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Neuropathological Criteria for the Diagnosis of Alzheimer's Disease: Are We Really Ready Yet?

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BALL, M. J. AND G. H. MURDOCH. *Neuropathological criteria for the diagnosis of Alzheimer's disease: are we really ready yet?* NEUROBIOL AGING **18**(S4) S3–S12, 1997.—The specific diagnosis of AD as a particular dementia from which a patient suffered assumes, debatably, a reasonably pure clinicopathological entity in which the same concatenation of lesions will not be encountered in others dying with a similar clinical disorder. Statistically complex computations such as multivariate analyses of morphometric data from our laboratory and similar attempts in Swedish and British series may not prove pragmatic for pathological confirmation. The Braaks' schema posits six stages in the evolution of AD. Unfortunately, application of this model to 50 British autopsies cannot reliably identify those cases clinically diagnosed as demented. Furthermore, lack of universal definition for each of the probable lesional subtypes augments the difficulty devising a quantitative consensus. Disease stage refers to a progressive increase in anatomical (geographic) extent of involvement, whereas, grade refers to a progressive increase in severity of affliction within any one site. There is only a tendency for stage and grade to progress in parallel. Nor is it obligatory that either always does progress. More energies should be concentrated upon determining which histopathological abnormality is most injurious to neuronal integrity. Dutch workers opine that in both normal aging and AD, claims of massive, neocortical nerve cell loss may have been based on inadequate morphometry and/or a loss of markers. Requiring urgent resolution is whether cellular changes seen in brains of aging normals represent merely the earliest phase of typical AD (and therefore a good model for Alzheimer pathogenesis), or rather reflect a totally different aging syndrome distinct from AD. We have proposed that abnormalities in the hippocampal formation (with or without neocortical neuronal lesions) may underlie a decline of all higher cognitive functions in senile dementia Alzheimer type. West and colleagues optical disector approach likewise shows that neurodegeneration associated within aging individuals' hippocampi is quantitatively and qualitatively distinct from the neuronal loss in AD. Clinical confreres' imprecision whether or when to term subtle cognitive loss "incipient AD" is understandably mirrored by residual neuropathological struggles to dichotomize such brains as "normative aging" distinct from "putative AD." © 1997 Elsevier Science Inc.

Alzheimer's dementia Morphometry Multivariate analysis Normative brain aging Hippocampal neuropathology Staging and grading

HISTORICAL REFLECTION

DESPITE enormous growth in our understanding of possible pathogenetic pathways leading to the cellular and molecular pathology of Alzheimer's disease (23), many investigators seem troubled by the lack of common agreement on a set of diagnostic criteria for the neuropathological definition of Alzheimer's disease. There is precious little published evidence that in any organ other than the brain, the severity of pathological affliction correlates closely with the measurable degree of clinical signs and symptoms. The amount of renal glomerular pathology, or the degree of hepatic cirrhosis shows only very weak predictive value in establishing a quantifiable diagnosis of any particular kidney or liver disorder. In longitudinal studies, despite increasing use of clinical scales attempting to grade the severity of Alzheimer's disease (49), neurologists continue to modify such rating schemata in efforts to resolve many ambiguities. The weightiness attached to one set of autopsy criteria for the neuropathological diagnosis of Alzheimer's disease, following publication of what have now become known as the Khachaturian criteria (38), has in some ways outdistanced the scientific rigour of that collectivized exercise [. . . one definition of a camel is: a horse designed by a committee], more likely reflecting the imprimatur of the National Institutes of Health (National Institute on Aging) that convened the panel of neuropathologists (including this author, M. J. B.) whose deliberations were recorded in that publication. More recently, additional valiant efforts have been made to refine a semiquantitative series of diagnostic pathological criteria, including: (i) a protocol developed from 15 participating Alzheimer research centers (48), and (ii) a primer to guide community pathologists in making the diagnosis of Alzheimer's disease (47). Mirra and colleagues suggest that neither neocortical tangles nor amyloid angiopathy are required for such a diagnosis, and advocate deriving an "age-related senile plaque score". These workers also admit, "Senile plaques have different appearances", and "The relationship of senile plaque type to cognitive impairment remains controversial. Some workers claim that diffuse plaques are more commonly encountered in nondemented elderly, whereas others maintain that diffuse plaques are most common in Alzheimer's disease".

Nearly three decades ago, British workers first reported an association between quantitative measures of dementia and

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changes in the cerebral cortex in elderly individuals (14), and despite some statistical concerns, this distinction soon became entrenched as a pathological basis by which to differentiate normative aging from Alzheimer's disease (60). Admirably, Tomlinson undertook a forthright review of this challenging assumption many years later (59), warning us, "The issue of what is accepted as Alzheimer's disease, whether for routine diagnostic or research purposes, needs to be rapidly settled for clearly the use of different criteria would have many undesirable results. Ideally, we need world-wide agreement on clinical criteria for the diagnosis of Alzheimer's disease at different ages, and pathological material should be exchanged to eliminate technical or interpretational differences as contributing to the present discrepancies." This pioneer was particularly puzzled by the American observation that 30% of Alzheimer cases above 74 years of age (and as many as 50% in older ages) may not show any neocortical neurofibrillary tangles (58). Tomlinson himself regarded such cases as "probably senile dementia Alzheimer type", but reserved judgment on their final position. From Tomlinson's own experience in 73 demented patients dying after a long duration in a psychiatric institution, such cases formed but 12% of the Alzheimer's disease category. In a later categorization of the brains of 100 demented individuals, he also found fully 12% showing insufficient morphological abnormality of any kind to explain the dementia.

