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Biochemical and Biophysical Research Communications 336 (2005) 84–92
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Induction of anti-inflammatory immune response by an adenovirus vector encoding 11 tandem repeats of Aβ1–6: Toward safer and effective vaccines against Alzheimer's disease

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Received 3 August 2005
Available online 19 August 2005

Abstract

Induction of an immune response to amyloid β-protein (Aβ) is effective in treating animal models of Alzheimer's disease. Human clinical trials of vaccination with synthetic Aβ (AN1792), however, were halted due to brain inflammation, presumably induced by T cell-mediated immune responses. We have developed an adenovirus vector as a "possibly safer" vaccine. Here, we show that an adenovirus vector encoding 11 tandem repeats of Aβ1–6 can induce an immune response against amyloid β-protein. Much higher titers against amyloid β-protein were observed when an adenovirus vector encoding GM-CSF was co-administered. Immunoglobulin isotyping revealed a predominant IgG1 response, indicating anti-inflammatory Th2 type. Immunohistochemical analysis revealed no inflammation-related pathology in the brain of mice immunized with the adenovirus vector. Induced antibodies strongly reacted with amyloid plaques in the brain, demonstrating functional activity of the antibodies. Thus, the adenovirus vector encoding 11 tandem repeats of Aβ1–6 may be a safer alterative to peptide-based vaccines.

Keywords: Alzheimer's disease; Vaccine; Adenovirus; Amyloid; Transgenic mice; Immunization; ELISA; Antibody; GM-CSF; Self protein

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder and the most common cause of dementia in the elderly. The cardinal pathologic changes in the brain include loss of neurons, neurofibrillary tangles (NFTs), and amyloid deposits in neuritic plaques (NPs) and cerebral blood vessels (cerebral amyloid angiopathy). To date, no satisfactory treatment is available for AD. The main constituent of amyloid plaques is amyloid β-protein (Aβ). Aβ is proteolytically produced from amyloid β-protein precursor (APP). Increasing evidence supports the notion that Aβ and its precursors play pathogenetic roles in AD. Causative mutations of familial forms of AD occur in three different genes (APP, presenilin 1 and 2). These genes have been shown to increase Aβ production, particularly, a longer form of Aβ, which consists of 42 amino acids (Aβ1–42). Aβ1–42 is considered to be highly amyloidogenic, and oligomeric forms of Aβ are neurotoxic [1,2]. Overexpression of mutant forms of APP in transgenic mice led to AD-like pathologies including amyloid plaques in the brain. Immunization of these AD mouse models with synthetic Aβ by repeated needle injection prevented or reduced Aβ deposits [3] and attenuated their memory and learning deficits [4,5]. These groundbreaking results led to clinical trials of immunizing AD patients with synthetic

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doi:10.1016/j.bbrc.2005.08.044

Aβ. These clinical trials, however, had to be halted due to brain inflammation, presumably induced by T cell-mediated immune responses [6–8].

In attempting to develop safer immunization modalities for AD treatment, we previously evaluated the efficacy of adenovirus vectors encoding either Aβ1–42 or the 99 amino acid carboxyl terminal fragment of APP (C99) in induction of immune responses, as well as, clearance of Aβ deposits in AD model mice [9,10]. In the previous study, we demonstrated that adenovirus vectors encoding either Aβ1–42 or C99 alone were ineffective in eliciting an immune response against the self-antigen Aβ; and that the hurdle of Aβ's low immunogenicity was overcome by co-administration of an additional adenovirus vector encoding granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF was essential for induction of an immune response against Aβ by adenovirus vectors encoding either Aβ1–42 or C99. Because Aβ1–15 has been identified as a B cell epitope [11–13] and Aβ6–28 contains a T cell epitope [13], we have chosen Aβ1–6 as the antigen to study with the aim of developing a safer vaccine.

Aβ is a self/plasma peptide. The induction of an immune response against self/plasma peptides is problematic because of its low immunogenicity. Hsu et al. [14] developed a new and efficient delivery system to induce an immune response against poorly immunogenic plasma peptides. They used multiple repeats of a small self-peptide (12 repeats of 10 amino acid residues of gonadotropin-releasing hormone) fused to the receptor-binding domain (Ia) of *Pseudomonas* exotoxin A (PEDI) as an immunogen. Immunization of female rabbits with this immunogen resulted in production of high-titer antibodies specific to the hormone and caused degeneration of the ovaries in marked contrast to immunization with a single copy of the hormone. In the present study, we took the same approach to test the immunogenicity of an adenovirus vector encoding multiple repeats of Aβ1–6. An adenovirus vector encoding Aβ1–42 was also used for comparison.

Materials and methods

Construction of expression vectors. cDNA for PEDI was synthesized using six pairs of overlapping nucleotides listed in Table 1. In these nucleotides, the native signal sequence was replaced with that of immunoglobulin Kappa light chain in order to increase the secretion of antigens from infected cells [10]. The DNA sequences were humanized using more frequently used codons in human and mouse in order to increase translation of its mRNA [10]. These pairs were annealed and double-stranded DNAs were made using heat stable *Pfu* DNA polymerase. The double-stranded DNAs were digested with appropriate restriction enzymes indicated in Table 1 and then cloned into pSP-NMC plasmid sequentially to produce pSP-PEDI. The pSP-NMC plasmid was constructed by replacing the multiple cloning site of pSP72 (Promega) with the *Not*I, *Sac*I, *Xho*I, *Xma*I, *Sfi*I, *Bsr*GI, *Sac*II, and *Eco*RI sites using the synthetic nucleotides that contained the above-mentioned restriction enzyme sites. After verifying the DNA sequence of the cloned PEDI, pSP-PEDI was used for cloning of cDNAs encoding multiple repeats of Aβ1–6 and Aβ1–42.

cDNA for Aβ1–42 was synthesized by PCR using two oligonucleotides, 5′-TTC CGC GGC GAC GCC GAG TTC AGA CAC GAC A-3′ and 5′-CCG AAT TCT AGG CGA TCA CCA CGC CGC CCA CC-3′, as primers and pCA-fSAβ [10] as a template. The PCR products were digested with *Sac*II and *Eco*RI, and cloned into the same restriction enzyme sites of pSP-PEDI to generate pSP-PEDI Aβ1–42.

