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Antibodies to Potato Virus Y Bind the Amyloid β Peptide

*IMMUNOHISTOCHEMICAL AND NMR STUDIES**

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Studies in transgenic mice bearing mutated human Alzheimer disease (AD) genes show that active vaccination with the amyloid β (Aβ) protein or passive immunization with anti-Aβ antibodies has beneficial effects on the development of disease. Although a trial of Aβ vaccination in humans was halted because of autoimmune meningoencephalitis, favorable effects on Aβ deposition in the brain and on behavior were seen. Conflicting results have been observed concerning the relationship of circulating anti-Aβ antibodies and AD. Although these autoantibodies are thought to arise from exposure to Aβ, it is also possible that homologous proteins may induce antibody synthesis. We propose that the long-standing presence of anti-Aβ antibodies or antibodies to immunogens homologous to the Aβ protein may produce protective effects. The amino acid sequence of the potato virus Y (PVY) coat protein is highly homologous to the immunogenic N-terminal region of Aβ. PVY infects potatoes and related crops worldwide. Here, we show through immunocytochemistry, enzyme-linked immunosorbent assay, and NMR studies that mice inoculated with PVY develop antibodies that bind to Aβ in both neuritic plaques and neurofibrillary tangles, whereas antibodies to material from uninfected potato leaf show only modest levels of background immunoreactivity. NMR data show that the anti-PVY antibody binds to Aβ within the Phe[4]–Ser[8] and His[13]–Leu[17] regions. Immune responses generated from dietary exposure to proteins homologous to Aβ may induce antibodies that could influence the normal physiological function of the protein and the development or progression of AD.

Despite great advances in our understanding of the genetics and molecular biology of Alzheimer disease (AD),[2] we do not fully understand why ~99% of people with the disease are affected. Although familial early-onset AD is caused by well described mutations in the amyloid β (Aβ) precursor (chromosome 21) and presenilins 1 and 2 (chromosomes 1 and 14) (1), these mutations are responsible for only ~1–2% of the cases of the disease. The most important genetic risk factor for the more prevalent (so-called sporadic) disease is the ϵ4 allele of apoE, which is well described and is responsible for ~40–60% of the inherited risk. However, the ϵ4 allele is likely not causative, as approximately one-third of people with the disease do not have the gene, and many people with the gene do not have the disease. (45% of apoE ϵ4 homozygotes do not get the disease by age 80 (2).)

Immunization with the Aβ peptide produces behavioral and histopathological improvement in transgenic mice bearing genes for human AD (3). In these transgenic mice, the Aβ vaccination paradigm is effective when administered either early in life, before onset of behavioral or structural evidence of the disease, or later, after disease onset (3). Because both active vaccination with the Aβ peptide and passive immunization with anti-Aβ antibodies have beneficial effects (4), the potential for AD therapy is under active investigation (4). This vaccination approach has been thwarted by the development of autoimmune meningoencephalitis in both mouse studies (5) and human trials in the United States and Europe (6). However, subjects who developed anti-Aβ antibody responses had improved cognitive function and activities of daily living (7) as well as clearance of the Aβ deposits (8). Hock and Nitsch (9) have concluded that "in humans . . . antibodies against Aβ-related epitopes are capable of slowing progression of AD." Currently ongoing Phase 3 clinical trials of Aβ immunotherapy must be completed before answers concerning the therapeutic value of this approach can be obtained.

We propose that the mechanisms demonstrated by the Aβ immunization paradigm may also be operating lifelong, without active or passive vaccination. Those individuals with higher levels of the presumed naturally occurring anti-Aβ antibodies may be protected from developing AD. Conflicting studies have been reported thus far on this possibility: increased (10–12), decreased (13–15), or unchanged (16) levels of anti-Aβ autoantibodies have been noted in studies of AD patients and control subjects. Moir *et al.* (17) found that circulating autoantibodies specific for Aβ oligomers are decreased in AD. It is not clear whether the studies discussed above measured total circulating

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[2] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β; PVY, potato virus Y.

anti-Aβ antibodies or only those antibodies that were not bound to circulating Aβ (18). Also, the presence of circulating anti-Aβ antibodies may very well be modified by the presence of disease; anti-Aβ antibody studies have not yet been completed in longitudinal studies of as yet unaffected subjects. It is also not clear if the assays applied in these studies were sensitive to cross-reacting antibodies. Thus, the active and passive Aβ immunization paradigm suggests that the presence of circulating anti-Aβ antibodies may influence the development of AD. In the absence of Aβ vaccination, exposure to an immunogen that bears significant amino acid sequence homology to Aβ could result in antibody production that has either protective or detrimental consequences (as illustrated by the studies mentioned above).

