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Neurobiology of Aging 28 (2007) 813–823

NEUROBIOLOGY OF AGING

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Dendrimeric Aβ1–15 is an effective immunogen in wildtype and APP-tg mice

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Received 23 February 2006; received in revised form 3 April 2006; accepted 9 April 2006
Available online 24 May 2006

Abstract

Immunization of humans and APP-tg mice with full-length β-amyloid (Aβ) results in reduced cerebral Aβ levels. However, due to adverse events in the AN1792 trial, alternative vaccines are required. We investigated dendrimeric Aβ1–15 (dAβ1–15), which is composed of 16 copies of Aβ1–15 peptide on a branched lysine core and thus, includes an Aβ-specific B cell epitope but lacks the reported T cell epitope. Immunization by subcutaneous, transcutaneous, and intranasal routes of B6D2F1 wildtype mice led to anti-Aβ antibody production. Antibody isotypes were mainly IgG1 for subcutaneous or transcutaneous immunization and IgG2b for intranasal immunization, suggestive of a Th2-biased response. All Aβ antibodies preferentially recognized an epitope in Aβ1–7. Intranasal immunization of J20 APP-tg mice resulted in a robust humoral immune response with a corresponding significant reduction in cerebral plaque burden. Splenocyte proliferation against Aβ peptide was minimal indicating the lack of an Aβ-specific cellular immune response. Anti-Aβ antibodies bound monomeric, oligomeric, and fibrillar Aβ. Our data suggest that dAβ1–15 may be an effective and potentially safer immunogen for Alzheimer's disease (AD) vaccination.

Keywords: Vaccine; Aβ immunization; Alzheimer's disease; Intranasal; Transcutaneous; Subcutaneous

1. Introduction

Alzheimer's disease (AD) is characterized by the deposition of cerebral amyloid-β (Aβ) protein, neuritic plaques, glial activation, and neurofibrillary tangles composed of phosphorylated tau [45]. Epidemiologic, pathologic, and genetic evidence demonstrates that Aβ has a pivotal role in the pathogenesis of AD [20]. In a seminal study, Schenk et al. demonstrated that immunizing PDAPP-transgenic (tg) mice by intraperitoneal injection with aggregated Aβ1–42 peptide and adjuvant resulted in the lowering of cerebral Aβ [42]. This was followed by our report of reduced cerebral Aβ levels in PDAPP-tg mice following intranasal immunization with Aβ1–40 peptide [29,54]. Soon thereafter, several reports demonstrated the importance of antibody-mediated clearance of Aβ and its role in improving cognition [4,11,21,36]. In addition, anti-Aβ antibodies have been induced using various adjuvants [8,25,31], DNA immunization [16,55], and intranasal immunization [28,48]. Together these encouraging animal data led to a multi-center Aβ vaccine clinical trial (AN1792) that was halted when approximately 6% of the subjects experienced symptoms of meningoencephalitis [17,39,41]. Interestingly, three autopsy case reports from subjects who received Aβ vaccination demonstrated brain regions with strongly reduced numbers of plaques compared to controls [13,32,38]. However, T cell infiltrates were present in the leptomeninges, perivascular spaces, and brain parenchyma in two cases, suggesting a T cell-mediated immune response to the Aβ1–42 vaccination. Therefore, Aβ immunotherapy has potential to clear Aβ in humans but more research is required to determine why a subset of patients

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doi:10.1016/j.neurobiolaging.2006.04.007

experienced adverse outcomes and how to avoid such events in future trials.

The B cell epitope(s) in humans [15], monkeys [26], and mice [1,29,34] is located within the Aβ1–15 region, whilst the T cell epitope has been mapped within Aβ15–42 [8,35]. Thus, Aβ fragments spanning the B cell epitope but not the T cell epitopes may be safer, as a potentially deleterious anti-Aβ cellular immune response may be avoided. There have been reports that shorter Aβ fragments stimulated a humoral immune response when conjugated to T cell helper epitopes [3], expanded with the addition of lysine residues [47], mutated [46] or presented as multiple copies [56]. However, there is a paucity of data regarding the T cell response to these immunogens.

The purpose of the current study was to determine if multiple copies of a short fragment of Aβ (dendrimeric Aβ1–15) would result in a humoral immune response, whilst avoiding a T cell immune response. Dendrimeric vaccines are composed of multiple copies of a peptide on a branched lysine core [2,6,51]. These peptides have the advantage of increasing the molecular weight and the number of epitopes present in the immunogen as well as reducing degradation of the short single Aβ1–15 peptides. We examined the use of dAβ1–15 immunization when administered intranasally (i.n.), subcutaneously (s.c.) and transcutaneously (TCI) in both wildtype and J20 APP-tg mice.

2. Methods

2.1. Animals

Eight-week-old B6D2F1 male mice were obtained from Taconic Farms (Germantown, NY, USA), with four mice in each treatment group. Heterozygous J20 APP-tg mice (APP_{sw} and $_{V717F}$) under control of the platelet-derived growth factor (C57BL/6 X DBA2 background) [37] were from our breeding colony. Vaccination was begun at 6.1 ± 0.1 months of age, during the initial stages of Aβ plaque deposition in brain, with five to six mice per treatment group. Mice were genotyped using PCR. All animal use was approved by the Harvard Standing Committee for Animal Use and in compliance with all state and federal regulations.

2.2. Immunization

Aβ1–40, Aβ1–42, and dAβ1–15 peptides were synthesized by the Biopolymer Laboratory, Center for Neurologic Diseases (Boston, MA, USA). The dendrimeric Aβ1–15 (dAβ1–15) immunogen consisted of 16 copies of the first 15 amino acids of Aβ on a lysine backbone. The dAβ1–15 peptide was diluted in distilled water at either 4 or 8 mg/ml. Circular dichroism analysis demonstrated a random structure, without α or β sheet structures (data not shown). A mixture of Aβ1–40 (3 mg/ml) and Aβ1–42 (1 mg/ml) in distilled water was incubated overnight at 37 °C. Synthetic Aβ1–42 assembles into a variety of structures in aqueous buffers, including low *n*-oligomers, ADDLs, protofibrils, and fibrils [53]. The solutions of synthetic Aβ used in this study probably contained a mixture of these assemblies, but biophysical analysis was not performed to determine the presence or relative abundance of these species. The immunogens were aliquotted and frozen at −20 °C. The adjuvant, mutant heat labile *Escherichia coli*, LT(R192G) (kind gift of J. Clements, Tulane University School of Medicine, New Orleans, LA, USA) [10], was mixed with immunogen just prior to immunization. Mice in control groups received either an equivalent dose of LT(R192G) or water alone, both without immunogen.

