal accumulation and aggregation of these products in certain cognitive brain regions during aging precede neuronal injury and AD pathology (Selkoe, 1999). Thus, prevention or elimination of amyloid plaques deposition may prevent or slow the onset of dementia because the presence of plaques correlates with the resulting dementia of AD (Naslund et al., 2000).

There is strong evidence that immune therapy is able to remove Aβ from the central nervous system (CNS), which ultimately improves the cognitive decline associated with the disease. Schenk and colleagues (Schenk et al., 1999) were the first group to report the cerebral Aβ lowering effect after immunization with the aggregated Aβ in an AD mouse model. They demonstrated in 1999 that monthly intraperitoneal (i.p.) injections of aggregated $A\beta_{1-42}$ (AN-1792) emulsified in complete Freund's adjuvant (CFA) prevented Aβ plaque deposition when given to young mice prior the onset of plaque formation and, clearly reduced plaque formation when administered to older mice after plaque formation had been initiated. Shortly thereafter, was reported that passive immunization with $A\beta_{1-42}$ specific antibodies via weekly i.p. injection for 6 months cleared amyloid plaques in PDAPP mice (Bard et al., 2000), suggesting that induction of serum antibody levels is a prerequisite for efficacy. Actually, many ongoing phase III trials on solanezumab and bapinezumab (two humanized anti-Aβ monoloclonal antibodies) are being tested to check out the efficiency of passive Aβ immunotherapy on the course of the Alzheimer's disease progression (Panza et al., 2011). In addition, several immune-based strategies have protected different AD transgenic mice

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta; i.n, intranasal; s.c, subcutaneous; CFA,complete Freund's adjuvant; IFA,incomplete Freund's Adjuvant; PLGA, polylactide-co-glycolide acid; PBS, phosphate-buffered saline; SAP, surface associated peptide; Sol, solution; MP, microparticles.

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models from cognitive impairment. Janus et al. (2000) demonstrated that i.p. injections of an $A\beta_{1-42}$ suspension in a TgCRND8 murine model of Alzheimer's disease reduced plaque burden and improved the behavior in the Morris water maze test. Morgan et al. (2000) reported that subcutaneous (s.c) injections of an $A\beta_{1-42}$ suspension in Tg2567 and PSAPP tg mice led to improvement in a radial-arm maze test of working memory and a modest reduction in plaque burden. These preclinical results largely justified the first Aβ immunotherapy clinical trial by Elan Corp in collaboration with Wyeth–Ayerst with their AN-1792 vaccine, a mixture of fibrillar $A\beta_{1-42}$ and the saponin adjuvant QS-21, on AD patients. Unfortunately, the phase II clinical trial was halted due to signs of acute meningoencephalitis in ~6% of AD patients (Schenk, 2002). Nevertheless, a study with a subgroup of these patients established, that antibodies against Aβ appear to slow cognitive decline (Bayer et al., 2005). The cause of meningoencephalitis in a subset of patients has not been definitively determined; however, the antibody titers did no correlate with the presence or severity of meningoencephalitis (Orgogozo et al., 2003). The presence of T lymphocyte infiltration in the leptomeninges, cerebrovasculature and cerebral neocortex provided support for the theory that the adverse response to the vaccine was due to a T cell-mediated autoimmune response (Schenk, 2002).

Since then, numerous studies have reported that the B cell epitope in humans, (Geylis et al., 2005) monkeys (Lemere et al., 2004) and mice (McLaurin et al., 2002) is located within the $A\beta_{1-15}$ region, while the T cell epitope has been mapped within the $A\beta_{15-42}$ (Cribbs et al., 2003). These observations have been used to design alternative immunogens, which contains the N-terminal antibody epitope but lack the more C-terminal T-cell reactive sites (T cell epitopes) for immunization (Lemere et al., 2007). These shorter Aβ fragments have been shown to lead an important immune response when were administered using different approaches such as: conjugated to other T cell helper epitopes (Agadjanyan et al., 2005), formulated as a thioredoxin-constrained B-cell epitope peptide (Moretto et al., 2007), administered as dendrimers and liposome vaccines (Muhs et al., 2007; Seabrook et al., 2007), expanded with the addition of lysine residues (Maier et al., 2006), combined with hepta-polylysine (MAP) in Tg2576 mice (Hu et al., 2008) and in rhesus monkeys (Li et al., 2005a) or when administered as a novel adenovirus vaccine (Zou et al., 2008).