There are at least three objectives for which such diagnostic criteria might be employed: (a) as a guide for community hospital pathologists, wishing to make a diagnosis of AD in their routine autopsy service. For this objective, the markedly elevated density of neurofibrillary degeneration in neurons of the dorsal raphe nuclei, when contrasted with tangle severity in controls, hints that a minimum number of histological sections, e.g., from the brain stem, could suffice for such purpose. (b) Forensic pathologists would like some histopathological criteria by which to claim knowledge in a courtroom that a brain by itself showed "unequivocal evidence of AD". It is highly unlikely there is such a precedent for confirmation of a clinical diagnosis in any other organs of the body. (c) A Research Protocol would derive considerable strength in providing a uniformly accepted, reproducible set of pathological criteria, which might then be utilized for epidemiological or cross-cultural studies, as well as for interlaboratory pooling of data emanating from multiple centers harvesting autopsied brains of demented patients.

These worthwhile goals must nevertheless be tempered with the cautionary comments that: (i) the diagnosis of dementia, to begin with, is possible only by a clinician, never by a pathologist or a radiologist, who cannot evaluate the cognitive status which existed during the life of that individual, and (ii) the specific diagnosis of Alzheimer's disease as a particular type of dementia from which a patient suffered assumes the existence of a reasonably pure clinicopathological entity, in which the same constellation of lesions will not be encountered in brains of any other people dying with a similar clinical disorder. It is by no means certain whether this is actually the case in any other organ systems. Additionally, can such a diagnosis commonly be standardized quantitatively? Or more likely, does a clinically informed pathologist evaluate the severity of histopathological lesions in light of the clinical picture already known, reaching a diagnosis only after this synthesis of information?

Methodological Hurdles

In searching for uniformly reproducible methods of quantifying the lesions which may underlie the pathological diagnosis of AD, neuropathologists encounter several methodological problems, which to date can only be partially resolved. These are discussed in some detail in the Appendix, q.v. infra (29,3,1,32,44,22,39,62, 46,21,2,20,55,31,27,40,28,33,65). Ideally our definition of the various lesion types should allow for reproducible recognition by independent observers. However the nature of the "Alzheimer process" makes this goal quite unattainable, in that to a variable extent, each of the known histological abnormalities, especially plaques and tangles, exists as a continuum having several morphological features. There is also an inverse correlation between the number of categories (subtypes) of each such lesion and the degree of interobserver variation, so that extensive (arbitrary?) subdivisions should be avoided; the optimal approach may be to choose a "reasonably reproducible" definition of a "limited" number of lesions. At the same time, however, merely defining a structural "lesion-type" by the ease with which it can be reproducibly recognized may not necessarily reflect a functionally significant biological entity. For example, "diffuse" senile plaques might well represent a heterogeneous grouping of lesions with vastly differing pathophysiological import.

Multivariate Analyses of Neuropathological Features

In serially sectioned hippocampal and ten neocortical regions from 45 Alzheimer's patients and 12 age-matched controls, our laboratory performed a multivariate analysis of morphometric data from 5 histopathological lesions (quantified in 1,941,667 microscopic fields), to learn that a diagnostic prediction regarding the brain of any single individual should be possible with a statistically calculated degree of certainty (10). The data from each of the five pathological indices were transformed to a mean of zero and a standard deviation of one (Z-transformation), so as to put each of the indices on an equal footing with the others. A method of ordination known as Principal Components Analysis was used to summarize the data onto component axes, performing an Eigenanalysis on the covariance matrix. This can be visualized as a rotation of the data in a multidimensional space defined by the variables, and produces new parsimonious axes called component axes. Consequently, two or three component axes should be sufficient to summarize a majority of the variation. To enhance the interpretation of the scattergram resulting from the first two components of this PCA, the results of a classification were superimposed, for which purpose we chose Ward's agglomerated method of cluster analysis, which groups individuals based on pair-wise distances (63). This study also disclosed a topographical pathway by which neurofibrillary tangles disseminate through the brains of patients with Alzheimer's disease, whereas we found no recognizable pattern in the ranking of senile plaque densities, suggesting that they reach a maximum ceiling in neocortical regions much earlier than tangles. Other workers have claimed that people whose brains show many plaques and many tangles are always demented, whereas those with many plaques but few if any tangles are not (11).

A clinicopathological correlation and diagnostic classification using multivariate data analysis were also presented by a Swedish team, in autopsied brains from 55 patients with various kinds of dementia and 19 nondemented aged-matched controls (4). This complex study suggested that nondemented aged cases could be separated histopathologically from the demented patients, removing from the clinical grey area those borderline cases in which neurologists utilized the term "possible Alzheimer's Disease". A British group combined data from 47 different neuropathological variables, including gross features of the brain and density and distribution of plaques and tangles, in a cluster analysis multivariate statistical method on 78 cases of Alzheimer's disease, in order

to identify possible pathological sub-types of this disorder (6). Surprisingly, familial cases of Alzheimer's disease did not cluster as a separate, pathologically identifiable group. Sixty-eight percent of their cases formed a group in which the distribution of plaques and tangles was restricted to a relatively few number of brain areas, whereas 15% of cases formed a smaller group in which the lesions were much more widely disseminated throughout the neocortex. It is uncertain whether such statistically complicated, computational approaches will prove widely helpful in the pragmatic need for pathological confirmation of Alzheimer's disease.

