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Aβ-Immunotherapy for Alzheimer's Disease Using Mannan–Amyloid-Beta Peptide Immunoconjugates

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ABSTRACT

In Alzheimer's disease (AD) the accumulation of pathological forms of the beta-amyloid (Aβ) peptide are believed to be causal factors in the neurodegeneration that results in the loss of cognitive function in patients. Anti-Aβ antibodies have been shown to reduce Aβ levels in transgenic mouse models of AD and in AN-1792 clinical trial on AD patients; however, the clinical trial was halted when some patients developed meningoencephalitis. Theories on the cause of the adverse events include proinflammatory "primed patients," a Th1-inducing adjuvant, and Aβ autoreactive T cells. New immunotherapy approaches are being developed to eliminate these putative risk factors. Mannan, which is recognized by pattern recognition receptors of the innate immune system, can be utilized as a molecular adjuvant to promote a Th2-mediated immune response to conjugated B cell epitopes. The N-terminus of Aβ was conjugated to mannan, and used to immunize mice with low concentrations of immunoconjugate, without a conventional adjuvant. Mannan induced a significant and highly polarized toward Th2 phenotype anti-Aβ antibody response not only in BALB/c, but also in B6SJL F1 mice. New preclinical trials in AD mouse models may help to develop novel immunogen–adjuvant configurations with the potential to avoid the adverse immune response that occurred in the first clinical trial.

INTRODUCTION

Alzheimer's disease (AD) is a most common form of dementia in the elderly and is characterized by a progressive loss of memory and general cognitive decline. The neuropathological features of the disease include neurofibrillary tangles (NFT), deposition of amyloid-beta (Aβ) in senile plaques, and neuronal loss in affected brain regions (Price and Sisodia, 1994). The amyloid cascade hypothesis that proposed a central role of Aβ deposition in the brain in the onset and progression of AD (Hardy and Higgins, 1992; Hardy and Selkoe, 2002), remains to be a rationale for therapeutic strategies (Golde, 2005). Thus, reduction of the level of Aβ in the brain may diminish learning and memory deficits observed in AD patients. Recently, several groups have demonstrated that active immunization of amyloid precursor protein (APP) transgenic (Tg) mice with fibrillar Aβ, as well as passive transfer of anti-Aβ antibodies, significantly reduced Aβ plaque deposition, neuritic dystrophy, and astrogliosis in the brains of these mice (Schenk *et al*., 1999; Bard *et al*., 2000; Morgan *et al*., 2000; Wilcock *et al*., 2004a). Improvements in learning and memory were also observed after either active or passive immunization of APP/Tg mice (Janus *et al*., 2000; Morgan *et al*., 2000; Dodart *et al*., 2002; Sigurdsson *et al*., 2004; Wilcock *et al*., 2004a; 2004b).

Based on these results, the AN-1792 vaccine clinical trial was initiated with AD patients, but was halted because a subset of participants developed meningoencephalitis. Although the results of the first vaccination of elderly AD patients with $A\beta_{42}$ peptide raised concerns about the safety of AN-1792 vaccine, follow-up studies suggest that anti-Aβ antibodies were capable of reducing AD pathology and, at least in some patients, diminishing the progressive cognitive decline associated with the disease (Hock *et al*., 2003; Nicoll *et al*., 2003; Ferrer *et al*.,

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2004; Bayer *et al*., 2005; Fox *et al*., 2005; Gilman *et al*., 2005; Masliah *et al*., 2005). Second-generation vaccines, which induce a Th2-polarized immune response or utilize nonself T-cell epitopes in the immunogen to amplify the antibody response to the B-cell epitope of Aβ, may provide safer alternatives for active immunization (Cribbs *et al*., 2003b; Agadjanyan *et al*., 2005; Cribbs and Agadjanyan, 2005). Previously, it was demonstrated that a Th2-type of humoral immune response in APP/Tg mice was therapeutically effective (Weiner *et al*., 2000). Thus, an adjuvant that can direct the immune response towards a Th2 phenotype may be critical for the design of a safe and effective immunotherapy for AD patients.

Mannan has been investigated extensively as a molecular adjuvant due to its ability to enhance both B- and T-cell immune responses (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 2000; Karanikas *et al*., 1997; Vaughan *et al*., 2000; Stambas *et al*., 2002a, 2002b, 2005). The adjuvant function is dependent on the ability to target the immunogen to antigen-presenting cells expressing receptors specific to mannosylated sugars. Mannose-binding receptors (MBRs) are expressed on dendritic cells, some endothelial cells, and macrophages (Engering *et al*., 1997a; Gröger *et al*., 2000; Linehan *et al*., 2000). In addition, Mannose-binding lectin (MBL) (Turner, 1996; Tenner, 1999; Vasta *et al*., 1999), which also has an opsonic function similar to complement C1q (homologue of MBL), binds to complement receptor type 1 (CD35) (Ghiran *et al*., 2000), and therefore stimulates phagocytosis of antigen conjugated to mannan. Dendritic cells are able to present very low concentrations of mannosylated antigen 100–1000 fold more efficiently than non-mannosylated antigen (Engering *et al*., 1997a). Besides promoting phagocytosis and antigen presentation, antigens conjugated to mannan may also provide a stronger signal to antigen-specific B cells by simultaneous triggering of BCR and CD21 and/or BCR and CD35 C' receptors (Molina *et al*., 1996; Kozono *et al*., 1998). Enhancement of immune responses against several mannosylated antigens, including peptide antigens, has been demonstrated (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 2000). Under certain conditions, mannosylated antigens induced strong Th2 type anti-inflammatory responses with high levels of IgG1 antibodies, IL4 and IL10 production (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 1996; Vaughan *et al*., 1999; Apostolopoulos and McKenzie, 2001).

