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Evidence for the presence of antibodies to cholinergic neurons in the serum of patients with Alzheimer's disease

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Summary. A blind study showing that serum from patients with Alzheimer's disease causes immunolysis of mammalian brain synaptosomes is reported. Control, aged-matched, sera were largely without effect. The immunolysis was directed mainly against cholinergic synaptosomes. The data presented support the hypothesis that autoimmune mechanisms may operate in the pathogenesis of Alzheimer's disease.

Key words: Alzheimer's disease – Immunolysis – Cholinergic neurones – Synaptosomes

Introduction

Senile dementia of the Alzheimer type (SDAT) is a major dementing disorder of old age, but the aetiology and pathogenesis of the disease remain obscure [23, 24]. Definitive diagnosis can only be made on histopathological evidence obtained at autopsy, or by biopsy, as highly characteristic pathological changes are seen in the disease [4, 26]. In particular, there is an abundance of neuritic plaques, neurofibrillary tangles and cerebrovascular amyloid deposits and neuronal loss in specific brain regions. Changes in a wide range of neurotransmitter systems have also been reported [17]. Various hypotheses have been proposed to explain the aetiology and pathology of Alzheimer's disease [17, 18]. However, to date no single hypothesis encompasses all known changes. The failure in the cholinergic system has been of particular interest and is linked to a loss of neurons in the basal forebrain and of cholinergic markers in the cerebral cortex [17, 24, 25, 29, 31]. The presence of amyloid deposits and cortical plaques in post-mortem brain tissue has suggested a link between autoimmune responses and the pathogenesis of the disease [18]. A blood-brain barrier deficit has been reported in many cases [2, 30], and amyloid deposits and neuritic plaques are often found associated with blood vessels. Moreover, antibodies inhibiting choline acetyltransferase activity and specifically binding to cholinergic neurons have been reported in sera from patients with Alzheimer's disease [7, 13, 14, 21] and these observations have raised the question of whether autoimmune factors might play a part in the pathogenesis of the disease [18]. We report the presence of antibodies in sera, from groups of American and Swedish patients with Alzheimer's disease, which are able to lyse mammalian synaptosomes in the presence of complement. This immunolysis is detected by the release of soluble cytoplasmic enzymes, such as lactate dehydrogenase and choline acetyltransferase, from the synaptosomes into the incubation fluid [11]. Immunolysis can only occur when the antibodies present recognise and bind to an antigen on the outer cell surface, thereby triggering the complement-mediated lytic action. The findings are discussed in terms of the significance of autoimmune factors in the pathogenesis of Alzheimer's disease.

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Subjects and methods

Serum from patients with Alzheimer's disease and controls

American patients and controls. All patients met NINCDS-ADRDA criteria for the clinical diagnosis of probable Alzheimer's disease [20]. The patients and controls were rated for clinical severity, employing the clinical rating scale of Reisberg et al. [22], and controls were age matched to the patients. All patients had a rating greater than 4 on the Reisberg scale, which has the range 1–7. This scale correlates with the mini-mental scale [6, 15] used for the Swedish patients. The age range was 52–88 years for the ten patients (mean age 72.3, SD 3.8 years, median 76 years). The mean duration of illness at the time of blood sampling was 3.5, SD 0.8 years, with a median duration of 2.5 years. Controls were healthy, elderly volunteers (over 65 years old) and in the cognitive tests they all scored 1 on the Reisberg scale.

Swedish patients and controls. The patients with Alzheimer's disease were all diagnosed after an exhaustive clinical evaluation using the DSM III criteria [3] and were all of high severity (mini-mental state score less than 17 on a scale 0–30 [15]). The age range was 58–88 years (mean age 73, SD 3.3 years, median 71.5 years for the eight patients). The duration of illness at the time of blood sampling was 6.6, SD 1.2 years, with a median duration of 6 years. Controls were aged matched to the patients and were also tested using the DSM III criteria. They showed mini-mental state scores greater than 25.

