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Encapsulation in biodegradable microparticles enhances serum antibody response to parenterally-delivered β-amyloid in mice

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Abstract

Poly(lactide-co-glycolide) (PLG) microspheres were tested as a parenteral delivery system for human β-amyloid (1–42) (Aβ), a potential immunotherapeutic undergoing assessment in Phase 1 studies for Alzheimer's disease (AD). Aβ was successfully encapsulated in PLG microspheres of average sizes of 3 or 15 μm diameter. Swiss Webster (SW) mice were injected by the sub-cutaneous (s.c.) or intra-peritoneal (i.p.) routes with 3–33 μg Aβ. Aβ-PLG microparticles (3 μm) induced dose-dependent antibody responses, which were maximal at 33 μg Aβ, while Aβ in phosphate-buffered saline (PBS) produced weak antibody responses at the same doses by both routes. Significantly increased antibody responses were seen for both small and large particle formulations given by the i.p. route in comparison to the s.c route. It was previously reported that passive immunisation with Aβ-specific antibodies cleared amyloid plaques in a mouse model of AD (Bard F, Cannon C, Barbour R, et al. Peripherally administered antibodies against amyloid β-peptide enter the nervous system and reduce pathology in a mouse model of Alzheimer disease. Nature Med 2000;6:916–19), an indication that induction of serum antibody is a prerequisite for efficacy.

Keywords: β Amyloid; Poly(lactide-co-glycolide); Alzheimer's disease; Adjuvants

1. Introduction

It is well established that Aβ peptide of 42 amino acids in length is a major component of insoluble β-amyloid plaques seen in the post-mortem brains of Alzheimer disease (AD) patients [1]. Recent evidence from human post-mortem brain tissue suggests that plaque density in the cerebral cortex may correlate with AD progression and that its deposition precedes clinical symptoms [2]. There is therefore a sound rationale to develop methods to prevent fibrillar Aβ formation or to hasten its metabolism to benign forms.

A novel way to successfully prevent Aβ deposition and to hasten clearance is to induce an immune response against Aβ. Using the amyloid β precursor protein expression driven by platelet-derived growth factor (PDAPP) transgenic mouse model of AD which over-expresses the human gene for APP (amyloid precursor protein), Schenk et al. showed that repeated parenteral immunisations of aggregated Aβ administered in CFA/IFA completely prevented plaque deposition when used as a prophylactic treatment [3]. In PDAPP mice that had formed AD-like neuropathology, therapeutic immunisation with Aβ significantly slowed the amount of plaque deposition, astrogliosis and neuritic dystrophy. Recent studies show that several mouse models of AD display memory loss, behavioural impairment and spatial learning deficits [4–6] and that these symptoms can be alleviated by Aβ immunotherapy [5,6].

Despite efficacy of Aβ in the PDAPP mouse, the co-administered CFA and IFA adjuvants are toxic in man. Alum (aluminium hydroxide) is currently the only

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FDA-approved adjuvant for routine human immunisation [7]. Several other promising adjuvants are however in phase II and III clinical trials. Subsequent steps in the development of an immunotherapy for AD may therefore be dependent on identifying new formulations of Aβ in adjuvants and/or delivery systems that have the potential to be approved for human use. A phase 1 study of an Aβ vaccine delivered by injection to AD patients with an as yet undisclosed adjuvant is currently underway with results due in 2001.

In the current study we formulated Aβ in PLG for testing as a parenteral delivery system. The rationale is based on a 20-year history of injected immunisation of mice and primates with antigens in PLG (e.g. [8–11]) and reviewed recently in [12]. Moreover, immunisations with antigens entrapped in PLG appears to have the potential to stimulate cell mediated (Th1 type) immunity [13] compared to the mixed Th1 and humoral (Th2 type) or polarised Th2 type immunity normally associated with alum. Injected PLG-entrapped antigens may also have the potential of simulating multiple immunisation without the need for boosters as a consequence of timed release of antigen in vivo (reviewed in [14]). In addition, PLG is approved in man for a number of peptide-based products including a monthly depot form of leuprolide acetate for prostate cancer and for endometriosis (Lupron Depot®) [15] and also for use as surgical sutures [16]. PLG manufacturing processes have been successfully scaled up, for example, for human growth hormone [17]. For these reasons PLG might be a good delivery system to take to the clinic should it show promise in animal studies.