Based on the above background, we microencapsulated the $A\beta_{1-15}$ fragment in polylactide-co-glycolide acid (PLGA) as a delivery system. PLGA was previously used by Brayden et al. (2001) to entrap the $A\beta_{1-42}$ peptide into PLGA biodegradable microspheres. The $A\beta_{1-42}$ PLGA microspheres elicited a strong antibody response in mice immunized by subcutaneous and intraperitoneal routes.

In our study, Balb/c mice were immunized by intranasal and subcutaneous routes with $A\beta_{1-15}$ loaded PLGA microparticles. Serum antibody levels against the toxic $A\beta_{1-40}$ fragment were measured by the ELISA technique and compared to those elicited after the administration of the $A\beta_{1-15}$ fragment in a Freund's adjuvant emulsion and in a solution of the peptide alone in phosphate-buffered saline (PBS).

With this strategy our challenge is to develop an easy, safe and effective vaccine in Balb/c mice immunized by subcutaneous and intranasal routes with the B cell epitope $A\beta_{1-15}$ as antigen entrapped into PLGA biocompatible and biodegradable microparticles.

2. Materials and methods

2.1. Reagents

$A\beta_{1-40}$ (molecular weight 4329.8 Da) and $A\beta_{1-15}$ (molecular weight 1826.86 Da) were supplied by Innovagen (Sweden). The polymer poly (D,L-lactide-co-glycolide) (PLGA) (Resomer® RG 503, Mw 33,900) with a copolymer ratio of 50:50 (lactic/glycolic (%)) and an intrinsic viscosity of 0.8 dl/g in $CHCl_3$ was supplied by Boehringer Ingelheim K.G. (Ingelheim, Germany). Bovine serum albumin (BSA), polyvinyl alcohol (PVA) (average MW 30,000–70,000), normal mouse IgG, affinity-purified goat anti-mouse IgG (peroxidase conjugate), Freund's Complete/Incomplete Adjuvant, TMB peroxidase substrate and TMB stop reagent, were supplied by Sigma Chemical Co. (St. Louis, MO, USA). 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

Balb/c mice aged 5 weeks (Harlan Interfauna Iberica S.L., Barcelona, Spain) were randomly divided into four groups of ten and housed in an animal room with free access to food and water throughout the study. All procedures with animals were in compliance with the Public Health Service Policy and supervised by the Institution Standing Committee for Animal Use in Laboratory (Register identification number: CEBA/4/2009/HERNANDEZ MARTÍN).

2.3. Preparation of $A\beta_{1-15}$ PLGA Microspheres

PLGA microspheres were prepared by a modification of the solvent evaporation/extraction technique using a w/o/w double emulsion system previously described (Igartua et al., 1997). Briefly, 200 mg PLGA 50:50 were dissolved in 4 mL dichloromethane and emulsified with 200 μl of a 5% w/v $A\beta_{1-15}$ aqueous solution by probe sonication for 30 s at 50 W (Branson Sonifier® 250). The primary emulsion (w/o) was poured into 20 mL 8% polyvinylalcohol and emulsified for 5 min at 9500 rpm using a turbine homogeniser (Ultraturrax® T-25) in order to obtain a double emulsion (w/o/w). Finally, to favour the removal of the organic solvent from the microparticles to the external phase, 40 mL of 2% aqueous isopropanol solution was added and the system was mechanically stirred for 2 h until dichloromethane evaporation. The resulting microparticles were separated by centrifugation, washed three times with distilled water and freeze-dried for 24 h.

2.4. Particle morphology and size analysis

Particle size distribution was determined by laser diffractometry (Coulter Counter® LS130 particle size analyzer, Amherst, MA, USA). Microparticle morphology was examined by scanning electron microscopy (SEM, Jeol® JSM-35 CF, Japan).

2.5. Determining the loading of $A\beta_{1-15}$ peptide in microparticles

Total peptide loading and surface associated peptide (SAP) were determined using the microBCA assay in a linear working range for peptide concentrations of 5–20 μg/mL. Total peptide loading was evaluated after disrupting the microparticles in 0.2 M NaOH. Surface associated peptide was measured in the supernatant after centrifuging a microparticles suspension in 20 mM PBS maintained under orbital rotation at 37 °C for 30 min.