Immunolysis test

This test was based on the method of Docherty et al. [11]. One millilitre of synaptosomes prepared from cerebral cortex of

Sprague Dawley rats [5] was pre-incubated (approx. 1 mg synaptosomal protein/ml) at 37°C in oxygenated phosphate-buffered Krebs-glucose medium (m*M*): NaCl, 140; KCl, 5.0; $MgCl_2$, 1.6; $CaCl_2$, 1.0; Na_2HPO_4/NaH_2PO_4, 20.0; glucose, 20.0) for 15 min before addition of serum alone, guinea-pig complement alone, or serum plus complement. Incubations were continued for 60 min, except where the time course was studied. Synaptosomes were then deposited by centrifugation (10000 g/30 s) and supernatants were decanted. The tissue fraction was resuspended and used to test levels of Na^+-dependent high-affinity choline uptake, as well as lactate dehydrogenase (LDH) and choline acetyltransferase (ChAT) content. The levels of ChAT and LDH were measured in the supernatant fraction.

Control and patient sera were treated identically throughout. Serum samples with added azide (0.02% w/v) were stored at 0–4°C. Both serum samples and complement were dialysed against incubation medium (2 × 1000 vol.) for 3 h under vacuum dialysis before use. Typically 100 µl serum sample and 50 µl guinea-pig complement were added to 850 µl synaptosome suspension. Fifty to 200 µl serum was used in dose-response studies. No LDH or ChAT was detected in any of the serum samples. ChAT was measured by the method of Fonnum [16] and LDH by the method of Vassault [27]. Glutamate decarboxylase (GAD) and high-affinity choline transport were measured by the method of Docherty et al. [11] and dopamine-β-hydroxylase (DBH) by the method of Joh et al. [19].

All patients' sera were tested interspersed with control sera, one control and one (or more) serum from a patient with Alzheimer's disease being tested together. Beyond this pairing, all the tests were made blind in each series (i.e. the sera were not identified as being control or patient in origin). In each test series a triplicate set of "no addition" controls were also run; these contained neither control nor patients' sera. They acted as controls for the serum or complement alone additions. The difference between paired patient and control sera tested against a particular synaptosome preparation was selected as the index of lysis.

The immunolysis test was found to be reproducible; a given serum produced the same degree of immunolysis when tested on separate occasions, even after several months' storage at 0–4°C. Triplicate samples gave very similar results.

Statistics employed

The initial statistical analysis of the data summarised in Table 1 was performed by using a Biomedical Data Processing Program 8V [8] to do a mixed models analysis of variance. This employed three between factors and one within factor (with fixed and random levels respectively), using each of the triplicate values in each analysis as a separate datum point. The analysis used the logarithms of each triplicate replication in the test groups as follows: (a) serum plus complement, (b) serum alone, (c) complement alone and (d) no addition, for both sera from patients with Alzheimer's disease and control sera. The logarithms of the triplicates were normally distributed. This analysis indicated that working with logarithms stabilises the variance and that none of the latter control conditions exerted a statistically significant effect on the parameters measured. The *F*-values generated by the program are given in Table 1.

Table 1. Summary of data on percentage choline acetyltransferase (ChAT) and lactate dehydrogenase (LDH) release and choline uptake inhibition (see Fig. 1)[a]

	ChAT release	LDH release	Choline uptake
American sera			
Alzheimer (10)[b]	5.4 (2.8)	3.4 (2.3)	(−) 18.8 (9.2)
Control (8)	0.2 (1.1)	0.5 (0.3)	(+) 1.9 (4.0)
Swedish sera			
Alzheimer (8)	12.8 (2.0)	5.1 (2.2)	(−) 23.4 (8.8)
Control (7)	3.3 (2.1)	1.1 (1.0)	(−) 1.6 (7.3)
Combined American and Swedish sera			
Alzheimer (18)	8.7 (2.0)	4.2 (0.9)	(−) 20.8 (4.8)
Control (15)	1.6 (1.2)	0.8 (0.4)	(+) 0.3 (3.9)
F values[c] *and statistical significance* (two tailed)			
All sera	8.88 $P < 2\%$	5.20 $P < 5\%$	4.88 $P < 5\%$
American sera	4.88 $P < 5\%$	8.17 $P < 2\%$	8.88 $P < 2\%$
Swedish sera	10.49 $P < 2\%$	7.18 $P < 2\%$	7.78 $P < 2\%$

[a] The individual data for each serum are shown in Fig. 1.
[b] Mean values (SEM) are given for the number of sera given in parentheses and represent percentage total enzyme release, or choline uptake inhibition, as defined in the legend to Fig. 1. For choline uptake, (−) indicates a diminished uptake, and (+) indicates an increased uptake due to serum addition
[c] *F* values were calculated by taking logarithms of the data in order to stabilise the variance