In this paper we report the development of a novel AD vaccine consisting of the N-terminus of Aβ (Aβ_{28}) conjugated to mannan. We demonstrated that low doses of Aβ_{28} conjugated with mannan, in the absence of conventional adjuvant, are capable of eliciting a robust Th2-type anti-Aβ immunity in mice. The antibodies were specific for the N-terminus and were judged functional based on strong binding to Aβ-plaques in brain tissue from an AD case.

MATERIALS AND METHODS

Preparation of peptides and conjugation with mannan

Aβ peptides spanning aa 1–42 (Aβ_{42}), 1–28 (Aβ_{28}), 1–15 (Aβ_{1-15}), 6–20 (Aβ_{6-20}), 11–25 (Aβ_{11-25}), and 16–30 (Aβ_{16-30}) of Aβ_{42} were synthesized at the UCI core facility (Cribbs *et al*., 2003b; Petrushina *et al*., 2003). The Aβ_{28} peptide with an N-terminal linker (n-CAGA) sequence was synthesized, and mannan–Aβ_{28} conjugate was prepared as previously described (Inman, 1993; Lees *et al*., 1996). More specifically, mannan from *Saccharomyces cerevisiae* (cat #M-3640; Sigma, St. Louis, MO) was further purified by passage over a Q Sepharose FF column. Purified mannan (10 mg/ml) was activated by addition of the organic cyanylating reagent 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) (25 μl/ml, 100 mg/ml in acetonitrile). After 30 sec 25 μl of aqueous 0.2 M triethylamine (TEA) was added. After another 2 min an equal volume of 0.5 M hexanediamine, pH 9, was added and the reaction allowed to continue overnight. The solution was then dialyzed against water, and the mannan concentration and the free amine content were determined as previously described (Lees *et al*., 1996). The amino-mannan was bromoacetylated using NHS bromoacetate and desalted by dialysis. The n-CAGA–Aβ_{28} peptide (5.0 mg/ml) was dissolved in 0.15 M HEPES, 2 mM EDTA, pH 7.3, and the free thiol content determined using Ellman's reagent. The peptide was combined with the bromoacetylated mannan at a molar ratio of 30 thiols/100 kDa of carbohydrate under a stream of nitrogen. After an overnight reaction, the solution was quenched by the addition of mercaptoethanol, concentrated with an Ultra 4 (10-kDa cutoff) device (Millipore, Bedford, MA), and free peptide removed by gel filtration on a Superose 12 column equilibrated with 0.1 M HEPES, pH 8. The void volume peak was pooled. The peptide content was determined from the UV spectrum and its calculated extinction coefficient. The final product contained 50 μM peptide and five molecules of peptides per 100 kDa of mannan formulated in PBS.

Immunization of mice

Six- to 8-week old mice of different immune haplotypes BALB/c) and APP/Tg 2576 animals were immunized four times (biweekly) subcutaneously (s.c.) with 100 μg of fibrillar Aβ_{28} in alum as previously described (Cribbs *et al*., 2003b). To evaluate the efficacy of mannan as an adjuvant we immunized BALB/c mice s.c. four times (biweekly) with 2.5, 5, or 10 μg of mannan–Aβ_{28} peptide or with 10 μg of fibrillar Aβ_{28} peptide without alum. Two months later, the BALB/c mice were boosted one more time with the same dose of the appropriate immunogen.

Detection of anti-Aβ_{42} antibodies

Eight to 9 days after each immunization, sera from mice were collected, and anti-Aβ_{42} antibodies, as well as their isotypes, were determined by ELISA (Cribbs *et al*., 2003b; Ghochikyan *et al*., 2003; Petrushina *et al*., 2003; Agadjanyan *et al*., 2005). To identify the B-cell antigenic determinant/s within Aβ_{28}, we used four overlapping 15-mer peptides (Aβ_{1-15}, Aβ_{6-20}, Aβ_{11-25}, and Aβ_{16-30}) for epitope mapping. Since adsorption of a peptide to an ELISA plate may mask some of the peptide's epitopes, we detected B-cell antigenic determinants using a competition ELISA as previously described (Cribbs *et al*., 2003b).

Detection of anti-Aβ T-cell proliferation and production of cytokines by immune splenocytes

Nine days after the last boost, BALB/c mice were sacrificed, and anti-Aβ T-cell responses were analyzed using splenocyte

cultures from individual mice. We used HL-1 serum-free synthetic medium (Cambrex, Baltimore, MD) for our T-cell stimulation assays, because it significantly decreases nonspecific activation of splenocytes, allowing measurement of T-cell activation (proliferation, cytokine production, and Th1 and Th2 subsets) more accurately. To detect proliferation of splenocytes, we restimulated individual culture of cells with $A\beta_{40}$ peptide and measured ^{3}[H]thymidine uptake, as described previously (Cribbs *et al*., 2003b). Data are presented as the Stimulation Index (SI), and were calculated for each mouse.

We used the ELISPOT technique to detect production of IFNγ-Th1) or IL4 (Th2) lymphokines, as well as TNFα (proinflammatory) cytokine in restimulated splenocytes from experimental mice. Experiments were conducted as recommended by the manufacturer (PharMingen, San Diego, CA) and as we described previously (Cribbs *et al*., 2003b). The colored spots were counted, and the results were examined for differences between stimulated and nonstimulated conditions for each experiment using one-way ANOVA and Tukey's posttest, Graph Pad Prism 3.03.