2.6. In vitro release assay

In order to carry out studies on peptide release from microspheres formulations, 10 mg of microspheres were placed in test-tubes containing 2 mL of 20 mM PBS and incubated at 37 °C under continuous orbital rotation. At regular time intervals up to 98 days, the samples were spun at 10,000 rpm for 10 min. The supernatant was collected and assayed for peptide quantification using the

Table 1
Immunization protocol in groups of mice ($n = 10$) immunized with the $A\beta_{1-15}$ peptide. i.n: intranasal; s.c: subcutaneous; CFA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant. Sol. Solution; MP: microparticles.

Group	Route	Schedule (days)	Formulation
Sol	s.c	0, 14, 28	Solution
Freund's adjuvant	s.c	0, 14, 28	CFA, IFA
MP s.c	s.c	0, 14, 28	Microparticles
MP i.n	i.n	0, 1, 2	Microparticles

microBCA assay. The peptide release study was continued after replacement with the same volume of fresh buffer. The test was performed in three batches of the formulation.

2.7. Immunization protocols

For antibody induction against $A\beta_{1-40}$, 40 female Balb/c mice were randomly divided into four groups of 10 and immunized with three 30 μg doses of the $A\beta_{1-15}$ antigen following the procedures described in Table 1. Blood samples were collected from the submandibular area of the mice at weeks 1, 3, 5, 6, 7, 8 and 9. Samples were centrifuged for 5 min at 3000 rpm. The supernatant was removed and transferred to a new tube. Sera were stored at −20 °C until assayed by ELISA for anti-$A\beta_{1-40}$ IgG.

2.8. Anti-$A\beta_{1-40}$ antibody determination by enzyme-linked immunoabsorbent assay (ELISA)

Serum anti-$A\beta_{1-40}$ antibodies were measured by an indirect ELISA analysis as previously described (Leverone et al., 2003). Briefly, 96-well plates were coated with $A\beta_{1-40}$ peptide (2 μg/mL) in 50 mM carbonate buffer solution, pH 9.6. A standard curve was included in column 1 and 2 by adding serial dilutions of murine IgG from 30.0 to 0.0 ng/mL in the same carbonate buffer solution. The plates were covered and incubated over night at 4 °C. Plates were then washed three times with Tween–PBS. Wells were then blocked with 5% goat serum and 1% BSA in Tween–PBS for 2 h followed by three washes with Tween–PBS. TBS was added to column 1 and 2. The rest of the columns were added with mouse serum samples diluted in blocking solution and incubated for 2 h at RT. The plates were washed three times with Tween–PBS and then incubated for 1 h with goat anti-mouse IgG-HRP at 1 μg/mL. Plates were again washed three times with Tween–PBS, developed with TMB peroxidase substrate for 5 min and stopped with the addition of TMB stop reagent. The OD values were measured at 450 nm on an automatic microplate reader (Multiscan EX, Labsystems, Helsinki, Finland). The mean and standard deviation (SD) of pre-immune sera were calculated on each plate to determinate the percentage of responding mice. Antibody-positive values were set as their mean + 2 fold SD.

2.9. Statistical analysis

Statistical analysis was completed with the InStat programme (GraphPad Software, San Diego, CA, USA). Differences among groups of animals at significance levels of 95% were calculated by the non-parametric Mann–Whitney *U*-test. In all cases, *P* values < 0.05 were regarded as significant. Normal distribution of samples was assessed by the Kolmogorov–Smirnov test.

