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Antibodies to Different Isoforms of the Heavy Neurofilament Protein (NF-H) in Normal Aging and Alzheimer's Disease

Lior Soussan,[1] Kirill Tchernakov,[1] Orit Bachar-Lavi,[1] Tamar Yuvan,[1] Eliyahu Wertman,[2] and Daniel M. Michaelson[*,1]*

[1]Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel; and [2]Department of Neurogeriatrics, Ezrat Nashim Hospital, Jerusalem 91001, Israel

Abstract

Sera of normal controls and of patients with neurological diseases contain antineurofilament antibodies. Recent studies suggest that biochemically and immunologically distinct subclasses of neurofilaments occur in different types of neurons. Alzheimer's disease (AD), the major cause of dementia, is associated with a marked degeneration of brain cholinergic neurons. In the present work we characterized the repertoire and age dependence of antineurofilament antibodies in normal sera and examined whether the degeneration of cholinergic neurons in AD is associated with serum antibodies directed specifically against the neurofilaments of mammalian cholinergic neurons. This was performed by immunoblot assays utilizing neurofilaments from the purely cholinergic bovine ventral root neurons and from the chemically heterogeneous bovine dorsal root neurons. Antibodies to the heavy neurofilament protein NF-H were detected in normal control sera. Their levels were significantly higher in older (aged 70–79) than in younger (aged 40–59) subjects. These antibodies bound similarly to bovine ventral root and dorsal root NF-H and their NF-H specificity was unchanged during aging. In contrast, the levels of IgG in AD sera that are directed against ventral root cholinergic NF-H were higher than those directed against the chemically heterogeneous dorsal root NF-H. Immunoblot experiments utilizing dephosphorylated ventral root and dorsal root NF-H and chymotryptic fragments of these molecules revealed that AD sera contain a repertoire of antimamalian NF-H IgG. A subpopulation of these antibodies binds to phosphorylated epitopes that are specifically enriched in ventral root cholinergic NF-H and that are located on the carboxy terminal domain of this molecule. The level of these anticholinergic NF-H IgG are significantly higher in AD sera than in those of both normal controls and patients with multi-infarct dementia.

Index Entries: Alzheimer's disease; normal aging; antibodies; neurofilaments; cholinergic.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Neurofilaments are composed of 3 different proteins whose approx mol wts are 200, 160, and 68 kDa, and that are commonly referred to as the high (NF-H), middle (NF-M) and low (NF-L) neurofilament subunits. These three subunits contain homologous domains at their aminoterminal end. The greater mass of NF-H and NF-M, compared to that of NF-L, is caused by long extensions of sequence on their carboxyterminal end (for a review, *see* refs. *1,2*). This carboxyterminal tail domain contains multiple repeats of the motif Lys-Ser-Pro, which appears about 10 times in NF-M and more than 40 times in NF-H *(3,4)*. These repeats are potential phosphorylation sites. Indeed all 3 neurofilament proteins are phosphorylated in vivo. This occurs in proportion to the number of Lys-Ser-Pro repeats, so that NF-H is the most extensively phosphorylated and NF-L the least *(5–7)*. Recent immunohistochemical and monoclonal antibody experiments have shown that antineurofilament monoclonal antibodies yield distinct patterns in different types of neurons, suggesting that the neurofilament proteins are a family of distinctly phosphorylated molecules whose occurrence and distribution are a function of cell type *(8–13)*.

Sera of normal controls and of patients with several neurological and immunological diseases contain antineurofilament antibodies *(14–21)*. In view of the suggestion that biochemically distinct subclasses of neurofilaments occur in different types of neurons, it is of interest to determine the specificity of antineurofilament antibodies in normal sera and in sera of patients with neurological diseases and to examine whether thee antibodies reflect the types of neurons that degenerate in each case. By utilizing neurofilaments isolated from the purely cholinergic electromotor neurons of the electric fish *Torpedo*, we have previously examined whether the degeneration of brain cholinergic neurons in Alzheimer's disease (AD) and in Down's syndrome, which is one of the hallmarks of these diseases *(22,23)*, is associated with the appearance of anti-*Torpedo* cholinergic neurofilament antibodies. This study showed that sera of AD patients and of Down's syndrome patients older than 30 contain specific anti-NF-H antibodies (IgG) that bind to epitopes highly enriched in *Torpedo* cholinergic NF-H *(24–26)*. The biochemical and immunological properties of mammalian cholinergic NF-H are expected to resemble those of the NF-H molecules against which the AD and Down's syndrome anti-NF-H IgG are directed more than *Torpedo* cholinergic NF-H. At present purely cholinergic NF-H cannot be isolated from the mammalian brain. Purely cholinergic mammalian NF-H can, however, be isolated from ventral root neurons.