In this study SW mice were immunised parenterally with microparticle formulations of Aβ in PLG and serum antibody responses assessed by ELISA. Serum antibody was shown to be a key correlate for efficacy in the PDAPP mouse since passive immunisation with Aβ antibodies can penetrate into the CNS and activate microglia to clear pre-existing plaques [18]. SW mice were therefore used as an initial immunological screen for candidate Aβ delivery systems in advance of the lengthy experiments required for subsequent demonstration of efficacy in the PDAPP mouse.

2. Materials and methods

2.1. Chemicals

Human Aβ (1–42) (molecular weight 4514 Da) was supplied by California Peptide Research Inc. (Napa, CA) (Lot MF0639) or by the American Peptide Company (Sunnyvale, CA) (Lot 5108TT) through automated peptide synthesis. PLG Resomer RG504 (50:50 lactide:co-glycolide, molecular weight 57000 Da, i.v. 0.49 dl/g) (Lot numbers 34014 and 35011) was supplied by Boehringer-Ingelheim (Frankfurt, Germany). Dichloromethane was obtained from Labscan (Cork, Ireland). Poly-vinyl alcohol (PVA) USP (85–89% hydrolysed) Lot NCO166 was supplied by Spectrum Quality Products (New Jersey, USA). PVA (Poval 217), lot number 481747, was obtained from Kuraray (Tokyo, Japan). ELISA 96 well plates were from Costar (Amsterdam, Netherlands) and the plate reader was from Molecular Devices (USA). For the Aβ ELISA, 21F12 and 3D6 monoclonal antibodies to Aβ were raised at Elan Pharmaceuticals (USA). Avidin-horseradish peroxidase was from Vector Laboratories. Peroxidase-conjugated goat anti-mouse IgG was from Chemicon (USA).

2.2. Preparation of Aβ-PLG microparticles

Microparticle formulations were prepared using a modification of the water-in-oil-in-water (w/o/w) double emulsion solvent evaporation process [19]. The hydrophobic human peptide Aβ (1-42) was dispersed in PBS at pH 7.4 to give a hazy monomeric suspension which was either used immediately ('fresh'), or stored overnight to produce an aggregated fibrillar form ('aggregated'). Aggregated Aβ is a mixture of oligomers in which the monomeric units are held together by non-covalent bonds. Alternatively, the peptide was dissolved in ammonium hydroxide (0.01 N) at pH 10 ('solution'). Under this condition, the Aβ is a monomeric soluble clear mixture of peptide units.

To prepare microparticles, approximately 0.8 g or 2.4 g PLG (Resomer® RG504 polymer) was dissolved in an appropriate volume of dichloromethane to give a 4% w/v polymer organic solution and stirred for 120 min. A water-in-oil emulsion was prepared by adding the resulting Aβ aqueous suspension or solution to the organic phase, followed by homogenisation at 9500 rpm for 1 min with a S 25 homogeniser. The primary emulsion was poured slowly into a solution of 240 ml of 3% w/v Poval PVA. For some batches of Aβ in PLG, 2% w/v PVA (US Pharmacopoeia) was used as the stabiliser for the secondary emulsion instead of Poval PVA and this led to higher overall loading (Table 1). Homogenisation was carried out at 13500 rpm for 2–6 min to produce small microparticles of average diameter of 3 μm. For large 15 μm microparticles homogenisation speed was 8000 rpm for 6 min. For both large and small particles the resulting water-in-oil-in-water secondary emulsion was stirred overnight to evaporate the dichloromethane, and microparticles were collected by centrifugation (15 min, 8000 rpm). The microparticles were then washed twice with chilled autoclaved de-ionised water, dried in a vacuum oven at room temperature for 72 h. For the formulation in which Aβ was ad-mixed with PLG, 1 g of unloaded small microparticles was blended with 2.5 ml of Aβ-ag-

gregated suspension (2 mg/ml) and dried for 60 min at room temperature. Upon resuspension in PBS, no Aβ was located in the supernatant following centrifugation (5 min, 3000 rpm) indicative of efficient adsorption to the surface of the polymer.

2.3. Particle morphology and size analysis

PLG microparticle morphology was examined by scanning electron microscopy. Samples were mounted on stubs and coated using a Polarian CE500 gold electron microscope coater. Samples were observed with a Hitachi 5-4300 field emission electron microscope. Particle size was determined by laser diffractometry using a Malvern Mastersizer S version 2.14. Duplicate samples were dispersed in filtered Tween-20 (0.1%w/v) and were sonicated for 5 min followed by analysis in a continuously stirred cell.