3. Results

3.1. Particle characterization

The $A\beta_{1-15}$ peptide was successfully entrapped into PLGA microparticles by the w/o/w emulsion solvent evaporation/

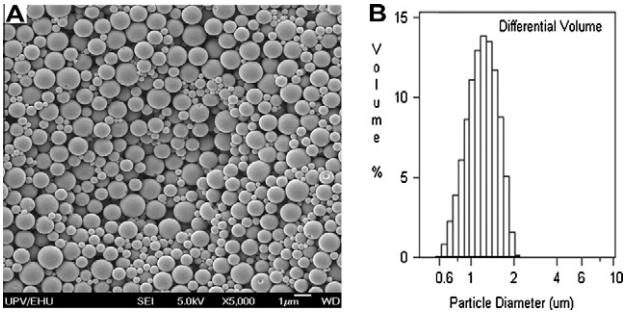

Fig. 1. Morphological characterization of the microparticles. (A) Scanning electron photograph (5000×) of $A\beta_{1-15}$ loaded PLGA microparticles. (B) Size distribution of PLGA microparticles.

extraction process resulting in smooth and sphere-shaped microparticles with a narrow size distribution (Fig. 1). The mean particle size post-lyophilisation was 1.22 ± 0.28 μm. The encapsulation efficiency (the percentage of initial peptide load entrapped into microparticles) and the surface-associated peptide (SAP) were 75.05 ± 4.17% and 59.81 ± 0.96%, respectively.

3.2. In vitro release assay

The next study was the release profile of the antigen from the PLGA microparticles.

As shown in Fig. 2, the release of $A\beta_{1-15}$ peptide followed a typical triphasic profile with an initial fast release "*burst effect*" about 65% of the total peptide loading after 24 h. During the next days a slow release rate was determined, followed by an increased and constant release rate up to the last days, when declined again. At the end of the study, approximately 90% of the total peptide was released.

3.3. Serum antibody response of $A\beta_{1-15}$ loaded microparticles

As featured in Fig. 3, the Freund's adjuvant emulsion elicited detectable antibody levels in 75% of treated mice three weeks after the first immunization, reaching 100% between weeks 5 and 7, and decreasing slightly until 88% at week 9. Mice immunized subcuta-

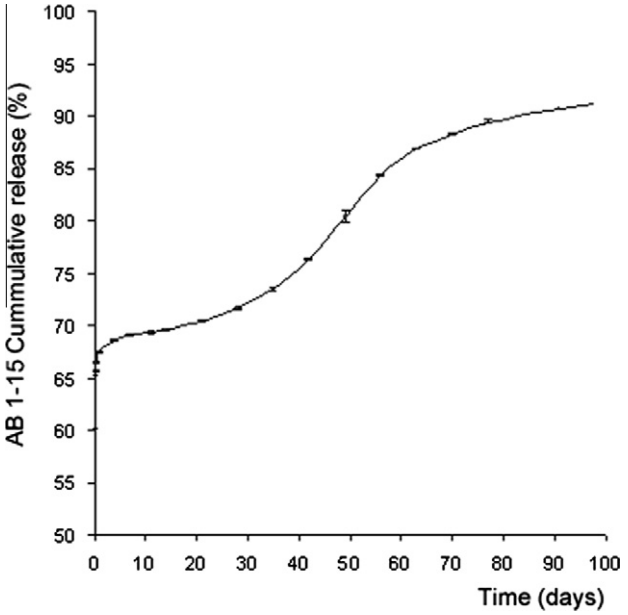

Fig. 2. Cummulative *in vitro* release profile of $A\beta_{1-15}$ peptide from PLGA microparticles. Values are represented as mean (± standard deviation, SD) for three batches of the formulation.

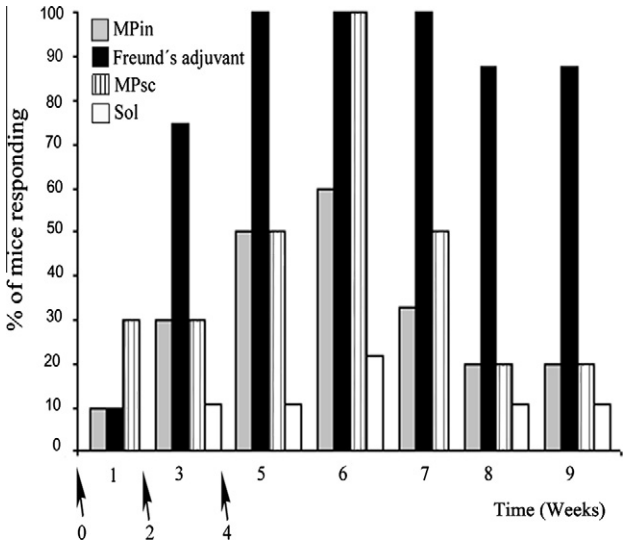

Fig. 3. Proportion of responding mice. Balb/c mice were subcutaneously immunized on weeks 0, 2 and 4 (see arrows on figure) with $A\beta_{1-15}$ microparticles, free peptide ($A\beta_{1-15}$) solution, or an emulsion of the $A\beta_{1-15}$ peptide in Freund's adjuvant. Intranasally immunized mice with $A\beta_{1-15}$ microparticles were immunized on days 0, 1 and 2.

