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Immunocytochemical profile of neurofibrillary tangles in Down's syndrome patients of different ages

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SUMMARY

Brains were obtained at autopsy from 24 patients with Down's syndrome, ranging in age from 13 to 71 years. Neurofibrillary tangle containing neurones of the hippocampus were stained using a Palmgren silver method and immunocytochemically (PAP) using antisera to paired helical filament protein, human tau protein and ubiquitin, as primary antibody. Counts of cells stained by each method were compared. In patients under 50 years of age, in whom only a limited number of tangle bearing cells were present, the number of profiles visualized with silver, anti-paired helical filament and anti-tau methods were similar. However, in patients over 50 years of age (and in certain of those under 50), in whom numerous tangles were present, the number of cell profiles visualized with silver and anti-paired helical filament methods were still similar though anti-tau detected fewer positive cells. This was because of the increased presence, in such patients, of extracellular tangles which had "lost" anti-tau immunoreactivity. Such data suggest that although tau protein forms a major antigenic determinant of neurofibrillary tangles in Down's syndrome (as it does in Alzheimer's disease) this protein may only decorate the basic paired helical filament protein skeleton, and is removed by macrophagic activity upon neuronal death. In all patients, anti-ubiquitin revealed fewer tangles than any other method. It is possible that ubiquitin may be

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present only transiently, within tangles perhaps following initial formation and lasting only as long as the normal protein degradation processes remain viable within the diseased neurone.

Key words: Down's syndrome; Neurofibrillary tangles; Immunocytochemistry

INTRODUCTION

Alzheimer's disease (AD) is characterized histopathologically by the presence of numerous senile plaques in the association areas of the neocortex and in the hippocampus and amygdala, and by the presence of many neurofibrillary tangles (NFT) within nerve cells, both in these cortical regions and also in subcortical regions such as the nucleus basalis, locus caeruleus and dorsal raphe (Mann 1988a).

However, the way in which both of these destructive lesions are formed still remains enigmatic. Immunocytochemical studies have shown that NFT share common epitopes with a wide variety of (normal) cytoskeletal and nonskeletal neuronal proteins. For example, NFT have been stained by antibodies directed against neurofilament proteins (Anderton et al. 1982; Gambetti et al. 1983; Rasool et al. 1984; Perry et al. 1985; Sternberger et al. 1985; Cork et al. 1986; Haugh et al. 1986; Miller et al. 1986), microtubules (Grundke-Iqbal et al. 1979), microtubule associated protein MAP2 (Kosik et al. 1984), and latterly tau proteins (Grundke-Iqbal et al. 1986; Iqbal et al. 1986; Ihara et al. 1986; Kosik et al. 1986; Delacourte and Défossez 1986; Wood et al. 1986; Bancher et al. 1987; Kowall and Kosik 1987; Défossez et al. 1988; Love et al. 1988). However, it now seems likely that monoclonals to neurofilament protein that bind to NFT do so because of a cross-reactivity with phosphorylated tau epitopes (Nukina et al. 1987) and it is possible that such cross-reactivity also accounts for the binding of MAP antibodies. NFT have also recently been shown to be recognized (Hyman et al. 1988; Love et al. 1988) by a monoclonal antibody, known as Alz50, which is directed against an antigen present in Alzheimer brain (Wolozin et al. 1986). This antigen is likely to be represented by a modified or aggregated form of tau (Nukina et al. 1988). Immunocytochemical evidence thus implies NFT to be derived (largely?) from tau proteins; direct protein chemical analyses (Kondo et al. 1988) confirm this impression and further indicate that it is the carboxyl third of tau that is embedded within the NFT (this portion also contains the site of phosphorylation, against which most anti-tau antisera are directed). Other recent work indicates NFT also to be ubiquitinated (Cole and Timiras 1987; Mori et al. 1987; Perry et al. 1987; Lennox et al. 1988; Love et al. 1988; Lowe et al. 1988).