2.4. Loading of Aβ in microparticles

Total protein loading of PLG microspheres was determined using a micro-titer plate micro-bicinchoninic (μBCA) assay protocol (Pierce, Rockford, IL) [20]. In triplicate, 10 mg of Aβ-loaded particles were added to a 3-ml solution of 1% sodium dodecyl sulphate in NaOH (0.1 M) and stirred on a shaker at 50 rpm for approximately 24 h at room temperature. The pH was then adjusted to pH 11.0 with hydrochloric acid (0.1 M) and the total protein content determined.

Aβ-specific loading was determined by a sandwich ELISA (modified from [21]). In brief, approximately 10 mg of Aβ-loaded microparticles was dissolved in a 3-ml solution of 1% w/v Tween-20 in 0.1 M NaOH at room temperature on a shaker at 50 rpm for approximately 24 h. Aβ samples and standards were then diluted 1:100 with 5 M guanidine/1% bovine serum albumen (BSA) followed by a 1:10 dilution with casein blocking buffer, comprising 0.25% casein and 0.05% sodium azide in PBS. The standards and samples had a final concentration of 0.5 M guanidine and 0.1% BSA.

The monoclonal antibodies 21F12 (Aβ 33–42) and biotinylated 3D6 (Aβ 1–5) were used as the capture and detector antibodies respectively in the ELISA. The 21F12 antibody was coated at 10 μg/ml into 96-well immunoassay plates overnight at room temperature. The plates were then aspirated and blocked with 0.25% human serum albumen in PBS for at least 60 min at room temperature. Following storage at 4°C under desiccation, the plates were then re-hydrated with wash buffer containing 0.05% Tween-20 in Tris-buffered saline. The samples and standards were added to the plates and incubated at 4°C overnight. The plates were washed at least three times with wash buffer between each step. Biotinylated 3D6 antibody was diluted to 2.5 μg/ml in a buffer containing 0.25% casein, 0.05% Tween 20 and 0.05% thimerosal. 3D6 antibody was then mixed in 0.05% Tween-20 in PBS at pH 7.4 and was incubated in the wells for 60 min at room temperature. Avidin-horseradish peroxidase was diluted 1:4000 in casein buffer, and added to the wells for 60 min at room temperature. The colorimetric substrate Slow TMB (3,3,'5,5' tetramethyl benzidine) — ELISA® (Pierce), was added and allowed to react for 15 min, after which the enzymatic reaction was stopped with addition of 1 M H_2S0_4. Reaction production was quantified using a Dynatech MR7000 spectrophotometer measuring the difference in absorbance at 450 and 650 nm.

2.5. Immunisations

SW mice (8–12 weeks old) were injected by the s.c. or i.p. routes with 200–250 μl of Aβ in PLG microparticle suspensions or with Aβ ad-mixed with PLG in sterile PBS. Doses of Aβ in PLG ranged from 3 to 33 μg per immunisation per mouse. Mice received either 33 μg Aβ in PBS or Aβ emulsified in CFA/IFA as negative and positive controls, respectively. Dosing of each formulation was carried out based on the actual amount of Aβ entrapped as measured by ELISA. For the CFA/IFA control, 33 μg Aβ was emulsified 1:1 (v/v)

Table 1
Characteristics of Aβ-PLG formulations

Formulation	Batch wt. (g)	Total protein (μg/mg)	Aβ ELISA (μg/mg)	% EE[a]	$D_{50\%}$ [b] (μm)
3 μm PLG[c]	0.8	Not determined	1.1 ± 0.2	23	2.7 ± 0.3
3 μm PLG[d]	2.4	1.4 ± 0.2	1.2 ± 0.1	22	2.5 ± 0.1
3 μm PLG[e]	2.4	4.0 ± 0.2	3.4 ± 0.2	68	3.1 ± 0.1
15 μm PLG[e]	2.4	4.0 ± 0.3	3.0 ± 0.1	60	14.8 ± 0.4

[a] % EE: % entrapment efficiency, i.e. actual loading of antigen divided by the theoretical loading (i.e. 0.5% w/w). Higher Aβ loading for the formulations (used in study referred to in Fig. 4) were achieved with 2% w/v PVA (US Pharmacopoeia) instead of 3% w/v Poval PVA.

[b] $D_{50\%}$: 50% of the particles were less than this size.

[c] Used in study referred to in Fig. 2.