Results

Basis for immunolysis test

In the present study the possible presence in human sera of antibodies to cholinergic neurons was investigated by attemping to cause specific complement-mediated immunolysis of cholinergic synaptosomes in preparations of rat brain cerebrocortical synaptosomes. We have previously demonstrated such specific immunolysis of the cholinergic subpopulation of synaptosomes by the addition of antibodies which recognise ChAT, together with complement, to mixed synaptosome preparations [9–11]. Lysis was monitored by following the release of the soluble enzymes ChAT and LDH, and by measuring changes in the extent of high-affinity choline transport by the synaptosomes after exposure to dialysed serum with or without complement, as described above.

In the extensive studies of Docherty et al. [9–11], a maximum of 10%–15% of the rat cerebrocortical synaptosomes was immunolysed by anticholinergic antibodies in the presence of complement, indicating that 10%–15% of the whole synaptosome fraction is derived from cholinergic neurons.

Action of sera from patients with Alzheimer's disease and controls

Ten sera from American patients with Alzheimer's disease were tested blind, randomly interspersed with eight American control sera, and sera from eight Swedish patients with SDAT were similarly tested blind with seven age-matched Swedish control sera. One control serum and one (or more) serum from a patient with Alzheimer's disease were tested together against a particular batch of freshly prepared rat cerebrocortical synaptosomes. In these tests the action of serum plus complement was compared with that of complement alone. It can

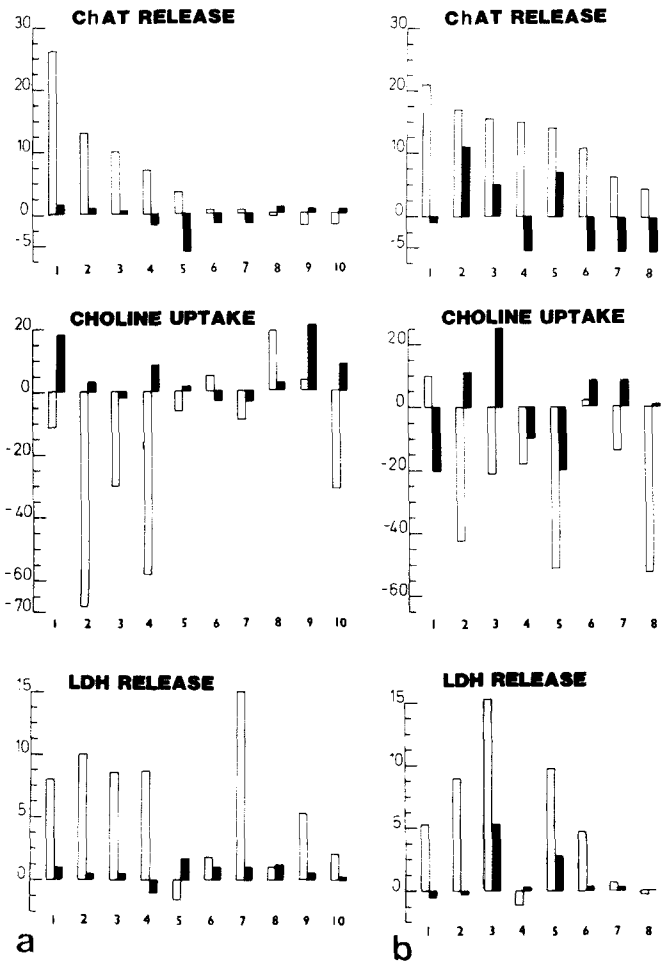

Fig. 1. Action of Alzheimer and control serum plus complement on choline acetyltransferase (ChAT) and lactate dehydrogenase (LDH) release, as well as on high-affinity choline uptake into rat cerebrocortical synaptosomes. **a** American, **b** Swedish patients and controls. The action of patient (□) and paired control sera (■) are shown as histograms and assigned a number for identification in the other tests on this figure an in tests reported in Tables 2–4. The *ordinates* for each parameter measured represent the difference between the action of serum plus complement and complement added alone to rat cerebrocortical synaptosomes expressed as a percentage of total enzyme present in the preparation which is released, or percentage of choline uptake in the absence of additions. Negative values indicate a larger action for complement alone in releasing the enzyme. For choline uptake, negative values indicate the degree of inhibition of the uptake process. The sera are arranged in a left-to-right sequence of increasing action in causing release of ChAT. All tests were done blind, and sera from one or more patients were always tested with control sera against each synaptosome preparation. The choline uptake studies employed 1.0 μ*M* (^{3}H)-choline (1 μC_i/ml) to select high-affinity uptake. The absolute levels of parameters measured in control, untreated, synaptosomes were: LDH: 1.68 μmol/mg; ChAT 157 μmol/mg; choline uptake 121 pmol/mg per 2 min