The Problem with Staging

Although the pathogenetic evolution of the lesions of Alzheimer's disease within any one brain cannot be serially reexamined in a longitudinal fashion, reconstruction of the likely sequence of events from cross-sectional investigation of many brains has provided investigators with a few recent insights into the likely temporal sequence that such lesions may well follow. In a morphometric study from our laboratory on the brains of 57 people (45 demented patients dying with Alzheimer's disease and 12 age-matched cognitively normal control subjects), we quantified the density of nucleolated neurons bearing neurofibrillary tangles of classical or mature senile plaques, nucleolated neurons with granulovacuolar degeneration of Simchowicz, of eosinophilic, rod-like bodies of Hirano, and of nerve cell numbers, both in the serially sectioned hippocampus and also in 10 neocortical regions (30). Utilizing multivariate analysis and rank ordering (including a Principal Component Analysis), our data revealed that the mesial temporal cortex is affected by Alzheimer lesions before any parts of the neocortex; and that the histopathological changes then progress from hippocampus through the other temporal gyri, followed later by frontoparietal and cingulate regions, and involving sensorimotor and visual cortices only very late in the disease. This geographical sequence was most recognizable for the ranking of the mean Adjusted Tangle Indices, and because neurofibrillary degeneration is very probably a histological precursor of neuronal death (25), such a progression of neurofibrillary tangle formation within the brain of any one patient suffering from Alzheimer's disease would also concur with the observation of Hubbard and Anderson (34) that in demented patients over 80 years of age the temporal cortex is the most atrophic area. However, our data did not show a recognizable pattern in the ranking of the neuritic Plaque Indices, suggesting that mature senile plaques reach a maximum density (ceiling) in the neocortical regions earlier in the course of the AD than do tangles. Hence, although plaques might be a more meaningful indicator of progression during earlier phases of the disorder, the severity of tangle formation may better describe the degree to which AD has affected the brain in the latter stages of Alzheimer's disease. Such a greater utility of tangle evaluation was in accord with the observation of Barcikowska and colleagues (11) that people whose brains show many plaques and many tangles are always demented, whereas those with many plaques but few if any tangles are not.

A German laboratory has published a tantalizing staging schema based on a study of 83 brains, including 8 cases clinically diagnosed as dementia but whose neuropathological examination failed to meet conventional criteria for diagnosis of fully developed AD, and 21 other cases from demented "old-aged" individuals, including four people with Down syndrome, nine demented people in their 80s and one demented 90-year-old woman (18). In that landmark study, the distribution of neuritic plaques varied widely not only within architectonic units but also from one individual to another. By contrast, neurofibrillary tangles as well as neuropil threads exhibited an apparently characteristic distribution pattern, prompting these investigators to differentiate six stages in the evolution of Alzheimer's disease. Photomicrographs, including several montages together with "shading" on gross anatomical diagrams and on line diagrams of mesial temporal lobe, enhance the appeal of their conclusion that the earliest "transentorhinal" stages [I and II] show either a mild or a severe affliction within the transentorhinal layer pre-alpha; that the "limbic" stages [III and IV] are marked by a conspicuous affliction of layer pre-alpha in both the transentorhinal region and the entorhinal cortex proper, in addition to mild involvement of the first sector in Ammon's horn, and that the last two "isocortical" stages [V and VI] display destruction of virtually all isocortical association areas (large numbers of ghost tangles, major dropout of neocortical neurons, etc.). A later publication by the same laboratory utilized a recently introduced statistical classification system to analyze the prevalence of each of these six stages in patients at different ages (between 21 and 100 years) in a sample of 887 brains from a routine autopsy service (50). This analysis of cross-sectional data to develop a dynamic longitudinal understanding suggested statistically a requirement of 16 years to progress from stage I to stage II, 14 years to evolve from stage II to stage III; 13 more years from stage III to stage IV; and 5 additional years from stage IV to stage V. Therefore, the total evolution of neurofibrillary changes in an Alzheimer brain might require up to 50 years of time.

Unfortunately, a very recent application of this widely quoted staging model of Alzheimer lesions to a British series of 50 autopsy brains has found that this staging scheme does not reliably identify those cases clinically diagnosed as dementia (66). In this prospective clinical study of cognitive function in the elderly, which used the Camdex Protocol for diagnosis of Alzheimer's disease, half of the cases had been diagnosed as demented and half were intellectually normal. Both quantitative morphometric data of neurofibrillary tangle and neuritic plaque densities in hippocampus, entorhinal cortex and cerebral isocortex, as well as CERAD Neuropathological Assessments (48) were obtained. Cases in Braak stages III and IV were as equally likely to have been demented as intellectually normal. Although cases in Braak stages V and VI were very likely to be demented, even there exceptions still occurred. The conclusions of the Cambridge group therefore raise doubt about whether the quantitative and regionally geographic staging of Alzheimer lesions within any one brain can be incorporated into a diagnostic, neuropathological definition of AD.

Even the maturational sequence of any one key lesion may confound the diagnostic challenge. In an intriguing study of brains of 195 nondemented individuals, 104 with autopsy-confirmed critical coronary artery disease and 91 age-matched controls without heart disease, by Sparks and colleagues (54), the longitudinal process of senile plaque formation based on their cross-sectional data rested on three assumptions: (a) the formation of a senile plaque is a dynamic process; (b) no pathological alterations are observable prior to initiation of the formation of plaques; and (c) the end-point of plaque formation is reached when older forms of plaques (e.g., with dense cores or neurites) are the predominant observable feature. With those assumptions, the Lexington group postulated five discernible steps comprising the dynamic process of plaque evolution: (i) the presence of neurons immunoreactive to PHF-tau; (ii) the deposition of neuropil threads also immunoreactive to PHF-tau; (iii) the deposition of pockets of diffuse β-amyloid material (preplaques) in the presence of these immunoreactive neurons and threads; (iv) the deposition of sufficient β-amyloid positive material to form argyrophilic plaques of the

"diffuse" variety only; and (v) the final step (a point near the end of the continuum), the formation of neuritic and dense-core argyrophilic plaques, in addition to the diffuse forms.

