Antibodies to Cholinergic Neurons in Alzheimer’s Disease

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Abstract: Alzheimer’s disease (AD) is associated with degenerative changes in nuclei of the basal forebrain which provide most of the cholinergic input to the cortex and hippocampus and with a reduction in presynaptic cholinergic parameters in these areas. Although the etiology and pathogenesis of AD are not known, several reports indicate the involvement of immunological mechanisms. In the present work we examined the existence of antibodies in sera of AD patients that bind specifically to cholinergic neurons. As antigens we employed the purely cholinergic electromotor neurons of the electric fish *Torpedo* which are chemically homogeneous and cross-react antigenically with human and other mammalian cholinergic neurons. Our findings show that immunoglobulins from sera of AD patients bind to a specific antigen (molecular mass 200 kilodaltons) in the cell bodies and axons of *Torpedo* electromotor neurons and that the levels of such antibodies are significantly higher in AD patients than in controls. The possible role of these antibodies in the cholinergic dysfunction in AD and their diagnostic potential are discussed. Key Words: Alzheimer’s disease—Cholinergic neurons—Antibodies—*Torpedo*. Chapman J. et al. Antibodies to cholinergic neurons in Alzheimer’s disease. *J. Neurochem.* 51, 479–485 (1988).

Alzheimer’s disease (AD), the most common cause of dementia, is characterized by a relentlessly progressive intellectual and personality decline. Brains of patients with AD contain characteristic extracellular senile plaques as well as intracellular neurofibrillary tangles (Katzman, 1976). These histopathological changes are particularly pronounced in cortical and hippocampal areas and in the nuclei of the basal forebrain which provide most of the cholinergic input to the cortex and hippocampus (Coyle et al., 1983). The severity of these degenerative changes correlates with the cognitive impairment in AD (Blessed et al., 1968) as well as with a reduction in central cholinergic activity (Francis et al., 1985). The cholinergic changes are manifested by dysfunction and death of neurons in the basal forebrain and by a concomitant reduction in presynaptic cholinergic parameters in the cortex and the hippocampus (Sims et al., 1983). The extent of the cholinergic deficit, its occurrence early in the disease, its correlation with the cognitive deficit in AD (Francis et al., 1985), and the known role of cholinergic mechanisms in higher cognitive functions, particularly memory (Bartus et al., 1982, Levin et al., 1987), all indicate a central role for cholinergic degeneration in the pathogenesis of AD.

Although the etiology and pathogenesis of the cholinergic degeneration in AD are not known (Schmidt, 1983) several reports indicate the involvement of immunological mechanisms. These include the presence of immunoglobulins (Igs) in senile plaques (Ishii and Haga, 1976; Eikelenboom and Stam, 1982), the presence of antibodies in AD sera which have been shown histochemically to react with neuronal tissue (Nandy, 1978; Watts et al., 1981; Fillit et al., 1985), and abnormally increased expression of HLA-DR antigens in brains of AD patients (Rogers et al., 1986; Poupard-Barthelaix et al., 1987; McGeer et al., 1987), and abnormally increased expression of HLA-DR antigens in brains of AD patients (Rogers et al., 1986; Poupard-Barthelaix et al., 1987; McGeer et al., 1987). Furthermore, the presence of immune complexes in the CSF of AD patients (Cameron, 1985) and defective cellular immune function have also been described (Skias et al., 1985; Singh et al., 1987).

In view of the marked cholinergic degeneration in

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Abbreviations used: AD, Alzheimer’s disease; Ig, immunoglobulin; kD, kilodalton; PBS, phosphate-buffered saline; PK, perikarya; SDS, sodium dodecyl sulfate.
AD and of the suggested involvement of immunological mechanisms in the disease we have explored whether sera of AD patients contain antibodies that bind to specific constituents of cholinergic neurons. Ideally this should be investigated with a preparation of homogeneous purely cholinergic human or other mammalian neurons. As such preparations are not available we employed the purely cholinergic electromotor neurons of the electric fish *Torpedo*. These cholinergic neurons are chemically homogeneous and contain many constituents that cross-react antigenically with human and other mammalian cholinergic preparations. These include well-characterized cholinergic molecules such as the nicotinic acetylcholine receptor (Patrick and Lindstrom, 1973) and gangliosides (Richardson et al., 1982) as well as other antigens that share common epitopes with specific brain proteins (Kushner, 1984). Our findings show that AD Igs bind to a specific polypeptide in the cell bodies and axons of the *Torpedo* electromotor neurons and that a high level of such antibodies supports the diagnosis of AD.

