Brief Definitive Report

Human Antibodies Reactive with β-Amyloid Protein in Alzheimer's Disease

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Summary

Four human B cell lines established by Epstein-Barr viral transformation of B cells from a patient with a clinical diagnosis of Alzheimer's disease (AD) were found to secrete antibodies that react with plaques and cerebrovascular blood vessels in AD brain in a staining profile characteristic of β-amyloid protein (β-AP) in AD brain. Two of these antibodies were shown to be reactive with a rare plaque in a normal brain. In these studies, immunofluorescence and avidin-biotin complex immunoperoxidase methodology were used to determine antibody reaction, and thioflavine S was used to double label amyloid and neurofibrillary tangles. The four antibodies also reacted with neurons in normal and AD brain. Absorption studies, dot immunoblots, and enzyme-linked immunosorbent assays with β-amyloid peptides 1–28 (β-A_{1-28}) and 1–40 (β-A_{1-40}) indicate the major determinant of the reactive epitope is located in the region of amino acids 1–28 of β-AP. However, inhibition studies demonstrate a significant contribution to the antigenic determinant by the 29–40 region of the β-A_{1-40}. These antibodies represent the first human autoantibodies against β-AP. The pathological significance of these autoantibodies is discussed.

In Alzheimer's Disease (AD), the neuropathological hallmarks include unusually high numbers of plaques and neurofibrillary tangles (NFT) in the hippocampus, neocortex, and amygdala (reviewed in references 1 and 2). One component of the NFT is τ protein, a microtubule-associated protein (MAP). The plaques contain β-amyloid protein (β-AP), a fragment of 42–43 amino acids of the β-amyloid precursor protein (APP). There is significant neuronal loss in specific areas of the AD brain. Marked astrocytosis also accompanies these changes. Recent genetic studies have shown a single amino acid substitution in the transmembrane portion of the APP in some familial AD (reviewed in reference 1). Both genetic and environmental factors are thought to be important. Our studies and those of others suggest that there may be circulating antibodies that are specific for antigens in AD brain (reviewed in reference 3).

Our approach to study AD has been to immortalize B cells from patients with AD, other neurodegenerative disorders, strokes, and age-matched controls by EBV. This approach was undertaken by us to circumvent the difficulties in defining the specificities of serum autoantibodies unique for AD patients. These difficulties include the low concentrations of relevant antibodies and the presence of other autoantibodies to cellular constituents in aged individuals. Our previous studies have identified unique anti-NFT and antineural antibodies (4–6).

We have derived multiple B cell lines from blood obtained on three separate occasions from a patient with a clinical diagnosis of AD. There was considerable variation in the numbers of B cell lines established from each sample. It is of interest to note that in one of the three blood samples, four cell lines were found to secrete antibodies reactive with plaques and blood vessels in AD brain in a staining profile characteristic of β-AP. The characterization of these four antibodies is described in this report.

Materials and Methods

Derivation of EBV-transformed B Cell Lines. EBV-transformed B cell lines were derived from a patient with a clinical diagnosis of AD at 69, 73, and 74 yr of age according to Gaskin et al. (4). On these three separate occasions, 522, 684, and 190 cell lines were established, respectively. Three of the four cell lines used in this paper contained only one class of light chains, suggesting they are monoclonal or oligoclonal. One of these has been cloned by forming hybridomas with K6H6/B5 obtained from American Type Culture Collection (Rockville, MD) according to Carroll et al. (7). The fourth cell line, MRE310, contained κ and λ chains. It was also fused with K6H6/B5. Two positive hybrids were cloned twice by the limiting dilution method. The cloned hybridomas secreted only κ chain.

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Immunocytochemistry. Initial screening of supernatants for reactivity on AD brain by immunofluorescence was done on cryostat sections from AD temporal cortex (middle temporal gyrus) rich in plaques and NFT as previously described (4, 6). Antibodies positive for plaques were also tested using the ABC immunoperoxidase method previously described (4). Positive plaque antibodies were tested for reaction on temporal cortex of five AD (57, 69, 73, 80, and 87 yr old) and four normals (50, 61, 65, and 67 yr old). To determine possible antibody labeling of NFT, plaque neurites, and neuritic processes, double labeling was carried out as previously described (6), using the human antibodies and rhodamine-conjugated anti-human antibodies as described above and using anti-τ-2 (Sigma Chemical Co., St. Louis, MO) at a 1:50 dilution and fluorescein-conjugated goat anti-mouse IgG.