Two overlapping oligonucleotides, 5′-TTC AGA CAC GAC GCC GAG-3′ and 5′-GTG TCT GAA CTC GGC GTC-3′, encoding Aβ1–6, were annealed and ligated to each other to produce tandem repeats of Aβ1–6. Two pairs of linkers with *Sac*II and *Eco*RI sites, 5′-GTT CCA CCG CGG CGA CGC CGA G-3′ and 5′-GCC GCG GTG GAA C-3′as one pair, and 5′-TAG AAT TCG GAA-3′ and 5′-TTC CGA ATT CTA CTC GGC GTC-3′ as the other pair, were ligated onto the tandem repeats of the oligonucleotides. After SacII and *Eco*RI digestion, the repeats of the Aβ1–6 sequence were cloned into the same restriction enzyme sites of pSP-

Table 1
Synthetic nucleotides for PEDI cDNA

Oligo	Sense (5′ to 3′) (overlaps are underlined)	Antisense (5′ to 3′) (overlaps are underlined)
DIA1	TT**GCGGCCGC**GC CAC CAT GAG CGT GCC CAC CCA GGG CTG GGC CTG CTG CTG CTG TGG CTG ACC GAC GCC AGA TGC GCC GAG GAG GCC T (strikethrough is *Not*I)	GG**GAGCTC**CG CAC GCC GTC CTT CAG GTC CAG CAC CAG GCC TTG GCG CAC TCG TTC CAC AGG TCG AAG GCC TCC TCG GCG CAT CTG GCG T (strikethrough is *Sac*I)
DIA2	CG**GAGCTC**CC GGA TGA GCG TGG ACC CCG CCA TCG CCG ACA CCA ACG GCC AGG GCG TGC TGC ACT ACA GCA TGG TGC TGG AGG GCG GCA ACG A (*Sac*I)	CC**CTCGAG**CC GGA TGG TCA GGC CGT CGC TGG TGA TGC TCA GGG CGT TGT CGA TGG CCA GCT TCA GGG CGT CGT TGC CGC CCT CCA GCACCA TG (*Xho*I)
DIA3	GG**CTCGAG**GG CGG CGT GGA GCC CAA CAA GCC CGT GCG GTA CAG CT (*Xho*I)	GC**CCCGGG**CC TGC CGG GTG TAG CTG TAC CGC ACG GGC TTG TTG G (*Xma*I)
DIA4	GG**CCCGGG**GC AGC TGG AGC CTG AAC TGG CTG GTG CCC ATC GGC CAC GAG AAG CCC AGC AAC ATC AAG GTG TTC ATC CAC GAG CTG AAC GCC GGC (*Xma*I)	CT**GGGC**AGCTT**GGGC**AG CAG CTC GTC GCC CAT CTC GAT GGT GTA GAT GGG GCT CAT GTG GCT CAG CTG GTT GCC GGC GTT CAG CTC GTG GAT GAA (*Sfi*I)
DIA5	CT**GGGC**AAGCT**GGGC**AG GGA CGC CAC CTT CTT CGT GCG CGC CCA CGA GAG CAA CGA GAT GCA GCC CAC CCT GGC CAT CAG CCA CGC CGG CGT GAG CGT GGT GAT GGC CCA G (*Sfi*I)	GT**TGTACA**CG CCG TCC AGG GGG TCC AGC AGG CAC AGC ACC TTG CCG CTG GCC CAC TCG CTC CAC CGC TTC TCC CGC CGG GGC TGG GTC TGG GCC ATC ACC ACG CTC ACG CC (*Bsr*GI)
DIA6	CG**TGTACA**AC TAC CTG GCC CAG CAG CGG TGC AAC CTG GAC GAC ACC TGG GAG GGC AAA ATC TAC CGG GTG CTG GCC GGC AAC CCC G (*Bsr*GI)	CG**CCGCGG**GG GAA GTG CAG CCG GTG GGA GAT CAC GGT GGG CTT GAT GTC CAG GTC GTG CTT GGC GGG GTT GCC GGC CAG CAC CCG GT (*Sac*II)

The restriction enzyme sites are in bold letters.

PEDI to produce pSP-PEDI (Aβ1–6)$_n$, where n represents the number of Aβ1–6 repeats found in the plasmid. The number of repeats and the DNA sequence were verified by DNA sequencing and restriction enzyme analysis. A clone, which had $n = 11$, was used to make an adenovirus vector.

After *Not*I and *Eco*RI digestion, each DNA fragment encoding a fusion protein of PEDI and Aβ1–42 from pSP-PEDI Aβ1–42, or PEDI and (Aβ1–6)$_{11}$ from pSP-PEDI (Aβ1–6)$_{11}$ was cloned into pCAGGSnxec to generate pCA-PEDI-Aβ1–42 or pCA-PEDI-(Aβ1–6)$_{11}$, respectively. The coding sequences of the fusion proteins were placed under the control of a cytomegalovirus enhancer and β-actin promoter in pCAGGSnxec. The pCAGGSnxec expression vector was constructed by placing oligonucleotides containing *Xho*I and *Eco*RI sites between the *Not*I and *Cla*I sites in pCAGGSnc [15] (see Fig. 1).