Detection of Aβ plaques in human brain tissues

Sera from immunized mice were screened for the ability to bind to Aβ plaques on tissue sections from an AD case as we described previously (Ghochikyan *et al*., 2003; Agadjanyan *et al*., 2005). Briefly, pooled sera (dilution 1:500) were added to the serial 50-μm brain sections of formalin-fixed frontal cortical tissue from patients with neuropathological and behavioral patterns typical of severe AD. Sections were pretreated with 90% formic acid, and exogenous peroxidase was quenched. As a negative control, we used the same dilutions of preimmune sera. As a positive control, monoclonal antihuman Aβ antibody 6E10 (Signet Laboratories, Dedham, MA) was used. Binding of antibodies to the brain sections was detected by Vectastain Elite ABC Mouse anti-IgG/biotin–avidin/HRP system (Vector Labs, Burlingame, CA) with DAB, according to the manufacturer's recommendations. A digital camera (Olympus, Japan) was used to collect images of the plaques at 20× image magnification.

RESULTS

Immunogenicity of Aβ$_{28}$ peptide

Prior to testing the $A\beta_{28}$–mannan conjugates, we first evaluated the immunogenicity of the peptide alone in different strains of mice. Previously, we showed that BALB/c mice recognized the B- and T-cell antigenic determinants of $A\beta_{42}$ within the first 28 aa of this peptide (Cribbs *et al*., 2003b). In fact, BALB/c mice of H-2^d immune haplotype generated high titers of anti-Aβ antibodies (Petrushina *et al*., 2003; Gevorkian *et al*., 2004), and the highest level of anti-Aβ T cell responses after immunizations with fibrillar $A\beta_{42}$ peptides (Cribbs *et al*., 2003b). We directly analyzed the immunogenicity of fibrillar $A\beta_{28}$ peptide in this strain of mice and compared it with that in mice of H-2^b (C57BL6), H-2^s (SJL), and H-2$^{b\times s}$ (B6SJL F1),

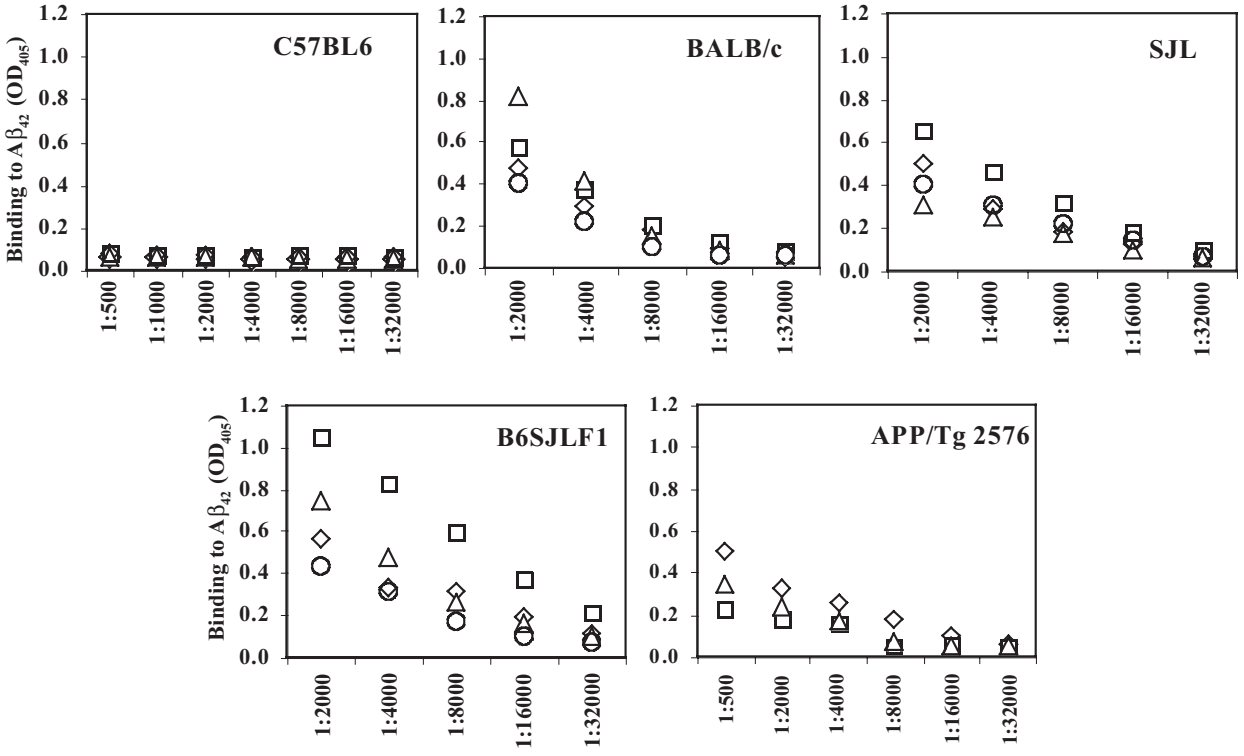

FIG. 1. The $A\beta_{28}$ sequence of the human $A\beta_{42}$ peptide is immunogenic in B6SJL F1, SJL, and BALB/c, but not in the C57BL/6 strain of mice (for each immune haplotype $n = 4$). Total Ig specific to $A\beta_{42}$-coated wells was detected in serum from individual mice after immunization and two biweekly boosts with fibrillar $A\beta_{28}$ formulated in alum, a Th2-type adjuvant.

immune haplotypes, as well as with antibody responses generated in APP/Tg 2576 animals, which have H-$2^{b\times s}$ background.