Inhibition Studies. Equal volumes of peptides (initially 400 ng/μl in 0.15 M NaCl) or 0.15 M NaCl only were added to diluted supernatants. After 2 h at room temperature, they were analyzed on tissue as described above. Peptides tested included the following β-amyloid peptides: β-$A_{1-28,glu-11}$ (Sigma Chemical Co.); β-$A_{1-28,gln-11}$ (Sigma Chemical Co., and Bachem Inc., Torrance, CA); β-$A_{1-40,glu-11}$ (Bachem Inc.); β-$A_{1-16,gln-11}$ (Sigma Chemical Co.), and β-A_{12-28} (Sigma Chemical Co.). Two human IgM antibodies (CAN15, which stains NFT, plaque neurites, and neuritic processes; and KKN351, which stains astrocytes [6]) were used as controls.

Dot Immunoassay. Aliquots, 10 μl of 1–4 μg peptide or BSA in 0.15 M NaCl or 0.15 M NaCl only, were dried onto nitrocellulose paper. Antibodies were serially diluted and allowed to react for 2 h at room temperature. Antibodies were also tested for reactivity to the cytoskeletal proteins bovine muscle myosin, porcine muscle actin, human epidermal keratin (all from Sigma Chemical Co.); bovine glial fibrillary acidic protein (ICN Biomedical Inc., Costa Mesa, CA); and porcine brain tubulin and MAPs purified as described (8). Experiments were done with 10 μl of 20 μg/ml, 200 μg/ml, and 1 mg/ml of cytoskeletal proteins in the dot blot. Human antibodies positive for cytoskeletal proteins were used as controls. Blots were scanned with a multimedia densitometer (Gilford Systems, Oberlin, OH). Peak heights and areas were calculated with the laboratory computing integrator (LC1-100; Perkin-Elmer Corp., Norwalk, CT). Antibodies were considered negative if <5% of the value of the positive control. MRE1 was used as a negative control.

ELISA. ELISAs with β-$A_{1-28,gln-11}$ or β-A_{1-40} and cytoskeletal proteins were done as previously described (6). 50 μl of peptides (5 μg/ml) or 100 μl of cytoskeletal proteins at 2–5 μg/ml was used. Peptides used in the inhibitive ELISA were incubated with the antibodies for 1.5 h at room temperature. As an added control, two antibodies with other reactivities (MRE1 and CAN15) were treated similarly. These antibodies failed to yield readings above the background.

Results

Antibodies Reactive with β-Amyloid Peptide–positive Plaques and Blood Vessels in AD Brain. Four antibodies from EBV-transformed B cell lines established from a single blood sample from a 73-yr-old female AD patient were found to react with AD temporal cortex in a profile characteristic of β-AP. Despite multiple cell lines being established on two other occasions, this pattern of reactivity was not noted. These four cell lines were MRE148, MRE267, MRE293, and MRE310. They have been found to secrete monoclonal IgM.

By either immunofluorescence or the avidin-biotin complex (ABC) immunoperoxidase method, the four antibodies stained plaques, neurons, and blood vessels with amyloid deposits in five cryopreserved AD brains. These five AD brains were selected because of the presence of different amounts of primitive plaques, classical plaques, compact plaques, diffuse deposits, and stellate deposits. In the case of immunofluorescence, thioflavine S was used to label β-AP in the plaques and blood vessels. In every section, there was complete overlap between immunofluorescence (red) and green fluorescence due to thioflavine S staining. The results of a representative experiment are shown in Fig. 1. In this experiment, MRE148