Construction and preparation of E1/E3-defective adenovirus vectors. E1/E3-defective adenoviral vectors were prepared using HEK293 cells and AdEasy Basic Kit (American Type Culture Collection, Manassas, VA). The DNA fragments containing the CMV enhancer/β-actin promoter, cDNA (for antigens), and β-globin poly(A) signal were isolated from pCA-PEDI-Aβ1–42 and pCA-PEDI-(Aβ1–6)$_{11}$ by *Sal*I and *Hin*dIII digestion and cloned into the *Xho*I (compatible with *Sal*I) and *Hin*dIII sites of pShuttle plasmid (AdEasy Basic Kit) to produce the adenovirus vectors. After homologous recombination between the shuttle plasmids and pAdEasy-1 in *Escherichia coli* BJ5183 cells, E1/E3-defective adenoviral vectors encoding PEDI-Aβ1–42 and PEDI-(Aβ1–6)$_{11}$ were prepared in HEK293 cells and designated as AdPEDI-Aβ1–42 and AdPEDI-(Aβ1–6)$_{11}$, respectively. After several rounds of passage in HEK293 cells, the adenovirus vectors were purified using the BD Adeno-X virus purification kits (BD Biosciences, Palo Alto, CA) and the titers of the purified adenovirus vectors were determined using the Adeno-X rapid titer kits (BD Bioscience) according to the manufacturer's protocols. Expression of the antigens in transduced HEK293 cells was determined by Western blotting using the enhanced chemiluminescence system and 6E10 antibody (Signet Pathology, Dedham, MA) for detection of PEDI-Aβ1–42 and PEDI-(Aβ1–6)$_{11}$. An adenovirus vector encoding murine GM-CSF (AdGM-CSF) [16] was prepared by ultracentrifugation over a cesium chloride gradient as previously described [17].

Experimental animals. Twelve-month-old Tg13592 mice were used. Establishment, propagation, and maintenance of the transgenic mouse line were previously described [18,19]. All of the Tg13592 mice used in this study had been backcrossed to C57BL/6 mice more than 10 generations. Tg13592 mice overexpress the signal-peptide plus 99-amino acid carboxyl terminal fragment of amyloid β-protein precursor and have a high level of plasma Aβ [18]. Five to six Tg13592 mice were used for each adenovirus vector vaccination with and without AdGM-CSF. Tg13592 mice were treated with PBS ($n = 4$) or AdGM-CSF ($n = 4$) as controls. C57BL/6 ($n = 7$) and Balb/C ($n = 7$) mice were used for AdPEDI-(Aβ1–6)$_{11}$ vaccination without AdGM-CSF. A transgenic mouse model of Alzheimer's disease, Mo/Hu APPswe PS1dE9 [20], was used to obtain brain sections for immunohistochemical staining to test the amyloid plaque immunoreactivity of sera obtained from mice subjected to adenovirus vaccination. All animal protocols used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham and the University of Illinois College of Medicine at Peoria.

Immunization protocol. Intranasal inoculation was carried out by pipetting 20 μl of adenovirus (1×10^8 PFU, Plaque-Forming Unit) into one of the nostrils of an anesthetized mouse with or without AdGM-CSF (1×10^8 PFU), followed by a booster every 3 weeks for 12 weeks for Tg13592 mice or every week for 4 weeks for C57BL/6 and Balb/C. Blood was collected through the tail 0 (preimmune), 6, and 12 weeks for Tg13592 mice, and 0 and 4 weeks for C57BL/6 and Balb/C after the initial immunization.

ELISA for determination of serum titers. After incubating blood at 4 °C for 3 h, the blood was centrifuged for 10 min at 10,000*g*. The serum was collected and frozen at −80 °C. Anti-Aβ immunoglobulin (IgM, IgG, IgG1, IgG2a, and IgG2b) titers were determined by enzyme-linked immunosorbent assay (ELISA) as described by Spooner et al. [21] with modification. Microtiter wells were coated with Aβ1–42 (5 μg/ml) in 50 mM carbonate buffer, pH 9.6, overnight at 4 °C and rinsed three times with washing buffer [phosphate-buffered saline (PBS) containing 0.05% Tween 20]. Microtiter wells were treated with blocking buffer (5.0% goat serum, 1% BSA, and 0.05% Tween 20 in PBS) for 2 h at room temperature. The serum samples were diluted with PBS and added to the microtiter wells. After incubation for 2 h at room temperature, the plates were washed five times with the washing buffer and incubated for 1 h with an appropriate horseradish peroxidase (HRP)-conjugated detection antibody. The detection antibodies were diluted at 1:2000 for anti-mouse IgG and IgM and 1:1000 for