To explore this hypothesis, we identified a naturally occurring protein that is highly homologous to the human Aβ peptide and that is a coat protein from a plant virus, potato virus Y (PVY) strain N (tobacco veinal necrosis) (BLAST, NCBI, and National Institutes of Health), to which humans are commonly exposed. PVY is an RNA virus and a member of the genus *Potyvirus* in the family Potyviridae (19, 20). It contains a single-stranded RNA molecule of 9 ± 7 kb, which is translated into a large precursor protein that is cleaved into 10 mature proteins (21, 22). PVY infects solanaceous crops (of the nightshade family) such as potatoes, peppers, tomatoes, cucumbers, and tobacco. Potatoes are the fourth largest food crop in the world. Infection with PVY limits crop yield but does not destroy all growth. PVY is found worldwide, and it is estimated that ~15% of potato crops are infected. It is likely that some potatoes consumed by humans are infected with PVY (23).

We report that antibodies to PVY bind to Aβ in solution and in tissue sections. Data are presented illustrating the biochemical nature of the binding of anti-PVY antibodies to the same region of Aβ as is bound by therapeutic antibodies to the Aβ protein.

EXPERIMENTAL PROCEDURES

Antibody Production—50 μg of peptide (27 amino acids from positions 52 to 77 of PVY with cysteine on the N terminus) was emulsified with 1:1 (v/v) Freund's complete adjuvant for the initial intraperitoneal injection, followed by a boost in Freund's incomplete adjuvant 2 weeks later, with monthly boosters thereafter. Mice were also inoculated with Aβ and infected and uninfected potato leaves. The positive control for PVY (catalog no. LPC20001, Agdia, Inc., Elkhart, IN) was resuspended in 10 mM citrate buffer containing 1 M urea and 0.1% β-mercaptoethanol, spun at 14,000 × *g* for 15 min to remove the particulates, and then dialyzed against phosphate-buffered saline. The first injection used Freund's complete adjuvant, followed by a booster 2 weeks later in Freund's incomplete adjuvant and then four more boosters at 1-month intervals with the latter adjuvant. Equal volumes of the sample and Freund's adjuvant were emulsified for the injections.

Enzyme-linked Immunosorbent Assay Screening—Plates were coated overnight with 10 μM Aβ or synthetic peptide in sodium bicarbonate buffer (pH 9.6). Plates were rinsed and blocked in 5% bovine serum albumin in Tris-buffered saline (50 mM Tris-HCl and 150 mM NaCl (pH 7.6)) and 0.001% Tween 20 (pH 7.6), followed by incubation either with serum from the mice injected with the synthetic peptide (35-1 through 36-3) or with serum from the leaf-injected mice (37-1 through 38-3) at dilutions of 1:20 (blue bar) and 1:50, respectively. Plates were developed with ABTS.

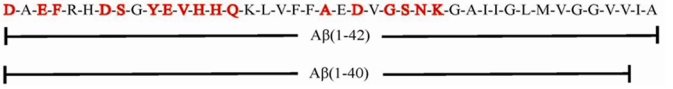

FIGURE 1. **Primary amino acid sequences of the Aβ-(1–40) and Aβ-(1–42) peptides.** The residues shown in *red* show homology to the PVY protein. The sequence of the N-terminal region of the PVY strain is H_2N-DDEFELDS*X*YEVHHQ*XXXXX*ANDTIDAGGSNK (where *X* is an unspecified amino acid on PVY) (BLAST, NCBI, and National Institutes of Health).