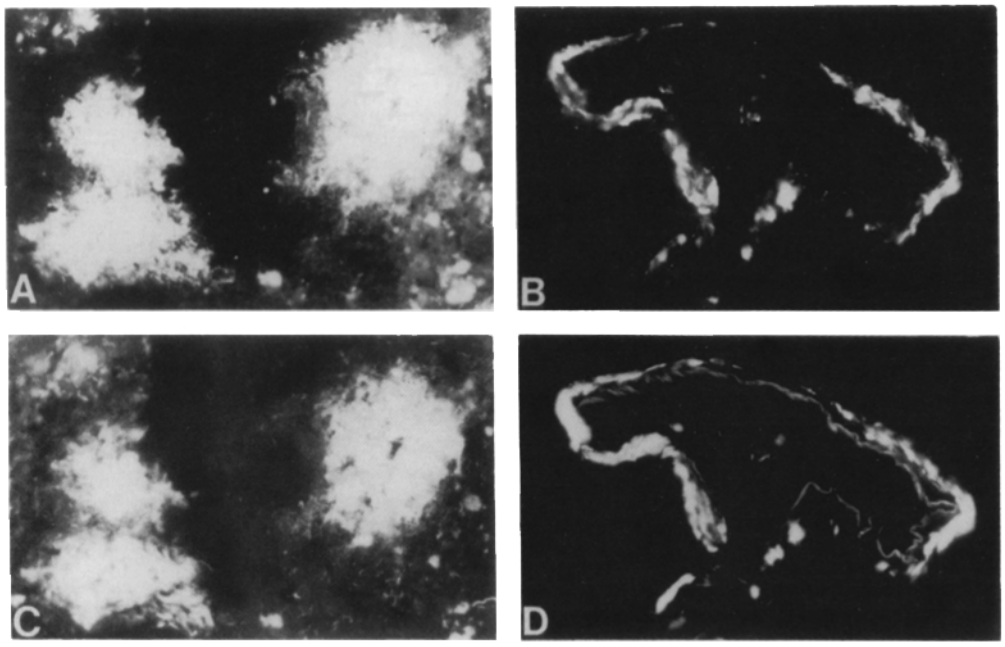

Figure 1. (*A*) Plaque labeling by antibody MRE148 on AD brain as detected by rhodamine-conjugated goat anti-human Ig fraction (red fluorescence). (*B*) Antibody MRE-148 reactivity with deposits in a cerebrovascular blood vessel in AD brain. (*C*) The same section as in *A* counterstained with thioflavine S, which emits green fluorescence (×290). (*D*) The same section of the blood vessel in *B* showing thioflavine S positive deposits (×145).