[d] Used in study referred to in Fig. 3

[e] Used in study referred to in Fig. 4.

with CFA alone for the first dose and then emulsified similarly with IFA for subsequent doses. Mice were immunised on days 0, 14 and 28 and bled on days 21 and 35 in two studies (Figs. 2 and 4). In a confirmatory study (Fig. 3), an extra dose was given at day 42 and subsequent bleeds were taken at days 49, 70 and 84. The mice studies adhered to the 'Principles of Laboratory Animal Care' (NIH publication no. 85-23, revised in 1985).

2.6. Anti-Aβ serum antibody detection

Aggregated $A\beta_{1-42}$ (100 μg) was added to 10 ml of 0.1 M phosphate buffer (1.7 mM NaH_2PO_4 and 98 mM Na_2HPO_4) at a pH 8.5. After mixing, 100 μl was added to each well of Costar 96 well ELISA plates. The plates were covered and incubated over night at room temperature. The wells were then aspirated to near dryness and washed. Specimen diluent (100 μl) containing BSA (0.6%), Triton 405 (0.05%), thimerosol (0.5%) was added to all wells except row A. Specimen diluent (150 μl) was added to row A. Negative control, positive control and test sera (1.5 μl) were added to appropriate wells in row A to give an initial 1/100 dilution. Serial dilutions resulted in 100 μl volume in the remaining wells. Plates were incubated for 1 h at room temperature and then washed four times with standard wash buffer containing 0.05% Tween-20 in Tris-buffered saline. One hundred microlitres of a 1/3000 dilution of peroxidase-conjugated goat anti-mouse IgG in specimen diluent was added to each well. After incubation for 1 h at room temperature, plates were washed four times in wash buffer. Pierce Slow TMB® (100 μl) was then added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 25 μl of 2 M H_2SO_4 and plates were read at 450 nm. Anti-Aβ serum IgG antibody titres were defined as the reciprocal of the dilution of serum giving one half the maximal optical density. Any samples not reaching 50% by the 218700 dilution were re-tested at higher dilutions.

2.7. Statistical comparisons

Comparisons of serum total antibody titres between immunised groups at the same or at different points were performed using one-way ANOVA by the INSTAT programme (GraphPad™, San Diego). In all cases, P values < 0.05 were regarded as significant.

3. Results

3.1. Characterisation of Aβ-PLG microparticles

The double emulsion process resulted in either small or large microparticles containing Aβ dependent on the homogenisation. Both types of particle were shown by scanning electron microscopy to be discrete and spherical (Fig. 1). The PLG formulation characteristics of the small and large microparticles are given in Table 1.

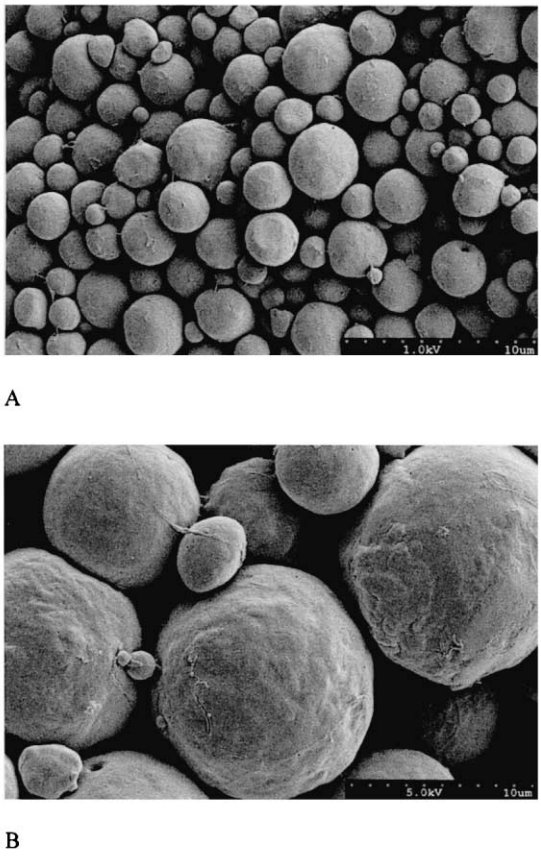

Fig. 1. Scanning electron micrographs of Aβ in PLG microparticles. Small (A) and large (B) particles are shown. Horizontal bars are 10 μm in each case.

