Duration and Specificity of Humoral Immune Responses in Mice Vaccinated with the Alzheimer’s Disease-Associated β-Amyloid 1-42 Peptide

CHAD A. DICKEY,1,2 DAVID G. MORGAN,1,2 SAGAR KUDCHODKAR,3 DAVID B. WEINER,3 YUN BAI,4 CHUANHAI CAO,4 MARCIA N. GORDON,1,2 and KENNETH E. UGEN4

ABSTRACT

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by overproduction of β-amyloid (Aβ), which is formed from amyloid precursor protein (APP), with the subsequent pathologic deposition of Aβ in regions of the brain important for memory and cognition. Recently, vaccination of murine models of AD that exhibit Aβ deposition has halted or delayed the usual progression of the pathology of AD. Our group has demonstrated that vaccination of a doubly transgenic mouse model (expressing mutant APP and presenilin-1) with the Aβ1-42 peptide protects these mice from the memory deficits they would ordinarily develop. This report further characterizes the Aβ1-42 peptide vaccine in mice. Anti-Aβ response time course indicated that at least three vaccinations (each 100 μg) were necessary to elicit a significant anti-Aβ titer. Subsequent vaccinations resulted in half-maximal antibody titers of at least 10,000, and these titers were maintained for at least 5 months after the final boost. Peptide binding competition studies indicated that the highest humoral responses are generated against the N terminus of the Aβ peptide. Also, measurement of specific murine Ig isotypes in Aβ-vaccinated mice demonstrated a predominant IgG1 and IgG2b response, suggesting a type 2 (Th2) T-helper cell immune response, which drives humoral immunity. Finally, lymphocyte proliferation assay experiments using Aβ peptides and splenocytes from vaccinated mice demonstrated that the vaccine specifically stimulates T-cell epitopes present within the Aβ peptide.

INTRODUCTION

It is estimated that during the next 50 years, the number of cases of Alzheimer’s disease (AD) in the US will increase from 4 million to 10 million or more, underscoring the pressing need for the development of novel effective therapies to combat this devastating disease. In AD, β-amyloid (Aβ) from the amyloid precursor protein (APP) is overproduced and deposited in extracellular plaques within the cerebral cortex and hippocampus, resulting in neurodegeneration with associated memory loss (Hardy, 1997; Selkoe, 2000). Recently, transgenic murine models have been utilized for vaccine studies with the Aβ1-42 peptide. In a landmark paper published in 1999, Schenk et al. reported that vaccination of an APP transgenic mouse model with the Aβ peptide resulted in a significant reduction in cerebral Aβ deposits. In a subsequent experiment, those investigators showed that the passive transfer of anti-Aβ antibodies (polyclonal or monoclonal) mediated the beneficial effect of vaccination as judged by a decrease in Aβ burden (Bard et al., 2000). Further studies by Basksai et al. demonstrated through multiphoton imaging that local application of Aβ antibody to the brains of live PDAPP mice resulted in clearance of cerebral Aβ plaques (Basksai et al., 2001). Other important work in the area of immune responses against Aβ is that from Solomon’s group in Tel Aviv. Her laboratory showed that vaccination of mice with engineered filamentous phage that express the Aβ3-6 epitope (EFRH) resulted in anti-Aβ antibodies of significant titer (Frenkel et al., 2000). Previously, Solomon’s laboratory demonstrated that monoclonal antibodies that recognize the amino region of Aβ could prevent the for-
mation of Aβ fibrils (Solomon et al., 1996) and cause disaggereation of already-formed Aβ fibrils (Solomon et al., 1997). Recently, our group (Morgan et al., 2000) as well as others demonstrated that Aβ1-42 peptide vaccination induced a significant antibody titer against Aβ along with amelioration of neural pathology and, importantly, protection from functional memory deficits that would normally have occurred in the transgenic mice used in these studies (Janus et al., 2000; Morgan et al., 2000). The study reported here further analyzes the immune responses to Aβ peptide vaccination. Specifically, in this report, we examined the time course of the anti-Aβ response to vaccination, confirmed the location of the major B-cell reactivities using truncated Aβ peptides in a competition assay, determined anti-Aβ isotype specificities as an indication of T-helper cell responses, and determined the ability of Aβ peptides to stimulate the proliferation of splenocytes from vaccinated animals. The results will aid in the development of vaccines targeting the Aβ peptide.