Immunocytochemistry—Hippocampal samples were obtained from patients with clinically and histopathologically confirmed AD (n = 3; ages 76–77) and aged-matched controls (n = 2; ages 48–67) (24). Tissue was fixed with methacarn (methanol/chloroform/acetic acid, 6:3:1) overnight at 4 °C, dehydrated, and embedded in paraffin. Endogenous peroxidase activity was eliminated by incubation in 3% H_2O_2 in Tris-buffered saline for 30 min. To reduce nonspecific binding, cells were incubated for 30 min with 1% normal goat serum in Tris-buffered saline. After rinsing briefly with 1% normal goat serum, cells were incubated overnight with primary antibody. Cells were stained with the peroxidase anti-peroxidase method (29) using 3,3′-diaminobenzidine as a chromogen (Dako Corp., Carpinteria, CA).

NMR Spectroscopy—Commercially prepared anti-potato virus IgG polyclonal antibody (Phyto Diagnostics, North Saanich, British Columbia, Canada) was obtained as a solution (5 mM) in 50% ammonium sulfate and 100 mM phosphate buffer. Uniformly ^{15}N-labeled Aβ-(1–40) and Aβ-(1–42) peptides (recombinant peptides) were prepared in monomeric form using procedures developed in our laboratory (25). In brief, this procedure involved disaggregation of the Aβ peptides (0.2–0.5 mg) by sonication in aqueous basic solution (pD 11, 0.2 ml, 10 mM NaOD), followed by mixing with cold (5 °C) phosphate buffer solution (pH 7.5, 0.6–1.0 ml, 5 mM) containing 0.50 mM predeuterated Na_2EDTA-d_{12} and 0.05 mM NaN_3. The amount of peptide and buffer varied in accordance with the desired peptide concentration (50–200 μM). To prevent aggregation, peptide solutions were kept cold (5 °C), and NMR spectra were obtained within 30 min of the sample preparation and at 5 °C. A Varian 600-MHz Inova spectrometer equipped with an HCN Bioprobe® was used for data acquisition, and the two-dimensional ^{1}H-^{15}N heteronuclear single quantum coherence experiments were recorded (on average) with 32 scans, 2048 complex points and the transmitter placed on the water signal (26). The sweep widths were 6373.5 and 2000.0 Hz in the F_1 and F_2 dimensions, respectively. Processing was done on PC or Octane-2 (Silicon Graphics) computers equipped with the Felix program (Accelrys).

RESULTS

Homology Sequencing—The amino acid sequences of Aβ and the PVY coat protein are shown in Fig. 1. The N-terminal domain of PVY is exposed to the exterior of the virion particle, enhancing the likelihood that it is immunogenic (27, 28). PVY

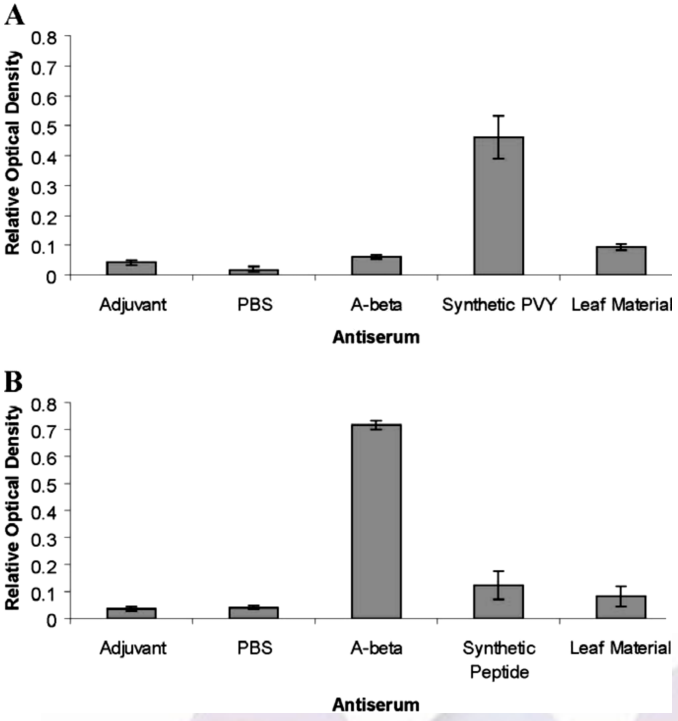

FIGURE 2. **Enzyme-linked immunosorbent assay data for plated synthetic peptide (PVY-(52–77)) probed with each of the specified antisera (*A*) and plated Aβ probed with each of the different antisera (*B*).** *PBS*, phosphate-buffered saline.