For experiments involving s.c. injections, 100 μg dAβ1–15 was mixed with 10 μg LT(R192G) and injected biweekly in a total volume of 100 μl. Intranasal vaccination was performed on a weekly basis as previously reported [48]. Briefly, immunogen plus 5 μg LT(R192G) were mixed and applied by pipet tip to the naris, allowing capillary action to draw the liquid into the nasal cavity. The total volume for all i.n. immunizations was 30 μl divided into two doses of 15 μl each (7.5 μl per naris), spaced at least 2 h apart. This minimized the amount of vaccine swallowed by the mice.

Transcutaneous immunization was adapted from a previous report [5]. Briefly, the dorsal surface of each mouse was shaved and allowed to heal for 24 h. Mice were anesthetized with ketamine:xylazine, the shaved skin was then hydrated with saline and the stratum corneum lightly disrupted with an emory board. The vaccine solution consisting of 100 μg immunogen and 25 μg of LT(R192G) in a volume of 50 μl was applied and allowed to absorb for 1 h, after which the mice were extensively washed with warm tap water. Vaccination was repeated biweekly.

2.3. Plasma and tissue collection

Blood was collected from the tail biweekly and the plasma frozen at −20 °C. One week following the final immunization mice were sacrificed by CO_2 inhalation. Blood was collected by cardiac puncture followed by perfusion with 10 ml Tris buffered saline (TBS). The spleen was aseptically removed and placed in RPMI (Invitrogen, Carlsbad, CA, USA) on ice for cell culture studies. The brain was removed and divided sagittally. One hemi-brain, as well as pieces of liver, kidney, and skin, were placed in 10% buffered neutral buffered formalin for 2 h, processed and embedded in paraffin. The other hemi-brain was snap frozen and stored at −80 °C for biochemical analysis of Aβ.

2.4. Anti-Aβ antibody ELISA

Plasma anti-Aβ antibodies were measured by ELISA as previously described [48], and included a mouse IgG (Sigma, St. Louis, MO, USA) standard curve. ELISAs for antibody isotypes and epitope mapping were performed as previously reported [25]. Briefly, quantitative Ig isotype-specific ELISAs were performed by the use of isotype specific

secondary antibodies for IgG1, IgG2a, IgG2b, IgA, and IgM (Zymed, San Francisco, CA, USA) and the addition of a standard curve of the appropriate isotype (Southern Biotechnology, Birmingham, AL, USA) to the standard immunoassay. Peptide competition assays to determine antibody epitopes were performed as previously described [44]. The following overlapping Aβ fragments (CND Biopolymer Laboratory) were used for antibody epitope mapping: Aβ1–15, Aβ1–7, Aβ3–9, Aβ7–12, Aβ11–25, Aβ26–42, and Aβ1–40. Diluted plasma samples were co-incubated with peptide fragments overnight and applied to Aβ1–40-coated ELISA plates.

2.5. Immunohistochemistry and image analysis

Twelve micrometre sections were cut from paraffin embedded tissue and immunostained using the ELITE ABC method (Vector Laboratories, Burlingame, CA, USA) as previously described [49]. The following antibodies and dilutions were used to examine T cells (CD5, 1:50, BD PharMingen, San Jose, CA, USA), B cells (CD45RC, 1:500, BD PharMingen), or activated microglia/macrophage, (MHC class II, 1:200 BD PharMingen; CD45, 1:5000, Serotec, Raleigh, NC, USA). Rabbit polyclonal Aβ antibodies DW14 1:1000 and R1282 1:1000 (gifts of D. Walsh and D. Selkoe, respectively, Center for Neurologic Diseases) were used to visualize various forms of Aβ deposition. Aβ40- and Aβ42-specific antibodies (1:1000) were obtained from Biosource (Camarillo, CA, USA). Positive controls (sections of spleen and brain from mice with experimental autoimmune encephalitis and aged APP-tg mice) and negative controls (normal immunoglobulin) were included. To screen plasma for antibody binding to AD plaques, paraffin-embedded human brain tissue was used as previously reported [27].

To quantify the percent area occupied by Aβ immunoreactivity in hippocampus, four equidistant sagittal sections per mouse were stained with Aβ polyclonal antibody, R1282, and images captured using a 4X objective on an Olympus BX50 microscope. Acquisition of images was performed in a single session using a SPOT camera (Sterling Heights, MI, USA) and the threshold of detection was held constant during analysis. Image analysis was performed using IP Lab Spectrum 3.1 Image Analyzer software (Fairfax, VA, USA).

2.6. Splenocyte proliferation assay

Splenocytes were isolated and harvested using standard methods as previously reported [44]. Aβ peptides were added to cultures in triplicate at a final concentration of 0, 0.5, 5 or 50 μg/ml. At 48 and 72 h, supernatants were collected and analyzed by ELISA for cytokines. To measure proliferation, 1 μCi of [^{3}H]-thymidine was added to cells at 72 h. Eighteen hours later, cells were harvested and thymidine incorporation determined using a liquid scintillation counter. A stimulation index was calculated using the following formula: CPM of well with antigen/CPM with no antigen.

2.7. Cytokine ELISA

Cytokine levels were measured in splenocyte supernatants using matching antibody pairs composed of capture and detection antibodies for IL-4, IL-10, and IFN-γ (BD PharMingen).

2.8. Aβ ELISA

Both soluble and insoluble brain Aβ levels were determined. For soluble Aβ levels, frozen hemi-brains were homogenized in four volumes of TBS with a protease inhibitor cocktail (Sigma). The samples were centrifuged at 60,000 rpm for 30 min at 4 °C. The supernatant was collected and stored at −20 °C. TBS insoluble Aβ protein was extracted as previously described [22] using 10 volumes of ice cold guanidine buffer (5 M guanidine-HCl/50 mM Tris, pH 8.0). ELISAs specific for human $Aβ_{40}$, $Aβ_{42}$, and total Aβ were performed (using antibodies kindly supplied by ELAN Pharmaceuticals) as previously described [54].