As shown in Figure 1, three biweekly injections with 100 μg of fibrillar human Aβ_{28} peptide formulated in a Th2-type adjuvant (Alum) induced anti-Aβ antibodies in all immune haplotypes except H-2^b. C57BL6 had not responded to immunizations with fibrillar Aβ_{28} at all, indicating that this immune haplotype did not recognize a T-cell epitope within this immunogen. B6SJL F1 animals induced the highest titer of anti-Aβ antibodies, whereas the levels of anti-Aβ antibody in SJL and BALB/c mice were moderate. However, a difference in the level of anti-Aβ antibody response between these groups is not significant ($p > 0.05$). Immunization of APP/Tg 2576 mice of H-$2^{b\times s}$ background induced the lowest level of anti-Aβ_{42} antibody response. These results were consistent with our previous findings that C57BL6 do not respond to Aβ_{28}, and that for APP/Tg 2576 mice human Aβ_{28} represents a self-antigen. However, collectively these data confirmed that wildtype mice of H-2^s, H-$2^{b\times s}$, H-2^d immune haplotypes, as well as APP/Tg 2576 mice recognized Aβ_{28} immunogen and produced anti-Aβ antibodies. Next, we tested the potency of mannan as an adjuvant in BALB/c wild-type mice.

Conjugation with mannan significantly enhances immunogenicity of the Aβ_{28} peptide

To determine the ability of mannan to enhance the immunogenicity of Aβ_{28} peptide, we vaccinated BALB/c mice with mannan–Aβ_{28} conjugate or fibrillar Aβ_{28}. The groups of experimental mice were injected with 2.5, 5, or 10 μg of Aβ_{28} conjugated with mannan, whereas control mice were immunized with 10 μg Aβ_{28}. Of note, we did not use any additional adjuvant, and prepared both immunogens in PBS. The sera collected from experimental and control mice were analyzed for the presence of anti-Aβ_{42} antibodies after one, two, and three biweekly boosts (Fig. 2). Three immunizations of mice with 10 μg of fibrillar Aβ_{28} were not sufficient to generate a detectable anti-Aβ_{42} antibody response. However, after a third boost this group of mice generated a low level of antibody response that was equal to that induced in animals immunized four times with 2.5 μg of mannan–Aβ_{28} conjugate. In contrast, mice immunized with 5 and 10 μg of mannan–Aβ_{28} induced low titers of anti-Aβ antibodies after the first boost. The second and third boosts with these doses of mannan–Aβ_{28} significantly enhanced anti-Aβ antibody production ($\geq$4–8 times). Of note, although 5 μg peptide induced higher response than 10 μg, the difference was not statistically significant. These mice were boosted one more time after a 2-month rest period. Anti-Aβ_{42} antibody responses were analyzed before and 9 days after the fourth boost (Fig. 3). After 2 months of rest, the level of anti-Aβ_{42} antibodies decreased slightly (32.5 $\pm$ 6% on average). As expected, a single boost enhanced a production of anti-Aβ_{42} antibodies with the largest increase detected in groups of mice immunized with 5 and 10 μg (data not shown) mannan–Aβ_{28}. We concluded that mannan was an effective molecular adjuvant that induced long-lasting anti-Aβ antibody responses in mice immunized with 5 and 10 μg of mannosylated Aβ_{28} peptide, although variability of humoral immune responses in individual mice was significant.

Mannosylated Aβ_{28} induced a Th2 polarized immune response

Antibody isotyping has been used as an indirect measure of the contribution of Th1 (IgG2a) and Th2 (IgG1) cytokines to the humoral response (Finkelman *et al*., 1990). In addition, it was demonstrated that the subclass of anti-Aβ_{42} antibodies might correlate with their therapeutic potential (Solomon *et al*., 1996; Bard *et al*., 2000; Dodart *et al*., 2002; McLaurin *et al*., 2002). Thus, we measured production of IgG1, IgG2a, IgG2b, and IgM anti-Aβ antibodies in the sera of immunized BALB/c mice. Both fibrillar Aβ_{28} and mannan–Aβ_{28} induced primarily IgG1 antibodies after immunization and three boosts (Fig. 4A). The ratio of IgG1 to IgG2a antibody in mice immunized with Aβ_{28} was 6. However, sera from mice immunized with mannan conjugated to Aβ_{28} significantly enhanced the highly polarized Th2 type immune response. The IgG1/IgG2a ratios in the sera of these animals increased to 15. Since Balb/c mice are