neously with microparticles based formulation elicited detectable antibodies levels in 100% of the mice at 6 weeks after the first immunization, decreasing to a 20% at 9 weeks. Between first and seventh weeks the percentage of responding mice oscillated between 30% and 50%. The percentage of intranasally immunized responding mice was always equal or inferior to that reached by the subcutaneous route at each week of the experiment. The highest percentages, 50% and 60%, were at weeks 5 and 6, respectively. The group of mice immunized with the free $A\beta_{1-15}$ solution, showed a poor response during the study. The maximum percentage of responding animals, 22%, was reached 6 weeks after the first immunization.

Fig. 4 represents anti-$A\beta_{1-40}$ antibody levels determined by ELISA from the serum of all groups of mice immunized with three doses of 30 μg of $A\beta_{1-15}$ peptide. Based on previous studies (Brayden et al., 2001), mice were immunized on weeks 0, 2 and 4 by the subcutaneous route. The group intranasally immunized with microparticles was immunized on days 0, 1 and 2 (Carcaboso et al., 2004). Microparticles based formulations reached higher immune response than the group immunized with the free peptide, however statistically significant differences were only observed between week 6 and 7. In all time point tested, the group of mice treated subcutaneously with microparticles elicited greater antibody levels than the group intranasally immunized with microparticles but the differences were not significant. The Freund's adjuvant group elicited higher immune response in all time point tested than the microparticle based formulations.

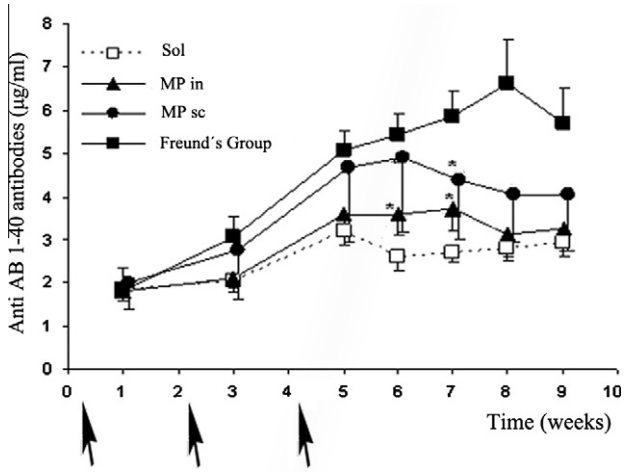

Fig. 4. Serum anti-$A\beta_{1-40}$ antibody levels determined by ELISA in immunized mice. Values given are mean ± SD for 10 mice per group. Differences between the solution group and the groups immunized with microparticles (i.n, or s.c) reached statistical significance at weeks 6 and 7 ($^*P < 0.05$).

4. Discussion

The present study was designed to investigate the immunogenicity of $A\beta_{1-15}$ loaded PLGA microparticles in Balb/c mice immunized by subcutaneous and intranasal routes with three 30 μg doses of the peptide. The results obtained showed that $A\beta_{1-15}$ fragment entrapped into PLGA microparticles enhanced immunogenicity when compared to the solution of the peptide alone in terms of percentage of responding mice and serum antibody levels against the toxic $A\beta_{1-40}$ fragment. Adyuvanticity of microparticles based formulation was compared to that obtained by Freund's emulsion. The PLGA formulation has a significant advantage in terms of adjuvant safety over the Freund's-based formulations. Despite Freund's adjuvants are very potent and have been widely utilized in Aβ immunization protocols (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999), they cannot be safely used in human, as they cause local tissue granulomas and abscesses at the site of injection.