2.9. Western immunoblotting

Conditioned media from Chinese hamster ovary (CHO) cells stably transfected to express mutant human APP (cell line 7PA2, kind gift of Drs. Dominic Walsh and Dennis Selkoe) or non-transfected CHO cells was centrifuged to remove cellular debris. The conditioned media was then incubated with plasma (1:50) from immunized (adjusted to 1 mg/ml of anti-Aβ antibody) or control mice. The monoclonal anti-Aβ antibody 6E10 (Signet Laboratories, Dedham, MA, USA) served as a positive control. A standard immunoprecipitation procedure using Protein G beads (Pierce, Rockford, IL, USA) was performed, with the products being electrophoresed on 16% Tris–glycine gels (Invitrogen) before transfer to nitrocellulose membranes. The anti-Aβ polyclonal antibody, R1282, was used to probe the blots and visualized using ECL (Pierce). A similar procedure was followed for synthetic Aβ except solutions of Aβ1–40 or Aβ1–42 were used at concentrations of 1.0, 0.5, and 0.1 μg/ml.

2.10. Statistical analysis

A Student's *t*-test or Kruskal–Wallis non-parametric ANOVA analysis was used to determine statistical significance between groups (pairwise or multi-group, respectively) using InStat (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Subcutaneous (s.c.) immunization with dAβ1–15 induces a moderate humoral immune response in wildtype B6D2F1 mice

B6D2F1 mice received three biweekly s.c. injections of dAβ1–15 plus LT(R192G). Using a specific ELISA, anti-Aβ

antibodies were detected after one injection, 2.0 ± 1.3 μg/ml (mean ± S.E.M.), and increased to ∼135 μg/ml 1 week after the final injection (Fig. 1A). Plasma from mice receiving dAβ1–15 plus LT(R192G), but not LT(R192G) alone, bound Aβ plaques in human Alzheimer's disease brain sections (Fig. 1B).

Antibody epitope mapping studies revealed that the antibodies recognized a dominant epitope within Aβ1–7 (Fig. 1C). Immunoglobulin isotype-specific ELISAs identified the principle anti-Aβ isotype as IgG1, with lower amounts of IgG2a and IgG2b (Fig. 1D). No IgA and low levels of IgM (19.8 ± 3.0 μg/ml) were detected.

Splenocyte cultures, established from mice immunized with dAβ1–15, LT(R192G) alone or untreated control mice, did not proliferate following in vitro stimulation with Aβ1–40, dAβ1–15, or Aβ1–15 (all stimulation indexes <2.0, data not shown).

3.2. Intranasal (i.n.) immunization with dAβ1–15 + LT(R192G) induces a robust humoral response in B6D2F1 mice

To assess the effectiveness of dAβ1–15 i.n. immunization, weekly administration of differing amounts of dAβ1–15 was performed in B6D2F1 mice. All mice receiving 25, 50, or 100 μg dAβ1–15 plus 5 μg LT(R192G) began producing anti-Aβ antibodies following four treatments (i.e. 4 weeks) (Fig. 2A). Plasma anti-Aβ titers reached ∼1300–2000 μg/ml after 10 weeks. IgG2b was the predominant immunoglobulin isotype, with lesser amounts of IgG1 and IgG2a (Fig. 2B). Minimal amounts of IgM were detected and IgA was not found. As seen with s.c. injections, the predominant antibody epitope was Aβ1–7 (data not shown). Splenocytes from mice immunized with 25 or 50 μg dAβ1–15 proliferated to a greater degree when stimulated in vitro with Aβ40 (S.I. ∼5 and 7, respectively) compared to mice receiving 100 μg (S.I. ∼2) as shown in Fig. 2C. IFN-γ, IL-10, and IL-4 were below the level of detection in culture supernatants.

To investigate if dAβ1–15 without adjuvant could induce an immune response, mice were given 50 μg of dAβ1–15 intranasally weekly for 8 weeks. The final antibody level was 27.6 ± 16.1 μg/ml, indicating the strong adjuvant effect of LT(R192G) in the earlier study. The predominant isotype was IgM (24.7 ± 7.1 μg/ml) with low levels of IgG1 (4.8 ± 2.8 μg/ml). Splenocyte proliferation studies showed no T cell reactivity upon restimulation with Aβ1–40, Aβ1–42, or Aβ1–15 (S.I. <2).

3.3. Transcutaneous (TCI) dAβ1–15 immunization results in a humoral immune response

Following four TCI applications (8 weeks of treatment) B6D2F1 mice receiving dAβ1–15 + LT(R192G) produced modest levels of anti-Aβ antibodies (∼30–40 μg/ml, Exper-