**MATERIALS AND METHODS**

**Preparation of antigens**

*Torpedo ocellata* were caught live off the coast of Tel-Aviv and maintained in sea water aquaria up to 3 months prior to use. Electromotor cholinergic cell bodies were purified from homogenates of freshly excised *Torpedo* electric lobes by density gradient centrifugation as described by Dowdall et al. (1976). Cholinergic nerve terminals (synaptosomes) were purified from homogenates of freshly excised *Torpedo* electric organs by differential and sucrose density gradient centrifugation as previously described (Michaelson and Sokolovsky, 1978). Electromotor nerves were excised and extracted by homogenization (10% wt/vol) in 10 mM phosphate buffer, pH 7.4, that contained 140 mM NaCl and 1% sodium dodecyl sulfate (SDS) (wt/vol). *Torpedo* cerebellum and olfactory bulb preparations were obtained by the same procedure used for purification of the electric lobe cholinergic cell bodies. *Torpedo* liver membranes were prepared by homogenization and centrifugation as described by Walker et al. (1982). Protein was assayed by the method of Lowry et al. (1951) as modified by Markwell et al. (1978), utilizing bovine serum albumin as standard.

**Collection of sera**

Patients (7 men; 15 women; age, 77 ± 6 years; mean ± SD) were diagnosed as suffering from AD by either clinical (n = 10) or research criteria (n = 12) (McKhann et al., 1984; Zemcov et al., 1984). The AD patients all had an insidious onset of the disease with a duration of at least 2 years and they were all severely demented with mini mental test scores below 60% of normal. Normal controls (11 men; 16 women; age, 75 ± 6) were age matched nondemented with no neurological or immunological diseases. Patients with multi-infarct dementia (7 men; 2 women; age, 75 ± 6); and Parkinson’s disease with dementia (5 men; 4 women; age, 74 ± 4); and nondemented patients with cerebrovascular disease (5 men; 2 women; age, 71 ± 6) and Parkinson’s disease (4 men; 1 woman; age, 70 ± 2) were diagnosed by standard clinical criteria.

**Immunoblot assay**

The antigen preparations (1 mg protein/ml) were boiled for 5 min in 100 mM Tris buffer, pH 6.8, which contained 1.8% SDS (wt/vol) and 3% mercaptoethanol (vol/vol). Polypeptides were separated by electrophoresis on 12 × 15 cm 10% SDS-polyacrylamide gels (50 μg protein/lane) utilizing a Bio-Rad Protean II Slab Cell (50 mA for 2.5 h) (Laemmli, 1970). The separated polypeptides were then transferred electrophoretically to nitrocellulose membranes (Bio-Rad Transblot Cell at 100 V for 2 h) (Towbin et al., 1979). The nitrocellulose sheets were cut into strips, overlaid (overnight, at 4°C) with 5% powdered milk solution in phosphate-buffered saline (140 mM NaCl in 10 mM phosphate buffer, at pH 7.4) (PBS), washed [3X in PBS + Tween 0.05% (vol/vol)], and reacted (2 h, at 25°C) with human sera diluted in PBS + Tween + *Torpedo* liver membranes (50 μg protein/ml). *Torpedo* liver membranes were used to reduce nonspecific binding. The nitrocellulose strips were then washed and the bound IgG or IgM was detected by either peroxidase-conjugated anti-human IgG or peroxidase-conjugated anti-human IgM (Sigma, 1:1000 in PBS-Tween for 1 h at 25°C). They were then washed and developed for 20 min with 0.01% H2O2 (vol/vol) and 0.6 mg/ml 4-chloro-1-naphthol in Tris 50 mM, pH 8.3, which contained 100 mM NaCl. The immunoblots were analyzed by a computerized densitometer (LKB model 2400) and the areas under the peaks recorded. The molecular weight of polypeptides on the SDS-polyacrylamide gels and following the immunoblot assay was determined from the position of marker proteins of known molecular weight. Proteins were visualized with either Coomassie Blue (SDS-polyacrylamide gels) or Ponceau S (nitrocellulose strips).