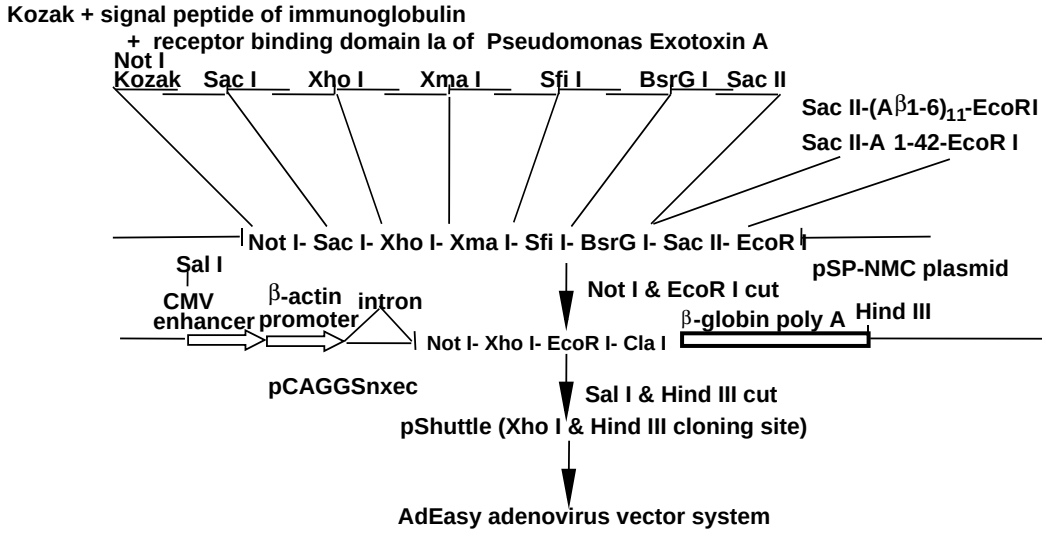

Fig. 1. Construction of adenovirus vectors.

anti-mouse IgG1, IgG2a, and IgG2b in the blocking buffer. The detection antibodies were purchased from Zymed (South San Francisco, CA). After washing the plates with the washing buffer, plates were incubated with TMB (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 15 min and the reaction was stopped with the addition of 1 N H_2SO_4. Optic densities at 450 nm were determined using a Microplate Reader (Labsystems, Finland). Anti-Aβ antibody titers in the mouse sera were determined using serial dilutions of 6E10 (monoclonal anti-Aβ antibody) as the standard. Therefore, the concentrations (μg/ml) of the serum titers presented here reflect the concentrations of 6E10 antibody, which produce the same ELISA readings, and may not accurately represent the absolute amounts. In the present study, the linear regression was found between 0.1 and 24 ng/ml of IgG. The minimum detectable anti-Aβ titer was 0.1 ng/ml, which was determined by adding two standard deviations to the mean absorbance obtained when the zero standard was assayed 32 times. Comparison of treatment groups was performed by ANOVA using the SigmaStat software (SPSS Science, Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

Immunoreactivity of antisera to monomeric and oligomeric Aβ. Western blot analysis was performed to evaluate the immunoreactivity of anti-sera to monomeric and oligomeric Aβ. Synthetic Aβ1–42 was purchased from US peptide. Oligomeric Aβ was prepared as described by Dahlgren et al. [22]. The peptide was dissolved in 1 mM hexafluoroisopropanol (Sigma) and then removed under vacuum in a Speed Vac (Savant, Holbrook, NY). The residual peptide was resuspended in dimethyl sulfoxide (Sigma) to a concentration of 5 mM. By adding phenol red free Ham's F-12 medium (Mediatech, Herndon, VA) to the resuspended peptide, the concentration was made to 100 μM and the peptide was kept at 4 °C for 24 h. The samples were diluted in NuPage sample buffer (Invitrogen, Carlsbad, CA) and separated by 16.5% Tris–Tricine SDS–PAGE. Western blotting was performed using induced anti-sera and an enhanced chemiluminescence system (Amersham, Arlington Heights, IL) as described previously [18].

Immunoreactivity of antisera to amyloid plaques in the brain. A 7-month-old-transgenic mouse (Mo/Hu APPswe PS1dE9) was sacrificed by intraperitoneal injection of sodium pentobarbital. The brain was fixed in 10% formaldehyde:90% alcohol. Six micrometer sections were prepared for immunohistochemistry. To test the immunoreactivity of sera from vaccinated mice with adenovirus vectors against amyloid plaques, the brain sections were stained with different dilutions of the sera by the avidin-biotin immunoperoxidase method using Vectastain ABC kit (Vector, Burlingame, CA). Endogenous peroxidase was eliminated by treatment with 3% H_2O_2 for 30 min. After washing with distilled water, the sections were blocked with 10% horse serum in 0.1 M Tris–saline (TBS) (pH 7.4) for 60 min at room temperature and incubated with diluted sera in 0.1 M TBS containing 10% horse serum for 16 h at 4 °C. The sections were rinsed in 0.1 M TBS containing 1% serum and incubated with biotinylated secondary antibody for 60 min at room temperature. After washing, the sections were incubated with Vectastain ABC reagent for 60 min at room temperature. Peroxidase activity was detected by treatment with 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin.

Detection of neuroinflammation in the brain. After immunization with adenovirus vectors for 12 weeks, mice were sacrificed and their brain sections were prepared as described [23]. The brain sections covered cortex, hippocampus, olfactory bulbs, and striatum. Immunohistochemistry was carried out by the avidin–biotin immunoperoxidase method using Vectastain ABC kit as described above. Rat anti-mouse Mac-3 (HIS24; BD Biosciences Pharmingen, San Diego, CA), rat anti-mouse CD45R/B220 (M3/84; BD Biosciences Pharmingen), rat anti-human CD3 (CD3-12; Serotec, Oxford, UK), and rat anti-mouse CD11b (M1/70.15; Serotec) were used as primary antibodies at the dilutions of 1:50 except CD11b 1:10.

Results

Construction of cDNA for multiple repeats of Aβ1–6

Hsu et al. [14] demonstrated that template-repeated polymerase chain reaction (TR-PCR) can be used to synthesize cDNA for multiple copies of a small peptide by two overlapping oligonucleotides. The number of repeats produced by TR-PCR for Aβ1–6 was less than 7 (data not shown). Therefore, we simply annealed and then ligated two overlapping oligonucleotides for Aβ1–6 to produce 11 tandem repeats of cDNA encoding Aβ1–6. Thus, the latter method is also possible to create multiple repeats of a small peptide.