Small microparticles had an overall mean diameter of 2.8 μm. Increasing the batch size from 0.8 to 2.4 g necessitated an increase in the total homogenisation time from 2 to 6 min in order to maintain the same particle size. Assessment of antigen content by ELISA studies showed that > 1 μg/mg of antigenically-active Aβ was entrapped in each of three batches made (Table 1). Altering the PVA grade and concentration from 3% w/v Poval to 2% w/v PVA USP was associated with a mean increase in Aβ loading from 1 to 3 μg/mg. The ELISA results obtained for the separate formulations used in each experiment correlated with total protein loading. This would suggest that $> 80\%$ of the suspension or solution loaded into small PLG microparticles was antigenically-active even in the absence of known protein stabilisers. The average total protein loading and average Aβ content of the larger particles was 4 and 3 μg/mg, respectively.

3.2. Serum antibody responses in mice to Aβ entrapped in PLG microparticles

Aβ entrapped in small 3 μm PLG microparticles at a dose of 33 μg induced anti-Aβ antibody responses after two s.c. immunisations. These titres were boosted after a third immunisation (Fig. 2). Each PLG formulation produced significant Aβ-specific serum titres, irrespective of whether the Aβ was in 'fresh', 'aggregated' or 'solution' form. The formulation in which 'fresh' Aβ was ad-mixed with PLG gave statistically lower responses at the first time point than 'fresh' Aβ encapsulated in PLG, but antibody responses were subsequently elevated to levels equivalent to entrapped PLG by the third bleed. Importantly, in this experiment the overall antibody responses at both time points to all four Aβ-PLG formats were no different than those detected for the positive control, 33 μg of Aβ in CFA/IFA (group E, Fig. 2). The formulation comprising small microparticles entrapping 'fresh' Aβ at pH 7.4 was selected for further testing.

Splenocytes were harvested from immunised mice at day 50 from the experiment cited in Fig. 2. Following culture for 48 h, the supernatants were harvested. The levels of interferon γ, interleukin-2 and interleukin-5 were determined by cytokine-specific ELISA as quantifiable markers of antigen-specific Th1 and Th2 T cell populations. Cytokine analysis showed moderate expression for all PLG formulations and low expression for Aβ in CFA/IFA (results not shown).

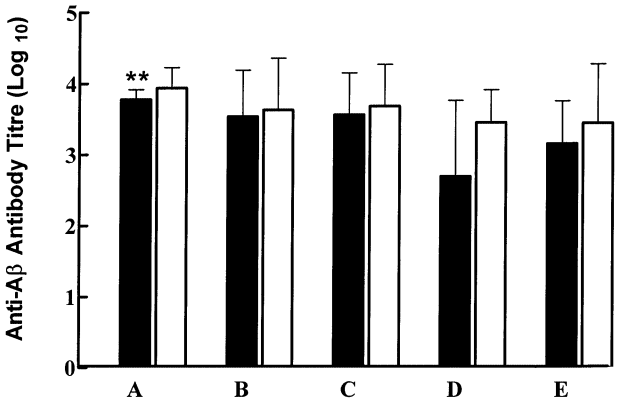

Fig. 2. Serum antibody responses to Aβ in PLG. Mice were immunised (s.c.) with 33 μg Aβ total dose on each of days 0, 14 and 28 and bled on days 21 and 35. Values given are means ± standard deviations with $n = 8$ in each group. Filled bars are day 21 and open bars are day 35. (A) Aβ in PLG ('fresh'), (B) Aβ in PLG ('aggregated'), (C) Aβ in PLG ('solution'), (D) Aβ mixed with unloaded PLG ('mixed'), (E) Aβ in CFA/IFA (positive control). Description of the different formats of Aβ used is in Section 2 and the formulation specifications are given in Table 1. (A) gave higher responses than (D) only at day 21 (** $P < 0.01$).