**Statistical analysis**

The values for the immunoblot peaks obtained with sera of the AD patients were compared to those of the other groups by means of a Wilcoxon’s rank order test. Proportions of patients in the AD group and in other groups that reacted positively with a given polypeptide band were compared by appropriate nonparametric statistical tests (Fisher’s exact test or x2 test) (Colton, 1974).

**RESULTS**

The possibility that AD sera contain antibodies that are directed against antigenic constituents of cholinergic cell bodies [perikarya (PK)] was examined by immunoblot assays utilizing control and AD Igs and cholinergic PK which were isolated from the electric lobe of *Torpedo ocellata* (Dowdall et al., 1976). Figure 1 shows the binding patterns of IgG (dilution 1:80) from the first group of cases examined (11 AD patients and 10 controls) to *Torpedo* PK polypeptides. As can be seen the AD IgGs interact with more PK antigens than do the controls. Careful examination of the immunoblots revealed the presence of a single polypeptide with an apparent molecular mass of approximately 200 kilodaltons (kD; PK200) which is recognized by most of the AD IgG (9/11) and by only few of the controls (2/10). The titers of the AD anti-PK200 IgG were such that most
were detectable at serum dilutions higher than 1:160. Examination, by Coomassie Blue staining, of the repertoire of Torpedo PK polypeptides revealed the presence of a prominent band at 200 kD (Fig. 1). Figure 2 represents the binding patterns of IgM (dilution 1:80) from 10 of the 11 AD patients presented in Fig. 1 and from the same 10 controls to Torpedo PK polypeptides. As can be seen the AD anti-Torpedo PK IgM bind with fewer antigens than do the corresponding IgG and their level is not higher than those of the controls. Furthermore the Torpedo PK antigen PK200 which is recognized by most of the AD IgG (Fig. 1) is apparently not recognized by the AD IgM (Fig. 2).

To evaluate the specificity of the anti-PK200 IgG to AD we performed the anti-Torpedo PK immunoblot assay with a large number of AD and control sera and with the sera of patients with other dementias, i.e., multi-infarct dementia and Parkinson's disease with dementia. The immunoblots were analyzed by a computerized densitometer (LKB model 2400) and the areas under the PK200 peak were recorded. Figure 3 presents the individual levels of anti-PK200 IgG of 22 AD patients, 29 normal age-matched controls, 9 multi-infarct dementia patients, and 9 Parkinson's disease patients with dementia. As can be seen the median of the anti-PK200 IgG of the AD patients is 0.43 (arbitrary units) whereas the medians of the normal controls and the other demented patients are 0.0. The observed difference between the AD patients and the other groups is statistically significant ($p < 0.01$ Wilcoxon's rank order test). Comparison of the average anti-PK200 IgG levels in the different groups revealed that AD sera contained higher antibody levels ($0.93 \pm 0.32$) (average $\pm$ SEM) than did the normal controls ($0.23 \pm 0.07$), and the patients with either multi-infarct dementia ($0.09 \pm 0.02$) or Parkinson's disease with dementia ($0.19 \pm 0.09$).

The extent to which the PK200 antigen is specific to Torpedo cholinergic neurons was investigated by examining its distribution in neural and peripheral Torpedo tissues. Figure 4 displays the binding of IgG from two AD patients to the cholinergic cell bodies, nerves, and nerve terminals of the Torpedo electromotor system, to Torpedo cerebellum and olfactory lobe, and to Torpedo liver membranes. As can be seen the AD IgG bind most markedly to the PK200 polypeptide of the cholinergic cell bodies, whereas liver membranes are completely devoid of this antigen. Cerebellum and olfactory lobe contain low levels of the 200-kD antigen. Examination of the distribution of PK200 within the electromotor neuron re-
Case study revealed that in addition to cholinergic cell bodies it is also present in their axons and that lower levels occur in the nerve terminals (Fig. 4). Similar results were obtained with three additional AD sera (not shown). These findings suggest that the PK200 antigen is enriched in cholinergic neurons of *Torpedo* brain and is not found in *Torpedo* liver.

