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Aberrant neurofilament phosphorylation in Alzheimer disease

(monoclonal antibodies/immunocytochemistry/peroxidase–monoclonal anti-peroxidase technique/senile dementia/neurofilament kinase)

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ABSTRACT **Alzheimer tangles, despite their location in neuronal perikarya, react immunocytochemically with monoclonal antibodies to phosphorylated epitopes of neurofilaments. Normal perikarya do not contain phosphorylated neurofilaments. The aberrant phosphorylation in both plaques and tangles seems to be largely restricted to individual phosphorylation sites among the many sites available in neurofilaments. It is suggested that the Alzheimer lesion involves an imbalance within specific kinases responsible for phosphorylation of different sites in neurofilaments.**

Excessive loss of memory and cognitive functions prior to the sixth decade of life defines Alzheimer disease clinically. Excessive loss of these functions after the sixth decade is considered senile dementia of the Alzheimer type. The pathologic corollary of these symptoms is the formation of neurofibrillary tangles and of senile plaques. Tangles are the result of a degenerative process in neuronal perikarya. They consist of neurofilaments that possess unusually high resistance to solubilization by detergents and appear on negative staining in electron microscopy as paired helical filaments. Plaques seem to be the result of degenerative processes in neurites. They contain paired helical filaments in an amyloid matrix.

Abundance of these changes, expecially when found in the hippocampus and other cortical areas as well, provides a pathologic diagnosis of Alzheimer disease. Loss of cognitive function and memory is a general symptom that may not necessarily be a reflection of these pathologic changes in every case. While most patients afflicted with these symptoms excessively will demonstrate the lesions, other disorders, not associated with plaques and tangles, may also lead to loss of memory, although less frequently. Furthermore, aging itself, even when associated with mild degrees of loss of cognitive function or memory, will reveal occasional plaques and tangles, but again with lesser frequency and density. In these cases, lesions are usually confined to the hippocampus. Other disorders, such as Down syndrome, exhibit lesions similar to those of Alzheimer disease.

We recently have shown (1) that, among a large number of monoclonal antibodies to brain homogenate, more than one-half were specific to identifiable individual structures, and more than one-quarter were specific for neuronal elements in the central and peripheral nervous system and failed, with rare exceptions, to stain nonneuronal elements (2). The neuron-specific antibodies could be divided into four groups on the basis of their immunocytochemical staining. Group I visualized cells and structures predominately in gray matter and reacted on electrophoretic immunoblots with isolated synaptic proteins. Group II visualized projection axons but not perikarya, dendrites, or proximal axons. Group III visualized perikarya, dendrites, and proximal axons and revealed little, if any, staining overlap with antibodies from group II. Group IV consisted of only two antibodies that revealed both elements detected by groups II and III. Despite differences in staining distribution, antibodies from both groups II and III reacted either with the 200-kDa neurofilament protein exclusively or with this protein and to a lesser extent with the 150-kDa protein and a 180-kDa protein. Each antibody within groups II and III gave a slightly different immunocytochemical staining distribution, which was attributed to "microheterogeneity," while the major nonoverlapping staining distribution between groups II and III was termed "macroheterogeneity."

Subsequent studies have shown (3) that the macroheterogeneity was posttranslational and depended on phosphorylation. Thus, antibodies from group II reacted exclusively with phosphorylated neurofilaments; those of group III, with nonphosphorylated epitopes in neurofilaments that are masked by phosphorylation; and antibodies from group IV, apparently with a more accessible, nonphosphorylated neurofilament epitope. In tissue sections, trypsin or phosphatase treatment alone had no effect on the immunocytochemical staining by antibodies from group II. However, trypsin followed by phosphatase reduced the staining. Trypsin treatment abolished the staining by antibodies from group III. However, the staining with these antibodies reappeared by subsequent phosphatase treatment but was converted to axonal staining—i.e., from a group III to a group II pattern. The data permitted the conclusions that neurofilaments in dendrites, perikarya, and proximal axons are nonphosphorylated and that phosphorylation occurs during transport along the axon. Furthermore, it was apparent that phosphorylated neurofilaments were more compact than nonphosphorylated forms.