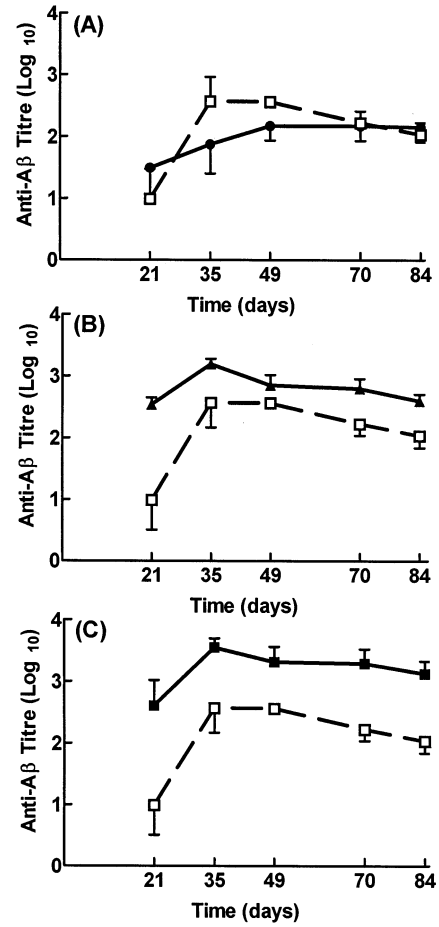

Fig. 3. Effect of immunising dose on antibody response to Aβ in PLG. Mice were immunised (s.c.) with Aβ ('fresh') in small microparticles on days 0, 14, 28 and 42, and bled on days 21, 35, 49, 70 and 84. Values given are mean ± standard deviations with $n = 8$ in each group at each time point. One way ANOVA comparison for average response of individual mice in each group over time: (A) □, Aβ in PBS (33 μg) vs. ○, Aβ in PLG (3 μg): NS. (B) □, Aβ in PBS (33 μg) vs. ▲, Aβ in PLG (10 μg) $P < 0.02$. (C) □, Aβ in PBS (33 μg) vs. ■, Aβ in PLG (33 μg) $P < 0.005$. Unloaded PLG microparticles and PBS/IFA solution controls ($n = 8$) gave no responses (data not graphed).

3.3. Effect of Aβ dose on the immune response to PLG-entrapped antigen

Having established that 33 μg Aβ entrapped in small PLG microparticles induced potent detectable serum antibodies after two immunisations, we then assessed the response to a range of doses of Aβ in PLG. A dose–response relationship was seen for 3, 10 and 33 μg Aβ in small PLG microparticles using 'fresh' antigen (Fig. 3). Responses to 33 μg Aβ in PLG were significantly increased by 5-8 fold over those seen to the same dose of peptide in PBS, (Fig. 3C), confirmation of the adjuvancy of the small PLG microparticle. Responses to 3 μg Aβ-PLG were weak and were not statistically different to those seen for 33 μg Aβ in PBS (Fig. 3A). The responses induced by 10 μg Aβ in PLG across all

timepoints taken together were statistically increased compared to the antibody level induced by 33 μg Aβ in PBS (Fig. 3B), suggesting that the threshold dose for response is between 3 and 10 μg Aβ in PLG. The responses induced by 10 μg Aβ in PLG were only slightly lower than those seen with 33 μg Aβ in PLG. The responses to the encapsulated antigen at the highest dose were present at 6 weeks after the fourth immunisation (Fig. 3C).

3.4. Effect of parenteral route of administration and particle size on the antibody response to Aβ entrapped in PLG

We examined whether Aβ-loaded large microparticles of average size 15 μm in diameter could give superior antibody responses to the 3 μm microparticles loaded with Aβ when mice were immunised with at a dose of 33 μg Aβ. The results suggest that antigen encapsulated in the small microparticles induced slightly increased responses to those of the larger microparticles at both day 21 and 35 when given by either the i.p. or the s.c. routes, respectively (Fig. 4), but this was not statistically significant. Antibody responses to Aβ in either particle sizes were however significantly increased when given by the i.p. route in comparison to the s.c. route (Fig. 4). The serum titres for the free antigen injected in PBS by the i.p. route (Fig. 4A) were weak and similar