Of course the earlier literature had already proposed a series of subtypes of senile plaques in schemata illustrating the possible temporal structural changes in the development of any single senile plaque (36,61). "Pre-plaques" and immature plaques may later become neuritic, then classical or mature, and then "burned-out" or compact varieties. Immunohistochemical analysis with antibodies to different phosphorylated and nonphosphorylated tau epitopes has likewise promoted the temporal concept of three stages in neurofibrillary degeneration in Alzheimer's disease (15): (i) tightly packed intracellular tangles within a pyramidal nerve cell; (ii) compact extracellular tangles shaped like an enlarged nerve cell without a clearly defined plasma membrane; and (iii) dispersed extracellular tangles whose paired helical filament core has by then lost the amino terminal epitopes, and then the phosphorylated epitopes. To date, the lack of universal, histopathological definition for each of these subcategories of plaques or tangles certainly augments the difficulty of devising a quantitative consensus for the pathological diagnosis of AD.

We must also take pains to distinguish carefully between disease "stage" and disease "grade". The stage refers to a progressive increase in the anatomical (geographic) extent of involvement. (The Braak classification scheme really deals primarily with this type of parameter.) The grade, on the other hand, refers to a progressive increase in severity or degree of affliction within any one individual site (e.g., number, size, or density of lesion). As with neoplasia, and most other disorders, there is only a tendency for stage and grade to progress in parallel. We may try to define a particular "subtype" of AD as one exhibiting a "characteristic course of progression" through certain stages and grades. For example, one subtype might proceed "low stage/low grade, to high stage/low grade, to high stage/high grade"; whereas yet another might evolve "low stage/low grade to low stage/high grade, to high stage/high grade". Nor is it obligatory that grade and/or stage always do progress. Is there, for example, a subtype of AD in which the worsening dementia is associated with a high grade while remaining at a low stage, e.g., a "pure" hippocampal dementia? (Vide infra, and (9)). Thus, at early and late times in any one patient's course, it may prove very formidable to categorize the brain as belonging to one specific subtype purely on morphological grounds alone. Clinical and genetic information (e.g., early onset; ApoE genotype) may assist us to achieve this objective.

Limitations in Lesional Subtyping

Recent histopathological investigations have discovered the major difficulties in superimposing a precise classification of subtypes of senile plaques upon the broad spectrum of features visualized microscopically. Using criteria of Delaere et al (27), Armstrong and colleagues studied the spatial pattern of diffuse, primitive, classic (cored) and compact (burnt-out) plaque subtypes of β-amyloid deposits in frontal and hippocampal cortex of nine case of Alzheimer's disease meeting the NINCDS/ADRDA clinical criteria (5). These workers admit particular problems in applying such a classification, firstly, because at least 10% of deposits in some brain regions fell intermediate in morphology between the diffuse and the primitive plaque varieties; and secondly, some classic plaques had undoubtedly been sectioned at their perimeter so that the core appeared to be absent, therefore becoming misclassified as small primitive plaques. A Japanese electronmicroscopic study comparing diffuse with primitive plaques in senile dementia Alzheimer type, utilizing pairs of ultrathin sections for EM and adjacent semithin sections immunolabelled for β-amyloid, found that in the frontal cortex a majority of the diffuse plaques had amyloid fibrils in part of but not in the entire β-amyloid immunoreactive area; whereas in contrast, in the temporal cortex of the same two 81-year-old demented patients, even the smallest diffuse plaques contained amyloid fibrils, with the amount of amyloid correlating significantly with plaque size (67). This group concluded that most of the diffuse plaques within frontal lobe remain as advanced diffuse plaques for a long time, and do not transform into primitive plaques, as do the diffuse plaques found in the temporal cortex.

Automated image analysis techniques applied to immunocytochemical brain samples are also being developed in order to circumvent the sometimes poor reliability and reproducibility of observers' routine microscopic classification of such lesions. Utilizing a discriminant function design, McKenzie and colleagues counted and sorted both diffuse and classic plaque-types in frontal lobe sections from 14 clinically diagnosed cases of Alzheimer's disease (45), and with the β-amyloid staining technique, classified each deposit on the basis of several morphological selection criteria, including size, degree of roundness, texture of the deposit, and presence of an internal area. A companion paper by this British team, detailing this computerized image capture and classification technique, acknowledges a misclassification rate of 9%, which could be considered reasonable because with two categories (classical and diffuse) the expected misclassification rate by chance would be 50% (28). Additionally, six operators obtained a much higher concordance between their results counting and classifying plaques with this automated approach than with the manual visual rating system.

The use of gray-scale images for computer-assisted image analysis of such data normally applies only to quantification of objects labeled with a single marker. Neuroscientists at the University of California Irvine extended this sort of analysis to double-labeled tissue sections in order to quantify dual labels separately based on their color characteristics, so as to analyze the resultant occurrence of overlap between two such labels (26). Their method for semiautomated color image analysis, which allows the identification of separate immunocytochemical labels (β-amyloid as brownish red, and PHF-tau within dystrophic neurites as a dark blue chromogen), was based on histogram mapping of hue saturation and value as well as overlapping feature detection algorithms. This approach apparently yields values for "total amyloid load" and "dystrophic neurite load," generates plaque histograms based on total size, and also subtypes plaques into diffuse/primitive and neuritic/classical categories. By adjusting the various feature criteria, these authors were able to achieve a highly promising agreement (correlation of 0.94) between a human observer and the optimal computer algorithm in classifying plaque subtypes on the three Alzheimer disease brains studied.

Which Lesion Harms the Neurons?