Alzheimer tangles are perikaryonal constituents. They have been shown by Selkoe *et al.* (4) to differ from normal neurofilaments in their resistance to solubilization by even extensive treatment with sodium dodecyl sulfate and, thus, can be considered highly compacted structures at least with regard to tertiary conformation. In contrast, normal perikaryonal neurofilaments, which are not phosphorylated, seem, according to our data, of noncompact configuration. It appeared, therefore, of interest to study phosphorylation of Alzheimer tangles and plaques and to examine the compactness of these structures with regard to susceptibility to dephosphorylation.

MATERIALS AND METHODS

This study includes two cases of Alzheimer disease, one case of Down syndrome, and a case of cerebral infarct. The first three cases exhibited progressive dementia and revealed, on autopsy, severe changes of the Alzheimer type in hippocampus and neocortex. The last case had only few changes of the Alzheimer type.

Paraffin sections were stained immunocytochemically (5) by using monoclonal first-layer antibodies, goat anti-mouse

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immunoglobulin, and ClonoPAP (peroxidase–monoclonal anti-peroxidase complex (3, 6). The first-layer antibodies were against phosphorylated neurofilament epitopes (antibodies 07-5, 03-44, 06-17, 04-7, and 06-68). Dephosphorylation of sections was carried out by treatment with trypsin for 10 min followed by phosphatase for 2.5 hr and again with trypsin for 10 min and phosphatase for 18 hr at concentrations of 400 μg/ml for both enzymes as reported (3).

RESULTS

Axons of normally appearing tissues in the four cases stained with all five anti-phosphorylated-neurofilament antibodies. The two cases of Alzheimer disease and the case of Down syndrome had extensive hippocampal lesions in which axons stained by these antibodies had been deleted. There were many tangles and plaques. The case of cerebral infarct had no tangles or plaques. In the Alzheimer and Down cases, tangles were stained by antibody 07-5 (Fig. 1). A lesser number of tangles and plaques were revealed by other antibodies that recognize phosphorylated epitopes.

Treatment with trypsin and phosphatase abolished the staining in tangles and plaques (Fig. 2).

DISCUSSION

The antibodies from group II, used in the present study, react with phosphorylated epitopes of neurofilaments. These antibodies reveal exclusively projection axons in normal-appearing rat (1), mouse, rabbit, and human brains and do not react with perikarya. The staining of perikaryonal constituents in Alzheimer disease and related disorders suggests, therefore, an aberrant and perhaps premature phosphorylation.

Treatment with trypsin and phosphatase completely abolished the staining in tangles and plaques. In contrast, such treatment caused a marked diminution of staining in normal axons but not complete abolition. It appeared, therefore, that Alzheimer tangles are more susceptible to trypsin/phosphatase treatments than are normal neurofilaments. Thus, at least with regard to susceptibility to these enzymes, Alzheimer tangles are not more compact than normal axonal neurofilaments, although they are more compact than the nonphosphorylated neurofilaments of normal perikarya (3).

Julien and Mushynski (7) have shown that there are 28 phosphorylation sites in the 200-kDa neurofilament protein. The likelihood that the different monoclonal antibodies to phosphorylated neurofilament epitopes react with different phosphorylation sites is reinforced by the slightly different staining distribution exhibited by these antibodies (1), by different developmental patterns revealed by them (8), by two-dimensional immunoblots (6), and by differences in degrees of susceptibility of neurofilaments in paraffin sections to treatment by trypsin and phosphatase when analyzed by different antibodies from group II. The finding that only antibody 07-5 reacted with all of the tangles, while the others reacted with only a few of them contrasts with the observations in normal tissues in which many axonal projections were revealed by any of these antibodies. Concurrent studies (10) have shown that tangles of aluminum poisoning in the rabbit, which morphologically resemble those of Alzheimer disease, are also phosphorylated. However, in contrast to the tangles of Alzheimer disease, they react better with antibodies 06-17 and 03-44 and not at all with antibody 07-5. Thus, although the lesions of aluminum intoxication and Alzheimer disease present similar morphologic features and although both lesions exhibit a shift of phosphorylation from an axonal to a perikaryonal location, the submolecular sites involved in the aberrant neurofilament phosphorylation process appear to be different. It is conceivable that this aberrant phosphorylation confers sufficient compactness to neurofilaments to prevent their further migration into the axon. Only one or few of the normal phosphorylation sites seem to be involved in this premature phosphorylation, in line with the impression that neurofilaments in Alzheimer tangles may be less compact than those in normal axonal projections.