be seen from Fig. 1 and Table 1 that the majority of sera from patients with SDAT caused greater release of ChAT and LDH into the incubation medium than the paired control with which it was tested. The difference between the mean values for ChAT or LDH release by grouped American SDAT patients or American control sera, without reference to pairing, also shows statistically significant enhanced release of the enzymes into the incubation medium (Table 1).

Essentially the same pattern of LDH and ChAT release, and depressed choline uptake, was obtained when differences between the addition of serum alone and serum plus complement (instead of complement alone and serum plus complement) were analysed for both American and Swedish sera. In this analysis of the data the differences in action between the two conditions were smaller (by about 20%). This was most likely due to the presence of residual active complement in some of the serum samples, because sera were not heat inactivated. It was for this reason that serum plus complement was compared with complement added alone, rather than serum plus complement being compared with serum alone. Heat-treated sera were not used routinely, as there was an inhibitory effect (15%) on choline transport by heated control sera when added alone.

Complement or serum added alone had only small effects on the immunolysis parameters and these were not additive, indicating that they exerted similar non-specific actions in common (Table 2). Moreover, "no addition" controls (see Subjects and methods) produced very small effects, commensurate with those caused by serum or complement added alone.

Specificity

The action of the sera appeared to be specifically directed towards cholinergic synaptosomes. Thus, neither GAD nor DBH were released after up to 2 h of incubation with selected American or Swedish SDAT patients' sera which effectively released ChAT in the same test and were known to be among the most active in causing cholinergic immunolysis (Table 2). This indicates that neither GABAergic nor noradrenergic synaptosomes are lysed by these sera. However, release of ChAT and reductions of high-affinity choline transport indicate *specific* immunolysis of cholinergic synaptosomes. It should be noted that complement or serum added alone produced relatively small effects (Table 2). These were commensurate with those produced in samples where no complement or serum was added (data not shown).

Dose-response correlation and time course of immunolysis

Increasing the amount of selected sera from American or Swedish patients with SDAT from 50 to 200 μl during a 1-h incubation caused a small but detectable increase in release of LDH and ChAT (Table 3). Time-course studies over the period 30–120 min showed that lysis was essentially complete after 60–90 min incubation for the selected American SDAT patients' serum studied but continued for Swedish SDAT patients' serum as judged by the release of ChAT and LDH (Table 4). Only one experiment (in triplicate) was performed in each case using sera selected as being among the most active in causing immunolysis in other tests.

Correlation between actions of sera on the three parameters measured

The majority of sera from American and Swedish patients with SDAT caused some degree of immunolysis of synaptosomes compared with relatively few of the control sera, based on the three indices employed (e.g. American SDAT patients' sera 6, 8, 10; Swedish SDAT patients' sera 7). Nearly all of the

Table 2. Specificity of immunolysis of synaptosome subfractions by sera from patients with Alzheimer's disease

Serum	Release of enzyme (% total released)								
	ChAT			GAD			DBH		
	S + C	S	C	S + C	S	C	S + C	S	C
AA	27 (5.7)	0 (0.3)	0 (2.1)	1 (0.8)	0 (0.5)	1 (0.9)	11 (3.7)	6 (2.0)	4 (0.3)
SA	12 (2.7)	4 (1.8)	2 (3.0)	3 (2.2)	1 (0.2)	1 (0.2)	nm	nm	nm
AC	2 (0.9)	1 (2.4)	3 (1.9)	2 (1.9)	4 (1.4)	2 (1.0)	nm	nm	nm