Although it is well recognized (see Mann (1988b) for review) that virtually all patients with Down's syndrome (DS), who survive past the age of 40 years, show in their brains the neuropathological characteristics of AD, it is not definitely known whether the NFT in DS, as well as displaying similar morphological features, also shares the

antigenic characteristics of those of AD. To our knowledge, only a few studies have investigated the immunochemical profile of the NFT in DS. In one, Anderton et al. (1982) noted NFT in a single elderly patient with DS to be reactive with 2 monoclonal antibodies (BF10 and RT97) directed against neurofilament proteins. In another study, Joachim et al. (1987) showed NFT in 2 patients (of unspecified age) to be immunoreactive to antibodies directed against both bovine tau and human fetal tau. Lennox et al. (1988) demonstrated NFT in 2 patients (also of unspecified age) to be immunoreactive to an antibody directed against ubiquitin protein.

In this study, we have investigated the immunocytochemical profile of NFT containing nerve cells of the hippocampus in patients dying, at different ages, with DS and compared the data with reported changes in AD. Furthermore, by observing how the stainability of NFT might vary, in DS, according to patient age, we are able to investigate the part played by each antigen in the formation of NFT. Hence we have stained NFT, in DS, using antibodies with determinants either unique to NFT (i.e., anti-PHF antisera) (Ihara et al. 1986; Persuy et al. 1985) or to human tau proteins (Défossez et al. 1988; Ihara 1988) or ubiquitin protein (Lowe et al. 1988).

MATERIALS AND METHODS

Brains were obtained at autopsy from 24 patients of age range 13–71 years dying with DS (see Table I and Mann (1988b); Mann et al. (1984, 1985, 1986, 1987) for clinical and pathological descriptions). From each of the formalin fixed brains, a standard block of mid-hippocampus was taken (at the level of the geniculate bodies) and from this a series of consecutive paraffin sections, cut at 5 μm thickness, was prepared. NFT were demonstrated by several techniques.

(i) A Palmgren (silver impregnation) method (Cross 1982).

(ii) A standard PAP method employing two anti-PHF antisera (Ihara et al. 1986; Persuy et al. 1985) as primary antibodies. Dilutions of 1/500 and 1/1 000 respectively were used. Sections stained with one of the anti-PHF antisera (Persuy et al. 1985) were counterstained with thioflavin S (fluorescent stain for amyloid).

(iii) A standard PAP method employing two anti-human tau antisera (Ihara 1988; Défossez et al. 1988) as primary antibodies. Dilutions of 1/200 were used in each instance.

(iv) A standard PAP method employing an anti-ubiquitin (Lowe et al. 1988) antiserum as primary antibody at a dilution of 1/100.

Full details concerning the characterization of these antisera have been presented previously by their originators (Ihara et al. 1986; Persuy et al. 1985; Défossez et al. 1988; Ihara 1988; Lowe et al. 1988).

TABLE 1

SELECTED CLINICAL AND PATHOLOGICAL DETAILS OF 24 PATIENTS WITH DOWN'S SYNDROME

Patient No.	Age/sex	Mental age/IQ (if known)	Karyotype (if known)	Neurological illness	Cause of death	Brain weight (g) (where recorded)
1	13 F	–		Nil	Lobar pneumonia A/V septal defect	1154
2	31 M	2/30	47XY21	Nil	Emphysema	1050
3	37 F	2/30	–	Nil	Obstruction of GI tract Diabetes mellitus	1045
4	38 M	–	–	Nil	Acute gastroenteritis	1185
5	40 F	–	–	Nil	–	–
6	41 M	–	–	Nil	Acute gastroenteritis	1245
7	42 F	–	–	Nil	–	–
8	42 M	–	–	Nil	–	–
9	43 M	–	–	Nil	Cor triloculare congestive cardiac failure	1295
10	47 M	–	–	Nil	Acute gastroenteritis	1200
11	48 F	–	–	Nil	Bronchopneumonia	1204
12	49 F	–	–	Nil	Ischaemic heart disease A/V septal defect	878
13	50 M	4/30	–	Nil	Ischaemic heart disease	–
14	52 M	–	–	Nil	Bronchopneumonia	1160
15	53 M	3/30	47XY21	Epilepsy	Bronchopneumonia Chronic bronchitis	870
16	58 M	3.8/< 30	47XY21	Nil	Bronchopneumonia	1020
17	58 M	3/30	47XY21	Epilepsy	Bronchopneumonia	970
18	59 M	4/30	47XY21	Nil	Bronchopneumonia	950
19	59 M	4/20	47XY21	Epilepsy	Bronchopneumonia	1018
20	60 F	< 2/< 30	47XX21	Nil	Bronchopneumonia	965
21	60 M	–	–	Epilepsy	Bronchopneumonia	920
22	64 M	2/< 30	47XY21	Nil	Bronchopneumonia	940
23	65 M	3.5/< 30	47XY21	Epilepsy	Bronchopneumonia	–
24	71 M	4.2/< 20	–	Epilepsy	Bronchopneumonia	1018