In the present study we examined whether the degeneration of brain cholinergic neurons in AD is associated with serum antibodies that are directed specifically against mammalian cholinergic neurofilaments. This was performed by immunoblot assays in which sera of AD patients and of controls were reacted with native, dephosphorylated, and proteolytically cleaved neurofilaments from cholinergic bovine ventral root neurons and from the chemically heterogeneous bovine dorsal root neurons.

Methods

Purification of Neurofilaments

Neurofilaments were prepared from freshly excised bovine ventral root neurons, dorsal root neurons, and spinal cord according to Carden et al. *(6)*. Protein was measured with the BCA Protein Determination Kit (Pierce, Oug-Beigerland, The Netherlands) utilizing bovine serum albumin as standard *(27)*.

Dephosphorylations and Chymotryptic Digestion of Neurofilaments

Dephosphorylation was performed according to Carden et al. *(6)*. Neurofilaments (2 mg/mL in 50 m*M* Tris-HCl, pH 8.0 containing 100 m*M* NaCl, and 1 m*M* $ZnCl_2$) were mixed with *E. coli* alkaline phosphatase (type III-N; Sigma, St. Louis, MO) at 20 U/mg of neurofilaments and incubated for 8 h at 37°C. Shorter times (4 min–4 h) yielded less extensive dephosphorylation. The extent of dephosphorylation was monitored by immunoblot assays utilizing monoclonal antibodies NE14 and N52 (Biomakor, Rehovot, Israel), which are directed against phosphorylated and nonphosphorylated NF-H epitopes, respectively *(28,29)*. Chymotryptic digestion of native neurofilaments was performed according to Chin et al. *(30)*. Neurofilaments (1.5 mg/mL) were incubated in 10 m*M* Tris-HCl, pH 7.5 containing 100 m*M* NaCl and 10 μg/mL chymotrypsin (sequence grade; Boehringer, Mannheim, Germany) at 25°C for 20 min. This resulted in the formation of 170 and 120 kDa carboxyterminal fragments of NF-H and NF-M, respectively.

Collection of Sera

Normal control sera were obtained from 114 volunteers aged 10–89, none of whom had any neurological or immunological diseases. Sixty five of these sera (age = 76 ± 6) were used as age-matched AD controls. AD patients (n = 48; age = 77 ± 6) were diagnosed as suffering from AD by research criteria *(31,32)*.

The AD patients had all experienced an insidious onset of the disease and had exhibited the disease for at least 2 yr. All were severely disturbed in activity of daily leaving, according to the clinical dementia ratings (CDR) *(33)* and had minimental test scores below 18 out of 30. Patients with multi-infarct dementia (MID) (n = 23; age = 77 ± 6.5) were diagnosed by standard clinical criteria.

Immunoblot Assay

The specified neurofilament preparations (2 µg native neurofilaments and 6 µg chymotrypsinized neurofilaments per lane) were electrophoresed on a mini-Protean apparatus (Bio-Rad, Richmond, CA), after which the separated proteins were transferred onto nitrocellulose sheets. The nitrocellulose sheets were cut to 30 strips, which were each incubated with either an AD or a control serum (dilution 1:80). The bound IgG were then detected by peroxidase-conjugated antihuman IgG (dilution 1:1000) as previously described *(25)*. The immunoreactions were terminated during the linear phase and the extents of binding of IgG to the neurofilament subunits were determined utilizing a computerized densitometer (LKB Model 2400). The levels of anti-NF-H IgG presented are the area, in arbitrary units, of the corresponding peaks of the scanned immunoblots. Control experiments in which the same sera were reacted with strips from each of the nitrocellulose sheets employed in every experiment ensured that the blots were identical, both in the efficacy of transfer of the neurofilaments from the gels to the nitrocellulose and in the intensity of their antineurofilament subunit immunoblot peaks.