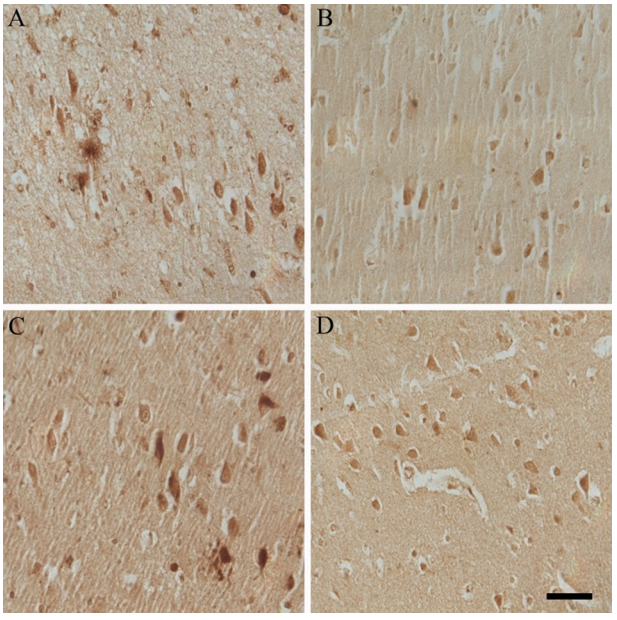

FIGURE 3. **Antibodies raised against PVY-(52–77) (*A*) and the leaf control (*C*) bind to neurofibrillary tangles, senile plaques, and neurons in AD (*B*) and to neurons in control cases (*D*).**

has 6 amino acids (at positions 60–65) that share a high homology to the N-terminal region of Aβ. This N-terminal region of Aβ (residues 1–40) has been demonstrated to be therapeutic in Aβ precursor protein-overexpressing animal models (29, 30). Also, it is this region (residues 4–10) of Aβ that is most highly immunogenic for B cells (31). The three-dimensional structure of the PVY protein is not yet known.

Enzyme-linked Immunosorbent Assay—To determine whether antibodies generated following vaccination with the PVY synthetic peptide labeled Aβ as well as the synthetic peptide, enzyme-linked immunosorbent assay screening was performed, which showed that antibodies made against the synthetic peptide had a high affinity for both the synthetic peptide and Aβ, whereas antibodies to the positive leaf control showed a weaker affinity than the synthetic peptide antibody for both Aβ and the synthetic peptide (Fig. 2). Lower levels of immunoreactivity were found using the antibodies to the control leaf material (Fig. 2).

Aβ is associated with senile plaques, neurofibrillary tangles, and neurons in AD (31); therefore, we tested whether mice inoculated with the PVY synthetic peptide develop antibodies that label Aβ in neuritic plagues as well as neurofibrillary tangles (Fig. 3). The synthetic peptide antibody recognized senile plaques, neurofibrillary tangles, and neurons. The positive control leaf antibody recognized neurofibrillary tangles, granulovaculolar degeneration, and neurons in AD cases (Fig. 3). Neuronal staining was observed in control cases for both the synthetic peptide and control leaf antibodies.

NMR Spectroscopy—To explore the binding between the Aβ peptide and the anti-PVY polyclonal antibody, we undertook NMR spectroscopic studies. The NMR peak assignments correspond to monomeric Aβ peptide (25), and the sample preparation protocol ensured that the Aβ peptides were monomeric at the beginning the NMR experiments. Aggregation during NMR data acquisition, particularly by the more aggregation-prone Aβ-(1–42) peptide, was prevented by acquiring the data at reduced temperatures (5 °C).

Fig. 4 shows the heteronuclear single quantum coherence NMR spectra of uniformly ^{15}N-labeled Aβ-(1–40) and Aβ-(1–42) peptides. The spectra of the peptides alone are superimposed with those containing 1:50 molar eq of the anti-PVY antibody. Heteronuclear single quantum coherence spectroscopy, which detects ^{1}H atoms directly attached to ^{15}N atoms, is a standard NMR experiment for proteins and provides a fingerprint for the backbone. The narrow chemical shift dispersion in the ^{1}H dimension (8.7 to 8.1 ppm) demonstrates that the peptides adopt predominantly monomeric, random, extended chain structures, consistent with previous studies (25, 33).