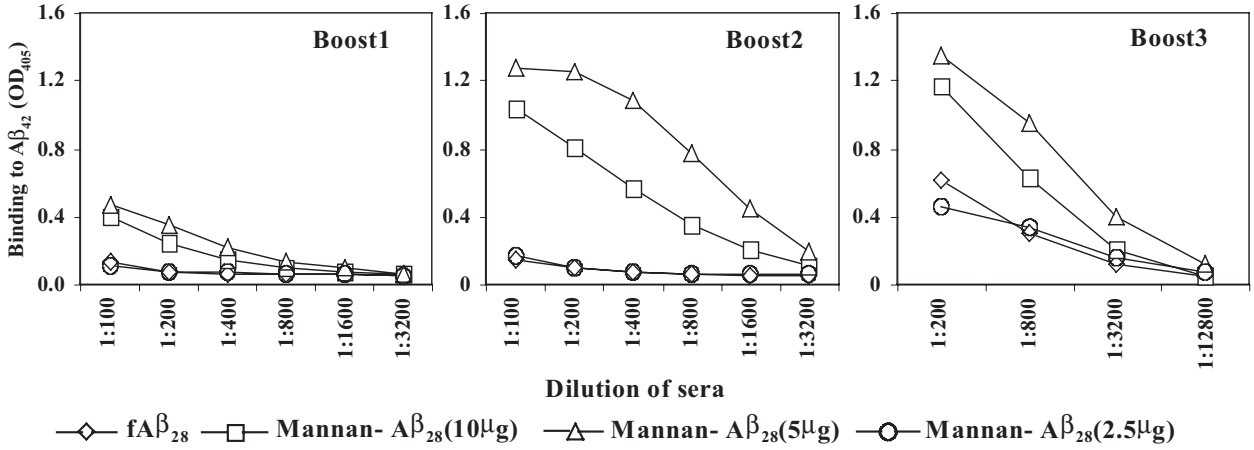

FIG. 2. Mannan enhanced immunogenicity of Aβ_{28} peptide. BALB/c mice were immunized and boosted one, two, or three times with the indicated dose of mannan–Aβ_{28} or fibrillar Aβ_{28}. Pooled sera were used for detection of binding to Aβ_{42}-coated wells. Representative ELISA data from three experiments are presented.

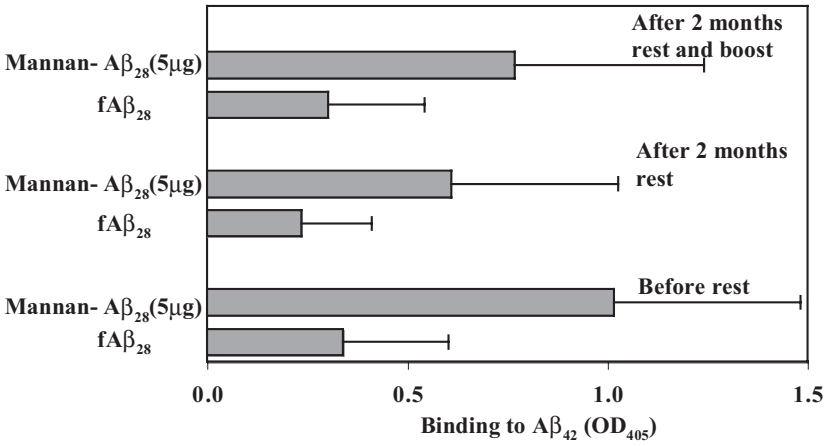

FIG. 3. Long-lasting anti-Aβ antibody responses in mice immunized with 5 μg of Aβ_{28} conjugated with mannan (the data with similar profile were obtained with 2.5 and 10 μg mannan-Aβ_{28}). After vaccination and three booster injections mice were rested for 2 months, and the immune response was recalled with the appropriate antigen. Individual sera from animals were diluted 1:250 and tested for detection of anti-Aβ_{42} antibodies in ELISA. The experiment was repeated with similar results.

Th2-prone, in order to demonstrate the real contribution of mannan in Th2 polarization of immune response we immunized B6SJL F1 mice with mannan–Aβ_{28} and measured production of IgG1 and IgG2a^b antibodies (Fig. 4B). The ratio of IgG1 to IgG2a^b antibody in immunized B6SJL F1 mice was 11 (Fig. 4C). Thus, mannan conjugation enhanced Th2-polarized anti-Aβ antibody responses, as has been observed after conjugation of mannan with other peptide immunogens (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 1996; Vaughan *et al*., 1999; Apostolopoulos and McKenzie, 2001).

To directly demonstrate a role of the mannan conjugation in Th1– and Th2-type immune responses, we analyzed the cellular immune responses in individual vaccinated mice. The splenocytes from immune mice were restimulated with Aβ_{40}, and the T-cell proliferation was analyzed (Fig. 5A). Both fAβ_{28} and mannan–Aβ_{28} induced robust T-cell proliferation with stimulation index of 8.2 and 7.7, respectively (Fig. 5A), although antibody responses in mice immunized with fAβ_{28} were significantly weaker than in mice administered with mannan–Aβ_{28} (Figs. 2 and 3). Next, we analyzed a production of Th1 (IFNγ) and Th2 (IL4) lymphokines by immune splenocytes isolated from mice immunized and boosted four times with 5 μg of mannan–Aβ_{28} and compared it with data obtained after vaccination with fibrillar Aβ_{28}. In addition, we detected a production of the pro-inflammatory cytokine TNFα that is expressed by activated macrophages, monocytes, neutrophils, lymphocytes, and natural killer cells and has been suggested to play a pivotal role in regulation of the synthesis of other pro-inflammatory cytokines (Arend and Dayer, 1995). Our data demonstrated that only a small number of splenocytes from mice immunized with the mannan-conjugated immunogen, but not fibrillar Aβ_{28}, produced this pro-inflammatory cytokine after *in vitro* restimulation with Aβ_{40} (Fig. 5B). On the contrary, the same immune splenocytes generated the highest number of cells producing IL4, and immunization of mice with fibrillar Aβ_{28} was less effective than vaccination of mice with Aβ_{28} conjugated with mannan.