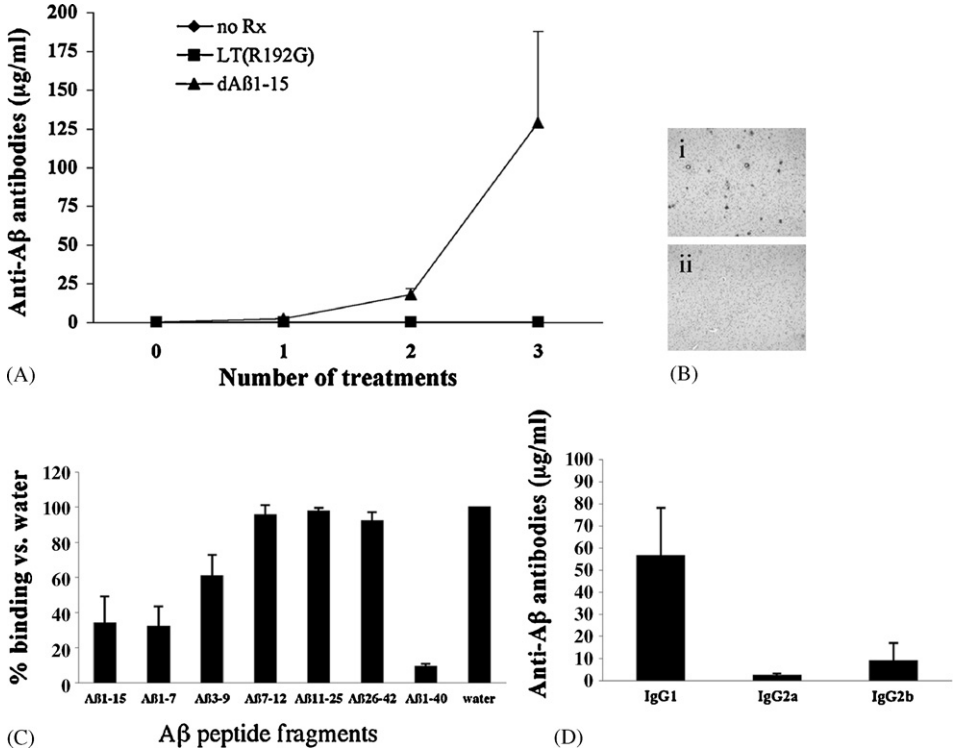

Fig. 1. dAβ1–15 induced a humoral immune response. B6D2F1 mice ($n = 4$) receiving three biweekly subcutaneous injections of dAβ1–15 plus LT(R192G) produced anti-Aβ antibodies (A). Plasma from mice receiving dAβ1–15 plus LT(R192G) (B, i) but not plasma from LT(R192G) immunized mice (B, ii) bound cerebral Aβ plaques in tissue from an AD subject. Adsorption ELISAs demonstrated that the antibodies recognized epitopes in the Aβ1–7 and Aβ1–15 region (C). Specific ELISAs demonstrated that IgG1 was the predominant isotype of anti-Aβ antibodies (D).

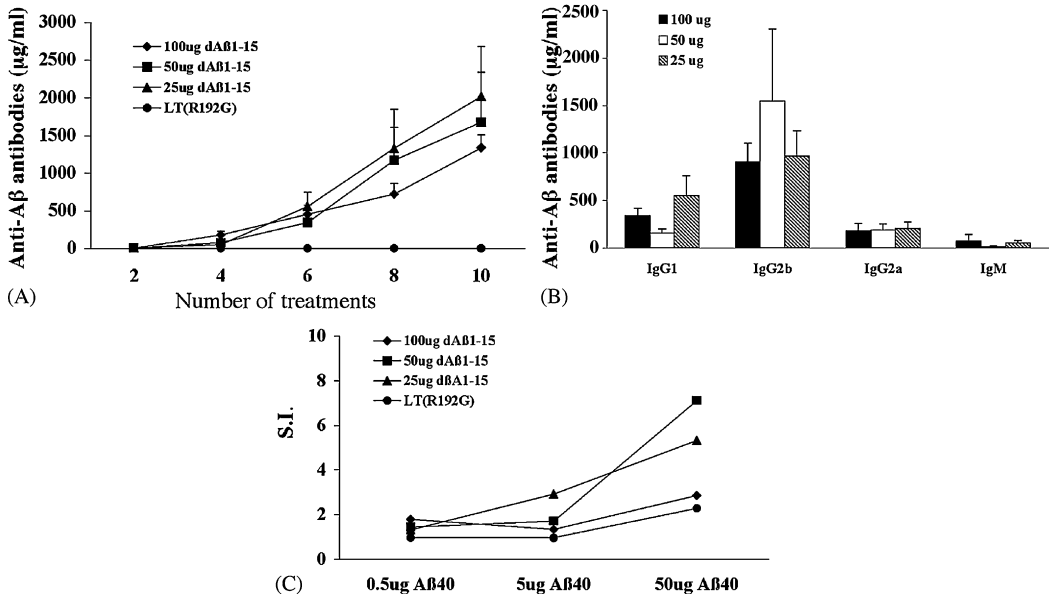

Fig. 2. Intranasal dAβ1–15 immunization in B6D2F1 mice resulted in a robust humoral immune response. Anti-Aβ antibodies were found in all B6D2F1 ($n = 4$) mice receiving i.n. treatments with either 100, 50, or 25 μg of dAβ1–15 plus 5 μg LT(R192G) (A). The main isotype was IgG2b (Th2-biased), with lower amounts of IgG1 (Th2) and IgG2a (Th1) (B). Splenocyte proliferation was measured by [3H] incorporation after 3 days in culture. Splenocytes isolated from mice receiving either 25 or 50 μg dAβ1–15 proliferated to a greater degree compared to mice receiving 100 μg dAβ1–15 (C). Stimulation index (S.I.) was calculated by the following formula: CPM of well with antigen/CPM wells with no antigen.