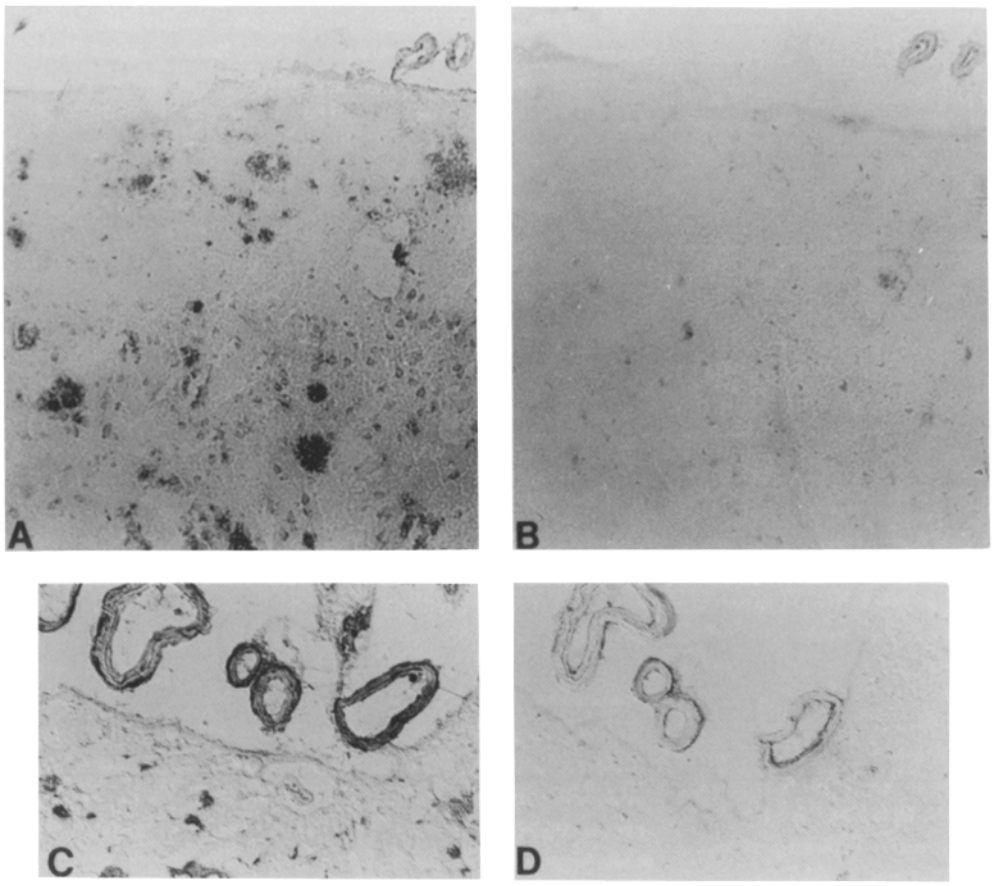

Figure 2. ABC reaction showing antibody MRE310 reaction with plaques and neurons in AD brain temporal cortex (*A*) and blood vessels and plaques in AD frontal cortex (*C*). *B* and *D* are the adjacent sections of *A* and *C*, respectively, and were reacted with MRE-310, which had been absorbed with β-$A_{1-28,gln-11}$ at 100 ng/μl. *B* and *D* demonstrate the loss of MRE310 antibody reactivity after absorption. (*A* and *B*) ×80; (*C* and *D*) ×115.

was shown to stain three plaques and a blood vessel with amyloid deposits by immunofluorescence (Fig. 1, *A* and *B*), which were identified by thioflavine S staining (Fig. 1, *C* and *D*). It is evident that there is complete overlap between both red and green fluorescence. All other human antibodies in our collection that are reactive with cellular constituents do not stain in this manner (see references 4 and 6). In addition, these antibodies were not reactive with NFT, plaque neurites, and neuritic processes that were identified with an anti-τ-2 mAb by double labeling. The staining of neurons in AD brains by these antibodies was seen by both immunofluorescence and the ABC method. The latter method does not have autofluorescence due to lipofuscin and allows better photography. Typical neuronal staining is shown in Fig. 2.

Neuronal staining with the four antibodies was also seen on all four normal brains that were studied. The blood vessels in these normal brains were nonreactive with either the antibodies or thioflavine S, indicative of the absence of β-AP. The staining of normal brain by these antibodies deserves further comment. Although the control brains were selected to contain few NFT and plaques, one of the normal brains (65 yr) contained an area with neurofibrillary tangles and a plaque confirmed by thioflavine S. Multiple sections were cut through this plaque. This plaque was reactive with the three antibodies, MRE148, MRE267, and MRE310, that were tested.

Identification of β-A_{1-40} as the Reactive Antigen. To demonstrate that the antibodies were against β-AP, the four antibodies were incubated with several peptides (200–250 ng/μl serially diluted twofold) before reaction on AD brain. Incubation with peptide β-A_{1-40} at 25 ng/μl resulted in complete loss of antibody reactivity whereas peptides β-$A_{1-28,glu-11}$ or β-$A_{1-28,gln-11}$ required 100–200 ng/μl (depending on which antibody) for complete inhibition. These experiments were done by both immunofluorescence and the ABC method. The latter method provided unambiguous results, as shown in Fig. 2, which demonstrates an absorption study with MRE310 and β-$A_{1-28,gln-11}$. The staining of plaques, blood vessels, and neurons in AD brain by MRE310 is completely abolished by β-$A_{1-28,gln-11}$ at 100 ng/μl. β-A_{1-40} was also used for similar experiments. 25 ng/μl was sufficient for complete absorption. Similar results were obtained with the other three antibodies. In general, it required four- to eightfold more when peptide β-A_{1-28} was used to completely block the staining. The degree of inhibition was generally proportional to the amount of the peptide used. No inhibition of antibody reactions on AD brain was found with β-A_{1-16} and β-A_{12-28} even at 750 ng/μl.

Reactivity with β-AP Peptides in the Dot Blot and in the ELISA. The reactivities of the four plaque-positive antibodies to β-A_{1-40}, β-$A_{1-28,glu-11}$, and β-$A_{1-28,gln-11}$ were also demonstrated by immunoblotting. A representative of three experiments is shown in Fig. 3. All four antibodies were reac-