Total IgG levels were measured by rate nephlometry (Beckman, Fullerton, CA, Immunohistochemistry Analyzer II).

Statistical Analysis

The values of the immunoblot data of the different groups were compared by means of a Wilcoxon rank order test *(31)*.

Results

Antineurofilament Antibodies in Normal Aging

The individual levels of IgG directed against bovine ventral root NF-H and bovine dorsal root NF-H in sera of normal controls aged 10–89 are shown in Figs. 1A,B. Regression analysis of these data revealed that the level of IgG directed against both of these antigens were age dependent and were about threefold higher than in sera of subjects aged 70–79 than in sera of younger subjects. This difference was statistically significant for cases aged 40–59 ($p < 0.02$: Wilcoxon's rank order test). The levels of these anti-NF-H IgG in sera of 80–89-yr-old controls were slightly lower than those of the 70–79-yr-old group. This difference, however, was not statistically significant. Measurement of the levels of antibovine spinal cord NF-H IgG of these sera revealed a similar age dependence (Fig. 1C). The levels of total IgG, unlike anti-NF-H IgG, did not change during normal aging and were the same for subjects aged 70–79 (12.2 ± 0.5 mg/mL: n = 31) and 40–59 (12.4 ± 0.7 mg/mL; n = 25) This suggests that the age dependence of anti-NF-H IgG is specific to these antibodies. Normal sera also contained IgG that react with NF-M and NF-L. However, the incidence and the levels of these IgG were markedly lower than those of anti-NF-H IgG (not shown).

The presently observed increase in the levels of antineurofilament antibodies is consistent with previous reports that the level of antineuronal autoantibodies increase with age *(35,36)*. The finding that for all these NF-H preparations the average levels of anti-NF-H IgG were about threefold higher in subjects aged 70–79 than in those of younger subject (*see* Fig. 1) suggests that the increase in anti-NF-H IgG levels during normal aging is not accompanied by a parallel increase in the levels of IgG that bind specifically to either ventral root cholinergic NF-H, dorsal root NF-H, or spinal cord NF-H.

Antineurofilament Antibodies in Alzheimer's Disease

The possibility that AD sera contain higher levels of antimammalian cholinergic NF-H IgG than controls was examined by immunoblot assay utilizing neurofilaments from bovine ventral root cholinergic neurons. Figure 2 shows the binding patterns of IgG (serum dilution 1:80) from a representative group of 4 AD patients, 4 MID patients, and 4 age-matched normal controls. As can be seen,