B-cell epitope specificity of anti-Aβ_{28} antibodies

To demonstrate the specificity of antibodies and to identify the B-cell antigenic determinant/s within the Aβ_{28} immunogen, we screened the antisera with a series of short overlapping peptides encompassing entire Aβ_{28} peptide using a competition ELISA assay (Cribbs *et al*., 2003b). Preincubation of antisera with 2.5 μM of the full-length Aβ_{42} peptide resulted in strong

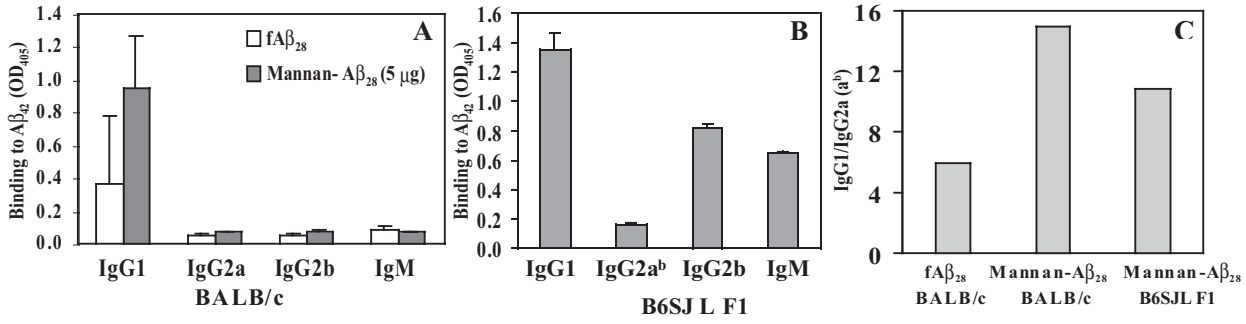

FIG. 4. **(A)** BALB/c mice immunized with mannan–Aβ_{28} (5 μg) or fibrillar Aβ_{28} formulated in PBS induced Th2-polarized anti-Aβ_{42} antibodies of IgG1 isotype (similar results were obtained with 2.5 and 10 μg mannan–Aβ_{28}). Sera for this assay were collected before the rest period (after the third boost) and diluted 1:250 prior to the detection of isotypes. These results were generated with individual mice. **(B)** Immunization of B6SJL F1 mice with mannan–Aβ_{28} also induced anti-Aβ_{42} antibodies of IgG1 isotype. **(C)** IgG1/IgG2a (a^b) ratio for BALB/c mice immunized with mannan–Aβ_{28} or fibrillar Aβ_{28} and B6SJL F1 mice immunized with mannan–Aβ_{28}.

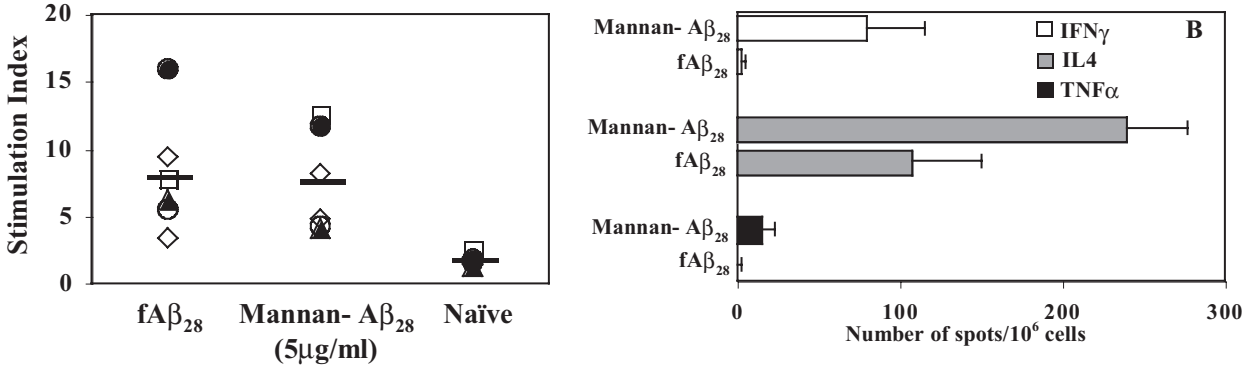

FIG. 5. Mannan–Aβ_{28} and fibrillar Aβ_{28}-induced Th2-type cellular immune responses in BALB/c mice. **(A)** Immune splenocytes isolated from individual mice immunized with mannan–Aβ_{28} or fibrillar Aβ_{28} and *in vitro* restimulated by Aβ_{40} peptide-induced robust T-cell proliferation. Data are presented as Stimulation Index (SI). **(B)** Production of Th1 (IFNγ) and Th2 (IL-4)-type cytokines, as well as pro-inflammatory TNFα by immune splenocytes isolated from mice immunized with 5 μg of mannan–Aβ_{28} or fibrillar Aβ_{28}. The ELISPOT technique was used as described in Materials and Methods, and data are presented as a delta between number of spots in activated with Aβ_{40} and nonactivated splenocyte cultures.

inhibition of antibody binding to Aβ_{42} on the plate (Fig. 6). At 2.5 μM, the A$\beta_{1–15}$ peptide was equally effective at blocking the binding of antibodies from mice immunized with fibrillar Aβ_{28} or the mannan–Aβ_{28} immunogen. Notably, A$\beta_{6–20}$, A$\beta_{11–25}$ or A$\beta_{16–30}$ peptides were ineffective (Fig. 6). Thus, vaccination with both fibrillar Aβ_{28} and mannosylated Aβ_{28} activated B cells specific to the B-cell epitope in the A$\beta_{1–15}$ peptide.