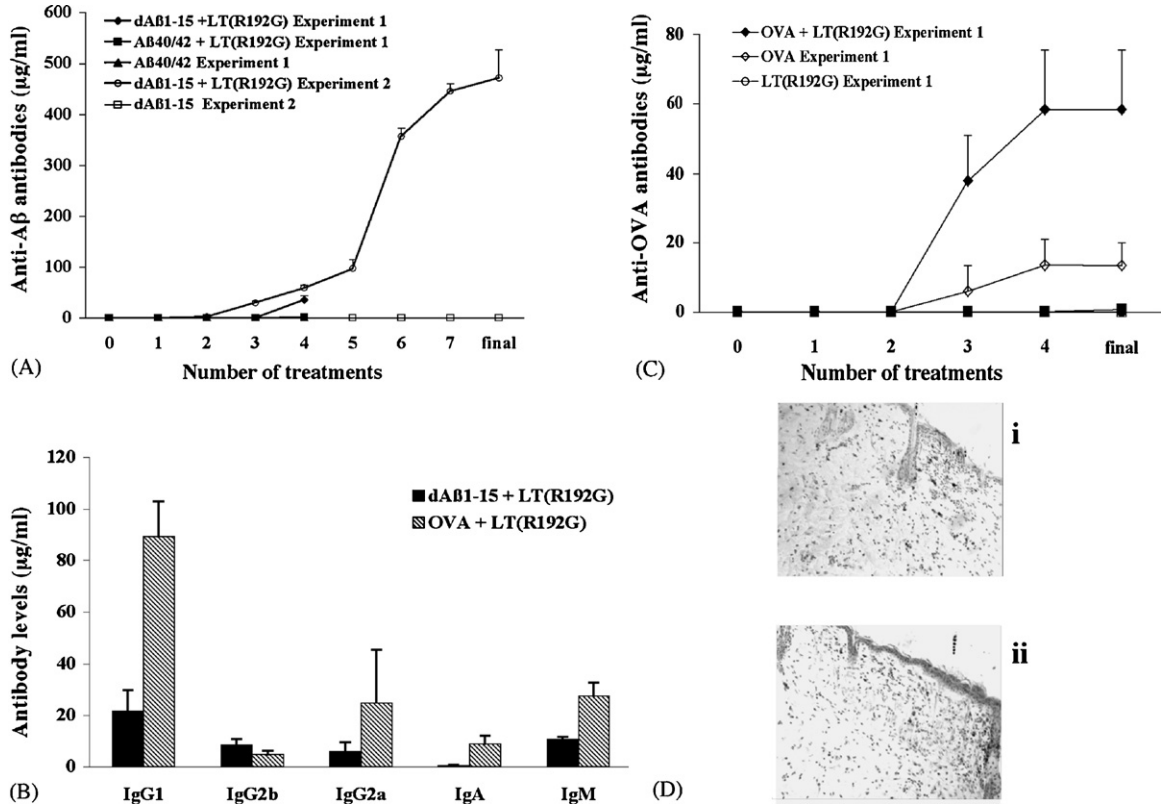

Fig. 3. Transcutaneous dAβ1–15 immunization induced a moderate humoral immune response. TCI with either dAβ1–15 (A) or OVA (B) resulted in the production of specific antibodies to the immunogens in B6D2F1 mice ($n = 4$). Increasing the number of immunizations over time resulted in greater levels of anti-Aβ antibodies (Experiment 2, A). TCI with Aβ40/42 peptide ± LT(R192G) or dAβ1–15 alone did not result in the production of anti-Aβ antibodies (A). Isotype-specific ELISAs demonstrate that IgG1 was the predominant isotype (C). Histological examination of the TCI skin site demonstrated that both LT(R192G) immunized mice (D, i) and dAβ1–15 + LT(R192G) immunized mice (D, ii) had a small number of CD5 positive T cells present, with no signs of a destructive immune response.

iment 1, Fig. 3A). No anti-Aβ antibodies were identified in plasma of mice immunized transcutaneously with full-length Aβ1–40/42 peptide with or without LT(R192G). Ovalbumin (OVA) was used a control immunogen and resulted in approximately twice as many specific antibodies (∼60 μg/ml) compared to anti-Aβ antibodies when combined with LT(R192G) (Fig. 3B). Immunization using OVA without adjuvant also led to the low production of anti-OVA antibodies of ∼10 μg/ml. IgG1 was the major isotype of both the anti-OVA and anti-Aβ antibodies, with lower levels of IgG2b, IgG2a, and IgM (Fig. 3C).

The skin sites that received TCI were macroscopically normal after four immunizations. Microscopic examination revealed low numbers of T cells and MHC II positive cells in untreated skin (data not shown). After treatment with LT(R192G) alone, there was a moderate increase in skin resident T cells and MHC II positive cells but there was no difference between mice receiving either dAβ1–15 plus LT(R192G) or LT(R192G) alone (Fig. 3D).

Splenocyte proliferation assays demonstrated a mild proliferative response to Aβ1–40 stimulation in mice receiving dAβ1–15 + LT(R192G) (S.I. = 4) compared to those receiving LT(R192G) alone (S.I. = 2.2), Aβ1–40/42 + LT(R192G) (S.I. = 3.0) or those receiving Aβ1–40/42 alone (S.I. = 2.2). The proliferative response was substantially higher in mice receiving OVA + LT(R192G) and subsequently stimulated with OVA (S.I. = 9.1).

Longer term TCI (eight treatments) was performed to determine if dAβ1–15 alone resulted in an immune response. Only B6D2F1 mice receiving both dAβ1–15 + LT(R192G) produced detectable amounts of anti-Aβ antibodies (Experiment 2, Fig. 3A). As before, the main isotype was IgG1 (235.9 ± 17.9 μg/ml), with lower amounts of IgG2b (58.3 ± 28.0), IgG2a (12.2 ± 7.0), and IgM (27.4 ± 6.1). There was a low proliferation (S.I. ∼3.0) of splenocytes when stimulated in vitro with Aβ1–40, similar to that seen with mice receiving four TCI treatments.

3.4. Intranasal dAβ1–15 immunization results in a humoral immune response in J20 APP-tg mice

Six-month-old J20 APP-tg mice received weekly intranasal immunization with either 100 μg dAβ1–15 + 5 μg LT(R192G), 5 μg LT(R192G) alone, or water for 6 months. Plasma anti-Aβ antibody levels were measured biweekly by ELISA. The dAβ1–15 dose was chosen as it was previously demonstrated that this dose gives an adequate humoral immune response but minimal T cell response in wildtype B6D2F1 mice. Approximately 50% of J20 APP-tg mice have plaques at 5–7 months of age, therefore immunization was begun at 6 months, the time of the first appearance of amyloid plaques [37]. Five of six mice receiving dAβ1–15 + LT(R192G) produced significant amounts of anti-Aβ antibodies (range ∼300–2000 μg/ml) (Fig. 4A). One mouse consistently produced low levels of anti-Aβ antibodies (maximum amount ∼10 μg/ml), thus it was excluded as a non-responder from further analysis of Aβ levels and pathology. The isotype of the anti-Aβ antibodies were found to be mainly IgG2b, with lower amounts of IgG2a and IgG1, as observed in wildtype B6D2F1 mice (Fig. 4B). Epitope mapping demonstrated that the anti-Aβ antibodies predominately bound a region within Aβ1–7 (data not shown).