With the anti-PVY antibody, several amide-NH peaks have different chemical shifts that are more confined to the polar 1–28 N-terminal residues and not within the hydrophobic 29–40 or 29–42 C-terminal peptide region. The residues showing the most pronounced chemical movements include Phe^{4}, Arg^{5}, Ser^{8}, Tyr^{10}, His^{13}, Gln^{15}, Lys^{16}, Leu^{17}, and Ser^{26} for the Aβ-(1–40) peptide and Phe^{4}, Arg^{5}, Ser^{8}, His^{13}, Gln^{15}, Lys^{16}, Leu^{17}, Ser^{26}, and Asn^{27} for the Aβ-(1–42) peptide.

Graphical depictions show that the ^{1}H and ^{15}N chemical shift differences are localized within two regions, Phe^{4}–Ser^{8} and His^{13}–Leu^{17}, which may constitute a binding pocket associated within PVY (Figs. 5 and 6). Control studies showed that the chemical shift movement upon addition of the anti-PVY antibody to the Aβ peptide solution was not caused by other components (such as ammonium sulfate) present within the antibody solution. Because these studies utilized commercially prepared anti-PVY antibody solutions, we were unable to conduct titrations at antibody concentrations greater than 1:50

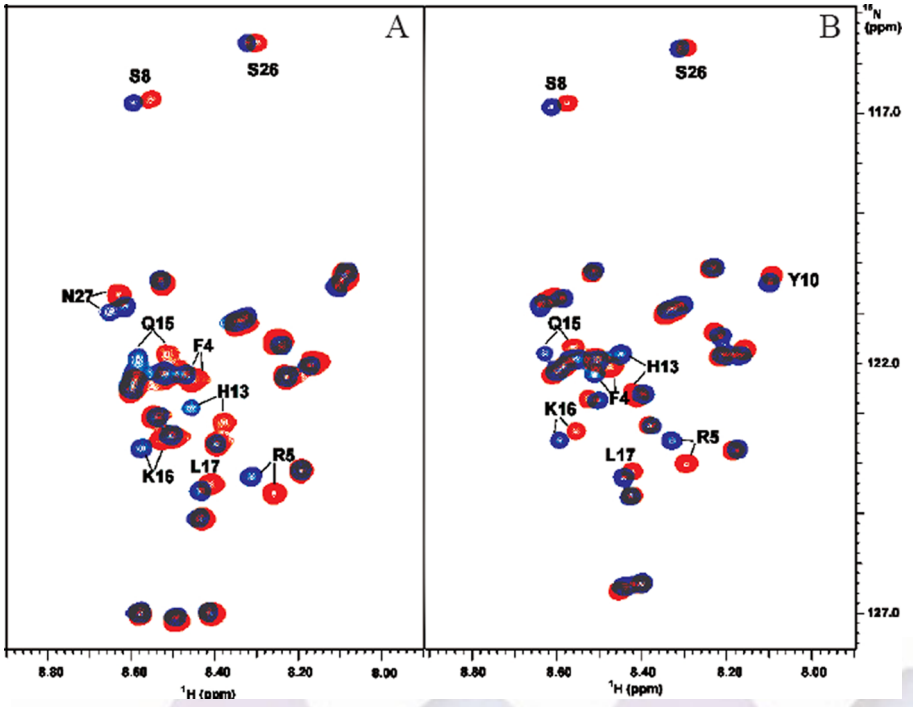

FIGURE 4. **Expanded ^{1}H-^{15}N heteronuclear single quantum coherence spectral regions showing the backbone NH signals of the Aβ-(1–40) and Aβ-(1–42) peptides (50 mM) in aqueous phosphate buffer (5 mM, pH 7. 3, 5 °C).** *Red* peaks correspond to the peptides alone, whereas *blue* peaks are the peptides plus 1:50 molar eq of anti-PVY IgG polyclonal antibody. The peaks undergoing significant chemical shift movements are labeled.