Confirmation of expression of fusion proteins, PEDI-Aβ1–42 and PEDI-$(Aβ1–6)_{11}$, in HEK293 cells

Adenovirus vectors, AdPEDI-Aβ1–42 and AdPEDI-$(Aβ1–6)_{11}$, were prepared in HEK293 cells. In order to confirm the expression of PEDI-Aβ1–42 and PEDI-$(Aβ1–6)_{11}$ from the vectors, HEK293 cells were transduced with the vectors and the cell lysates (20 μg of protein for each sample) were subjected to Western blotting using 6E10 for PEDI-Aβ1–42 and PEDI-$(Aβ1–6)_{11}$. PEDI-Aβ1–42 and PEDI-$(Aβ1–6)_{11}$ were observed as 32 and 40 kDa proteins, respectively, as expected (Fig. 2). In addition to 32 kDa protein (presumptive PEDI-Aβ1–42), approximately 4 and 60 kDa

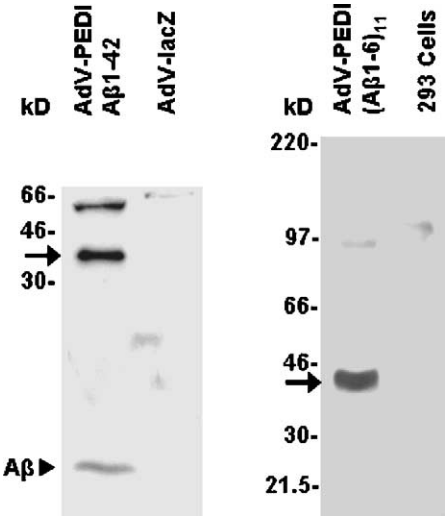

Fig. 2. HEK293 cells express fusion proteins, PEDI-Aβ1–42 or PEDI-$(Aβ1–6)_{11}$, after transduction with AdPEDI-Aβ1–42, or AdPEDI-$(Aβ1–6)_{11}$, respectively. Cell lysates from HEK293 cells transduced with AdPEDI-Aβ1–42 or AdPEDI-$(Aβ1–6)_{11}$ were subjected to PAGE followed by Western blotting using 6E10 antibody for detection of PEDI-Aβ1–42 or PEDI-$(Aβ1–6)_{11}$. As controls, cell lysates from HEK293 cells either non-transduced or transduced with an adenovirus vector encoding β-galactosidase (AdV-lacZ) were used. The arrows indicate protein fragments with expected sizes of the fusion proteins. The arrowhead indicates a presumptive degradation fragment of PEDI-Aβ1–42.

fragments were detected as minor bands. These fragments can be a degradation fragment and dimer of PEDI-Aβ1–42, respectively.

PEDI-(Aβ1–6)$_{11}$ is sufficient for induction of an immune response to Aβ by adenovirus without GM-CSF

Anti-Aβ antibody titers in the sera from Tg13592 mice were determined by ELISA at each of three time points (week 0, 6, and 12) using serial dilutions of 6E10 antibody as the standard (Table 2 and Fig. 3). After the third vaccination of AdPEDI-(Aβ1–6)$_{11}$ with or without AdGM-CSF (week 6), anti-Aβ antibodies were readily detectable in three out of six Tg13592 mice (5.38 ± 1.18 μg/ml) and two of six Tg13592 mice (2.34 ± 0.86 μg/ml), respectively. Thus, it is possible to induce immune responses against Aβ without GM-CSF. GM-CSF seems to boost the serum titers as well as the number of mice that responded (Table 2). The following immunization, however, does not significantly increase the serum titers for AdPEDI-(Aβ1–6)$_{11}$ immunization (Fig. 3). None of the Tg13592 mice ($n = 6$) immunized with AdPEDI-Aβ1–42 had discernible anti-Aβ titers, in up to 12 weeks, irrespective of co-administration of AdGM-CSF. Anti-Aβ antibody titers from Tg13592 mice vaccinated with AdGM-CSF alone (four mice) or with PBS (four mice) were indiscernible.

Induced anti-Aβ antibodies are immunoreactive to Aβ oligomers as well as Aβ deposits in the brain

Immunoreactivity of anti-sera induced by adenovirus vectors against monomeric, oligomeric, and aggregated Aβ was determined by Western blot analysis. Monomeric and oligomeric Aβ species were prepared by the method described by Dahlgren et al. [22] and separated by 16.5% Tris–Tricine SDS–PAGE. When 6E10 antibody was used, Aβ monomer (4 kDa), trimer (12 kDa), and tetramer (16 kDa) were visualized as major species (Fig. 4). Aggregates of Aβ (>46 kDa) were barely detected at the stacking gel by 6E10 (Fig. 4).

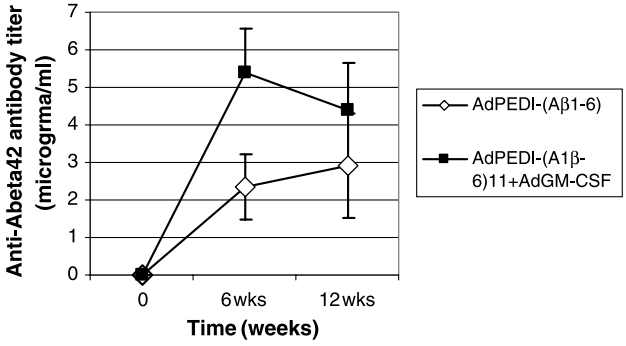

Fig. 3. Induction of anti-Aβ antibodies in Tg13592 mice after vaccination with AdPEDI-(Aβ1–6)$_{11}$ combined with or without AdGM-CSF as an adjuvant. Serum titers for anti-Aβ antibodies were determined by ELISA using anti mouse IgG antibody at the indicated time points; pre-serum (week 0), 6 weeks, and 12 weeks after the initial vaccination. The error bars are SEM.