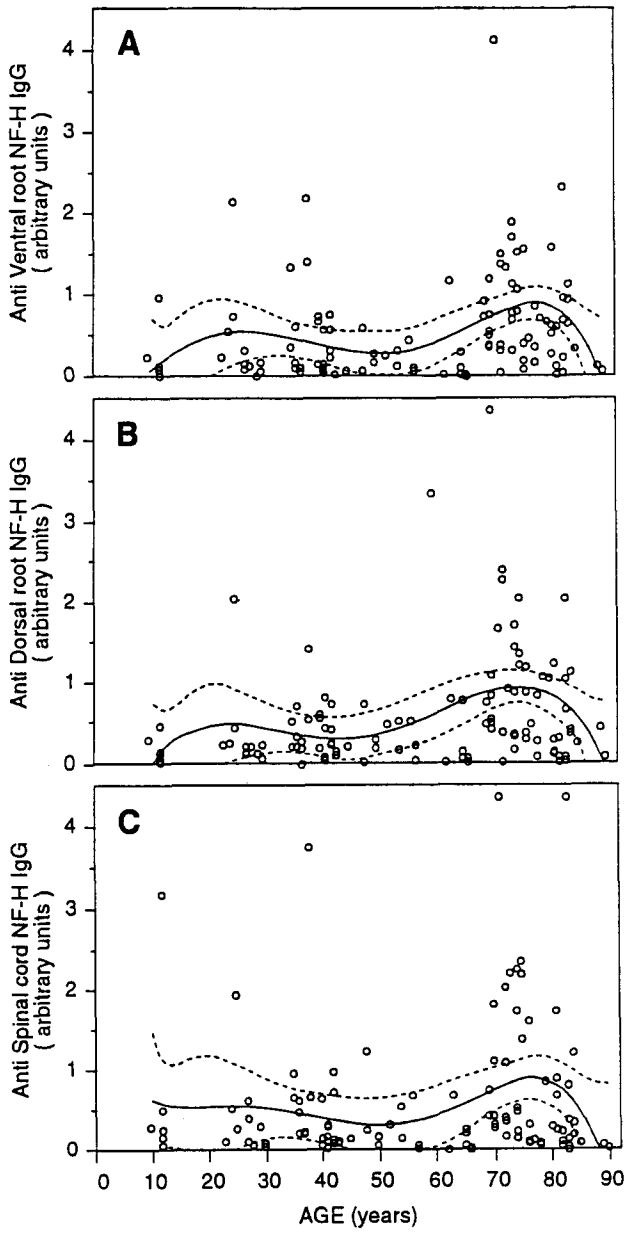

Fig. 1. The age dependencies of the levels of antibovine ventral root NF-H IgG **(A)**, antibovine dorsal root NF-H IgG **(B)**, and antibovine spinal cord NF-H IgG **(C)** in normal human sera. Sera (dilution 1:80) from 114 subjects (age range 10–89) were immunoreacted with blots of the indicated neurofilaments as described in Methods. Results presented are the area in arbitrary units, of the anti-NF-H immunoblot peaks of the individual subjects. The curves presented correspond to results of regression analysis with the 95% confidence intervals indicated by the broken lines.

the reaction on NF-H was stronger than on NF-M and NF-L. Comparison of the antiventral root cholinergic NF-H IgG levels of the cases presented revealed that the reaction of the AD sera was strongest, whereas that of the normal controls was the weakest. To evaluate the prevalence of antiventral root cholinergic NF-H IgG in AD, MID, and normal control sera, we performed an immunoblot assay on sera from 48 AD and 23 MID cases and from 65 age-matched normal controls. The results thus obtained revealed that the average levels of antiventral root NF-H IgG in AD and in MID sera were about two-fold higher than those of normal control sera (Table 1), and that these differences between AD patient and normal controls and between MID patients and normal controls were both statistically significant ($p < 0.03$, Wilcoxon rank order test).

The extent to which the NF-H epitopes recognized by AD IgG are specific to bovine ventral root cholinergic NF-H was examined by immunoblot assays in which the same AD and control sera were reacted with neurofilaments of the chemically heterogeneous bovine dorsal root neuron. Figure 3 shows the binding patterns of antidorsal root neurofilaments IgG of the same representative group of 4 AD, 4 MID patients, and 4 age-matched controls whose antiventral root neurofilaments IgG are depicted in Fig. 2. As can be seen, the profiles obtained with the dorsal and ventral root neurofilaments differed markedly; normal control IgG reacted more strongly with dorsal root NF-H than did the AD and MID sera, whereas the opposite was observed with ventral root NF-H (compare Figs. 2 and 3). Measurements of the levels of antidorsal root NF-H IgG in the same 48 AD, 23 MID, and 65 normal controls sera whose antiventral root NF-H IgG were determined, revealed that the individual levels of IgG to dorsal root NF-H were indeed significantly lower in AD and MID sera than in normal control sera (Table 1). The levels of AD and control IgG that are directed specifically against mammalian cholinergic NF-H were evaluated by subtracting the level of antidorsal root NF-H IgG of each serum from its antiventral root NF-H IgG level (Fig. 4 and Table 1). This revealed that about 60% of the AD and MID sera had IgG that bind more to mammalian cholinergic NF-H than to chemically heterogeneous mammalian NF-H, whereas most of the normal control IgG bound less to cholinergic NF-H (Fig. 4). This difference was significant ($p < 0.001$).