To further analyze the potential therapeutic efficacy of anti-Aβ antibodies generated in response to the Aβ_{28}–mannan conjugate vaccine, we also determined binding to amyloid plaques in human brain tissue. We used pooled sera from mice immunized with mannan–Aβ_{28} and observed that this antiserum bound to amyloid plaques on the brain sections of cortical tissues from a severe AD case (Fig. 7). Preimmune sera from these mice and irrelevant immune sera did not bind to the amyloid plaques (data not shown). These data suggest that anti-Aβ antibodies raised after immunizations with Aβ_{28}–mannan conjugate were potentially functional, as it was demonstrated previously with antibodies specific to fibrillar Aβ_{42} (Schenk *et al*., 1999; Bard *et al*., 2000; Janus *et al*., 2000; Morgan *et al*., 2000) or A$\beta_{1–15}$ peptide fused with foreign T-cell epitope (Agadjanyan *et al*., 2005).

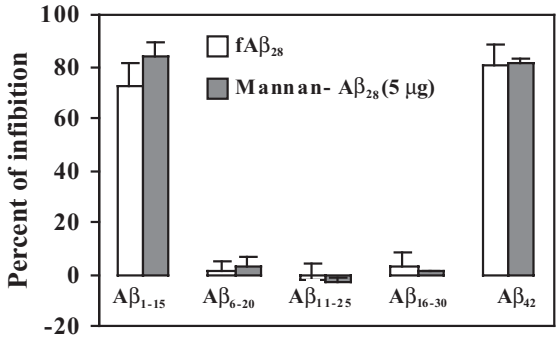

FIG. 6. Mannan–Aβ_{28}, as well as fibrillar Aβ_{28} induced predominantly antibodies specific to the N-terminal region of Aβ_{42}. Mapping of B-cell epitopes was conducted by competition ELISA as we previously described (Cribbs *et al.*, 2003b). Individual sera from immune mice (final dilution 1:250) were collected and preincubated with A$\beta_{1–15}$ A$\beta_{6–20}$, A$\beta_{11–25}$, A$\beta_{16–30}$, or Aβ_{42} peptides with the final concentration of each peptide being 2.5 μM before binding to Aβ_{42}-coated wells. Representative ELISA data from two experiments.

DISCUSSION

Anti-Aβ-immunotherapy is a novel strategy to induce antigen-specific humoral immune responses therapeutic for AD. Although the failure of the first anti-Aβ-immunotherapy clinical trial was disappointing, the follow-up studies indicate that Th2-prone immune response may be more beneficial and safer than a Th1 response. Mannan has previously been shown to be a potent molecular adjuvant enhancing both B- and T-cell immune responses, as well as antigen uptake and presentation (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 2000; Engering *et al*., 1997a, 1997b; Karanikas *et al*., 1997; Gröger *et al*., 2000; Linehan *et al*., 2000; Vaughan *et al*., 2000; Stambas *et al*., 2002a, 2002b, 2005). This molecular adjuvant not only enhanced antibody responses specific to appropriate peptide attached to it (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 2000), but under certain conditions also induced Th2-polarized immunity (Okawa *et al*., 1992; Apostolopoulos *et al*., 1995, 1996; Vaughan *et al*., 1999; Apostolopoulos and McKenzie, 2001). Taking advantage of this property of mannan, we designed a novel AD vaccine that will induce Th2-prone immune responses directed to the Aβ peptide. The data presented here further support the previous observations and demonstrate that mannan conjugates can enhance Th2-polarized immune responses to the Aβ_{28} peptide immunogen.

Previously, we have demonstrated that Aβ_{28} peptide possessed both B- and T-cell antigenic determinants of Aβ_{42} in BALB/c mice (Cribbs *et al*., 2003a). In this study, we confirm

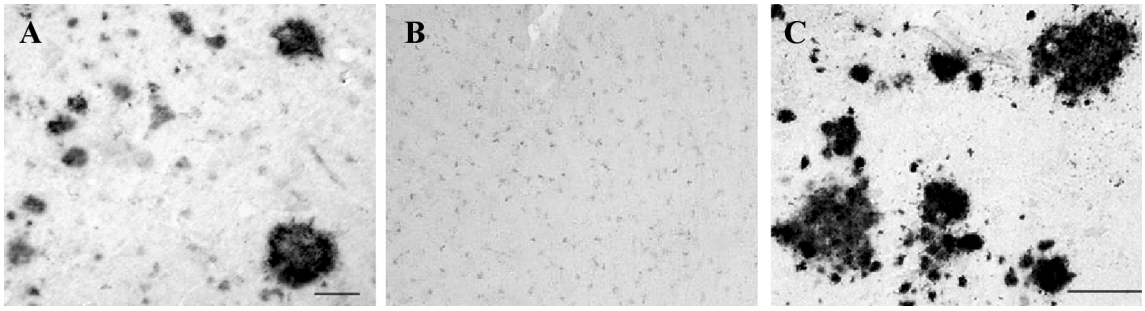

FIG. 7. Mannan–Aβ_{28} induced potentially therapeutic anti-Aβ_{1-15} antibodies, which are capable of binding to amyloid plaques in human brain tissue **(A)**. Coincubation with Aβ_{28} peptide blocked this binding **(B)**. 6E10 antibodies were used as a positive control **(C)**. Picture represents binding of 1:500 diluted pooled sera to a 50-μm brain section of formalin-fixed cortical tissue from an elderly individual with neuropathological and behavioral patterns typical to severe AD. Original magnification, $\times$20.