RESULTS

(i) Prevalence and distribution of NFT in hippocampus

Group 1. In patients 1, 2 and 7, no NFT whatsoever were demonstrable, using any staining method, within any of the CA areas or subiculum.

Group 2. In patients 3–6, 9, 11–13, a variable number (but usually only a few) NFT were present. These were generally present in CA4/5 region (soleplate) of Ammon's horn, though in most patients, an occasional NFT was also seen in CA1 and subiculum. In patients 6, 11 and 13, however, a moderate number of NFT was present in these latter two regions and these patients displayed the highest count of tangles in this group. In all patients, NFT were clearly localized within a neuronal cell membrane; extracellular NFT were not seen.

Group 3. In patients 8, 10, 14–24, numerous NFT were seen throughout the hippocampus but chiefly within CA1 and subiculum, with many fewer NFT in CA4/5 and only occasional ones in CA2/3. Although most NFT were again bounded by a cell membrane, in many instances (and this varied much from patient to patient), other NFT were apparently freely present within the neuropil with no associated nerve cell membrane. These have been variously referred to as extracellular or ghost tangles (Rasool et al. 1984).

(ii) Staining patterns

(a) Neurofibrillary tangles

In each patient intracellular NFT were strongly and similarly stained using either silver, anti-PHF or anti-tau (Fig. 1a) methods. Occasional cells however showed patchy areas of immunoreactivity, with tau antisera, with areas of NFT material strongly stained admixed with areas of unstained NFT (Fig. 1b). Many intracellular NFT stained strongly with anti-ubiquitin (Fig. 1c) though some cells showed only a weak immunoreactivity and others were completely non-reactive (Fig. 1d).

Extracellular tangle material on the other hand, stained less strongly with both silver (sometimes not at all with silver) (Fig. 1e) and both of the anti-PHF antisera (Fig. 1f), but not at all with either of the anti-tau (Fig. 1c) or the anti-ubiquitin (Fig. 1g) antisera. Generally, the NFT material was less compact than that within intracellular tangles and bundles of filaments were apparently separated by neuropil. Clumped areas of anti-ubiquitin immunoreactivity were often seen around extracellular NFT material (Fig. 1g).

In the anti-PHF/thioflavin S double stained sections intracellular NFT showing strong anti-PHF activity were only weakly fluorescent whereas extracellular NFT with weak anti-PHF immunoreactivity were strongly fluorescent. Again, "intermediate" cells were seen in which separate areas of strong anti-PHF activity and weak fluorescence were observed along with areas of weak anti-PHF activity and strong fluorescence.

In addition, both of the anti-tau antisera stained (weakly) a variable number of other neurones, chiefly within areas CA3 and CA4/5 of hippocampus. Such staining was