The antigenic properties of neurofilaments are affected by their degree of phosphorylation *(37–39)*. We therefore examined the possibility that dephosphorylation affects the binding of AD IgG to ventral root and dorsal root NF-H. In accordance with

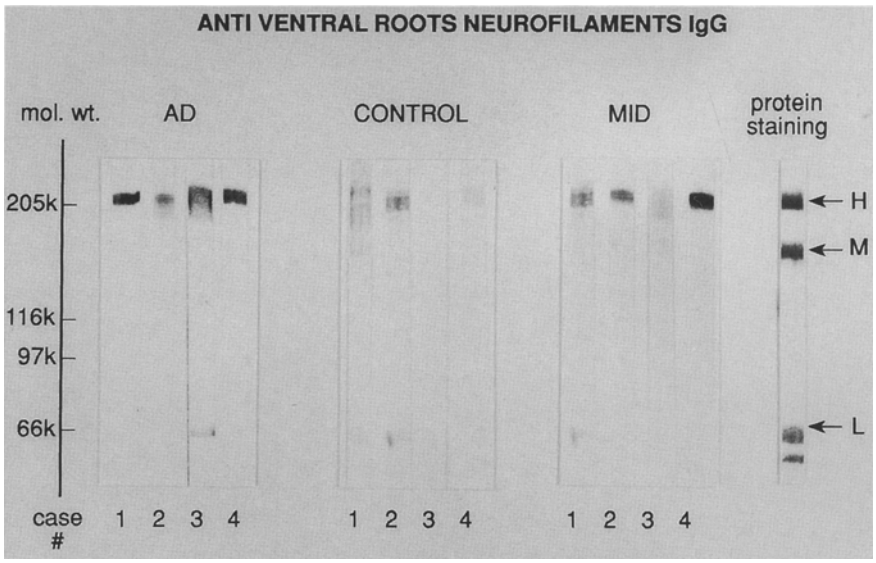

Fig. 2. Immunoblot assays of IgG directed against bovine ventral root cholinergic neurofilaments in sera (dilution 1:80) of Alzheimer's disease (AD) and multi-infarct dementia (MID) patients and of normal controls (control). The lane on the right corresponds to protein staining of the blotted neurofilaments with Amido black. The position of the heavy, medium, and light neurofilament subunits are indicated respectively by H, M, and L.

Table 1
Antineurofilament Antibodies

		Native NF-H			170 kDa chymotryptic fragment of NF-F		
Sera	*n*	Anti-VNF-H IgG	Anti-DNF-H IgG	Anti-VNF-H IgG minus anti-DNF-H IgG	Anti-VNF-H IgG	Anti-DNF-H IgG	Anti-VNF-H IgG minus anti-DNF-H IgG
AD	48	0.65 ± 0.14^a	0.43 ± 0.12^a	0.22 ± 0.01^c	1.11 ± 0.27^b	0.17 ± 0.04	0.95 ± 0.25^d
Normal controls	65	0.34 ± 0.09	0.80 ± 0.16	-0.46 ± 0.01	0.52 ± 0.12	0.21 ± 0.05	0.31 ± 0.10
MID	23	0.86 ± 0.25^a	0.32 ± 0.11^a	0.54 ± 0.04^c	0.62 ± 0.29	0.37 ± 0.10	0.25 ± 0.25

The levels of IgG (in arbitrary units) to NF-H isolated from axons of bovine ventral root cholinergic neurons and of bovine dorsal root neurons and to the 170 kDa chymotryptic fragments of these molecules were measured by immunoblot assays as described in Methods. The difference between the antiventral root NF-H (VNF-H) and antidorsal root NF-H (DNF-H) IgG levels was calculated for each case. Results presented are the average ± SEM for each group. The result of the AD and MID cases were compared to those of the normal controls by means of a Wilcoxon rank order test.