PLGA was selected as delivery system based on the following reasons. Firstly, we have extensive experience in the use of biocompatible and biodegradable PLGA microparticles as delivery system for synthetic peptides and proteins (Gutierro et al., 2002; Mata et al., 2007; Rosas et al., 2002). Secondly, PLGA has been approved in humans as a depot formulation for protein-based products (Plosker and Brogden, 1994). Thirdly, it has been shown that the administration of microencapsulated model antigens often induces a potent and perdurable humoral immune response (Igartua et al., 1998). Finally, PLGA microparticles can release the antigen in a pulsed profile, depending on microparticles size and lactide to glycolide ratios (Cleland, 1999), which can reduce the number of immunizations (Brayden et al., 2001; Rajkannan et al., 2009).

We selected the $A\beta_{1-15}$ peptide as immunogen because numerous studies have reported that the T cell epitope is mapped within the $A\beta_{15-42}$ fragment (Cribbs et al., 2003), while the B cell epitope in humans (Geylis et al., 2005), monkeys (Lemere et al., 2004) and mice (McLaurin et al., 2002) is located within the $A\beta_{1-15}$ region. Such shorter Aβ fragments have been shown to elicit an appropriate immune response when administered in different formulations (Agadjanyan et al., 2005; Hu et al., 2008; Li et al., 2005a; Maier et al., 2006; Moretto et al., 2007; Muhs et al., 2007; Seabrook et al., 2007; Zou et al., 2008). These approaches elicited high antibody levels that recognized the toxic soluble (Agadjanyan et al., 2005) and the aggregated (Moretto et al., 2007) forms of the $A\beta_{1-42,}$ as well as the soluble form of the $A\beta_{1-40}$ fragment (Seabrook et al., 2007). In all the cases studied, Th2 response predominated over Th1. Although highly effective at inhibiting mayor diseases requiring cellular immune response (Finkelman et al., 1990), Th1 pro-inflammatory response may increase the risk of induction an autoimmune reaction and has been linked with autoimmune disease progression and severity (Swanborg, 2001). On the other hand, Th2 response induces B cells to promote the production of antibodies, suppress the activation of Th1 cells and can inhibit experimental autoimmune encephalomyelitis (Aharoni et al., 2000). Experimental evidence indicates that the clearance of Aβ plaques from the brain is dependent on the Aβ antibody and not

on T-cell mediated mechanisms (Cribbs et al., 2003). Thereafter, the induction of Th2 response may be more beneficial and safer than a Th1 response, in order to clear Aβ plaques from the brain and eliminate, or at least attenuate, the potential adverse events initiated by infiltration of auto-reactive T cells and inflammatory-induced cerebral vascular microhemorrhages. Hence, making Aβ immunotherapy successful requires production of strong antibody responses without Th1–type immunity. The experimental data obtained with the $A\beta_{1-15}$ fragment showed that this sequence could be an optimal immunogenic peptide of vaccine against AD. Immunizing with $A\beta_{1-15}$ may be less likely to be recognized as a self-antigen and therefore, safer than full-length Aβ as an immunogen. This is especially relevant because of the cessation of the first Phase2 human clinical trial. In addition, immunizing with $A\beta_{1-15}$ rather than $A\beta_{1-40/42}$ could significantly reduce the cost of immunization, because it's synthesis is less expensive than of 40/42 residue peptide (Leverone et al., 2003).