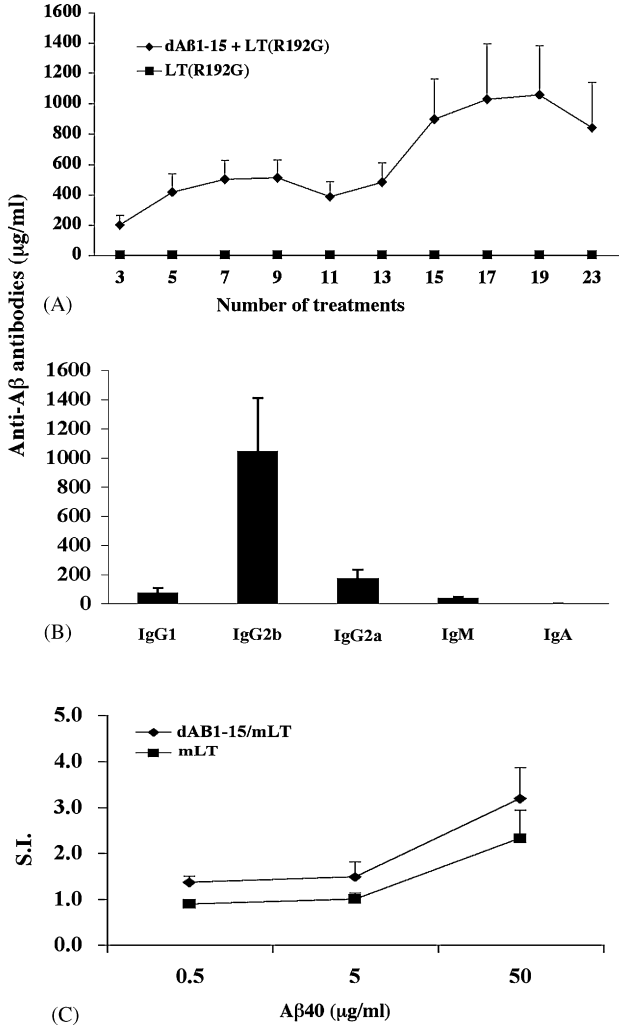

Fig. 4. Intranasal dAβ1–15 immunization of J20 APP-tg mice resulted in a robust humoral immune response. Anti-Aβ antibodies were induced in five of six J20 APP-tg mice receiving i.n. dAβ1–15 immunization but not in H_2O ($n = 5$) or LT(R192G) ($n = 6$) treated mice (A) bleed biweekly. Immunoglobulin isotype specific ELISAs demonstrated an isotype profile similar to that seen in B6D2F1 mice (B). Splenocytes isolated from both dAβ1–15 and vehicle control LT(R192G) immunized mice demonstrated a low stimulation index (S.I.) after in vitro stimulation with Aβ40 peptide (C), indicating an extremely low T cell-mediated immune response.

Splenocyte cultures demonstrated minimal proliferation in response to Aβ40 stimulation (S.I. ∼3.2) (Fig. 4C).

3.5. Plasma antibodies from dAβ1–15 immunized mice recognize Aβ

Several methods were used to determine Aβ binding properties of anti-Aβ antibodies from dAβ1–15 immunized

mice. ELISAs were performed using wells coated with either Aβ1–40 or Aβ1–42. Using a ratio of the binding to Aβ42/Aβ40, plasma from i.n. immunized wildtype mice had a ratio of 1.8, whilst plasma from i.n. immunized J20 APP-tg mice had a ratio of 1.3. Therefore, by ELISA, the antibodies preferentially bound Aβ42 compared to Aβ40. Soluble synthetic Aβ40 and Aβ42 and plasma were mixed in solution and immunoprecipitated to determine antibody binding. Plasma from i.n. or TCI immunized B6D2F1 mice and from i.n. immunized J20 APP-tg mice immunoprecipitated more Aβ42 than Aβ40 (data not shown), thus confirming our observations by ELISA.

The conformation of synthetic Aβ may differ from natural Aβ, therefore conditioned media from 7PA2 cells, known to produce monomers and oligomers of both Aβ1–40 and Aβ1–42 but not aggregates [52], was examined using immunoprecipitation. Antibodies from all dAβ1–15 immunized mice were able to bind Aβ monomers and oligomers (Fig. 5).

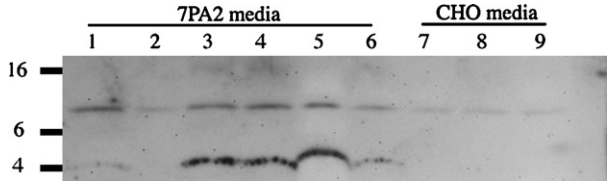

Fig. 5. Anti-Aβ antibodies induced by dAβ1–15 bind soluble Aβ. Plasma from immunized and control mice was used to immunoprecipitate 7PA2 conditioned media containing Aβ monomers (~4 kD) and dimers (~8 kD). The monoclonal antibody 6E10 was used as a positive control (lane 1) and plasma from an un-immunized mouse (lane 2) was a negative control. Plasma from dAβ1–15 immunized mice were investigated, lane 3 s.c. immunized, lane 4 i.n. immunized, lane 5 TCI and lane 6 J20 APP-tg i.n. immunized. The same plasma from lanes 3–5 were used in CHO conditioned media as a control. Bands at 4 and 8 kDa in 7PA2 media show that antibodies can recognize monomeric and dimeric Aβ, whilst those same bands are not seen in un-immunized mice or CHO media.

3.6. Cerebral Aβ levels in J20 APP-tg mice following dAβ1–15 immunization

Cerebral Aβ levels were examined both biochemically and using quantitative immunohistochemistry in J20 APP-tg mice after receiving dAβ1–15 immunization. LT(R192G) alone or water. Immunization was started at 6 months of age, the commencement of Aβ deposition in J20 APP-tg mice. Neither TBS (soluble) nor guanidine soluble (TBS insoluble) Aβ*x*-40 or Aβ*x*-42 were significantly altered as determined by ELISA (Table 1). However, the dAβ1–15 immunized group had the lowest amount of guanidine soluble Aβ*x*-42. In addition, this group had the highest level of plasma Aβ. Quantitative immunohistochemistry showed a significant decrease in the %area immunoreactivity of Aβ (using

Table 1
Cerebral and plasma Aβ levels in J20 APP-tg mice

Treatment	Aβ*x*-40 TBS soluble[a]	Aβ*x*-40 guanidine soluble[a]	Aβ*x*-42 TBS soluble[a]	Aβ*x*-42 guanidine soluble[a]	Plasma Aβ1-total[b]
dAβ1–15	7.9 ± 1.2[c]	53.0 ± 10.8	76.7 ± 8.8	622.8 ± 122.6	139.3 ± 50.9
H20	13.4 ± 2.0	41.4 ± 4.9	65.2 ± 3.3	859.1 ± 140.0	42.9 ± 9.8
LT(R192G)	5.8 ± 0.7	54.6 ± 18.6	66.6 ± 7.3	1279 ± 393.2	107.7 ± 30.9

[a] ng/mg of tissue.
[b] pg/ml of plasma.
[c] Mean ± S.E.M.