MATERIALS AND METHODS

Animals and vaccination regimen for time course analysis

Eight 5-month-old mice transgenic for the presenilin (PS1) gene were bled prior to any vaccination and subsequently vaccinated with the Aβ1-42 peptide (100 μg in complete Freund’s adjuvant). These mice were then bled 7 to 10 days after each vaccination, and the blood was centrifuged to collect sera. Mice were boosted 2 weeks after the initial vaccination and monthly for 7 months (100 μg of Aβ1-42 in incomplete Freund’s adjuvant for vaccine boosters 2 through 5 and in mineral oil for vaccinations 6 through 9) (Schenk et al., 1999). Sera were also collected 5, 9, and 14 months after the final Aβ1-42 vaccination.

ELISA methodology

An ELISA analysis was performed in 96-well plates as previously described (Morgan et al., 2000). Briefly, wells were coated with 250 ng of Aβ1-42 peptide (BACHEM, Torrance, CA) and incubated at 37°C for 1 h. They were then washed and blocked overnight with 5% nonfat dry milk in PBS. After washing of the wells, primary sera from transgenic mice vaccinated with Aβ1-42 were added in duplicate at an initial dilution of 1:2048 and diluted serially in PBS to 1:4,194,304. These plates were incubated at 37°C, then washed 10 times and blocked for 30 min at 37°C. After the plates had been washed four times, anti-mouse IgG (generated against the whole immunoglobulin molecule) conjugated with horse-radish peroxidase (HRP) (Sigma Chemical Co., St. Louis, MO) diluted 1:5000 was added to the plates that were then washed and developed with (3,3′,5,5′-tetramethylbenzidine HCl) TMB (Sigma). The plates were analyzed spectrophotometrically at 450 nm. Half-maximal antibody titers were determined by dividing the highest OD150 value in the dilution range of each sample by 2. The values for the six positive mice were averaged, and the standard deviation was calculated.

Peptide competition inhibition assay

Plates were coated with Aβ1-42 peptide and blocked as described above. Sera from three mice with high anti-Aβ antibody titers were each diluted 1:500 in PBS. Four concentrations (at 10-fold decreases in concentrations beginning at either 0.001, 0.01, or 0.1 μg/ml) of the following Aβ peptides (corresponding to the amino acid range) were added to individual tubes of antisera: 1-16, 10-20, 20-29, 29-40, 1-40, or 1-42. A peptide from collagen II (H2N-KNNQKSEPLIGRKKT-COOH) was also used at different concentrations as a nonspecific control. The tubes with experimental or control peptide + antisera were incubated at 37°C for 1 h. The peptide-treated sera were subsequently added in triplicate to plates coated with the Aβ1-42 peptide (at 5 μg/ml, 50 μl per well). The plates were then analyzed for binding as described in the ELISA protocol presented above. The OD values were converted to a percentage of the OD value obtained in the presence of the control peptide or in the absence of any peptide.

Immunoglobulin class determination assay

Plates were coated with Aβ1-42 peptide and blocked as previously described. Several sera with known high titers were selected and pooled. This pool, along with normal mouse serum, was then diluted 1:1048, 1:4096, and 1:16,384 and added to wells in duplicate. Specific isotypes were determined through the use of a kit containing anti-mouse Ig-subclass-specific HRP-conjugated secondary antibodies (Zymed), and 2.2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Zymed) was used as the developing substrate. These plates were read spectrophotometrically at 405 nm at 10 and 20 min after addition of the sub-