Rat cerebrocortical synaptosomes were incubated with the serum (100 µl) for 2 h at 37°C as described in Subjects and methods. Antibiotics (penicillin and streptomycin) were present (200 IU/ml). AA = serum from American patient no. 2 of Fig. 1; SA = serum from Swedish patient no. 7 of Fig. 1; AC = American control serum no. 2 of Fig. 1.
Values are mean (SEM) of three determinations. nm = not measured; S = serum alone; C = complement alone; S + C = serum plus complement, added; GAD = glutamate decarboxylase; DBH = dopamine-β-hydroxylase.
All values were corrected for "no addition" controls

Table 3. Dose-dependence of immunolysis of rat cerebrocortical synaptosomes by serum from patient's with Alzheimer disease

Dose	Serum								
	AA			SA			Control sera		CU
	LDH	ChAT	CU	LDH	ChAT	CU	LDH	ChAT	
50 µl	7.3 (0.4)	6.8 (1.9)	172 (14)	6.8 (2.4)	8.8 (2.1)	185 (13)	7.0 (1.9)	7.2 (0.1)	112 (20)
100 µl	8.2 (0.7)	9.3 (0.8)	138 (3)	8.7 (0.9)	10.7 (1.5)	149 (11)	8.1 (2.2)	7.5 (1.3)	138 (11)
150 µl	8.9 (2.9)	10.7 (2.8)	89 (12)	8.1 (2.8)	10.5 (2.4)	147 (11)	8.0 (2.2)	6.9 (1.4)	136 (16)
200 µl	10.8 (1.6)	13.2 (1.7)	125 (11)	10.3 (0.2)	9.0 (1.0)	97 (9)	7.2 (2.0)	6.4 (1.0)	150 (11)

Rat cerebrocortical synaptosomes were incubated with serum (50–200 µl) plus complement for 60 min at 37°C described in Subjects and methods. Data for LDH and ChAT represent percentage of total present which was recovered in the incubation medium. The high-affinity choline uptake (CU) data represent picomoles of choline transported per sample per 2 min. Values represent difference between serum plus complement and complement alone.

Data are mean ± SEM from three determinations: AA = serum from American patient no. 2 of Fig. 1; SA = serum from Swedish patient no. 8 of Fig 1. Control sera were SC no. 3 and no. 2 of Fig. 1. The data for control are mean of results from these two sera

Table 4. Time course of immunolysis of rat cerebrocortical synaptosomes by serum from patients with Alzheimer disease

Min	Enzyme release (% total release)							
	AA		SA		SC		Complement only	
	LDH	ChAT	LDH	ChAT	LDH	ChAT	LDH	ChAT
30	5.4 (1.8)	15.1 (1.9)	5.5 (0.5)	15.4 (2.2)	7.8 (11)	7.3 (0.3)	4.4 (0.3)	6.7 (0.4)
60	7.0 (0.4)	13.5 (1.4)	8.0 (1.2)	13.8 (1.6)	7.0 (2.1)	6.8 (0.9)	5.3 (0.6)	6.4 (1.0)
90	7.0 (0.8)	17.5 (0.4)	10.8 (0.4)	12.0 (0.4)	10.8 (0.4)	7.3 (1.4)	5.1 (1.1)	6.6 (0.9)
120	7.5 (0.8)	17.0 (1.7)	16.1 (0.9)	18.3 (0.9)	7.3 (1.2)	6.2 (0.8)	5.4 (0.4)	7.1 (0.5)

Rat cerebrocortical synaptosomes were incubated with the serum (100 µl) for the period indicated as described in Subjects and methods. Antibiotics (penicillin and streptomycin) were present (200 IU/ml). AA = serum from American patient no. 1 of Fig. 1; SA = serum from Swedish patient no. 3 of Fig. 1; SC = Swedish control serum no. 3 of Fig. 1. Values given in the first six columns represent the release obtained when synaptosomes were incubated with sera plus complement. Data are mean (SEM) of three determinations

sera from patients with Alzheimer's disease changed one or the other parameter.

For the American group of patients with SADT, there was a general correlation between the amount of LDH and ChAT released by the sera and the extent of the decrease in high-affinity choline uptake (Fig. 1). Thus, six of the ten patients showed correlation between the magnitude of response of all three parameters, whilst four showed correlation between only two parameters. For five of the eight Swedish patients, all three parameters correlated, and three patients showed only two parameters correlating.