A sense that some biochemical definition of Alzheimer's disease might exist in autopsy material is aided by an analysis of cholinergic markers, neuropeptides, and amines and their metabolites from identical specimens across 10 neocortical regions in a recent sample of 47 cases of Alzheimer's disease and 5 normal elderly (13). The choline acetyltransferase activity across the neocortex of the AD cases was highly correlated with Clinical Dementia Ratings, as assessed by the 7-point CDR scale of Hughes (35), whereas none of the amines or metabolites or the neuropeptides related significantly to dementia severity. The strong association of functional impairment with the cholinergic deficits was

independent of age. None of the neocortical regions examined exhibited a consistent pattern of differential (enhanced) susceptibility to this diminished cholinergic activity. Although the relationship of the mean temporal neocortex choline actyltransferase activity to the CDR global clinical score of these Alzheimer's disease cases was highly significant ($p = 0.003$), with a correlation coefficient of just -0.46, only 21% of the variance in the cholinergic marker can be attributed to the clinical dementia severity. When age at death as a covariant was incorporated into an analysis of covariance for all the neurochemicals assayed, the most significant age-corrected decrements in mean neocortical values (between AD cases and controls) were found for the cholinergic markers (ChAT, $p < 0.001$; AChE, $p < 0.001$).

Most pathological criteria for the postmortem diagnosis of Alzheimer's disease will, naturally, rely upon histopathological rather than neurochemical parameters. Further energies should therefore be focused upon determining which histological abnormality is most harmful to neuronal integrity, because that particular lesion would then most likely find usefulness in a reproducible diagnostic protocol. Ultrastructural evaluation of frontal cortex from the brain of an 81-year-old man with senile dementia Alzheimer type autopsied 2 hours postmortem showed diffuse senile plaques (devoid of both amyloid core and swollen neurites) with only occasional scattered bundles of amyloid fibrils, and the neuropil between these many diffuse plaques appeared virtually normal, morphologically (68). Using double-label immunohistochemistry to reexamine amyloid deposits with antibodies to β-amyloid and PHF-Tau by conventional and confocal microscopy, a postmortem survey of four elderly controls (ages 71–91) and 12 AD patients (56–89 years) has found that in control brains diffuse plaques rarely contain PHF-tau positive profiles, and only a small number of PHF-tau positive dystrophic neurites, whereas, a very dense network of PHF-tau positive dystrophic neurites extends throughout the neocortex in the Alzheimer diseased brains, permeating nearly all neuritic as well as diffuse plaques (53). If soluble β-amyloid is a normal metabolic product of nerve cells, and abnormally phosphorylated PHF-tau is a pathological neuronal marker, much caution must be exercised in choosing whether to incorporate β-amyloid, immunoreactivity, amyloid fibrils, or PHF-tau co-deposits in any histological search for a set of pathological criteria for diagnosis.

One of our earliest studies (in serially sectioned hippocampus from brains of 18 mentally normal people and eight Alzheimer demented patients) found a high negative correlation between density of nerve cells and numbers of neurons both with tangles and with granulovacuolar degeneration (7). Because the greatly augmented numbers of neurons afflicted by neurofibrillary and/or granulovacuolar degeneration were found within a severely shrinking population of surviving pyramidal hippocampal cells, (in which for example, the pathophysiological significance of 200 neurons with tangles and 600 neurons with granulovacuolar change may be far greater in a cubic millimeter containing only 3,000 nerve cells than in a cubic millimeter of more than 7,000 neurons) these data raise yet another potentially confounding covariable, i.e., the neuronal population in which such lesions are occurring. Clearly, with an up to 40% smaller nucleolus in the nucleus of tangle-bearing neurons than in their immediate non-tangle neighbours (25), any neuron containing a typical neurofibrillary tangle in its perikaryon has almost certainly been operating at a considerably reduced metabolic rate. Reinforcing the notion of tangling as a neuronally harmful phenomenon, Stojanovic et al, using computer-enhanced image analysis on 500 hippocampal neurons in ten Alzheimer brains and six of our age-matched controls, showed the average lipofuscin content in the tangle-bearing neurons in the Alzheimer brains is only 10% of total perikaryal area, whereas in tangle-free neurons in the Alzheimer brains and in aged controls, lipofuscin occupies 31% and 33% of cellular area (56). The safe storage of neurotoxic metabolic byproducts, as measured by intrinsic lipofuscin autofluorescence, appeared three times more abundant in neurons without tangles.

Even the intuitively appealing concept that intracytoplasmic tangle formation, especially if spread also to neocortex, might be an ideal diagnostic marker for cognitive deterioration is not yet fully established. Hyperphosphorylation, diminishing the microtubule binding capacity of tau, destablizes microtubles and may enhance the formation of paired helical filaments constituting the neurofibrillary tangles. However, a recent study of intracellular tangles containing full-length tau, which failed to immunolabel using phosphorylation-dependent anti-tau antibodies suggests that hyperphosphorylation of tau may not in fact be obligatory in the formation of neurofibrillary tangles (16). Equally curious is the result of an image analysis examination of the size of the immunocytochemically detected nerve cell Golgi apparatus in hippocampal neurons of the CA1 area of Alzheimer patients by Salehi and coworkers (52). Although the size of the Golgi apparatus of eight Alzheimer patients (ages 54–88 years) was significantly reduced compared to that in six nondemented age-matched controls, there was no significant correlation between size of Golgi apparatus and the presence or absence of intracytoplasmic neurofibrillary tangles, or the density of extraneuronal tangles surrounding each measured nerve cell. In addition, there was no significant decrease in the nerve cell profile area of tangled neurons either. Thus, although the protein synthetic or secretory function of the neurons was decreased in both tangled and tangle-free nerve cells in the demented patients' hippocampi, the presence of intracellular or extracellular tangle formation did not affect the extent to which the protein synthetic ability of the nerve cells in this area was reduced. If the formation of neurofibrillary degeneration and the reduced metabolism in Alzheimer's disease are two independent phenomena (which in some areas sometimes affect the same neurons), the pathologists' choice of a "tangle severity index" as a diagnostic criterion for Alzheimer's disease may not prove sufficiently hardy.