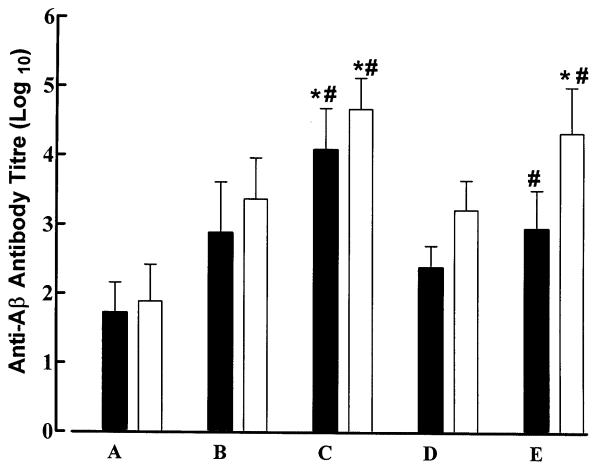

Fig. 4. Comparison of large and small microparticles encapsulating Aβ administered by different routes over time. Mice were immunised (s.c. or i.p.) with 33μg Aβ ('fresh') on days 0, 14 and 28, and bled on days 21 and 35. (A) Aβ in PBS (i.p.), (B) Aβ in 3 μm PLG (s.c.), (C) Aβ in 3 μm PLG (i.p.), (D) Aβ in 15 μm PLG (s.c.), (E) Aβ in 15 μm PLG (i.p.). Values given are means ± standard deviations with $N = 7–8$ in each group. Filled bars are day 21 and open bars are day 35. Unloaded 15 μm PLG control microparticles ($n = 8$, i.p. route) gave no responses (data not graphed). * $P < 0.01$ for Aβ-PLG given by the i.p route (C, E) in comparison to the same PLG formulation given by the s.c. route (B, D) at the same time points. # $P < 0.001$ for Aβ-PLG given by the i.p route (C, E) in comparison to Aβ in PBS given by i.p. route (A) at each of the two matched time points.

to those seen to Aβ in PBS by the s.c. route (Fig. 3A). The effectiveness of the particle as an adjuvant for Aβ was again clearly detected since both types of PLG entrapped formulations induced significant antibody responses when administered by the i.p. route at both timepoints. The antibody responses for Aβ entrapped in large and small microparticles by either the i.p. or s.c. routes were significantly enhanced over those detected to Aβ in PBS by either route.

4. Discussion

This study has demonstrated that immunisation of mice with β-amyloid (1–42) can stimulate strong antibody responses when encapsulated in PLG microparticles. In the first report demonstrating that immunisation with Aβ attenuated AD-like pathology in the PDAPP mouse [3], the adjuvant combination used was CFA followed by IFA given in regular injections over twelve months. Unfortunately, despite very potent efficacy, Freund's-type adjuvants cannot be used safely in man as they have a propensity to cause local tissue granulomas and abscesses at the site of injection [22].

PLG was selected as a possible delivery system for the following reasons. Firstly, it has a history of approval in man as a depot for protein-based products [15]. Secondly, PLG has also been successfully tested in animals as a vaccine adjuvant by injected [8–14] and by mucosal routes [23,24]. Thirdly, there is consensus that immune responses at the level of serum antibody can be obtained for antigens delivered in injected PLG microparticles which are of the same order as those achieved with the same dose of particular antigen adsorbed to alum [25,26]. Fourthly, injected PLG formulations have been shown to induce either polarised T helper cell type 1 (Th1) or mixed Th1/Th2 responses, dependent on the antigen, dose and route of administration [13,27]. In contrast, alum-based vaccines have a tendency to stimulate polarised Th2 responses [28], a feature that would favour its use in vaccines directed against extracellular pathogens including certain parasites and many bacteria, but less so against intracellular pathogens such as viruses and mycoplasma. Finally, an important feature for PLG microspheres is that they can release antigen in a pulsed format from one formulation administered in a single dose, dependent largely on blends of microsphere sizes and ratios of poly(lactide) to glycolide [11,14,29]. In-built formulation features that can mimic the required booster formats offer potential for reducing the number of immunisations. In the case of Aβ in PLG, an encouraging finding therefore was that serum titres were present 6 weeks after a total of four immunisations, an improvement in the dosing schedule over the multiple injections required for the antigen in CFA/IFA in the PDAPP mouse [3].

In SW mice we have confirmed that an adjuvant is useful in conjunction with Aβ since the antigen gave weak antibody responses even at doses as high as 33 μg in PBS administered by either the s.c. or the i.p. routes. By comparison, mice injected by either the s.c. or i.p. routes with 1μg of potent immunogens in solution, e.g. inactivated pertussis toxin and filamentous haemagglutinin, will mount a vigorous antibody response 2 weeks after two immunisations, even in the absence of an adjuvant [30].

Both the low loading and the low loading efficiency of Aβ in both sizes of particle were typical of that seen for most peptides encapsulated in PLG [31]. Altering the PVA grade and concentration was associated with an increase in antigen entrapment in particles and this was attributed to an increase in the stability of the secondary emulsion. Increased loading efficiency may be achieved by altering the solvent or by using a different method of preparation such as spray-drying. Upon injection, Aβ-PLG microparticles gave responses that were comparable to those seen with CFA/IFA after an equivalent number of doses. These data are similar to that seen by others for model antigens such as bovine serum albumen in PLG by the s.c. route in which the adjuvancy of the microspheres was also reported to be equivalent to that seen with CFA [32]. In agreement with Johansen et al. [33], the overall immune responses to the larger 15 μm diameter particles were not better than those achieved with the 3 μm size particles. It would appear therefore that the optimum particle size for parenteral immunisation for Aβ is within a wide diameter range of the micron scale. The fact that the smaller sized particles tended to induce a slightly increased response could be due to a number of factors, including increased particle uptake in the 3 μm size range by macrophages, increased surface area for antigen presentation and a faster release rate.

