22pter→q11, which can be represented as dic(22;22)(q11;q11) or 22pter→q11::q11→22pter (four copies of D22S9), but can also result from an interstitial duplication of the 22q11 region (three copies of D22S9). The latter may explain the few reported CES cases that lack an extra chromosome (*10*). Our conclusion can extend only to the patients tested, as a random selection of subjects would be impossible because of the small numbers available. However, the selection was made only on the basis of availability, and, although most are from the more severely affected end of the CES spectrum, one (I.G.) was mildly affected and does show four copies of D22S9.

In this study we applied densitometric and statistical analysis of Southern blots to examine genetic conditions that are not amenable to study by standard cytogenetic methods. A second condition for which probe D22S9 could be useful is DiGeorge syndrome, which is characterized by aplasia of the thymus and parathyroids, and which may be associated with a small deletion at 22q11 (*11*). A similar approach may prove useful in assessing the chromosomal composition of tumors, since the need to culture such tissue is eliminated.

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A Neuronal Antigen in the Brains of Alzheimer Patients

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A monoclonal antibody was prepared against pooled homogenates of brain tissue from patients with Alzheimer's disease. This antibody recognizes an antigen present in much higher concentration in certain brain regions of Alzheimer patients than in normal brain. The antigen appears to be a protein present in neurons involved in the formation of neuritic plaques and neurofibrillary tangles, and in some morphologically normal neurons in sections from Alzheimer brains. Partial purification and Western blot analysis revealed the antigen from Alzheimer brain to be a single protein with a molecular weight of 68,000. Application of the same purification procedure to normal brain tissue results in the detection of small amounts of a protein of lower molecular weight.

ALZHEIMER'S DISEASE IS A NEURODEGENERATIVE disorder characterized clinically by progressive loss of intellectual function. This impairment of function appears to be correlated with numbers of neuritic plaques in the neocortex and with loss of presynaptic markers of cholinergic neurons (*1*). Neuritic plaques are composed of degenerating axons and nerve terminals, often surrounding an amyloid core and usually containing reactive glial elements (*2*). Another characteristic pathologic feature of Alzheimer's disease, the neurofibrillary tangle, is an intraneuronal mass composed of normal intermediate filaments and paired helical filaments (PHF) with unusual properties (*3*).

In studying the topographic distribution of plaques and tangles in the brains of Alzheimer patients, we noted that the lesions occur with high frequency in regions receiving cholinergic innervation from the ventral forebrain (*4*). This cholinergic cell group appears to be extremely vulnerable to the disease process, and evidence that cholinergic nerve terminals participate in plaque formation has been presented (*5*). To better define the relation between ventral forebrain cholinergic neurons and the lesions of the Alzheimer brain, we have prepared monoclonal antibodies to homogenates of ventral forebrain tissue taken at autopsy from four patients with Alzheimer's disease. The resulting antibodies were screened on the basis of their ability to differentiate brain tissue from patients with Alzheimer's disease and from normal subjects in both immunochemical and immunocytochemical procedures.

Antibodies were initially assayed according to their ability to bind to brain homogenate that had been immobilized onto polyvinyl plates (1 μg per 50-mm diameter well) by drying at 37°C for 1 hour. Antibody binding was detected with peroxidase-conjugated goat antibody to mouse immunoglobulins. Those antibodies that showed greater than a 50% increase or decrease in binding to homogenates of Alzheimer brain relative to normal tissue were studied further. One of these antibodies, Alz-50, is described below.

Initial assays showed that the binding of Alz-50 was highly selective for brain tissue from Alzheimer patients. Figure 1 shows that 0.33 μg of temporal cortex homogenate from Alzheimer patients gave an optical density only slightly lower than 10 μg of temporal cortex homogenate from normal patients. From these data we conclude that the antigen is elevated 15 to 30 times in the temporal cortices of the Alzheimer patients. Alz-50 reactivity was similarly elevated in the nucleus basalis and hippocampus. These areas, cortex, nucleus basalis, and hippocampus, are all known to contain neuritic plaques and neurofibrillary tangles in brains of patients with Alzheimer's disease. Brain areas less affected by the disease, such as caudate, thalamus, or cerebellum demonstrated little or no reactivity.