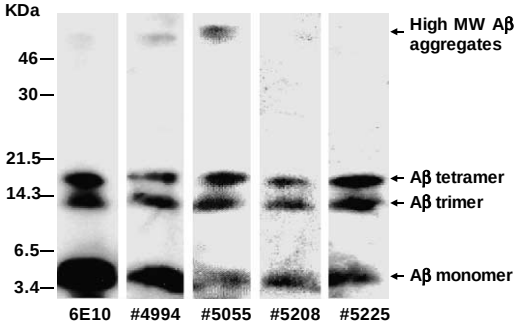

Fig. 4. Immunoreactivity of anti-sera against oligomeric Aβ. Anti-sera, #4994 and #5055, were obtained from Tg13592 mice 6 weeks after immunization with AdPEDI-(Aβ1–6)$_{11}$ plus AdGM-CSF. Anti-sera, #5208 and #5225, were from Tg13592 mice 6 weeks after immunization only with AdPEDI-(Aβ1–6)$_{11}$. Aβ oligomers are separated by 16.5% Tris–tricine SDS–PAGE and, after blotting to a PVDF membrane, are detected by using the indicated anti-sera or 6E10 antibody as a control, using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

All the sera (#4994, #5055, #5208, and #5225) from Tg13592 mice immunized with AdPEDI-(Aβ1–6)$_{11}$ reacted strongly with Aβ trimer, tetramer, and mono-

Table 2
Characterization of anti-sera induced by adenovirus vectors

Strains/immunogens	No. of mice with Aβ42 antibodies	Antibody titers (μg/ml)	Ig isotypes of Aβ42 antibodies
Tg13592			
AdPEDI-(Aβ1–6)$_{11}$	2/6	$2.34 \pm 0.86^{*}$	IgG1, IgG2b
AdPEDI-(Aβ1–6)$_{11}$ + AdGM-CSF	3/6	$5.38 \pm 1.18^{*}$	IgG1, IgG2b
AdPEDI-Aβ1–42	0/6	N/A	N/A
AdPEDI-Aβ1–42+AdGM-CSF	0/6	N/A	N/A
C57BL/6J			
AdPEDI-(Aβ1–6)$_{11}$	4/7	$4.69 \pm 0.62^{**}$	IgG1, IgG2b
Balb/Cj			
AdPEDI-(Aβ1–6)$_{11}$	7/7	$4.63 \pm 0.55^{**}$	IgG1

*,**Sera were collected 4** and 6* weeks after the initial vaccination.

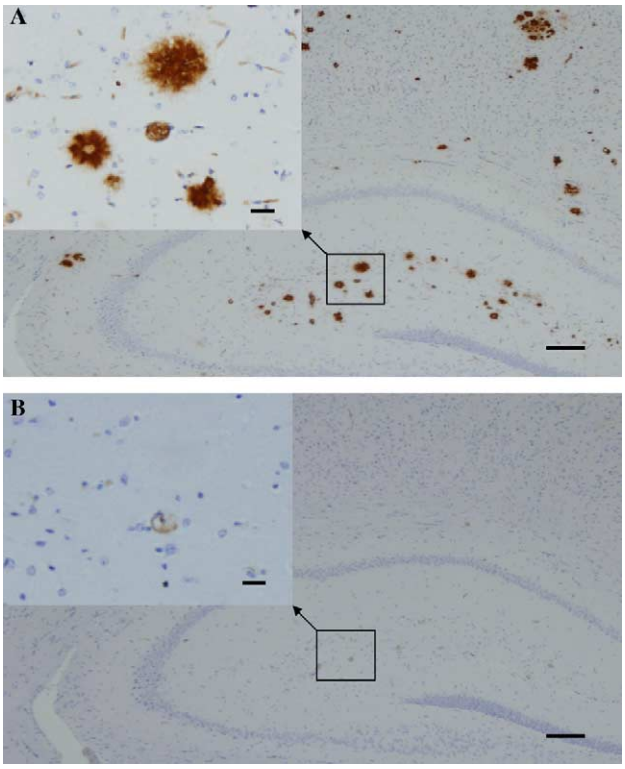

Fig. 5. Sera from Tg13592 mice immunized with AdPEDI-$(A\beta1–6)_{11}$ react with amyloid plaques in the brain. Serial brain sections from a 7-month-old Mo/Hu APPswe PS1dE9 mouse were stained with (A) #4994 serum (week 6) from a Tg13592 mouse vaccinated with AdPEDI-$(A\beta1–6)_{11}$ plus AdGM-CSF and (B) serum (week 6) from a Tg13592 mouse treated with PBS. The insets are high power magnifications of the indicated area by boxes. Scale bars indicate 25 μm for the insets and 150 μm for the low power fields.

mer (Fig. 4). All the anti-sera tested reacted weakly with aggregated Aβ (>46 kDa) at the stacking gel. Thus, the observed bands by the anti-sera were similar to those by 6E10 antibody.

Brain sections from a 7-month-old Mo/Hu APPswe PS1dE9 mouse were immunostained with 6-week sera from Tg13592 mice subjected to AdPEDI-$(A\beta1–6)_{11}$ vaccination with (#4994) or without AdGM-CSF (#5225) using the avidin-biotin immunoperoxidase method. Both of the 6-week sera reacted with amyloid plaques in the brain (Fig. 5), whereas there was no plaque detected by 6-week sera obtained from mice treated with PBS (Fig. 5). These findings demonstrate the specificity of anti-sera against amyloid deposits in the brain.