[a] $p < 0.03$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] $p < 0.0001$.

previous reports on other mammalian neurofilament preparations *(6,7)* dephosphorylation with alkaline phosphatase increased the electrophoretic mobility of the NF-H and NF-M proteins of ventral root neurons and of dorsal root neurons (Fig. 5). Control experiments revealed that the binding of monoclonal antibody NE14, which recognizes phosphorylated NF-H epitopes *(28)*, was abolished by dephosphorylation of ventral root neurofilaments, whereas that of monoclonal antibody

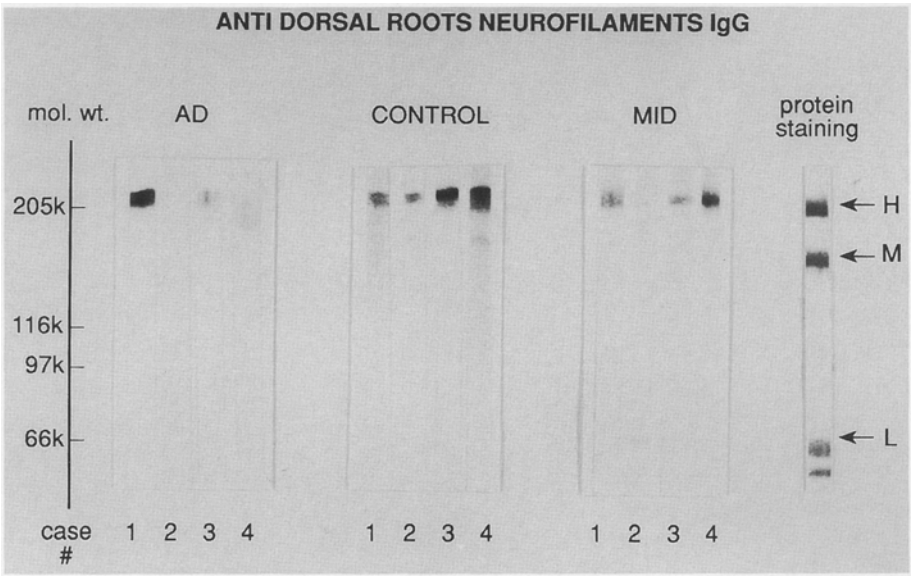

Fig. 3. Immunoblot assays of IgG directed against bovine dorsal root neurofilaments in sera (dilution 1:80) of Alzheimer's disease (AD) and multi-infarct dementia (MID) patients and of normal controls (control). The lane on the right corresponds to protein staining of the blotted neurofilaments with Amido black. The position of the heavy, medium, and light neurofilament subunits are indicated, respectively, by H, M, and L.

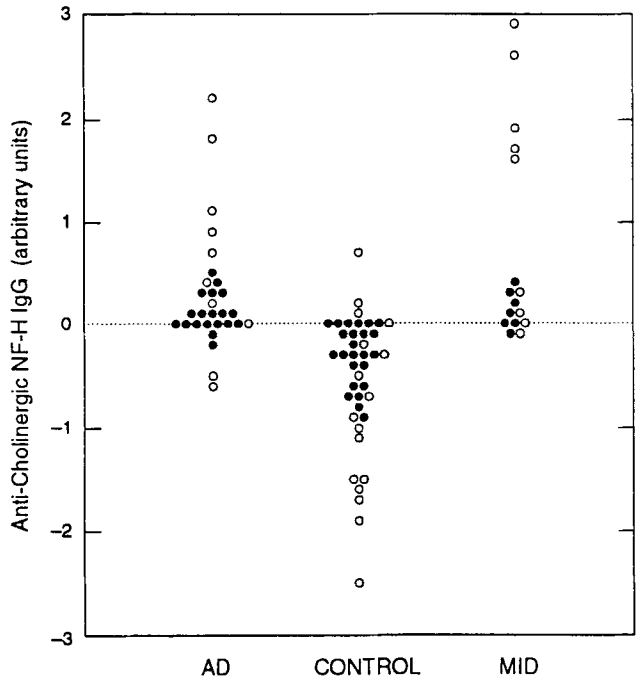

Fig. 4. Individual levels of IgG to cholinergic NF-H in sera of AD and MID patients and of normal controls. Results presented are the values of the antiventral root cholinergic NF-H peak minus the antidorsal root NF-H peak of the individual cases. Empty circles represent a single case, whereas each of the filled circles corresponds to two cases.