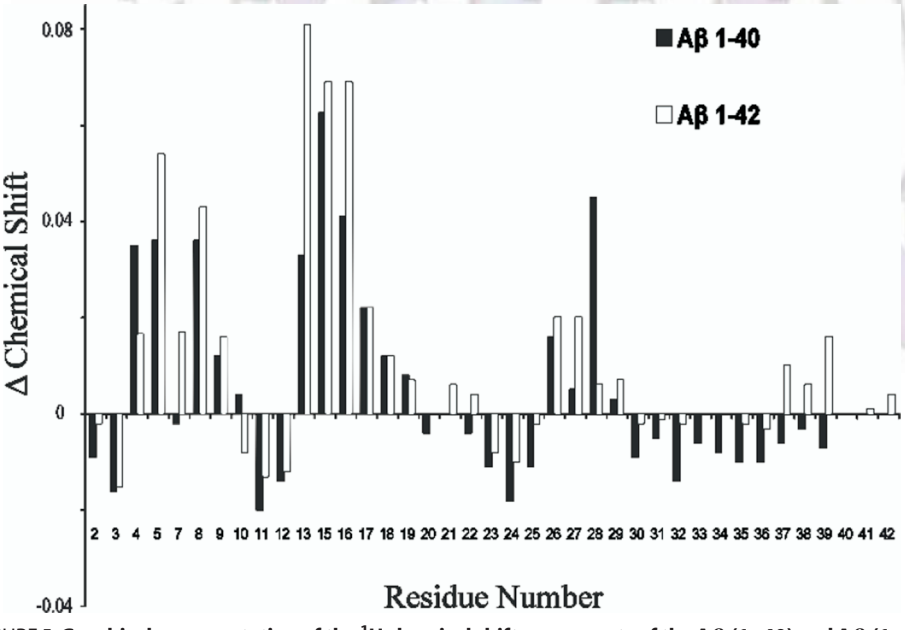

FIGURE 5. **Graphical representation of the ^{1}H chemical shift movements of the Aβ-(1–40) and Aβ-(1–42) peptides with PVY.**

molar eq relative to peptide. However, even at these low concentrations, the anti-PVY antibody induced significant chemical shift movements, indicative of binding to the monomeric Aβ peptide.

DISCUSSION

The immunological approach to AD treatment has received great attention in animal and human studies since the original observations of Schenk *et al.* in 1999 (3). However, the role of immunological processes operating over a lifetime in determining who gets the disease has not been widely considered. If anti-Aβ antibodies are beneficial in Aβ-overexpressing transgenic mice and humans with AD, then the presence of antibodies possessing the ability to bind Aβ may prevent or delay the onset of disease. Aβ-binding antibodies may develop through natural mechanisms, as autoantibodies often develop with aging. Alternatively, anti-Aβ antibodies may be effectively produced through immunological responses to immunogens bearing sequence homology to Aβ, such as PVY.

The aggregation and assembly of the Aβ protein into amyloid deposits are major neuropathological hallmarks of AD. The two predominant forms of Aβ are *X*-40 and *X*-42, with the latter protein being more aggregation-prone and whose overproduction has been linked to many familial forms of AD. The Aβ peptide is a normal physiological constituent that, from age-related micro-environmental changes, can undergo a conformational conversion from soluble monomeric random structures into aggregated β-pleated sheet structures, with the latter forming neurotoxic soluble aggregates (such as AD diffusible ligands) and protofibrils and eventually precipitating as mature amyloid fibrils. It is now thought that methods for preventing the Aβ conformational conversions and fibril formation could ameliorate the effects associated with Aβ-induced neurotoxicity in AD. Because monomeric and oligomeric species of Aβ exist in equilibrium in tissue culture medium (34) and because the soluble oligomers are now thought to be the major culprit and resistant to proteolysis (35–37), the Aβ monomer may be the best therapeutic target for binding by an amyloid inhibitor. Current FDA-approved AD drugs include acetylcholinesterase inhibitors and an *N*-methyl-D-aspartate antagonist that improves cognition and behavior but does not reduce amyloid burden or delay progression.