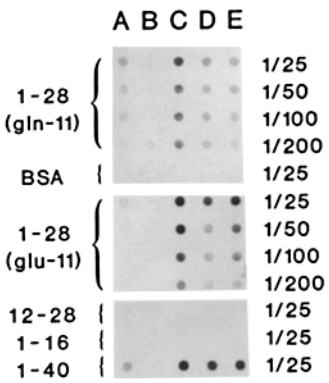

Figure 3. Dot immunoassay of synthetic β-$A_{1-28,gln-11}$, β-$AP_{1-28,glu-11}$, β-A_{12-28}, β-A_{1-16}, and β-A_{1-40} with selected MRE antibodies. The antigen is indicated on the left and the dilution of antibody on the right. Lanes *A–E* designate the antibodies used: lane *A*, MRE293; lane *B*, MRE1; lane *C*, MRE148; lane *D*, MRE267; and lane *E*, MRE310. MRE1 is an IgM antibody that shows no reaction on brain. The other four antibodies react with plaques and blood vessels in AD brain and neurons in normal and AD brain.

tive to β-A_{1-40} in the dot blot. Three of the antibodies, MRE148, MRE267, and MRE310, were more reactive with β-$A_{1-28,glu-11}$ whereas MRE293 was more reactive with β-$A_{1-28,gln-11}$. These results suggest that MRE293 recognizes a somewhat different epitope than the other three antibodies. The four antibodies were nonreactive to β-A_{12-28} and β-A_{1-16}.

By densitometric scanning, MRE148 was most reactive to β-A_{1-40}. The blotting intensity of MRE148 to β-A_{1-40} is arbitrarily set as 1 and used as a reference. MRE310 was next in this reactivity with 0.80, followed by MRE267 with 0.71, and then by MRE293 with 0.30. Similarly, scanning of the reactivities of antibodies at a 1:25 dilution to β-$A_{1-28,glu-11}$ resulted in MRE148 (1.0) > MRE310 (0.59) > MRE267 (0.47) > MRE293 (0.15), whereas antibody reactivities to β-$A_{1-28,gln-11}$ were MRE148 (0.70) > MRE267 (0.39) > MRE 310 (0.36) > MRE293 (0.27). Scanning of the dots against β-A_{12-28} and β-A_{1-16} yielded negative results. MRE1, which is an IgM with an unknown antigenic reactivity and without antibrain activity, was used as a negative control in these experiments.

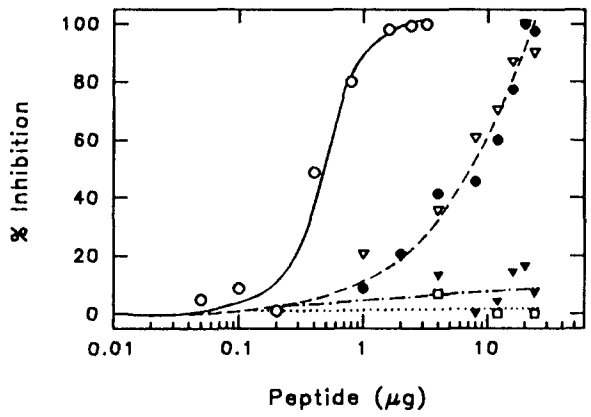

Figure 4. ELISA to measure the ability of β-A_{1-40} (○), β-$A_{1-28,glu-11}$ (▽), β-$A_{1-28,gln-11}$ (●), β-A_{1-16} (□), and β-A_{12-28} (▼) to inhibit the binding of MRE310 to β-A_{1-40}. The inhibitory potencies of both 1–28 peptides were similar and thus a single curve has been drawn through these points.

The β-amyloid antibodies were negative on dot blots using the cytoskeletal proteins myosin, actin, keratin, glial fibrillary acidic protein, tubulin, and MAPs as antigens at concentrations up to 10 μg/dot. They were also negative in an ELISA using the same panel of cytoskeletal proteins.

The four anti-β-amyloid antibodies were reactive with β-A_{1-28} and β-A_{1-40} in the ELISA. Inhibition studies were also done. In a typical experiment, as shown in Fig. 4, MRE310 binding to β-A_{1-40} was blocked with various β-amyloid peptides. The inhibitory potencies of β-$A_{1-28,glu-11}$ and β-$A_{1-28,gln-11}$ were similar. Thus, a single curve was fitted through these points. With β-A_{1-40}, 0.5 μg yielded 50% inhibition. With β-A_{1-28}, 7 μg was needed. Both β-A_{1-16} and β-A_{12-28} at 24 μg did not significantly inhibit in the ELISA. Similar results were found with the other three antibodies in this ELISA system. These data are in agreement with the inhibition results obtained in immunohistochemical studies described in the previous section. The results from the two inhibition assays document that there is a significant contribution to the reactive epitope by the sequence 29–40 in the β-A_{1-40}.