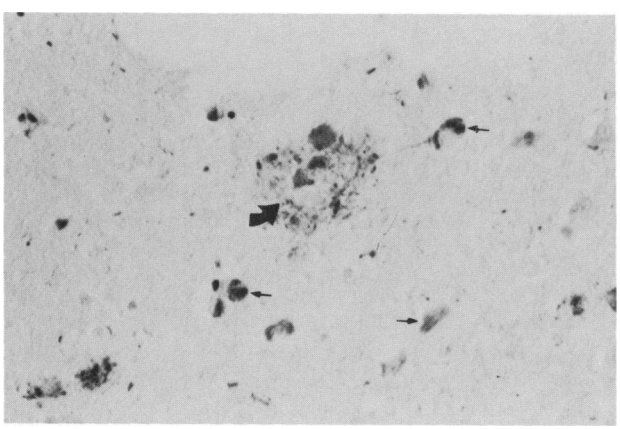

FIG. 1. Alzheimer disease; paraffin section from case 1 was stained with 07-5 monoclonal antibody to phosphorylated neurofilament epitopes, diluted 1:24,000. Plaques (curved arrow) as well as tangles (small arrows) are stained, even though the latter are perikaryonal structures.

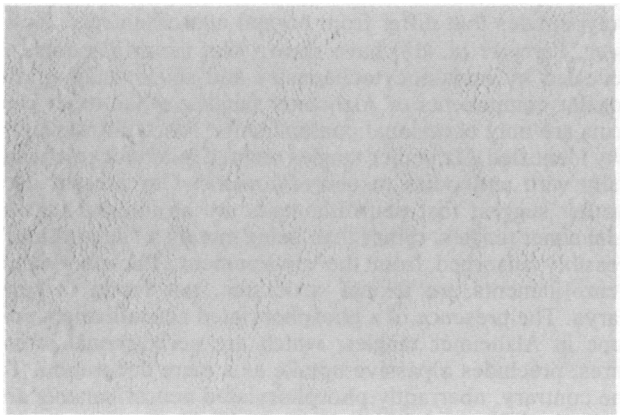

FIG. 2. Section adjacent to that in Fig. 1, treated with trypsin and phosphatase and then with 07-5 monoclonal antibody to phosphorylated neurofilament epitopes, diluted 1:24,000.

The preferential phosphorylation of only one or a few phosphorylation sites in Alzheimer disease suggests that a specific neurofilament kinase is involved and that there exist different kinases for different phosphorylation sites. If the Alzheimer lesion, indeed, is due to unbalanced involvement of only one or few highly specific kinases, then a search for a specific inhibitor may have therapeutic potential. Such an inhibitor may be an analog to the binding site of the kinase with a specific neurofilament region that mediates transfer of phosphate to this site.

While the present work includes only a limited number of cases, another study (11), with additional cases of Alzheimer disease, also revealed phosphorylated tangles restricted to the 07-5 site.

It has sometimes been questioned whether Alzheimer tangles are primarily composed of neurofilaments or whether their formation is due to a disturbance in other proteins. Wisniewski *et al.* (12) suggested that neurofilaments merely provide a passive "decoration" of Alzheimer tangles. Indeed, Igbal *et al.* (13) feel that isolated Alzheimer tangles provide

polypeptides that differ from normal neurofilaments. However, Perry *et al.* (14) have shown that neurofilaments, as revealed by immunocytochemistry and silver staining, are regular components of Alzheimer tangles, while other proteins are only occasional contaminants. Also, ultrastructurally identified Alzheimer tangles reacted immunocytochemically with antibodies to neurofilaments. Our present data further suggest that neurofilaments are an integral part of Alzheimer tangles, rather than being merely a "decoration," possibly adsorbed from the environment. Phosphorylated neurofilaments are axonal structures, not found in perikarya. The presence of a phosphorylated neurofilament epitope in Alzheimer tangles, which are perikaryonal structures, precludes a passive uptake as a mere decoration. To the contrary, aberrantly phosphorylated neurofilaments are an integral feature of Alzheimer tangles.