The immunocytochemistry of Alz-50 on

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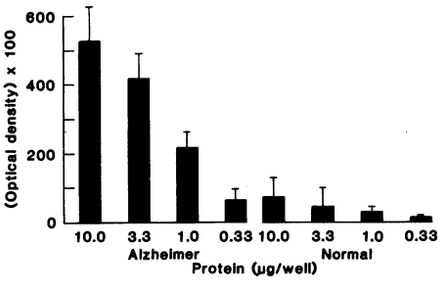

Fig. 1. Quantitation of Alz-50 reactivity in temporal cortex of patients that had died of Alzheimer's disease and normal individuals. Alzheimer's disease cases were typical in both clinical and neuropathologic features (*3*). Brains were obtained from normal individuals dying in hospital from lung or heart disease. These patients were not demented prior to death and had no history of neurologic or psychiatric disease; neuropathologic studies failed to reveal any significant pathology. The reactivity in the brains of the Alzheimer patients is estimated to be 15 to 30 times greater than in the brains of the normal subjects. Various amounts of antigen (x axis) was dried onto 90-well polyvinyl plates (NUNC, Germany). Nonspecific binding of protein to the antigen was blocked by incubating the plates with 0.01*M* TBS, *p*H 7.4, plus 5% dried milk for 1 hour. Alz-50 was diluted 1:5 in blocking solution and incubated overnight at 4°C. Unbound antibody was removed by washing five times with 0.02% Tween-TBS. Peroxidase-coupled goat antibody to mouse immunoglobulins (Kirkegaard & Perry) was diluted 1:100 in blocking solution, added to the plates, and incubated for 1 hour at room temperature. After five washes with 0.02% Tween-TBS, reactivity was visualized with 2,2′-azino-di′-3-ethyl-benzthiazoline solution (ABTS) (Kirkegaard & Perry). Results are expressed as means, with standard deviations indicated. Numbers of cases were ten for Alzheimer and six for normal.

Formalin-fixed tissue was dramatic and was consistent with the hypothesis that Alz-50 was highly selective for neuronal components in Alzheimer brain (Fig. 2A). Many neurons in the pyramidal layer of the hippocampus were stained. The antigen was present in cell bodies and in neurites. Figure 2C shows that plaques were strongly stained by the antibody. The staining was confined to the neuritic meshwork present in plaques. Darkly stained neurons and plaques were found throughout Alzheimer hippocampus and cortex. By contrast, there was virtually no staining of normal brain (Fig. 2B). This pattern of specificity was observed in a total of eight brains from Alzheimer patients and five brains from normal subjects.

To determine the relation between Alz-50 staining of neurons and the presence of neurofibrillary tangles we used a double staining technique. Vibratome sections of Formalin-fixed Alzheimer tissue were reacted with Alz-50, and reactivity was visualized with the use of peroxidase conjugated goat antibody to mouse immunoglobulins. 4-Chloronaphthol was used to visualize the peroxidase reaction; this compound, a peroxidase substrate, yields a product that precipitates in aqueous solution but is soluble in organic solvents. The tissue section was photographed and the 4-chloronaphthol was removed by dehydration and xylene treatment. Finally, plaques and tangles were stained with thioflavine S, a sensitive histologic reagent for the demonstration of these lesions, and the section was photographed again.

Comparison of the staining patterns (Fig. 3) revealed that many neurons were stained both by the antibody and by thioflavine S. However, several neurons were darkly stained by the antibody and did not appear to contain neurofibrillary tangles. A small fraction of neurons that contained tangles and were thioflavine-positive were not positive for Alz-50 (Fig. 3, A and B). The staining of plaques by Alz-50 was also studied by this method. All plaques bound both Alz-50 and thioflavine. In addition, these results confirmed the neuritic nature of the antibody staining; staining was present in the neuritic periphery of the plaques but absent in the amyloid core.

Simple biochemical experiments suggest that the Alz-50 antigen is distinct from PHF, the major tangle component. Unlike PHF, the Alz-50 antigen is largely soluble in 0.01*M* tris-buffered saline (TBS) and completely soluble in TBS containing 5% sodium dodecyl sulfate (TBS-SDS) (*6*). Solubility was tested by vortexing Alzheimer cortex homogenate for 2 minutes in TBS or TBS-SDS. The homogenate was then centrifuged at 10,000*g* for 10 minutes. Supernatant and pellet were separated, and the pellet was washed twice by homogenizing and centrifuging as above. The supernatant and pellet were homogenized in water and various amounts of each sample (10, 3, 1 μg per 50 μl) were dried onto polyvinyl plates. The presence of antigen was determined by an enzyme-linked immunosorbent assay (ELISA). PHF reactivity was monitored by means of an antibody to PHF, antibody 704.1 (*10*). Alz-50 reactivity was found in