The $A\beta_{1-15}$ peptide was successfully entrapped into PLGA microparticles by the w/o/w emulsion solvent/evaporation extraction process. When observed under scanning electron microscopy (SEM) the microparticles appeared spherical with a smooth uniform surface and a narrow size distribution as shown in Fig. 1. We obtained $A\beta_{1-15}$ PLGA microparticles of 1.22 ± 0.28 μm diameters. Microparticle sizes obtained in our study differ significantly to those obtained by Brayden et al. (2001) when $A\beta_{1-42}$ was successfully entrapped in PLGA microparticles by the double emulsion technique resulting in either small or large microparticles (3 or 15 μm diameter) depending on the homogenization procedure (Brayden et al., 2001). Larger particle sizes (2–12 μm) were also obtained by Rajkannan et al. (2009), when they microencapsulated by the same w/o/w double emulsion solvent evaporation method the B-cell epitope $A\beta_{1-12}$, the T-cell epitope $A\beta_{29-40}$ and the full $A\beta_{1-42}$ (Rajkannan et al., 2009). Size is one of the parameters that can affect the immune response elicited by the administration of polymeric spheres (Gutierro et al., 2002). It is well accepted that the immunostimulatory activity of microparticle vaccines is based on the engulfed fraction (size dependent), which is transported to the lymph nodes, processed and finally antigen fragments are presented to T helper lymphocytes. The optimum particles size for parenteral immunization of Aβ peptide could be within a wide diameter range of the micron scale. Brayden et al. immunized Swiss Webster mice subcutaneously with 3 or 15 μm diameter $A\beta_{1-42}$ PLGA microspheres (Brayden et al., 2001). The results obtained suggested that antigen encapsulated in 3 μm PLGA microspheres induced slightly increased responses to those of the 15 μm. According to previously published reports (O'Hagan et al., 1993), it seems that there is a optimal size range for the phagocytosis of the spheres by macrophages, which could be from 1 to 2 μm. These cells are well known for their ability to act as antigen presenting cells, so a better phagocytosis could result in a better stimulation of the immune system. Gutierro et al., elicited higher serum IgG antibodies when immunized parenterally Balb/c mice with BSA entrapped in 1 μm diameter PLGA microspheres than with the administration of BSA entrapped in 500 and 200 sized nanospheres (Gutierro et al., 2002). Differences on the total serum IgG response induced by particles of different sizes did not result in differences on the IgG1 or IgG2a-type immune response, suggesting that the antigen processing and presentation is similar in all cases tested for PLGA particles.

The encapsulation efficiency (the percentage of initial peptide load entrapped into PLGA microparticles) was 75.05 ± 4.17%. This percentage is similar to the 70.46% obtained by Rajkannan et al., when $A\beta_{1-12}$ was entrapped into PLGA microparticles with a similar w/o/w double emulsion solvent evaporation process. However, differs from the 60.93% to 65.98% obtained whit the $A\beta_{29-40}$ and the full $A\beta_{1-42}$ (Rajkannan et al., 2009). The hydrophobic nature of the $A\beta_{29-40}$ and the full $A\beta_{1-42}$ peptides may have lesser tendency to be retained in the inner aqueous phase, thereby, the encapsulation efficiency of the peptide decreases as the hydrophobicity increases.

Surface-associated peptide represents the fraction of the peptide initially released from microparticles. Therefore, surface-associated peptide release profile does not depend on the polymeric degradation properties but on its own diffusion and solubility properties. Our high surface-associated peptide percentage obtained (59.81 ± 0.96%) could be due to the high amphiphilicity of the $A\beta_{1-15}$ fragment, since according with our experience, normal values with other synthetic peptides oscillate between 10% and 35% (Carcaboso et al., 2004; Gutierro et al., 2002). It remains unclear whether antigens must be properly entrapped in order to enhance immune response. Entrapped antigen into the PLGA formulation is likely to be more stable given the protection required against aggressive environments and degradation enzymes. However, it has been found that antibody responses were also seen when PLGA microspheres were simple ad-mixed with $A\beta_{1-42}$ (Brayden et al., 2001), suggesting that PLGA may act in part with a similar adjuvant mechanism than aluminium hydroxide for $A\beta_{1-42}$ peptide.

Our next experiment was designed to study the *in vitro* release profile of the $A\beta_{1-15}$ peptide from the PLGA microparticles. As can be observed in Fig. 2, the microparticles formulation containing de $A\beta_{1-15}$ peptide showed a typical triphasic profile. The "sustained release" profile of the antigen from the PLGA microparticles was postulated to be the key to reduce the number of inoculations (Cleland, 1999), and depends both on the molecular weight of the polymer and on the lactide:glicolide ratio (Langer et al., 1997). The high initial $A\beta_{1-15}$ release ("*burst effect*") observed after the first hours (about 65% of the total peptide loading) is usually attributed to the amount of peptide close to the surface (Igartua et al., 1998). Our high calculated surface-associated peptide percentage (59.81 ± 0.96%) could contribute to the high "*burst effect*" observed.