FIG. 1. Time course of antibody response after Aβ vaccination. The graph demonstrates the average half-maximal antibody titers ± SEM (see text for description of titer determination methods) against Aβ1-42 in PS1 mice as a function of the number of vaccinations with the peptide, as well as age in months. Titers were determined 2 weeks after each monthly inoculation. The arrows indicate when the vaccine inoculations were administered. The first data point represents the value of the preimmune sera, with the first vaccination given the following day.
| AA No. | H\textsubscript{2}N\textsuperscript{+} | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | COO\textsuperscript{-} |
|-------|------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1–42  | D A E F R H D S G Y E V H H Q K L V F F | A E D V G S N K G | A I I G L M V G G V V I A |
| 1–16  | D A E F R H D S G Y E V H H Q K | | |
| 10–20 | Y E V H H Q K L V F F | | |
| 29–40 | F A E D V G S N K G | | |
strate, and the relative amounts of the different murine Ig isotypes binding to the immobilized Aβ1-42 peptide were determined.

**Lymphocyte proliferation assay using Aβ peptides**

Two Balb/C mice vaccinated as part of the antibody time-course analysis presented in this paper were utilized in LPA analyses. Mice had half-maximal anti-Aβ1-42 antibody titers of at least 5000 at the time of this analysis. Each mouse was boosted intraperitoneally with 100 μg of the Aβ1-42 peptide. There days after the boost, the mice were sacrificed, the spleens were excised, and a single viable spleen cell population was prepared with a Stomacher-80 apparatus (Seward/Lab System, Inc). Red blood cells were lysed, and the viability of the cell population was determined to be approximately 92% by trypan blue exclusion.

Two Aβ peptides were utilized to test for lymphocyte proliferation activity; i.e., Aβ1-40 (which is freely soluble in aqueous buffer) and Aβ1-42 (which initially needs to be solubilized in ammonium hydroxide, pH 8.5). Each peptide was solubilized appropriately and added to triplicate wells with 5 × 10⁵ spleen cells at concentrations of either 5 or 1 μg/ml in R10 (RPMI1640 medium supplemented with 10% FBS). Cells were incubated and stimulated in the presence of peptides for 3 days. After incubation, 1 μCi of [³H]thymidine was added, and the plates were incubated for 15 h before termination of the reaction. Cells were lysed, and specific radioactivity was measured in the lysate. The stimulation index (SI) was determined by dividing the average spleen cell proliferation (measured as cpm) in response to different concentrations of Aβ1-40 or Aβ1-42 peptides by the average proliferation (cpm) induced by R10 medium alone as a negative control: SI = average cpm (Aβ peptides)/average cpm (R10).

**RESULTS**

No significant anti-Aβ humoral immune response developed until after the third vaccination with the Aβ1-42 peptide. After the third vaccination, 50% maximal titers began to increase significantly, and they plateaued at approximately 15,000 following the sixth vaccine booster (Fig. 1). Additionally, a 50% maximal titer of approximately 10,000 was demonstrated 5 months after any further Aβ1-42 vaccination (Fig. 1) with a gradual decrease through the lifespan of the mice, yielding detectable titers 14 months after any further boosting.

Binding efficacy and specificity of sera from immunized mice was measured through the use of several truncated Aβ peptide fragments in a competitive binding inhibition assay. Three sera were selected from mice vaccinated with the Aβ1-42 peptide. All of the sera were chosen on the basis of demonstration of a high anti-Aβ titer in previous ELISA analysis (data not shown). These samples were diluted 1:500 and separately incubated with four 10-fold dilutions (the starting dilution was determined from previously collected data) of six peptide variants from Aβ (Table 1) and a non-Aβ peptide from collagen II. These samples were then added in triplicate to wells coated with Aβ1-42 for ELISA screening as described above. The values shown in Figure 2 are an average ± SD of the three serum samples.