Rasool *et al.* (15) have produced an antiserum to isolated Alzheimer tangles that does not react with normal neurofilaments. They suggest (16) that Alzheimer tangles are a highly modified form of neurofilaments or another neuronal protein, "presumably a result of extensive posttranslational modifications." The present studies confirm an abnormal posttranslational processing of neurofilaments. If orderly phosphorylation that involves all of the phosphorylation sites described by Julien and Mushynski (7) is essential for the formation of normal, compact axonal neurofilaments (3), the selected phosphorylation of a single site, as now observed in Alzheimer tangles, may result in irregular and incomplete assembly, morphologically identified as tangles, and immunocytochemically revealed by presentation of new epitopes (15) in addition to those shared with normal neurofilaments.

Two monoclonal antibodies to the 200- and 150-kDa neurofilament proteins (9), respectively, as used by Rasool *et al.* (15) failed to react with isolated Alzheimer tangles, although they did react *in situ*. Presumably, their reaction *in situ* may have been with a component of perikarya not actively associated with Alzheimer tangles. The failure of some antibodies to neurofilaments to react with Alzheimer tangles is expected, however, even if Alzheimer tangles are exclusively a product of neurofilaments. Only one of the antibodies to phosphorylated neurofilament epitopes reacted with Alzheimer tangles in the present study, and none of them reacted in normal perikarya. Also, most antibodies to nonphosphorylated forms of neurofilaments are masked from reaction with phosphorylated axonal neurofilaments (3), and some of them may be masked equally from reaction with phosphorylated Alzheimer tangles.

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1. Sternberger, L. A., Harwell, L. W. & Sternberger, N. H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1326–1330.
2. Ostermann, E., Sternberger, N. H. & Sternberger, L. A. (1983) *Cell. Tissue Res.* **228,** 459–473.
3. Sternberger, L. A. & Sternberger, N. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6126–6130.
4. Selkoe, D. J., Ihara, Y. & Salazar, F. J. (1982) *Science* **215,** 1244–1245.
5. Sternberger, L. A., Hardy, P. H., Jr., Cuculis, J. G. & Meyer, H. G. (1970) *J. Histochem. Cytochem.* **18,** 315–333.
6. Sternberger, L. A. (1985) *Immunocytochemistry* (Wiley, New York), 3rd Ed., in press.
7. Julien, J.-P. & Mushynski, W. E. (1982) *J. Biol. Chem.* **257,** 10467–10470.
8. Goldstein, M. E., Sternberger, N. H. & Sternberger, L. A. (1982) *J. Neuroimmunol.* **3,** 203–217.
9. Anderton, B. H., Breinburg, D., Downes, M. J., Green, P. J., Tomlinson, B. E., Ulrich, J., Wood, J. N. & Kuhn, J. (1982) *Nature (London)* **298,** 84–86.
10. Troncoso, J. C., Sternberger, L. A., Sternberger, N. H., Hoffman, P. N. & Price, D. L. (1985) *J. Neuropathol. Exp. Neurol.* **44,** 332.
11. Cork, L. C., Altschuler, R. J., Struble, R. G., Casanova, M. F., Price, D. L., Sternberger, N. H. & Sternberger, L. A. (1985) *J. Neuropathol. Exp. Neurol.* **44,** 368.
12. Wisniewski, H. M., Merz, P. A. & Igbal, K. (1984) *J. Neuropathol. Exp. Neurol.* **43,** 643–656.
13. Igbal, K., Grundke-Igbal, I. & Wisniewski, H. M. (1985) *Trans. Am. Soc. Neurochem.* **16,** 165.
14. Perry, G., Rizzuto, N., Autilio-Gambetti, L. & Gambetti, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3916–3920.
15. Rasool, C. G., Abraham, C. A., Anderton, B. H., Haugh, M., Kahn, J. & Selkoe, D. J. (1984) *Brain Res.* **310,** 249–269.
16. Rasool, C. G., Anderton, B. H., Kuhn, J., Ihara, Y. & Selkoe, D. J. (1983) *J. Neuropathol. Exp. Neurol.* **42,** 335–345.