Discussion

In the present study, four human mAbs were identified to be reactive with β-AP in AD. This was supported by immunohistochemical studies and ELISAs with β-amyloid peptides as substrates. These antibodies stained all plaques and neurons in both normal and AD brains. This reactivity differs from the reactivity of antibody 161 described by Simpson et al. (9), which also reacts with plaques and neurons. However, Ab 161 did not react with the birefringent material in the plaques when Congo red was used to identify β-amyloid in the plaque and the antibody also stained the nuclei of the neurons. Thus, these four antibodies represent the first human autoantibodies against β-AP.

The nature of the reactive epitope was explored. The reactive epitope appears to reside in the region of amino acids 1–28 of the β-A_{1-40}. Further analysis suggests that the reactive epitope is conformational in that there is a significant contribution to the antigenic determinant by the region 29–40. In comparison with β-A_{1-40}, four- to eightfold greater concentrations of β-A_{1-28} were required in immunohistochemical studies to block the staining of amyloid plaques. It appears that the conformational nature of the reactive epitope is conserved in the amyloid plaques in situ. This epitope also differs from those recognized by xenogeneic antibodies raised against β-A_{1-28} (10–12). Thus, these antibodies recognize a unique epitope on the β-AP and their reactions represent an added example of uniqueness of epitopes reactive with autoantigens.

>6,000 EBV-transformed β cell lines from both normals (1,705 from six individuals), patients with AD (2,834 from six individuals), and other diseases (1,825 from six individuals) have been screened for their reactivity against AD brain sections. Only four were found to react with the β-AP. These four lines were derived from the blood of a single bleed from a single individual with AD. It appears that these anti-β-

amyloid antibody–secreting B cells in the circulation are rare. The fact that these cell lines were established from only one of the three bleeds underscores the importance of repeated studies in a single individual when peripheral blood is used for the generation of human cell lines or hybridomas secreting specific antibodies of interest.

The role of β-AP in the pathogenesis of AD has been reviewed (1, 2). It appears that an excessive production of APP and the generation and accumulation of β-AP in the brain are characteristic of AD and related disorders. Recently β-AP was reported to be found in various tissues and in circulation (13, 14). The mechanism for its selective accumulation in the AD brain remains to be elucidated. The presence of circulating β-AP may preclude the detection of circulating anti-β-amyloid antibodies and provides an explanation for our inability to detect these antibodies in the patients' sera (n = 10). Whether these antibodies can be detected in the cerebrospinal fluid of AD patients remains to be determined. The availability of these cell lines allows us to determine whether there are extensive mutations in the V regions by sequencing the mRNA encoding for these regions. The existence of extensive mutations in these regions would indicate the process is antigen driven. The presence of aggregated β-AP may be relevant to this process in that aggregated proteins have been known to break humoral tolerance.

The presence of complement components in plaques in AD brain has been well documented (reviewed in references 15–18). The presence of C1q in plaques is indicative of complement activation via the classical pathway. The detection of Ig in the plaques has been reported. The Ig deposits do not frequently colocalize with C1q deposits. Recently, Rogers et al. (17) have obtained evidence that β-AP binds C1q and activates complement in an Ig-independent manner. However, these observations do not exclude the possibility that anti-β-amyloid antibodies may participate in complement fixation in vivo and contribute to the pathogenesis of AD. In this regard, the bound Ig may be solubilized as the consequence of complement fixation. Relevant to this discussion is the recent observation by Levine et al. (19) that in a murine model in which α virus is produced in brains of adult mice with severe combined immunodeficiency with little central nervous system (CNS) pathology, intraperitoneal injections of specific antibodies clear α virus infection from brain neurons. It has been difficult to demonstrate the presence of the injected antibodies in the brain. Thus, our understanding of immune clearance in the CNS is incomplete and the extrapolation of mechanisms in other organ systems to the CNS should be carried out with caution. Therefore, a pathogenetic role of anti-β-amyloid antibodies cannot be dismissed at the present. Because of the rare incidence of these antibody-secreting lines and since all four lines were generated from a single patient, one cannot conclude that this antibody is disease related. However, it is now feasible to generate antiidiotypic antibodies to these anti-β-amyloid antibodies. The staining of Ig deposits in the AD brain by these antiidiotypic antibodies would add support to a pathogenetic role of these antibodies.

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