Once $A\beta_{1-15}$ PLGA microparticles were obtained and characterized *in vitro* in terms of size, encapsulation efficiency, surface-associated peptide and peptide release profile, our next step was to evaluate the immune response when administered by subcutaneous and intranasal routes to Balb/c mice (Figs. 3 and 4). We selected subcutaneous and intranasal administrations because both routes have been associated with a predominant Th2 immune response profile appropriated for AD immunotherapy (Agadjanyan et al., 2005; Lemere et al., 2002; Seabrook et al., 2004). In addition, intranasal immunization has the advantages of being painless and easily administered compared to injections.

As featured in Fig. 3, data reveal the advantage of the microparticles administered by subcutaneous or intranasal routes over the administration of the solution of the peptide alone, since at each time point of the experiment the percentage of responding mice was higher in the $A\beta_{1-15}$ loaded microparticle based formulation when compared with the solution of the peptide alone. When microparticles were administered subcutaneously, detectable antibody levels were elicited in 100% of treated mice at six weeks of the first immunization, the same percentage that was found in the group treated with the complete/incomplete Freund's adjuvant emulsion. Our data reveal that the adyuvanticity of microspheres following subcutaneous administration is comparable to that of Freund's complete/incomplete adjuvant as occurred in the Brayden et al. study when they immunized mice with $A\beta_{1-42}$ entrapped in PLGA microspheres (Brayden et al., 2001).

Fig. 4 represents anti-$A\beta_{1-40}$ antibody levels determined by ELISA from the serum of all groups of mice immunized with three 30 μg doses of $A\beta_{1-15}$ peptide in different formulations. In all time point tested, the group of mice treated subcutaneously with microparticles elicited greater anti-$A\beta_{1-40}$ antibody levels than the group

intranasally immunized, but the differences were not statistically significant between both groups. $A\beta_{1-15}$ loaded microparticles (subcutaneous or intranasally administered) elicited higher antibody levels when compared with the peptide alone group, reaching significant differences between weeks 6 and 7. The Freund's adjuvant group overcame the microparticle based formulations in all time point tested. Taken together our data reveal the advantage of the microparticle based formulation over the administration of the peptide alone solution, since percentage of responding mice and serum antibody levels are higher and close to those obtained by the Freund's group.

Interestingly, our proposed immunization protocol detected $A\beta_{1-40}$ antibody levels in the serum of mice immunized with three 30 μg doses of the $A\beta_{1-15}$ peptide, reducing the risks associated to the full-length Aβ, the dose, and the number of immunizations of the regimens proposed by other studies that employed short fragments of the Aβ as antigen (Agadjanyan et al., 2005; Hu et al., 2008; Li et al., 2005a,b; Maier et al., 2006; Moretto et al., 2007; Muhs et al., 2007; Rajkannan et al., 2009; Seabrook et al., 2007; Zou et al., 2008). Almost all of these regimens used multicopies of the $A\beta_{1-15}$ fragment as immunogen in order to improve its poor immunogenicity; either directly synthesized to a new T cell helper epitope or directly linked by a tandem repeat. This might lead to the creation of a new B cell epitope, and so to the production of newly unknown antibodies. Furthermore, the direct linkage could cause the recombination of multiple copies of $A\beta_{1-15}$, and affect its spatial structure and the exposure of the B cell epitope. Additionally, we have found detectable antibodies anti-AB_{1-40} in the serum of mice stimulated by the B-cell epitope $A\beta_{1-15}$, despite the fact that has been demonstrated by epitope mapping studies that the full length $A\beta_{1-42}$ and the T-cell $A\beta_{29-40}$ epitopes showed greater affinity to bind with the $A\beta_{1-42}$ antigen than that of the antibodies induced by the B-cell epitope as the $A\beta_{1-12}$ (Rajkannan et al., 2009).

In conclusion, our study shows for the first time that $A\beta_{1-15}$ can be successfully entrapped into PLGA microparticles by the w/o/w emulsion solvent extraction process. Resulting 1 μm $A\beta_{1-15}$ loaded microparticles enhanced immune response in terms of percentage of responding animals and serum IgG levels against the toxic $A\beta_{1-40}$ fragment when compared to the solution of the peptide alone, reaching immunostimulary properties comparable to the potent Freund's complete adjuvant. Our initial results indicate that immunotherapy with $A\beta_{1-15}$ loaded PLGA microparticles could be a promising approach for the future development of a safe vaccine against Alzheimer's disease.

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