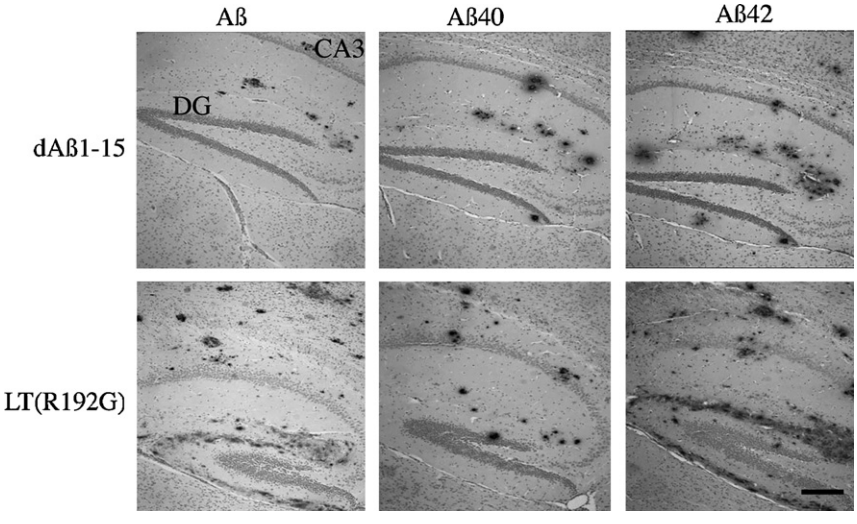

Fig. 6. Immunohistochemical analysis of dAβ1–15 immunized J20 APP-tg mice. A significant reduction in Aβ deposition was observed in J20 APP-tg mice immunized with dAβ1–15 compared to those immunized with LT(R192G) (Table 2; $p < 0.05$). Hippocampus from dAβ1–15 and LT(R192G) i.n. immunized mice were examined for total Aβ, Aβ40, and Aβ42 using specific antibodies. Magnification bar, 100 μm.

Table 2
Hippocampal Aβ immunoreactivity in J20 APP-tg mice

Treatment	%Area Aβ[a]	%Area Aβ40	%Area Aβ42
dAβ1–15	1.8 ± 0.4[b,^]	1.1 ± 0.2	3.1 ± 1.0^
H20	10.3 ± 2.2	1.5 ± 0.3	8.3 ± 2.7
LT(R192G)	9.8 ± 1.7	2.1 ± 0.4	10.2 ± 1.8

[a] %Area immunoreactivity.
[b] Mean ± S.E.M.
^ $p < 0.05$ compared to H20 or LT(R192G) treated mice.

a general polyclonal anti-Aβ antibody) in the hippocampus of the dAβ1–15 treated group (1.8 ± 0.4%) compared to LT(R192G) (9.8 ± 1.7%) or water (10.3 ± 2.2%) treated mice ($p < 0.05$). A significant reduction in Aβ42 immunoreactivity ($p < 0.05$) but not Aβ40 was also found (Table 2). Immunohistochemistry results are shown in Fig. 6, demonstrating a reduction in total Aβ, Aβ40, and Aβ42. Microglial activation (CD45) and reactive astrocytes (GFAP) were reduced overall and confined to areas of remaining Aβ plaques in mice receiving dAβ1–15 immunization (data not shown). T and B cells were absent from brain sections (data not shown).

4. Discussion

The purpose of these studies was to investigate the use of smaller Aβ fragments, the most effective route, and the optimal dosage for a successful immunogen for Aβ vaccination. This strategy of targeting the Aβ B cell epitope whilst avoiding an Aβ-specific T cell epitope may be safer than using full-length Aβ1–42 peptide and perhaps, may avoid some of the adverse sequelae seen in the AN1792 clinical trial, which used fibrillar, full-length Aβ1–42 [17,39]. We have demonstrated in both wildtype and APP-tg mice that dAβ1–15 + LT(R192G) i.n. immunization induced an effective immune response. As well, we have established that TCI may be a viable administration route for Aβ immunization.

Based on the observation that the predominant B cell epitope is Aβ1–7 [1,15,26,34], we constructed dAβ1–15, composed of 16 copies of the Aβ1–15 on a lysine tree. We demonstrated previously that Aβ1–15 peptide is not an effective primary immunogen but can be used to boost the immune response after a priming injection with full-length Aβ in wildtype mice [30]. Others have reported that a multivalent peptide composed of four copies of Aβ1–5, Aβ3–9 or Aβ5–11 contiguous to the T cell helper epitope of OVA was successful in eliciting an antibody response, which lowered cerebral Aβ [3]. A recent study further supports this strategy as adding a pan HLA DR-binding epitope to the Aβ1–15 fragment increased the immune response in wildtype mice [1]. Other strategies to limit the T cell response have incorporated the Aβ B cell epitope in a phage vector [14], mutated the Aβ fragment [46] or constructed a multiple antigen tree composed of four copies of Aβ1–33 [56]. To date, there has been very limited data reported on the T cell reactivity to Aβ peptide fragment vaccines in APP-tg mice, which is important if T cell-mediated autoimmunity is to be avoided. This may be difficult to avoid as many humans harbor a small population of Aβ-reactive T cells [35] and the use of animals and computer prediction programs for antigen recognition may not accurately predict the outcome of vaccination in humans. The use of transgenic mice expressing human HLA class II haplotypes have clearly demonstrated the variability in immune response to full-length Aβ peptide [9] In addition, we demonstrated a difference in the humoral immune response to Aβ vaccination in different mouse strains [48].