Ad PEDI-$(A\beta1–6)_{11}$ effectively induces immune responses in Balb/C and C57BL/6 mice without AdGM-CSF

We previously reported that GM-CSF is essential for induction of immune responses against Aβ by adenovirus encoding Aβ [9,10]. To confirm our current observation in non-transgenic mice as well as in another mouse strain, we immunized C57BL/6 and Balb/C mice weekly with only AdPEDI-$(A\beta1–6)_{11}$ and, after fourth immunization, serum titers were determined. Anti-Aβ antibodies (4.69 ± 0.62 μg/ml serum) were detected in 4 of 7 C57BL/6 mice and all of the Balb/C mice ($n = 7$) had high serum titers (4.63 ± 0.55 μg/ml) (Table 2). The serum titers in C57BL/6 and Balb/C are higher than that in Tg13592 mice immunized with only AdPEDI-$(A\beta1–6)_{11}$ ($P < 0.05$).

IgG1 predominates in induced anti-Aβ antibodies

To quantify the immunoglobulin isotypes of the anti-Aβ antibodies in the mice immunized with only AdPEDI-$(A\beta1–6)_{11}$ or with both AdPEDI-$(A\beta1–6)_{11}$ and AdGM-CSF, ELISA was performed, using isotype specific antibodies (IgM, IgG1, IgG2a, and IgG2b) with 6E10 antibody as a standard (Table 2, Fig. 6). The induced anti-Aβ antibodies are predominantly of the IgG1 isotype (Fig. 6). Compared with IgG1 and IgG2b, the levels of IgG2a were negligible (<0.2 μg/ml at week 4 for Balb/C and C57BL/6 or at week 6 for Tg13592) and those of IgM were negligible, also. In mice, the production of IgG1 is primarily induced by Th2 cytokines, while IgG2a is produced through Th1 cytokines. The IgG1/IgG2a ratios for Balb/C and C57BL/6 are 8.7 ± 2.4 and 12.1 ± 3.3 (mean ± SEM), respectively, and those for Tg13592 mice are 9.3 ± 3.7 with AdGM-CSF and 20.9 ± 9.2 without AdGM-CSF. Thus, these results indicate that intranasal vaccination of AdPEDI-$(A\beta1–6)_{11}$ induces highly Th2-polarized immune responses in mice.

No inflammatory changes in the brain

To examine the possible inflammation in the brain, immunohistochemistry for Mac3, CD3, CD45R, and CD11b was performed to identify macrophages, T cells, and B cells, and microglia, respectively. Inflammatory infiltration of macrophages and leukocytes was not identified in cortex, hippocampus, olfactory bulbs, and striatum. No differences in the morphology and the

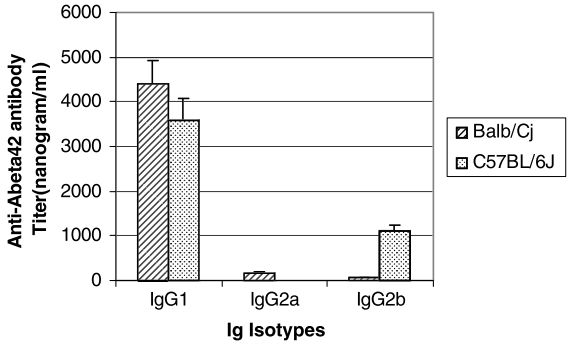

Fig. 6. Isotypes of anti-Aβ antibodies induced by intranasal administration of AdPEDI-$(A\beta1–6)_{11}$ in C57BL/6 and Balb/C mice. Isotype specific titers for anti-Aβ antibodies were determined by ELISA, using anti mouse IgM, IgG1, IgG2a, and IgG2b antibodies 4 weeks after the initial vaccination. The error bars are SEM.

staining pattern were observed by immunohistochemistry between the Tg13592 mice immunized with AdPEDI-(Aβ1–6)$_{11}$ and AdGM-CSF, and those treated with PBS in the brain.

Discussion

Good vaccines are immunogens that are safe and elicit a long-lasting protective immunity against a disease with a minimum number of inoculations. Genetic immunization is the approach for eliciting immune responses against specific proteins by expressing genes encoding the proteins in an animal's own cells. The substantial antigen amplification and immune stimulation resulting from antigen presentation in vivo can induce a solid immunity against the antigen. Viral vectors as gene-based vaccines show much higher levels of antigen expression compared with DNA plasmid vectors. Adenoviruses were originally developed for gene therapy and, later, were recognized and developed by a number of investigators as potentially excellent vaccine vectors because they induce specific immune responses against the gene products delivered by adenoviruses [24,25]. We also utilized adenovirus vectors as safe and effective vaccines [9,10,17,26,27]. Because clinical trials of the Aβ peptide-based vaccine (AN1792) were halted due to T cell-mediated brain inflammation [6–8], responsive, yet safe, vaccines for Alzheimer's disease are gravely needed. In this study, we have tested the potential of an adenovirus vector encoding eleven tandem repeats of Aβ1–6, AdPEDI-(Aβ1–6)$_{11}$, as a possibly safer alternative to Aβ peptide-based vaccines. We used Aβ1–6 as an antigen to polarize the immune response toward an anti-inflammatory Th2 phenotype, because Aβ1–15 has been demonstrated to be a B cell epitope, and because a T cell epitope is found within Aβ6–28 [11–13]. Intranasal administration of AdPEDI-(Aβ1–6)$_{11}$ in mice efficiently induced antibodies against Aβ. The IgG1/IgG2a ratios of the antibodies range from 8 to 20. These ratios indicate a highly Th2-polarized immune response, as expected. Furthermore, immunohistochemical analysis revealed no inflammatory signs in the brain of mice immunized with the vector. Thus, AdPEDI-(Aβ1–6)$_{11}$ may be a safe alternative to Aβ peptide-based vaccines.