N52, which is directed against nonphosphorylated NF-H epitopes *(29)* was unaffected by dephosphorylation (Fig. 5). Similar results were obtained with dorsal root neurofilaments (not shown). The binding of IgG (dilution 1:80) from a representative group of 5 AD patients to native and dephosphorylated ventral root neurofilaments is depicted in Fig. 5. As can be seen, only one of the three AD sera that had antinative ventral root NF-H had IgG that recognized dephosphorylated NF-H. Of the two cases that had no antinative NF-H IgG, one had IgG that recognized dephosphorylated NF-H. Comparison of the levels of antidephosphorylated ventral root NF-H IgG in a large number of AD (n = 48), normal control (n = 65), and MID sera (n = 23) to their antinative ventral root NF-H IgG levels revealed that most of the AD and MID antiventral root NF-H IgG bound to phosphorylated epitopes. Furthermore, unlike with the native molecules, there were no differences between the binding of AD, MID, and normal control IgG to dephosphorylated ventral root and dorsal root NF-H (not shown).

The finding that most of the AD antiventral root NF-H IgG bind to phosphorylated epitopes prompted us to examine whether the sensitivity and specificity of the measurement of anticholinergic NF-H IgG could be improved by utilizing the highly phosphorylated carboxyterminal domain of NF-H as anti-

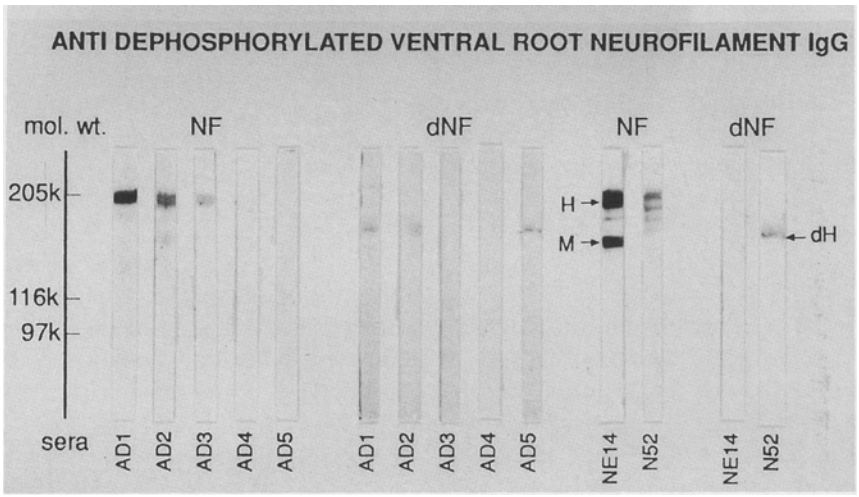

Fig. 5. Immunoblot assays of IgG in AD sera directed against phosphorylated and native ventral root neurofilaments. Neurofilaments were dephosphorylated as described in Methods. N52 and NE14 are antineurofilament monoclonal antibodies that are directed respectively against nonphosphorylated and phosphorylated NF-H epitopes. The position of the heavy and medium neurofilament subunits NF-H and NF-M are indicated by H and M. The position of NF-H following dephosphorylation is indicated by dH.