Most Aβ immunization studies have utilized injectable vaccines, often in complete Freund's adjuvant [21,36,42]. We have previously demonstrated that i.n. immunization with full-length Aβ peptide plus LT(R192G) effectively generates a humoral immune response in both B6D2F1 mice [25] and APP-tg mice [43]. Intranasal immunization has the advantages of being painless and easily administered compared to injections. It also tends to induce a more Th2-biased immune response. Transcutaneous immunization is similarly easy to administer, painless and leads to the induction of systemic immunity as seen by several studies [18,33,40]. Therefore, we sought to determine whether these routes of administration would lead to an effective anti-Aβ humoral immune in response to dAβ1–15 immunization.

Subcutaneous immunization with dAβ1–15 + LT(R192G) resulted in a relatively weak humoral immune response in B6D2F1 mice, accompanied by limited in vitro splenocyte proliferation. In contrast, intranasal immunization resulted in greater anti-Aβ antibody levels. Lower doses of i.n. dAβ1–15 resulted in a greater humoral and cellular immune response as we have previously reported following immunization with full-length Aβ in B6D2F1 mice [44]. The reasons for increased immune response to lower amounts of immunogen are not known but may be a prozone effect [50] or induction of tolerance by greater amounts of antigen [12]. The splenocyte proliferation seen with the highest restimulation dose of Aβ40 in the wildtype mice suggests that a T cell eptiope may be located in the dAβ1–15. This may be due to the three amino acid difference in the amino-terminus between rodent and human Aβ, therefore the wildtype mice may respond to human Aβ as a foreign antigen. Nevertheless, we used the higher amount of dAβ1–15 to i.n. immunize APP-tg mice to avoid the T cell response seen with the lower immunogen amounts. There was minimal splenocyte proliferation seen in the J20 APP-tg mice following i.n. dAβ1–15 immunization, suggesting minimal T cell immunity. Intranasal immunization of J20 APP-tg mice significantly reduced the amount of cerebral Aβ plaques and attending pathology as determined by immunohistochemistry. Biochemically, there was no significant decrease in cerebral Aβ, though there was a trend for lower insoluble Aβ*x*-42. Therefore, using dAβ1–15 in J20 APP-tg mice resulted in significantly fewer Aβ plaques but the biochemical levels of Aβ were not changed enough to reach significance. This may be due to the variability in the cerebral Aβ levels in 12-month-old J20 APP-tg mice as

can be appreciated by the large SEM reported in all treatment groups and the small number of mice per group. In addition, it should be noted that the biochemical studies were performed on the entire hemi-brain for each mouse whereas immunohistochemical quantification was restricted to an AD-related brain region, the hippocampus. However, these data are consistent with a recent report examining a vaccine based on Aβ1–30 peptide, which did not show a decrease in Aβ levels as measured by ELISA but did demonstrate a reduction in smaller Aβ plaques [46]. Therefore, these data suggest that dAβ1–15 immunization may decrease plaque burden and its attending pathology but may have less of a robust effect on overall Aβ levels in brain. Future studies to determine if reducing plaque burden by dAβ1–15 immunization in APP-tg mice is sufficient to improve cognitive performance will be informative.

Transcutaneous immunization has been shown to be an effective delivery route for various experimental vaccines [19] at least partially due to the dense population of skin resident antigen presenting cells, including Langerhans cells, dendritic cells, etc. [23]. Transcutaneous immunization with dAβ1–15 plus LT(R192G) induced a moderate humoral immune response, however no immune response was detected following immunization with Aβ1–40/42 peptide or dAβ1–15 without adjuvant. The lack of an immune response may be due to the presence of larger aggregates in the full-length Aβ preparation, which do not effectively cross the skin in sufficient quantities to induce an immune response. Splenocyte proliferation was low following dAβ1–15 immunization compared to OVA immunization, indicating a minimal Aβ-specific T cell response. Thus, it appears that dAβ1–15 is an effective transcutaneous immunogen but requires the addition of adjuvant. Based on the lack of inflammation in the skin, it appears that the induction of the immune response occurred in lymphoid tissue, perhaps by antigen presenting cells transporting dAβ1–15 to the draining lymph node similar to a recent report using TCI to deliver an experimental HIV vaccine [5]. Further research is required to determine if the incorporation of dAβ1–15 into a patch or a combination of i.n. and TCI immunization would further enhance humoral immunity.

Regardless of the route of immunization, s.c., i.n. or TCI, the resulting anti-Aβ antibodies bound Aβ, as measured using several different experimental approaches. Plasma from immunized but not non-immunized mice bound Aβ plaques in brain tissue from AD patients. Using a specific ELISA and immunoprecipitation of synthetic Aβ, we demonstrated that antibodies bound both Aβ40 and Aβ42, with a preference for Aβ42. This may be because Aβ42 forms fibrils to a greater degree than Aβ40, thereby exposing different binding sites. The preferential binding to Aβ42 may explain why cerebral levels of Aβ42 were more strongly reduced compared to Aβ40. Plasma from immunized mice recognized both Aβ monomers and oligomers in 7PA2 conditioned media. As oligomers may be the toxic moiety and have a detrimental effect on cognition [7,24,52], the removal of this species may be more beneficial than the overall removal of Aβ.

In conclusion, we have demonstrated that dAβ1–15 was an effective immunogen when administered via subcutaneous, intranasal or transcutaneous routes. A robust humoral immune response, resulting in predominantly Th2-biased immunoglobulins (i.e. IgG1 and IgG2b), with low T cell reactivity was seen, suggesting that this immunogen may avoid an autoreactive T cell response. A caveat to these studies is that the immune response in mice may not mimic that seen in humans, as thus far, active Aβ immunization has not induced meningoencephalitis in APP-tg mice as it did in humans. Future studies in non-human primates would be informative regarding the safety and efficacy of dAβ1–15. However, our current data suggest that dAβ1–15 may have potential as a safe and effective AD vaccine because of its strong humoral response and low Aβ-specific cellular immune response.

Acknowledgements

This work was supported by NIH grant AG20159 (C.A.L.) We thank Drs. D. Walsh, D. Selkoe, J. Clements, and Elan Pharmaceuticals for supplying reagents and cell lines. We gratefully acknowledge J. Sears, A Hyungh, and D. Li for excellent technical assistance.

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