Our NMR data demonstrate that the anti-PVY antibody binds to monomeric Aβ. With our NMR sample preparation

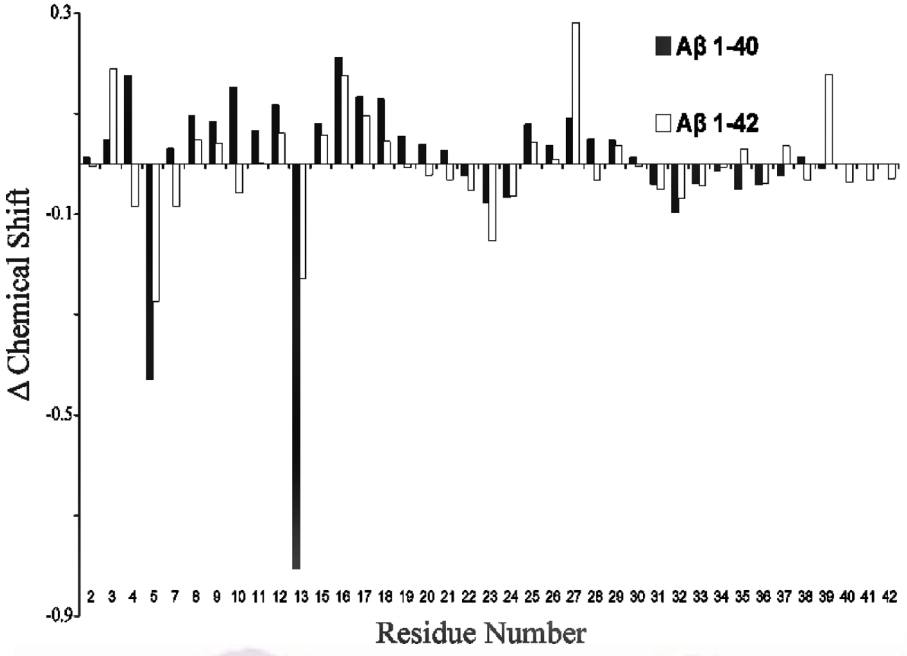

FIGURE 6. **Graphical representation of the ^{15}N chemical shift movements of the Aβ-(1–40) and Aβ-(1–42) peptides with PVY.**

protocol (25), Aβ is monomeric, and the lack of any line width changes is consistent with the anti-PVY antibody binding to monomeric peptide. However, it is possible that the anti-PVY antibody could also be binding with small amounts of soluble Aβ aggregates, and further work to investigate this possibility is currently under way in our laboratory. In contrast, significant line width reductions were seen with binding to human serum albumin, due to binding with Aβ oligomers (38). This is exceptional given that the majority of proteins or small molecules that reportedly bind to the Aβ peptide target the soluble aggregates or early-stage amyloid fibrils (39, 40). ApoE ϵ3 is an endogenous inhibitor of Aβ aggregation that binds to pre-nuclear Aβ oligomers and blocks production of the nucleation steps in amyloid formation (41). More recent NMR studies showed that nicotine (42), human serum albumin (38), and the Aβ-binding alcohol dehydrogenase (43) bind with soluble Aβ oligomers but not the monomers. Because the binding we detected was promoted with substoichiometric amounts (1:50) of the anti-PVY antibody, a stronger binding may occur at higher antibody concentrations. The binding seems localized within the Phe^{4}–Ser^{8} and His^{13}–Leu^{17} Aβ peptide regions. The importance of the central hydrophobic region for β-aggregation has been previously noted (44–47), and the core of the amyloid β-strand structure is composed of Leu^{17}–Ala^{21} (48). A major advantage of the NMR approach is that it provides atomic level details of protein structure and dynamics in solution that are not available with other low resolution techniques. NMR provides site-specific structural data that assist in the development of specific amyloid inhibitors that select for the monomeric form of the Aβ peptide. Recent work in mice demonstrated that a 56-kDa soluble Aβ-(1–42) assembly may be the actual culprit for initiating neuronal loss and memory deficits (49); thus, an inhibitor with any therapeutic value must prevent formation of this or other toxic Aβ aggregates (39). It is generally though that inhibitors that select for monomers or dimers are good starting points.

These results show promise that the anti-PVY antibody may be an effective means of regulating Aβ behavior, particularly because such a small relative molar ratio of antibody showed significant interaction with the peptide. However, because a polyclonal antibody was used, we saw only a solution average of the various forms of PVY present. As such, further work is under way to obtain a monoclonal antibody that can be used to perform more conclusive and quantitative experiments, including determination of a binding constant and epitope-binding domains.