In temporal lobe biopsy samples of 13 demented patients presenting before age 65 with histologically confirmed Alzheimer's disease and a mean duration of illness of 3.4 years, quantitative morphometry showed that a much more severe loss of synapses (quantified from electronmicrographs) than of nucleolated nerve cells (counted on semithin sections) was found at this early stage of the Alzheimer disease process (24). Dutch workers feel that both in normal aging and also in Alzheimer's dementia, various claims in the literature of massive, neocortical nerve cell loss may have been based on inadequate morphometry and/or a loss of markers rather than a genuine loss of nerve cells (57). If global neocortical cell loss does not take place in the brains of Alzheimer patients (51), it could transpire that nerve cell atrophy or shrinkage (with a proportional increase in the percentage of smaller neurons) may be a clinically extremely relevant phenomenon. How to reproducibly measure this shift, however, is yet another major research challenge.

Even the particular regional locale in which potentially important histological lesions make their appearance might determine whether they are linked to functionally significant alterations. Using β-amyloid staining and a novel methamine silver technique, Mann and colleagues found that in elderly Down syndrome patients, while the process of amyloidosis affects many areas of gray matter, it seems only in cerebral neocortex (but not in cerebellum) that such deposits are widely affiliated with a neuritic (paired helical filament) change, which in turn may be marking the process of clinically significant neurofibrillary degeneration (42).

Despite the recent burgeoning of evidence for the role of Apolipoprotein E genotype as a risk factor for Alzheimer's disease, the same Manchester laboratory, examining brains of 20 elderly patients with Down syndrome by monoclonal antibodies BC05 and BA27, has surprisingly shown no significant difference in the amount of β-amyloid deposition in the brain, either as β-amyloid 42 or β-amyloid 40, respectively, in those Down patients possessing the ApoE-E4 allele when compared to those without it (43).

The struggle to optimize pathological markers for a diagnostic definition of Alzheimer's disease is yet further complicated by those significant minority of demented patients' brains in which still other pathological lesions abound. Early data from our own laboratory failed to support the then popular notion that Alzheimer lesions were the morphological basis for the dementia frequently seen in Parkinson's disease (8). The reverse situation, in which variable numbers of Lewy inclusion bodies are spotted throughout subcortical and even neocortical regions of the brain of patients with typical Alzheimer's disease, is equally frustrating. A more than semantic controversy persists about the frequency of the so-called "Lewy body variant of Alzheimer's disease", versus "diffuse neocortical Lewy body disease". In some eastern American centers, this latter entity may constitute up to 25% of all organic dementias, an impression seemingly not shared by academic institutions in other parts of the continent. While fully 71% of a series of 48 cases of Alzheimer's disease had ubiquitin-positive neocortical Lewy bodies (37), there was no association between these cortical Lewy inclusions and either senile plaques or neurofibrillary tangles. How to integrate an assessment of these Lewy inclusions, as well as other confounding but probably "neuro-injurious" lesions such as Pick bodies and multiple microinfarctions into a multivariate definition of the substrata for Alzheimer diagnosis, is obviously a growing conundrum.

Brain Aging in the Very Old

If a universally accepted definition for the pathological criteria by which to diagnose Alzheimer's disease is ever developed, perhaps the single most important issue to be resolved will be whether the cellular changes seen in the brains of aging normal people represent merely the earliest phase of "typical" Alzheimer's disease, and therefore comprise a good model for Alzheimer pathogenesis, or rather whether these reflect a totally different constellation of events comprising an aging syndrome "distinct" from Alzheimer's disease. Analysis of 32 brains of demented Swiss patients ages 62–102 years has shown that both the total amount of β-amyloid immunostained tissue per square millimeter of temporal neocortex and the total number of senile plaques (of all histological varieties) per square millimeter correlated significantly with the duration of dementia [as documented both by family observation of symptom onset and by first Mini-Mental Status scores less than 23 out of 30; $p < 0.05$, < 0.01, respectively (12)]. These authors conclude that demented patients with Alzheimer's disease acquire more and more senile plaques in their cortex, the longer their disease lasts. When considering the process of formation, evolution, and possible disappearance of senile plaques, their data suggest that plaques either persist once they have formed, or that they keep forming more quickly than they disappear. Data from a London, Canada study indicate, by contrast, that in normal aging senile plaques do not accumulate progressively (41). Modified Bielschowsky silver-stained sections of inferomesial temporal cortex were surveyed from the brain of 402 people ages 30–92 years, who had no history of dementia or neurological disorders, and no neuropathological evidence for neurological disease (as control, ten of the cases showing senile plaques randomly selected were immunostained for β-amyloid as well). As expected, the presence of any senile plaques correlated strongly with the age of the patient at time of death ($p < 0.0001$), with the prevalence rising from 1% below age 50, in successive decades, to greater than 70% of those brains over 80 years of age. Surprisingly, however, neither the mean nor the maximum density of senile plaques per square millimeter showed any correlative increase with age. A separate linear regression analysis performed on those individuals 65 years or younger ($n = 39$), an age group expected to contain very few demented individuals, still showed no increase in senile plaque density with aging, ruling out the possibility that the overall result could have been biased by the exclusion of demented patients. In most of these brains, all the senile plaques were of the diffuse variety. In only 9% of the total (37 cases), small numbers of neuritic plaques were also observed. Mackenzie's conclusion challenges the common belief that senile plaques progressively accumulate in some people as part of the normal aging process. Rather, it appears they may develop over a limited time period, after which their number stabilizes at some constant level. When the density of the neuritic variety of plaques was expressed as a proportion of the total number of senile plaques counted, however, a significant positive correlation with age was in fact observed ($r = 0.339$; $p < 0.05$). Perhaps in normative aging changes in senile plaque morphology may be more important than total senile plaque numbers. Nevertheless, the initial formation of senile plaques could be related to some relatively sudden change in cerebral environment, which is followed by the establishment of a new steady state accompanied by a stable number of plaques. If progressive plaque formation does occur during the duration of Alzheimer's disease, but not with the normative aging process, how can we safely assume an identical pathophysiological cascade is at work in both scenarios?