An Aβ plaque load in the brain is the most definitive marker of AD diagnosis, and its clearance has been used to evaluate the efficacies of therapeutic means in AD mouse models. It has been reported that antibodies whose epitopes reside in Aβ1–11 can invoke Aβ plaque clearance in AD mouse models; and that Aβ plaque binding correlates with a clearance response; but the ability of antibodies to capture soluble Aβ does not necessarily correlate with clearance efficacy [11,28,29]. Recently, oligomeric Aβ is thought to be more neurotoxic than fibrillar Aβ and oligomeric Aβ plays an important role in the pathogenesis of AD [1,2]. Consequently, we have tested the immunoreactivity of sera raised by AdPEDI-(Aβ1–6)$_{11}$ against Aβ oligomers and Aβ plaques in the brain by Western blotting and immunohistochemistry, respectively. All of the anti-sera tested reacted with oligomeric Aβ, as well as Aβ plaques (Figs. 4 and 5), suggesting that anti-sera induced by AdPEDI-(Aβ1–6)$_{11}$ may be effective in removing Aβ deposits and detoxifying Aβ oligomers. We are currently testing this modality in transgenic AD mouse models. The preliminary data indicate that nasal administration of AdPEDI-(Aβ1–6)$_{11}$ is effective in reducing Aβ load in the brain. Thus, AdPEDI-(Aβ1–6)$_{11}$ may be a useful alternative to Aβ peptide-based vaccines in clearing the Aβ load in the brains of AD patients.

We previously related that GM-CSF is essential for induction of an effective immune response by an adenovirus vector encoding Aβ1–42 (AdfKSAβ) [9,10]. Here, we have shown that an adenovirus encoding 11 tandem repeats of Aβ1–6 without GM-CSF is sufficient for induction of an immune response against Aβ. The hurdle of Aβ's low immunogenicity seems to be overcome by the tandem repeats of Aβ1–6, rather than the PEDI sequence fused to Aβ1–6, because AdPEDI-Aβ1–42 was unable to induce an immune response even with GM-CSF (Table 2) and because tandem repeats of a small self-peptide is reported to permeate self tolerance [14].

We previously demonstrated that nasal administration of an adenovirus vector encoding Aβ1–42 (AdfKSAβ) with GM-CSF induces a Th2 response in Tg13592 and C57BL/6 mice [9,10]. This observation is consistent with those of other investigators reporting the production of predominant IgG1 and IgG2b by synthetic Aβ1–40/42, regardless of the administration routes (intranasal or intraperitoneal) and adjuvants (Freund's or heat-labile enterotoxin) [30,11,12,31]. Aβ1–42, however, contains a T cell epitope but Aβ1–6 does not [13]. Therefore, we make a comparison between AdfKSAβ encoding Aβ1–42 and AdPEDI-(Aβ1–6)$_{11}$ encoding Aβ1–6 in the IgG1/IgG2a ratios. The IgG1/IgG2a ratio for AdPEDI-(Aβ1–6)$_{11}$ without AdGM-CSF (20.9 ± 9.2) is greater than that for AdfKSAβ with AdGM-CSF (5.8 ± 1.6) in Tg13592 mice ($P < 0.05$). Although the mean of the IgG1/IgG2a ratio for AdPEDI-(Aβ1–6)$_{11}$ with AdGM-CSF (9.3 ± 3.7) is 1.6 times greater than that for AdfKSAβ with AdGM-CSF in Tg13592 mice, the difference is not significant. In C57BL/6 mice, the IgG1/IgG2a ratio for AdPEDI-(Aβ1–6)$_{11}$ without AdGM-CSF (12.1 ± 3.3) is greater than that for AdfKSAβ with AdGM-CSF (6.7 ± 1.6) ($P < 0.05$). Thus, it is possible to further polarize the immune response toward an anti-inflammatory Th2-type by use of only AdPEDI-(Aβ1–6)$_{11}$.

Leverone et al. [30] reported that Aβ1–15 is less immunogenic than Aβ1–40/42 for intranasal immunization of the synthetic peptides and suggested the use of

Aβ1–15 as a safer, more cost-effective "boosting" immunogen following the initiation of vaccination by Aβ1–40/42. It is preferable not to use Aβ1–42, because in a clinical trial of AN1792, one out of 18 patients who had meningoencephalitis developed the symptoms with one immunization [8]. AdPEDI-$(A\beta1–6)_{11}$ produced much higher anti-Aβ titers (4.39 ± 1.18 μg/ml) than AdPEDI-Aβ1–42 (indiscernible) in Tg13592 mice. Furthermore, AdPEDI-$(A\beta1–6)_{11}$ did not require GM-CSF, but GM-CSF is essential for induction of an immune response by vaccination with AdfKSAβ encoding Aβ1–42 [9,10]. Thus, AdPEDI-$(A\beta1–6)_{11}$ not only overcame low immunogenicity of Aβ1–15, but also generated a more robust Th2 response than Aβ1–42-based vaccines. Taken together, these data indicate that the adenovirus vector encoding 11 tandem repeats of Aβ1–6 is a safer and more robust immunogen than Aβ1–42-based vaccines.

Acknowledgments

We thank Karen Minter for her help with preparing the manuscript. This study was supported in part by the National Institutes of Health (NS43947) and the Alzheimer's Association of America (ZEN-03-5834).

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