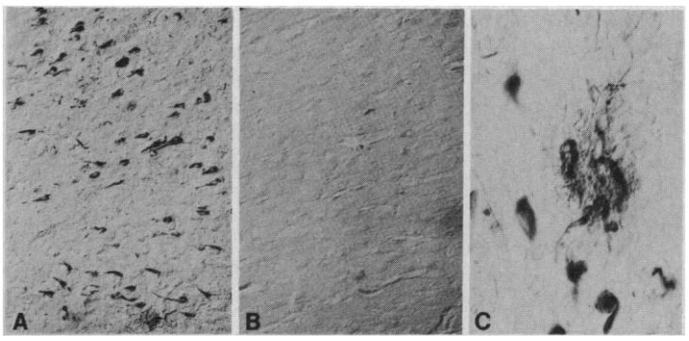

Fig. 2 (left). (A) Immunocytochemistry of Alz-50 staining in the pyramidal layer of hippocampus from a brain affected by Alzheimer's disease. Vibratome sections (40 μm) were cut from Formalin-fixed brain. The tissue was washed twice in TBS, incubated for 30 minutes in 0.25% Triton X-100–TBS, washed once in TBS, and incubated for 30 minutes in dried milk (blocking solution) for 1 hour at room temperature to prevent nonspecific binding of antibody. Alz-50 was diluted 1:5 in blocking solution and incubated with the tissue sections overnight at 4°C. Unbound antibody was removed by washing in TBS. Peroxidase coupled goat antibody to mouse immunoglobulin G was diluted 1:100 in blocking solution and incubated with the tissue sections for 1 hour. Antibody was visualized by incubating the sections for 8 minutes in a 0.1*M* tris solution, *p*H 7.4, containing 0.45 mg/ml of diaminobenzidine and 0.44 m*M* hydrogen peroxide. The tissue was then washed in TBS, dehydrated, and mounted. Magnification ×12.5. (B) Alz-50 staining in hippocampus from a normal brain. (C) A plaque from frontal cortex of a brain affected with Alzheimer's disease. Magnification ×25.

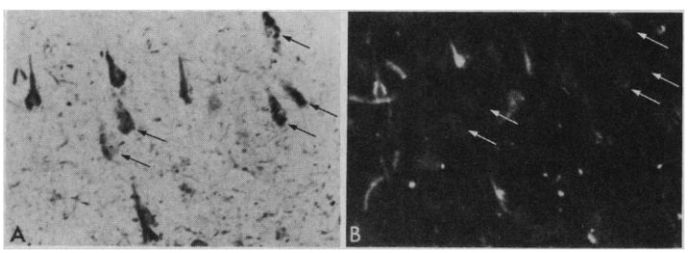

Fig. 3 (right). (A) Immunocytochemistry of Alz-50 in hippocampus from a brain affected by Alzheimer's disease. Arrows point to neurons that react with Alz-50 (A) but do not stain with thioflavine S (B). For details on the immunocytochemistry see legend to Fig. 1. The peroxidase reaction was developed by using 0.2 mg/ml of 4-chloronaphthol instead of diaminobenzidine (as in Fig. 2). Microscopy was performed by using phosphate-buffered saline (PBS)–glycerol instead of dehydration. Magnification ×12.5. (B) Thioflavine S histochemistry of the same section from (A). Note the absence of thioflavine S staining of neurons (arrows). To remove the 4-chloronaphthol the section was dehydrated, whereupon the 4-chloronaphthol dissolved in the xylene, and then subsequently rehydrated. The section was then incubated in a 0.01% thioflavine S solution in Formalin and rinsed by dipping three times in 80% ethanol (fluorescence microscopy; magnification ×12.5.)

the TBS supernatant and was quantitatively recovered in the TBS-SDS supernatant, whereas PHF reactivity remained in the pellet after TBS-SDS extraction. Thus, Alz-50 immunoreactivity is soluble and segregates away from PHF immunoreactivity.

Enzyme experiments confirmed this result. Before we dried the Alzheimer brain homogenates onto polyvinyl plates, we treated them for 0, 20, or 60 minutes with trypsin or alkaline phosphatase. An ELISA was used to measure the sensitivity of the Alz-50 epitopes to the treatment. Unlike the PHF, the Alz-50 epitope was highly sensitive to trypsin. This supports the hypothesis that the Alz-50 antigen is not PHF, and also demonstrates that the antigen is a protein.