Tabira and co-workers (50) in Japan have developed an oral vaccine for AD using a recombinant adeno-associated viral vector carrying Aβ cDNA. The vaccine reduced Aβ deposits without causing lymphocytic infiltration in the brain. It was proposed that mucosal immunity leads to safer immunological reactions to the vaccine. The lifelong development of antibodies that cross-react with Aβ following dietary exposure (such as PVY) to enhance clearance and inhibit aggregation may be less likely to elicit an autoimmune condition than late-life active vaccination because of the chronic development of the antibody response and the involvement of the immune system in the intestine, which is less likely than parenteral administration to elicit a T cell response (32). The immune system of the intestine enhances Th2 responses and suppresses Th1 responses, leading to relatively less cell-mediated immunity (32, 51). It has been proposed that immune mechanisms involving Th2-dependent responses would be the safest for an Aβ immune response in the setting of AD because Th2-dependent mechanisms produce antibodies that are less likely than those produced by Th1 responses to produce inflammation (52). The oral route of vaccination has also been used in studies in transgenic AD mice using transgenic potatoes expressing five tandem repeats of Aβ-(1–42). Mice immunized with Aβ with this edible vaccine made antibodies against Aβ and had reduced Aβ plaques in the brain (53).

The mechanisms by which anti-Aβ antibodies may have a therapeutic effect include the following: 1) entry into the brain and binding to oligomeric and fibrillar Aβ with microglial activation, eliciting Fc receptor-mediated phagocytic mechanisms of removal of antibody-antigen complexes (52); 2) antibody-mediated solubilization of fibrillar Aβ (32, 54); 3) stabilization of the Aβ monomer, thus preventing the subsequent association into the soluble aggregates; 4) binding of Aβ to antibody in the circulation, enhancing clearance of Aβ from the brain (the peripheral sink hypothesis) (55); 5) altered proteolysis of Aβ

(the ability of proteases that degrade Aβ (angiotensin-converting enzyme, neprilysin, endothelin-converting enzyme, plasmin, and insulin-degrading enzyme) (56) may be altered by binding of Aβ to antibodies); and 6) hydrolysis of Aβ by circulating autocatalytic IgM antibodies, as reported recently in studies of AD cases and controls by Taguchi *et al.* (12). Lifelong exposure to cross-reacting antibodies (such as PVY) that bind to Aβ may have protective effects through all of these mechanisms. This work is in keeping with current efforts to develop a safe and effective vaccine for AD (52). Novel immunogens have been developed that include the B cell epitopes of Aβ (the N terminus) but lack T cell-reactive sequences (57). The absence of a cellular immune response may provide for a safer therapy.

Plant viruses are found throughout the world, frequently infect crops used for human consumption, and have no known effects on human health. We propose that the development of antibodies to PVY following oral exposure is protective against the development of AD because of the beneficial effects of binding of the antibody to the Aβ protein. A model for this interaction may be supplied by the relationship between vaccinia infection (related to cowpox) and the resultant immunity to variola (smallpox). There are naturally occurring proteins other than PVY that bear significant homology to Aβ and that may influence the development of AD. For example, several proteins of *Enterococcus* contain sequences homologous to Aβ (NCBI and National Institutes of Health). The mechanism we propose may influence the pathophysiology of other conditions as well. Antibodies developed in response to naturally occurring plant or animal viruses, bacteria, or other agents may interact with protein trafficking in the brain and blood to influence handling and deposition of pathological proteins. This approach may be valuable for AD immunotherapy because of the relatively low inflammatory potential with intestinal immunogen delivery and the efficacy of antibody binding to pathogenic Aβ monomers.

It is of interest to note as well that circulating antibodies against both unphosphorylated and phosphorylated Tau proteins have also been observed (58), and active immunization with a phosphorylated Tau epitope in P301L tangle model mice reduced brain aggregated Tau and slowed progression of behavioral deficits (59). Also, antibodies generated against soluble oligomeric Aβ have been shown to neutralize oligomers of the prion protein and α-synuclein, suggesting that shared epitopes of these pathogenic proteins may play a role in several neurodegenerative illnesses (52, 60).

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