Antibody responses were also seen when PLG microparticles were simply ad-mixed with Aβ and this would suggest that externally-adsorbed antigen retains immunogenicity. Whether antigens must be properly entrapped in order to detect an enhanced immune effect upon injection is unclear. The results of the current study agree at least in part with those reported for antigen adsorbed to a polylamellar-substrate PLG particle in which significant antibodies titres have been detected for parenterally-administered adsorbed antigen [34]. While surface-bound antigen may make a significant contribution to the immune response to Aβ, the entrapped formulation is likely to be more stable given the protection offered by the particle. It is possible that a combination of PLG-entrapped Aβ with a surface adsorption process may yield more potent immune responses than the entrapped formulation reported here. The preliminary data would suggest that, similar to when other antigens are adsorbed to aluminium hydroxide, PLG may act in part as a with a similar adjuvant mechanism for Aβ since physical entrapment was not an absolute requirement to generate an antibody response. This has been shown clearly in the case of nasally-administered liposomes in which successful immunisation was achieved, not only with an influenza antigen entrapped in liposomes, but also when free antigen was administered nasally even at later time-points than control un-entrapped liposomes [35]. Thus, particles such as PLG microspheres may have a dual mechanism of enhancing responses to entrapped antigens, the first based on co-delivery of antigens into antigen presenting cells for immuno-stimulation and the second based on a classical adjuvant alum-like depot sustained release mechanism for Aβ. Yet preliminary data using Aβ adsorbed to aluminium hydroxide revealed a surprisingly poor adjuvant effect in mice (results not shown), so it is apparent that few assumptions or predictions can be made with confidence about immunotherapy with this antigen.

The adjuvant effect of PLG was detected in 76/80 (95%) of mice immunised with 10–33 μg Aβ-PLG in various formats. Mice immunised with antigen in PBS gave highly variable responses, which were significantly lower than for PLG-entrapped material at comparative doses. Administration by the i.p. route gave significantly higher titres than by s.c., and this is most likely due to a higher density of dendritic cells in the peritoneal cavity. The rationale for dosing by the i.p. route arose as a result of previous work [14] in which it was shown that it was a particularly effective route for stimulating immune responses for antigens in PLG.

The Aβ formulation in CFA/IFA adjuvant has been shown to slow plaque deposition, neuritic dystrophy and gliosis in the PDAPP mouse [3]. In the PDAPP mouse, major histocompatibility complex class II molecules in microglia and monocytes were activated by the Aβ vaccine [18,3]. Furthermore, Tan et al. [36] have shown that freshly solubilized Aβ can increase CD40 expression on cultured microglia from mice. It is likely therefore that antibody may be transported through the blood–brain barrier from the serum as whole antibody or active fragments [37] possibly resulting in an association with microglia adjacent to amyloid fibrillar plaques. Recently Bard et al. [18] showed that passive immunisation with polyclonal and monoclonal antibodies against Aβ could reduce plaque burden upon therapeutic immunisation of the PDAPP mouse, an effect which may be mediated through Fc microglial receptor-mediated endocytosis. This result is pertinent to our data with PLG in SW mice, since it strongly indicates that serum antibody induction by Aβ is a prerequisite for efficacy.

In conclusion, these studies show for the first time that Aβ in PLG microparticles produces a significant antibody response in mice after parenteral administra-

tion. It was confirmed that both small and large microparticles were effective by two parenteral routes and that surface-bound Aβ may contribute in part to the overall response. The PLG formulation of Aβ has a significant advantage in terms of adjuvant safety over the Freund's-based formulation used in our previous Aβ vaccine studies in PDAPP mice [3]. It remains to be shown if Aβ-PLG microparticles have comparable efficacy in this animal model of AD. Because of the relationship between serum antibody and efficacy in mice, our current approach in decision making for progressing to man is to examine adjuvant/delivery system formulations of Aβ in primates using serum antibody as the read-out.

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