Three of the six Aβ peptides (10-20, 20-29, and 29-40) failed to exhibit an inhibitory effect on the binding efficacy of antisera to immobilized solid-phase Aβ1-42 peptide, indicating that there was likely no active role for these regions in stimulating humoral immunity. The Aβ1-16 peptide, when mixed with specific immune antisera at a concentration of 10 μg/ml, resulted in a 50% reduction in binding of antisera to the immobilized Aβ1-42. Even at 1 μg/ml, Aβ1-16 exhibited ~30% binding. Incubation of either the Aβ1-40 or the Aβ1-42 peptide with antisera resulted in complete inhibition of binding to the immobilized Aβ1-42 peptide. However, the Aβ1-40 peptide was 100% effective at 10 μg/ml but not 1 μg/ml, whereas the Aβ1-42 peptide completely inhibited binding at 1 μg/ml (Fig. 2). These findings may indicate the potentially important role of the two hydrophobic C-terminal amino acids (41-Ala and 42-Thr) in inducing protective anti-Aβ immune responses. However, the necessity for Aβ to be in a fibrillar form to induce pathology and mediate protective immunity has not been established unequivocally. Addressing this debate are recent findings by Sigurdsson and colleagues, who demonstrated that vaccinations of a AD transgenic mouse with a nonfibrillar, soluble nonamyloidogenic peptide resulted in a significant decrease in Aβ burden in the mouse (Sigurdsson et al., 2001).

In our studies, the immunoglobulin isoforms in sera from PS-1 mice vaccinated with Aβ1-42 were determined through the utilization of HRP-conjugated anti-mouse Ig-subclass specific antibodies. The IgG1 and IgG2b isoforms were detected in the greatest amounts in the antisera pool. The IgG2b isotype accounted for the highest level and was maintained throughout the entire dilution range examined (Fig. 3). Although detectable, IgG2a was very low in comparison with the other two isoforms. The production of IgG1 and IgG2b is indicative of predominantly a type 2 (Th2) T-helper cell re-

![FIG. 2. Logarithmic representation of percent binding of antisera from vaccinated mice to immobilized Aβ1-42 after competition with various truncated Aβ peptides. Values represent the mean percent binding of three individual serum samples ± SEM. Concentration points indicate percent binding ± SEM. The peptide designation is as follows, with the numbers indicating the amino acids included from the Aβ1-42 parent peptide: (+) 1-16, (○) 10-20, (■) 20-29, (▲) 29-40, (▼) 1-40, and (●) 1-42.](image-url)
response, which drives humoral immune responses. All of the sera pooled in the sample measured for these analyses were collected after the sixth inoculation, presumably long after class switching from IgM to IgG had occurred. As expected, IgM levels were very low. Likewise, IgG3 isotype levels were very low (see Fig. 3).

Figure 4 demonstrates the T-cell proliferative activity using two Aβ peptides (1-40 and 1-42). The results indicated that the peptides specifically stimulated proliferation of spleen cells from mice vaccinated with the Aβ1-42 peptide. The two peptides appeared to be equipotent in their ability to stimulate lymphocyte proliferation with stimulation indexes (SIs) of at least 12 with the 5 μg of peptide and of at least 5 with 1-μg of peptide stimuli. An SI above 5.0 is considered to be very significant and is indicative of stimulation of a strong T-cell epitope. Therefore, our results with the Aβ peptide establish the presence of strong T-helper cell epitopes within several relevant Aβ peptides. The measurement of cytokines released in response to Aβ peptide stimulation from the LPAs is currently being performed. Preliminary results indicate a predominant release of interleukin-4 (IL-4) but not interferon-gamma (IFN-γ) (data not shown). Analysis of cytokine release will further characterize the relative contributions of Th2 (T-helper 2 lymphocytes, which drive antibody production) or Th1 (T-helper 1 lymphocytes, which drive cytotoxic T cells) responses to overall cellular immunity.

**DISCUSSION**

Because of the recent success of vaccination with the Aβ1-42 peptide in decreasing cerebral amyloid burden (Schenk et al., 2000; Weiner et al., 2000) and memory deficits (Janus et al., 2000; Morgan et al., 2000) in murine models of AD, a more comprehensive understanding of the immune responses in these animals is warranted.
Mice transgenic for the PS1 gene that were vaccinated with the Aβ1-42 peptide generated significant anti-Aβ1-42 antibody responses after three inoculations at monthly intervals, the antibodies reaching their maximal titer plateau after the sixth vaccination. This half-maximal antibody titer was maintained at an average of 14,600 for the remainder of the vaccine regimen and was sustained well beyond cessation of vaccine boosts, with only a 30% decrease 5 months after the last vaccination. At 8 months after the final boost, 35% of the maximal average titer was maintained, and even at 14 months beyond the final vaccine boost, there was still a significant amount of serum antibody present. This indicates a relatively long-lived antibody response after the Aβ1-42 peptide vaccination.