Neurofibrillary degeneration, on the other hand, may occur across a more continuous spectrum encompassing the normal aging process and early Alzheimer's disease. Bouras and colleagues investigated brains of 61 nondemented geriatric patients, ages 49–101 years, with no history of neurological or psychiatric disorders (17). Evaluation during life with the Mini-Mental State Examination had shown no signs of memory impairment in any of these people. Sections of superior frontal, inferior temporal and hippocampal cortex were surveyed for senile plaque and neurofibrillary tangle counts with anti-β-amyloid antibody, anti-tau antibody, and thioflavine S staining techniques. The amount of amyloid deposition did not correlate with increasing age. However, in all three regions analyzed, 8 of these 61 brains (13%) were characterized by a severity of neurofibrillary degeneration (expressed as percentage of total neurons showing a tangle) greater than one standard deviation above the mean for the total population. Within layer II of the entorhinal cortex, as many as 22.6% of all neurons showed neurofibrillary tangles, whereas the other 53 cases with "low" tangle counts never showed a density of tangle formation more than 9.6% of all neurons in entorhinal layer II. There was no correlation between tangle and plaque densities in any of the three areas quantified, and the eight cases with high tangle densities did not show comparably higher plaque counts. Although these eight patients never demonstrated any temporospatial disorientation or cognitive memory impairment, the authors speculate whether they might represent a group "with increased risk for the development of Alzheimer's disease". This observation could represent "the neuropathologic correlate of incipient dementia", whereas the bulk of the cases were merely a reflection of changes seen in "normal brain aging".

Along similar lines, Brady and Mufson investigated the hippocampal formation and anterior parahippocampal gyrus with a monoclonal antibody against the PHF-tau protein in 6 normal and 19 Alzheimer diseased brains, to survey the topographic distribu-

tion of PHF-tau containing profiles (19). There was a paucity of immunoreactive neuropil in the normal hippocampal complex, but in most Alzheimer disease cases a prominent PHF-tau immunoreactive neuropil in the outer two-thirds of the dentate molecular layer, and dense staining of neuropil in subfield CA1. Neuropil and nerve cell body staining displayed distinct laminar patterns within the entorhinal cortex. In general, the density of neurite staining in the neuropil appeared inversely proportional to the immunoreactivity within dendritic and somal compartments. The patterns of PHF-tau staining observed in the hippocampal complex of the Alzheimer disease cases coincides with patterns of well characterized afferent fiber pathways to these regions, further supporting our own group's earlier conjecture that histopathological abnormalities in the hippocampal formation (with or without neocortical neuronal lesions) may underlie the decline of all higher cognitive functions in senile dementia of the Alzheimer type (9).

In a stereological estimate of total nerve cell numbers in the major subdivisions of the hippocampal cortex of 45 nondemented control subjects between ages 13–101 years, West and coworkers utilized the optical disector technique to show neuronal dropout related to normal aging, and then compared this nerve cell loss in 14 of these controls with similar data obtained from 7 patients dying with Alzheimer's dementia (64). Within the CA1 region of hippocampus, there was almost no nerve cell loss in the normal control group (mean = 14,080,000 neurons), whereas a significant loss of nerve cells occurred in three sectors of the hippocampal formation in the Alzheimer brains, most pronounced in CA1 where an average of 68% of nerve cells were lost (mean = 4,400,000). These workers conclude that the neurodegenerative process associated with normative aging is qualitatively different from that occurring in Alzheimer's disease, which may not be an inevitable consequence of aging.

From our own laboratory, previously published quantitative criteria for the diagnosis of Alzheimer's disease had been propounded by contrasting a cohort of 36 demented patients, ages between 47–89 years, with 8 control subjects between 56–91 (10). However, only four of those demented people (ages 81, 82, 83, and 89 years) and only two controls (ages 80 and 91) died in their ninth (or 10th) decade of life (7). Obviously those guidelines e.g., that the serially sectioned hippocampus must contain (a) 20 tangle-bearing nucleolated neurons per cubic millimeter; and/or (b) at least 55 nucleolated neurons per cubic millimeter with granulovacuolar degeneration; and/or (c) a population of less than 5,600 nucleolated nerve cells per cubic millimeter (67) now merit thorough reevaluation in a prospectively assessed cohort of "old-old" subjects past age 85. At the Alzheimer Disease Core Center in Portland, we are fortunately tracking a cohort of more than 150 "super-normal" control subjects in whom risk factors for dementia such as hypertension or diabetes have been eliminated, through the Oregon Brain Aging Study (Dr. Jeffrey Kaye, P.I.), funded by the Veterans Administration. Repeat neuropsychological testing is confirming excellent preservation of cognitive functioning for most of these individuals often until very near the time of death. Although morphometric evaluations have been completed from the serially sectioned hippocampi of only seven cases accessioned to date, we are already struck by how frequently the hippocampal pyramidal layer shows focally severe but microscopically extremely restricted neurofibrillary and granulovacuolar degeneration, with afflicted neurons usually confined to Rose's H1 field and to the glomerular substance of Arnold within the entorhinal cortex. If senile plaques are observed in various neocortical samples, these are nearly always only of the "diffuse" variety. Because our clinical colleagues are in frequent disagreement about the possible presence of very subtle cognitive decline, and whether this should be termed "normal aging" or "incipient Alzheimer-type dementia", it is hardly surprising that our neuropathological thrust to categorize such brains as "normative aging" in distinction from "putative Alzheimer's disease" is similarly uphill.