gen in the immunoblot experiments. This was performed by chymotryptic digestion of ventral root and dorsal root neurofilaments that, in accordance with previous publications *(30)*, truncated the NF-H molecules to a 170 kDa carboxyterminal fragment that contained the Ly-Ser-Pro phosphorylation site (not shown). The average levels of AD ($n = 48$), MID ($n = 23$), and normal controls ($n = 65$) IgG directed against the 170 kDa carboxyterminal fragment of ventral root and dorsal root NF-H are presented in Table 1. As can be seen, the average level of AD antiventral root NF-H IgG thus measured was about twofold higher than those of both the normal controls and the MID patients ($p < 0.01$). In contrast, the levels of IgG of the three groups that bind to the carboxyterminal domain of dorsal root NF-H were similar (Table 1). The levels of IgG that bind specifically to the carboxyterminal domain of ventral root cholinergic NF-H were evaluated by subtracting the level of IgG of each serum that bind to the carboxyterminal fragment of dorsal root NF-H, from the level of its IgG that are directed against the carboxyterminal fragment of ventral root NF-H. This revealed that the level of AD IgG directed specifically against the carboxyterminal tail domain of mammalian cholinergic NF-H was more than threefold higher than those of either the normal controls or the MID patients (Table 1). Thus, AD sera contain IgG that are directed specifically at the highly phosphorylated carboxyterminal domain of ventral root NF-H, and whose levels are markedly and significantly higher than those of both normal controls and MID patients.

Discussion

The present results show that the levels of anti-NF-H IgG increase during normal aging and that sera of AD patients contain additional anti-NF-H IgG. These IgG are directed against phosphorylated epitopes specific to bovine ventral root cholinergic NF-H, and their levels in AD sera are markedly and significantly higher than in sera of both age-matched normal controls and MID patients.

Comparison of the present results to those obtained by experiments in which *Torpedo* cholinergic NF-H was utilized as antigen *(25,40)* reveals that bovine cholinergic NF-H is a better probe than *Torpedo* cholinergic NF-H for detecting the AD specific anticholinergic NF-H IgG. The method of detection of these antibodies is further improved when the highly phosphorylated tail domain of bovine cholinergic NF-H is used in the immunoassay. This antigen, unlike the whole NF-H molecule, enables the detection of a subpopulation of anti-NF-H IgG and the demonstration that it is indeed specific to AD (Table 1). This marked improvement in specificity paves the way for future refinements in

which smaller fragments of the carboxyterminal tail domain of bovine ventral root NF-H will be used in the immunoassay. Several studies have shown that sera and cerebrospinal fluid of AD patient contain brain reacting antibodies, some of which are directed specifically against cholinergic neurons (*41,42*, and Chapters 8 and 21 of this issue). In view of the present findings, it is possible that at least some of these antibodies are directed at specific NF-H isoforms.

One of the hallmarks of neurodegeneration in AD is loss of central cholinergic neurons *(23)*. It is therefore possible that the AD antibovine cholinergic NF-H IgG are associated with the cholinergic degeneration in this disease and that they are directed against human cholinergic NF-H epitopes similar to those of bovine cholinergic neurons. The levels of antichemically heterogeneous NF-H IgG, but not of IgG directed specifically against cholinergic NF-H, were found to increase during normal aging (Fig. 1). Together with the AD data, these findings suggest that the levels and repertoire of antineurofilament antibodies reflect the extent and specificity of neuronal degeneration. Accordingly, neuronal degeneration that occurs either during normal aging or in AD may result in exposure and release of normal intracellular constituents, such as neurofilaments, and in the subsequent triggering of an immune response and of antibody synthesis. However, an alternative possibility should also be considered. AD is associated with aberrant phosphorylation of neurofilaments and of other cytoskeletal proteins *(43–46)*. We have recently shown that ventral root NF-H contains more than twofold more phosphorylated serine residues than does dorsal root NF-H *(47)*. Thus, since the AD specific antiventral root NF-H IgG bind to phosphorylated epitopes, it is possible that the specificity of these antibodies is caused by a cross reaction of antibodies that were generated in vivo against an abnormal antigen such as hyperphosphorylated neurofilaments or tau. Further studies of the binding of AD and control sera to neurofilaments and to homogenates of defined AD and control brain areas are needed for unravelling the endogenous human antigen against which the AD specific antibovine ventral root NF-H IgG are directed.

The question as to whether the anticholinergic NF-H antibodies are involved in the pathogenesis of AD was not examined in this study. Support for a role for such antibodies in the pathogenesis of AD is provided by recent animal model studies *(48–50)* that revealed that prolonged immunization of rats with cholinergic NF-H induces specific neuronal derangements and cognitive deficits.

Acknowledgments

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