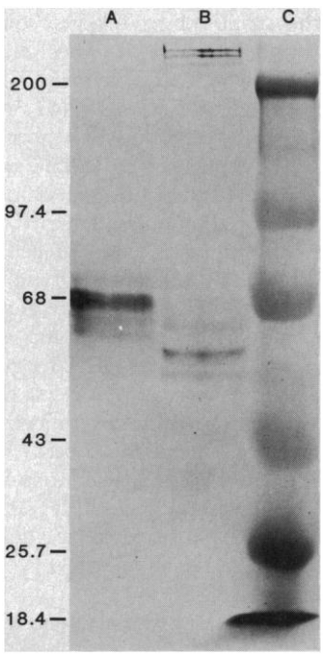

Fig. 4. Western blot of (A) Alzheimer and (B) normal temporal cortex fractions from the void volume of a Sepharose 6B column; (C) prestained molecular weight standards (Bethesda Research Laboratories). Temporal cortex (2.5 g) was homogenized into 10 ml of PBS and centrifuged at 20,000*g* for 20 minutes. The supernatant was run through a Sepharose 6B column and the void volume was collected. Protein determinations were performed and the samples were run on a 10% SDS-PAGE gel. Fifteen micrograms of protein was in the Alzheimer sample (A) and 45 μg of protein was in the normal sample (B). The protein was transferred to nitrocellulose for 3 hours at 125 mA; the buffer contained 19.2 m*M* glycine, 2.5 m*M* trizma base, and 20% methanol at *p*H 8.3. The antibody reaction was developed as in Fig. 2, except that phosphatase-coupled antibody was used instead of peroxidase-coupled antibody. Color development was achieved by means of BCIP/NBT (Kirkegaard & Perry). Omission of the incubation with Alz-50 completely abolished staining.

The Alz-50 epitope is not phosphatase sensitive; this does not prove that the Alz-50 antigen is not phosphorylated, although it is of interest because many antibodies to neurofilament epitopes that identify neurofibrillary tangles fail to react after phosphatase treatment. In addition, this result suggests that phosphorylation of the epitope does not account for the ability of the antibody to distinguish between Alzheimer and normal brain.

A Western blot of the Alz-50 antigen was obtained by first purifying the Alz-50 antigen in native form on a Sepharose 6B column. Temporal cortex homogenate from Alzheimer patients or normal subjects was centrifuged at 27,200*g* for 20 minutes, and the supernatant was run through the Sepharose 6B column. An immunoreactivity profile was obtained by diluting each fraction 1:100 in water, drying 50 μl of diluted fraction onto polyvinyl plates, and assaying by ELISA. The profile from Alzheimer brain revealed a single immunoreactive peak at the void volume. By contrast, no immunoreactivity was seen in the column fractionation of normal brain at this dilution. At a lesser dilution, normal brain immunoreactivity was seen at the void volume. This result supports the quantitative data shown in Fig. 1 demonstrating 15 to 30 times as much immunoreactivity in the Alzheimer brain as in the normal brain.

Samples of supernatant from Alzheimer and normal cortex were then studied by the Western blot technique (Fig. 4). The major band in Fig. 4, lane A, has an apparent molecular weight of 68,000 (68K). In Fig. 4, lane B, the reactivity from the normal void volume fraction was brought out by loading onto the polyacrylamide gel three times as much protein as was used for the Western blot of the Alzheimer void volume fraction. The major band has an apparent molecular weight of 59K. A doublet at 245K is also present.

These results show that in TBS the antigen recognized by Alz-50 is either aggregated or is part of a large complex. When dissociated, the epitope occurred as a single 68K protein that was distinctly different from the 59K antigen from normal brain. Several relevant proteins have subunit molecular weights in the 68K range: in the cytoskeletal family there are neurofilament and tau proteins (*7*), in the cholinergic family there is choline acetyltransferase (*8*). The Alz-50 antigen is therefore unlikely to be neurofilament, tau protein, or choline acetyltransferase, because none of these proteins seems to be elevated in concentrations 15 to 30 times in the brains of Alzheimer patients. Further, if the 68K Alzheimer type protein is related to the 59K protein, then the Alz-50 antigen is unlikely to be neurofilament, tau protein, or choline acetyltransferase. The relation among the proteins detected is questionable: the relatively large amounts of samples of normal brain tissue needed to detect any reactivity raises doubts about the specificity of the antibody binding. Some monoclonal antibodies to tangles react with proteins in the 59K to 68K range (*9*), but none of these show quantitative differences between normal and Alzheimer brain. The points raised above suggest that the epitope recognized by Alz-50 is a novel antigen.

Our results demonstrate that Alz-50 recognizes a protein present in neuronal terminals in plaques and in most neurons with tangles. The surprising finding is that Alz-50 immunoreactivity appears to precede the deposition of neurofibrils and PHF's to form tangles. Thus Alz-50 may recognize a precursor to tangle formation. Alternatively, Alz-50 immunoreactive neurons may be affected by Alzheimer pathophysiology but may not form classical tangles. The biochemical data showing that Alz-50 is not PHF antigen is consistent with this discordance between the presence of Alz-50 and the presence of neurofibrillary tangle. The function and identity of the proteins that are recognized by Alz-50 remains to be elucidated.

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