Optimistic Outlook

It may eventually transpire that cognitive deterioration in the human brain, like its histopathological counterpart(s), follows a slowly progressive slope of worsening over time, very much like cumulative atherosclerosis in the human aorta, and justifying distinctive clinico-pathological names only when significant symptomatology results from such lesion(s). Once the precise causative agent or agents for Alzheimer's disease have been discovered, it will retrospectively be easy to define crisply the pathological criteria associated with that "trigger".

In actuality, the present search for robust "clinico-pathological" correlations to validate neuropathological definitions may be inherently problematic. If a much worse lesional burden is needed to meet the Khachaturian criteria of an older patient (38), how can we simultaneously assume such lesions are occurring on a background of age-related decrements in physiological reserve? Yet it truly is every bit as plausible that the "simplified" (e.g., less "plastic") circuitry of an aged person's brain could be more resistant to the functional consequences being inflicted by such pathogenic events. How can both notions be right?

While such vital queries are being addressed, federal and other biomedical funding agencies must not diminish support for multidisciplinary research attacks on Alzheimer's disease merely because some international panel has yet to hammer out a universally lauded set of pathological criteria for diagnosis. The sheer energy which can be mobilized toward such an objective will surely, in itself, enrich the milieu in which we shall unravel the pertinent pathogenetic cascade by which structural and neurochemical aberrations accumulate in the human brain, which when responsible for cognitive decline, can through judicious pharmacological intervention be rendered reversible and even preventable.

APPENDIX

Density of histopathological lesions should ideally be expressed per unit area or preferably per unit volume of tissue analyzed. However, like many other organs, the brain swells when immersed in an aqueous fixative such as formaldehyde due to the hypertonicity of the organ (29), and subsequently, shrinkage occurs due to dehydration and paraffin embedding. Whereas brain volume itself may on average increase 8.5% during fixation in 10% formalin, individually measured changes range from a reduction of 13% to an increase of 25% (3). The need to derive appropriate correction factor(s) is overshadowed by a bewildering variety of histochemical and immunological staining techniques by which such key lesions as senile plaques and neurofibrillary tangles can be demonstrated. A steady stream of publications continues to debate the various merits of modified silver impregnation techniques for demonstrating tangles and amyloid plaques (1), whereas other comparisons of different methods of tissue processing (in which three silver stains and four immunohistochemical dilutions of a β-amyloid antiserum were used) indicate that senile plaque differentiation can be compromised by certain tissue processing and staining protocols (32). At least some comfort is to be had from one interlaboratory histopathologic comparison, in which we showed that very different staining methodologies may yield quite comparable quantitative diagnostic results (44).

The vagaries of sampling also interfere with reproducibility. Because in any one brain it remains unclear where in the regional

evolution the Alzheimerization process has reached, it is most difficult to decide precisely how to sample for the severity of lesions to establish a diagnosis. Sampling issues affecting quantification of plaques and tangles also influence attempts at analysis of nerve cell numbers and dendritic extent both in normal aging and in brains of Alzheimer victims (22). Personal preference for a particular staining methodology to visualize pertinent lesions accurately also plays some role. A French study comparing several different stains for plaques and tangles concluded the highest counts were obtained using a modified Bielschowsky method (39), whereas another European team concluded the thioflavine S approach detected up to 60% more plaques and 50% more tangles than the Bielschowsky method (62). An American consortium found that interlaboratory variations between commonly chosen staining techniques may be the major confounder which limited agreement amongst 18 medical centers (46).

In theory, computer-assisted quantification should offer notable improvements in interobserver reliability and test–retest reproducibility. However, before any automated techniques can be applied to such lesions as senile plaques, careful attention must be paid to stereological principles so as to avoid biased counts. Techniques devised to transform profile counts into particle numbers rely on certain approximate assumptions, for example, that nuclei are round, or that the largest diameter profiles represent the largest particles. To the degree that such approximations diverge from reality, conclusions drawn may be biased (21). In our own laboratory, the well established Abercrombie correction factor has been successfully utilized (2). A more general Correction Factor may also be employed (20).

Some North American neuropathologists have recently become enchanted with the stereological "disector" technique, promulgated in Scandinavia, which permits an unbiased estimation of true particle number by comparing counts in two parallel microscopic 'sections' (55,31). The disector (=two sections) approach has as yet not been widely applied to senile plaque counting, in part because of the enormous variability in observed size of these lesions, possibly between 2 and 200 microns in diameter (27). A recent Finnish study has shown very close agreement between counts from a single microscopic section and disector stereological counts (40).

Despite such limitations, micro computer-based image analysis has been employed with some success, at least in enumerating senile plaques (28). Hibbard and McKeel have utilized noninteractive computer imaging to count senile plaques in silver-stained sections by the arrangement and intensities of pixels within the feature boundary (33). Any increased speed of survey by which a much more representative portion of gray matter could be analyzed with such computer-assisted techniques must not overshadow the probable sacrifice in precision of quantitation, where the critical need is to establish comparability between reproducible manual counts and newly devised computer methods (65).

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