this observation by demonstrating production of anti-Aβ_{42} antibodies in wild-type mice of H-2^{d}, H-2^{s}, and H-2$^{b\times s}$ immune haplotypes immunized with 100 μg of Aβ_{28} peptide formulated in alum, which is a Th2-type adjuvant (Fig. 1). Of note, previously several groups, including us (Das *et al*., 2003; Petrushina *et al*., 2003; Seabrook *et al*., 2004; Kutzler *et al*., 2005), demonstrated that C57BL6 mice respond to fibrillar Aβ_{42} poorly. The results generated here indicate that H-2^{b} immune haplotype does not respond to Aβ_{28} peptide immunization, suggesting that these mice may not recognize a T-cell epitope within this peptide. While further investigation of these data is required in order to demonstrate an exact mechanism for the lack of response in H-2^{b} mice, it does emphasize the significant hurdle facing development of a small epitope vaccine in humans, which have multiple MHC haplotypes. We chose the Aβ_{28} peptide as a prototype immunogen to test the effectiveness of mannan as a molecular adjuvant to break tolerance against a self-peptide. We hypothesized that mannan should not only enhance antigen uptake and presentation, but may also induce better crosslinking of B-cell receptors, which amplifies the signal to Aβ specific B cells (Reth and Wienands, 1997; Wagle *et al*., 2000). The data presented here showed that very low doses of mannan–Aβ_{28} induced activation of B cells and generation of anti-Aβ_{42} antibodies after only one boost (Fig. 2). Even 2.5 μg of mannan–Aβ_{28} was active after two additional boosts with mannan–Aβ_{28}. In fact, the level of the humoral immune response in this group was similar to that generated in BALB/c mice immunized with 10 μg of fibrillar Aβ_{28} (Fig. 2). To check the longevity of the immune responses, mice from all groups were allowed to rest for 2 months. Importantly, mice boosted with mannan–Aβ_{28} responded to a single recall injection with the same antigen, suggesting that significant immunological memory was present in these mice (Fig. 3). The specificity of these antibodies was confirmed by a competition ELISA in which antisera from immune mice were preadsorbed by small overlapping linear peptides Aβ_{1-15}, Aβ_{6-20}, Aβ_{11-25}, or Aβ_{16-30}. Consistent with previous studies with fibrillar Aβ_{42} vaccine (Cribbs *et al*., 2003b), antisera raised in mice immunized with mannan–Aβ_{28} was specific only to Aβ_{1-15} (Fig. 6) and bound to amyloid plaques in cortical tissue from an AD patient (Fig. 7).

Next, we tested the Th1 and Th2 phenotype of humoral immune responses by analyzing isotypes of anti-Aβ antibodies generated in vaccinated mice. Antibody isotyping has been used as an indirect measure of the contribution of Th1 (IgG2a) and Th2 (IgG1) cytokines to the humoral response (Finkelman *et al*., 1990); thus, we measured anti-Aβ_{42} IgG2a and IgG1 antibodies in the sera of immune mice. Data indicated that both mannan–Aβ_{28} and fibrillar Aβ_{28} in PBS induced highly polarized Th2-type humoral immune responses (IgG1/IgG2a ratios were equal to 15 and 6, respectively) (Fig. 4). To confirm that mannan–Aβ_{28} induces Th2-type immune responses not only in typically Th2-prone Balb/c mice, we measured the production of anti-Aβ_{42} IgG2a^{b} and IgG1 antibodies in B6SJL F1 mice immunized with mannan–Aβ_{28} and showed that IgG1/IgG2a^{b} ratio is 11. Analysis of T-cell responses supported these results. More specifically, immune splenocytes from BALB/c mice immunized with mannan–Aβ_{28} after restimulation with Aβ_{40} peptide induced robust T-cell proliferation and generated substantial amounts of CD4^{+}Th2 cells, as well as a higher percent of cells producing IL4 (Th2) than IFNγ (Th1) cytokines (Fig. 5). Thus, mannan–Aβ_{28} induced predominantly anti-inflammatory Th2 type immune responses in BALB/c mice immunized with mannan–Aβ_{28} peptide. Th1 cytokines (IL12, IL18, and IFNγ) have been implicated in many autoimmune disorders, whereas Th2 type responses (IL-4, IL-10, and TGFβ) in some cases have been shown to attenuate cell-mediated immunity and inhibit autoimmune disease (Smeltz and Swanborg, 1998; Aharoni *et al*., 2000; O'Shea *et al*., 2001; Swanborg, 2001; Weiner and Selkoe, 2002). Therefore, the bias of the anti-Aβ immune responses towards a Th2 phenotype may be potentially beneficial for AD patients. As stated above, we are currently investigating neuropathological changes in APP/Tg 2576 mice vaccinated with mannan–Aβ_{28}, and preliminary data suggest that these animals generated robust anti-Aβ antibody production that can clear/inhibit AD-like pathology in the brains of these animals (Petrushina *et al*., 2006). The development of second-generation vaccine candidates, which promote a Th2-mediated immune response and the removal of the self T-cell epitope of the Aβ from the immunogen, may help to develop novel immunogen–adjuvant configurations which reduce the risk of adverse events that occurred during the first clinical trial in AD patients.

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