The competitive inhibition assay allowed the determination of the specific region of Aβ1-42 that is likely responsible for mediation of protective immunity. Of the peptide truncations used (which encompassed the entire Aβ1-42 peptide), Aβ1-16 was the only truncated Aβ peptide that blocked binding of antibodies to the immobilized Aβ1-42 peptide, indicating that this region contributes significantly to B-cell responses after Aβ vaccination. More interestingly, the Aβ1-42 peptide was more effective than Aβ1-40 in blocking serum antibody binding to immobilized Aβ1-42 peptide, despite the fact that there is only a 2-aa difference at the c terminus between the two peptides. It has previously been described that Aβ1-42 forms fibrils more readily than Aβ1-40, which is attributed to the presence of the two additional hydrophobic residues A and T at positions 41 and 42, respectively (Jarrett and Lansbury, 1993; Snyder et al., 1994). This could result in the folding of Aβ1-42 into the β-sheet conformation more readily, resulting in more rapid polymerization of the fibrils. Therefore, the immune system might recognize Aβ in its fibrillar form more effectively, suggesting a conformational binding preference rather than binding to a purely sequence-specific region.

The important role of humoral immunity was further fortified using isotype-specific mouse antibodies. A Th2 response induced by the vaccine was indicated by the generation of large amounts of IgG1 and IgG2a, similar to previous findings (Weiner et al., 2000). However, other T-cell activity cannot be completely excluded because of the detection of small amounts of IgG2a. Additionally, measurements were made after class switching had occurred from IgM to IgG. Our data also indicate the ability of our Aβ vaccination regimen to stimulate T-cell responses. The Aβ1-40 and Aβ1-42 peptides appeared to be equipotent in stimulating T-cell proliferation, indicating the presence of T-cell epitopes in the Aβ peptide (see Fig. 4). Further analysis of potential cellular immune responses against Aβ1-42 through vaccination is important to determine. It is relevant because such cellular immune responses could play some accessory role in protective immunity even though protection against Aβ deposit in the brain through vaccination depends in the brain through vaccination does appear to be mediated by antibodies. Likewise, potential cellular immune responses, through Aβ vaccination or other vaccines, may have a deleterious effect, as noted in conditions such as multiple sclerosis or HTLV-I-associated myelopathy.

In summary, this report further characterizes the immune responses elicited by vaccination of mice with an Aβ1-42 peptide. The results indicate that at least three subcutaneous vaccinations with the Aβ peptide are necessary to elicit humoral immune responses. Competition ELISA studies with Aβ peptides of various lengths confirm the presence of major B-cell epitopes in the amino region (first 16 amino acids) of the peptide that are likely involved in protective immune responses. Specific immunoglobulin isotype analysis of antisera from vaccinated animals suggests a strong Th2 immune response, which mediates potent humoral immune responses. Lymphocyte proliferation assays using splenocytes from Aβ1-42 peptide-vaccinated animals also demonstrated the ability of the Aβ peptides to elicit potent T-cell proliferative responses. The observations reported in this paper will have implications for further vaccine development against AD that target the Aβ protein. This includes information potentially important for the development of novel vaccine strategies against this disease such as the DNA technology. The findings reported here may also be important for the development of passive antibody immunotherapies against AD as well as other neurodegenerative diseases.

ACKNOWLEDGMENTS

This work was supported in part by a National Institutes of Health/National Institute on Aging grant (AG18478) to K.E.U., M.N.G., and D.G.M.

REFERENCES


Address reprint requests to:
Dr. Kenneth E. Ugen
Department of Medical Microbiology and Immunology
University of South Florida College of Medicine
MDC 10
12901 Bruce B. Downs Boulevard
Tampa, FL 33612

E-mail: kugen@hsc.usf.edu