The correlation between ChAT release, choline uptake and LDH release was statistically assessed by calculating Spearman rank correlation coefficients. The coefficient for the correlation between ChAT release and choline uptake inhibition for both American and Swedish sera taken together was -0.419 ($P < 0.05$). Between ChAT and LDH release it was 0.409 ($P < 0.05$) and between choline uptake and LDH release it was -0.262 (not significant). The coefficients of determinacy for the significant correlations are about 17%. Thus, for a given patient's serum, change from normal for one parameter was always accompanied by change in one or both

of the other parameters, but the linear relationship between the parameters accounts for only 17% of the variability in the data. The significance levels quoted are for a two-tailed test.

Discussion

The data presented here suggest that the sera from groups of American and Swedish patients with SADT contain antibodies able to cause complement-mediated immunolysis of synaptosomes isolated from rat cerebral cortex and subsequently incubated at 37°C. This immunolysis appeared to be principally of the cholinergic subpopulation of synaptosomes, since soluble ChAT was released along with LDH, and high-affinity choline uptake was substantially reduced. In contrast, soluble GAD and DBH, which are markers for GABAergic and noradrenic synaptosomes respectively, were not released. It has previously been shown that specific complement-mediated immunolysis of cholinergic synaptosomes is caused by adding complement together with antibodies which recognise ChAT [9–11], whilst the GABAergic subpopulation can be lysed with antibodies recognising GAD plus complement [10] and noradrenergic terminals with DBH antibodies plus complement [12]. Lysis of cholinergic synaptosomes with anti-ChAT released some 10% of total LDH present [9–11], which is close to the 10%–15% of LDH released by the more active sera in the present study.

Immunolysis of synaptosomes by antibodies and complement requires the corresponding antigen to be both present and accessible at the outer surface of the synaptosomes. In the present study, the nature of the antigen allowing specific immunolysis of the cholinergic subpopulations by sera from patients with Alzheimer's disease remains uncertain. However, the effectiveness of ChAT antibodies in causing such immunolysis when added to brain synaptosomes [9–11] suggests that some form of ChAT itself could be the antigen that allows immunolysis to occur. It has previously been reported [13] that IgG antibodies in sera from the American patients with Alzheimer's disease are reactive with magnocellular (presumed cholinergic) neurons in rat and human basal forebrain and can inhibit ChAT enzymatic activity. These findings suggest an autoimmune response to cholinergic neurons might be involved in the pathogenesis of the disease. Others have also reported the presence in the CSF of patients with Alzheimer's disease of antibodies which specifically recognise cholinergic neurons in rodent brain [21]. These were detected by immunocytochemistry and their binding was blocked by preincubation with anti-acetylcholine antibodies [21]. However, Watts et al. [28], using indirect immunofluorescence, were able to find no selective binding of antibodies in sera or CSF from patients with Alzheimer's disease to neurons in frozen brain sections. In another study [7] anticholinergic antibodies were detected in the sera of patients with Alzheimer's disease using ELISA assays and immunoblotting employing specific cholinergic antigens.

Our own studies, using the very sensitive immunolysis assay technique, also indicate the presence of anticholinergic antibodies in sera from patients with Alzheimer's disease. This suggest that the blood-brain barrier may be breached in this disease [2, 30], perhaps in selected regions bearing plaques, since brain tissue is not normally accessible to immune responses when the blood-brain barrier is intact [18]. Antibodies recognising cholinergic nerve terminals, formed as a result, could act in an autoimmune fashion to cause further plaque formation in selected regions of cerebral cortex, hippocampus and amygdala, as cholinergic terminals are lysed [18]. This would correlate with the observed retrograde degeneration of cholinergic neurons [18] and could lead to the observed death of the cholinergic cell bodies in the nucleus basalis [24, 31] from which the lysed axons and terminals project. Evidence that autoimmune mechanisms involving anti-vascular antibodies may well play a key role in causing injury to the blood-brain barrier in Alzheimer's disease has recently been published [14].

The other neurotransmitter changes known to occur in Alzheimer's disease may be involved by similar autoimmune processes, or they could be related to other aspects of the primary disease process [17, 18]. However, the degeneration of noradrenergic terminals seen in Alzheimer's disease [1, 17] does not appear to be associated with the appearance of antibodies recognising noradrenenergic neurons in the serum of the patients studied here, since no DBH was released during the observed immunolysis of cerebrocortical synaptosomes. This latter enzyme is specific to noradrenergic synaptosomes and is released during complement-mediated immunolysis induced by DBH antibodies in the presence of complement [12].

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