**DISCUSSION**

The findings reported in the present study indicate that Igs from the sera of AD patients bind to an antigen with a molecular mass of approximately 200 kD which is enriched in the cell bodies and axons of *Torpedo* electromotor neurons. These Igs were detected far more frequently in AD patients than in healthy controls or patients with other common causes of dementia. The anti-PK200 antibodies may thus serve as a much needed serological marker for AD and as a tool for probing the role of immunologic antineuronal mechanisms in this disease.

An evaluation of the diagnostic potential of the anti-PK200 IgG in differentiating AD patients from normal age-matched controls, from patients with other common causes of dementia (multi-infarct dementia and Parkinson’s disease with dementia), and from patients with other relevant neurological dis-
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The immunological trigger for the formation of anti-PK200 IgG and the extent to which these antibodies are involved in the development of cholinergic dysfunction in AD are not yet known. Future studies of the correlation between clinical, histological, and neurochemical parameters in AD and the levels of anti-PK200 antibodies in the sera and CSF of the patients may reveal whether these antibodies have a significant role in the disease. In addition, should the control cases with elevated anti-PK200 IgG levels

| TABLE 1. Evaluation of the diagnostic potential of anti-PK200 IgG |
|-----------------------------|-----------------------------|
| Sera                  | Anti-PK200 levels (arbitrary units) | Anti-PK200 >0.2 |
| AD (n = 22)             | 0.93 ± 0.32                  | 18/22 (82%)    |
| Normal controls (n = 27) | 0.23 ± 0.07a                 | 8/27 (29%)     |
| Multi-infarct dementia (n = 9) | 0.09 ± 0.02a       | 2/9 (22%)      |
| Parkinson's disease with dementia (n = 9) | 0.19 ± 0.09a  | 3/9 (33%)      |
| Cerebrovascular disease (n = 7) | 0.48 ± 0.3b     | 2/7 (29%)      |
| Parkinson's disease (n = 5) | 0.20 ± 0.17a      | 1/5 (20%)      |

The levels of anti-PK200 IgG were measured by immunoblot assays as described in Materials and Methods. Results presented are peak areas ± SEM of the indicated number of cases. The values of the AD patients differed significantly from those of the other groups.

*p < 0.01, †p < 0.02, Wilcoxon's rank sum test.

The p values correspond to a comparison of the Alzheimer data to those of the other groups by means of an appropriate statistical test (Fisher's exact test or x² test).

(Table 1) develop AD this may suggest that the production of these antibodies is an early and possibly significant event in the disease. An alternative way to assess the importance of anti-PK200 antibodies in neuronal degeneration would be to monitor the effect of such antibodies in animal models. Recent findings from our laboratory indicate that immunizing rats with Torpedo cholinergic cell bodies results in the production of anti-Torpedo PK200 antibodies which is accompanied by learning deficits and by biochemical and histological changes in their brains (in preparation).

The present findings indicate that the Torpedo 200-kD protein which is recognized by IgG from AD patients is specific to cholinergic cell bodies and axons (Fig. 4). The identity of this protein is as yet unknown. It is of interest to note that neurofilaments are composed of 200-kD, 150-kD, and 68-kD proteins and that the 200-kD and the 150-kD constituents cross-react antigenically with the paired helical filaments, which are a major component of the neurofibrillary tangles in degenerating AD neurons (Anderton et al., 1982). It is therefore tempting to suggest that the 200-kD protein which is recognized by the AD IgG is a component of the neurofilaments in Torpedo cholinergic neurons. Previous immunoblot and immunohistological studies have reported the presence of antibodies to mammalian brain neurofilaments in sera of normal subjects and AD patients (D’Angelo and D’Angelo, 1986; Plioplys et al., 1987). However, contrary to the present findings the AD and control levels of these antibodies were found to be similar (Sotelo et al., 1980; Gajdusek, 1985; Stefansson et al., 1985; Karcher et al., 1986). It should be noted that the 200-kD neurofilament proteins represent a family of proteins that are distributed heterogeneously in the CNS (Shaw and Weber, 1982; Peng et al., 1986). The present findings therefore suggest that the AD Igs recognize antigenic sites on the 200-kD neurofilament protein which are specific to cholinergic neurons. This hypothesis is currently being investigated in our laboratory.

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