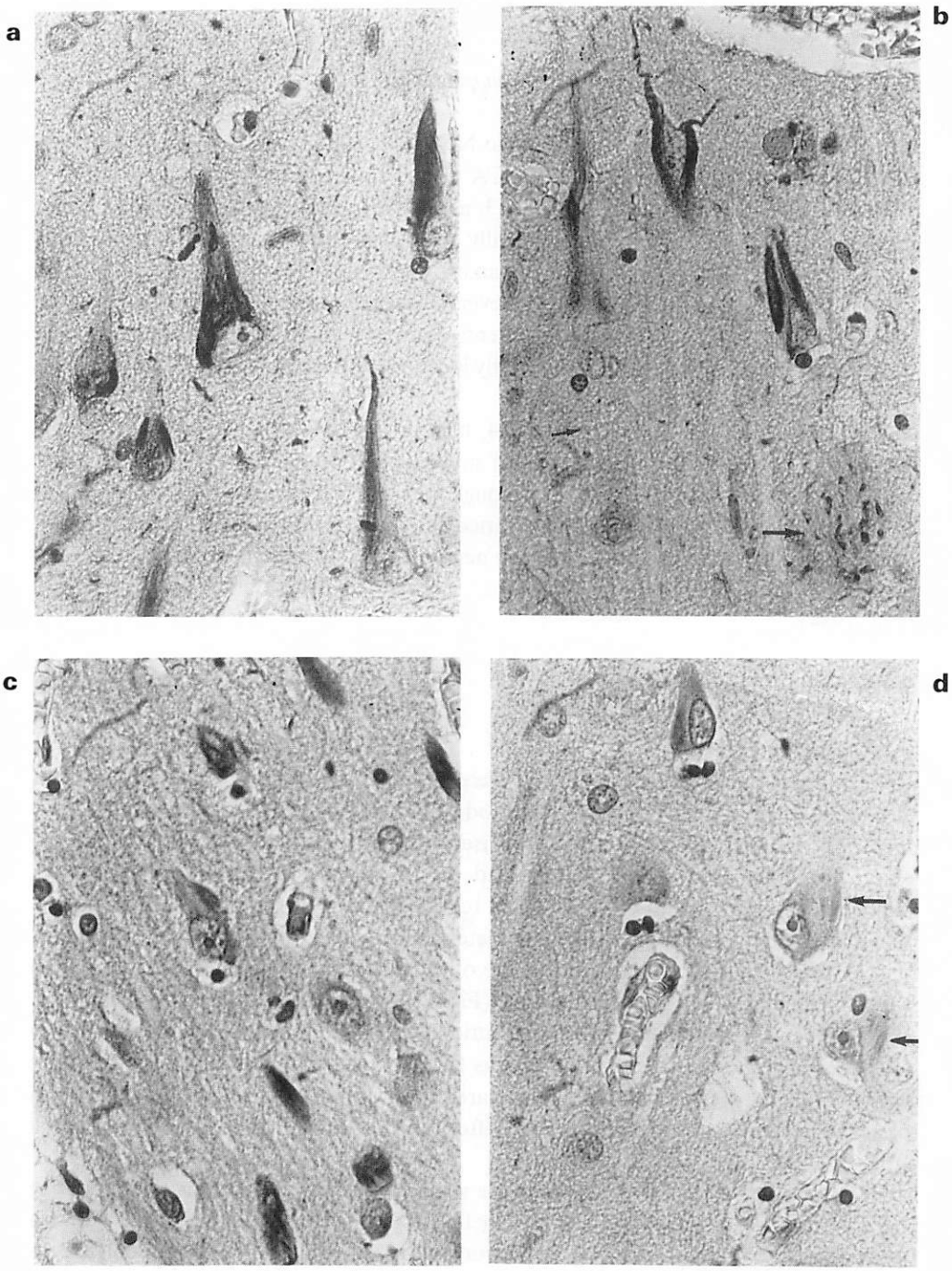
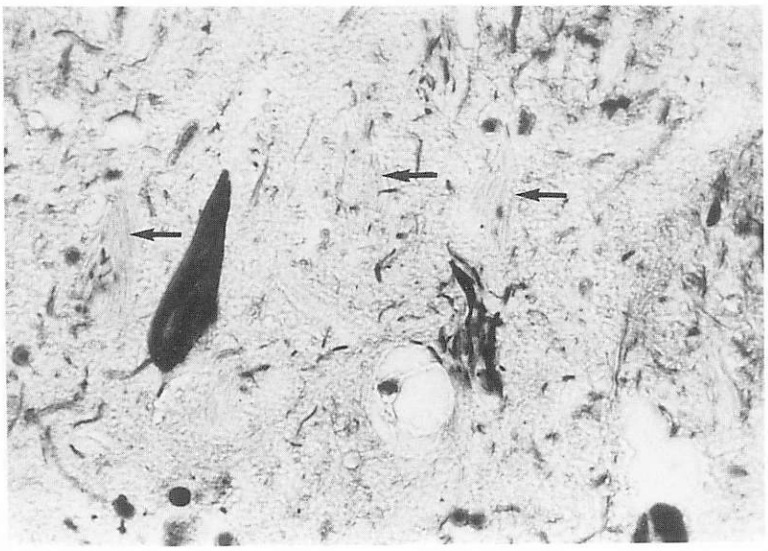
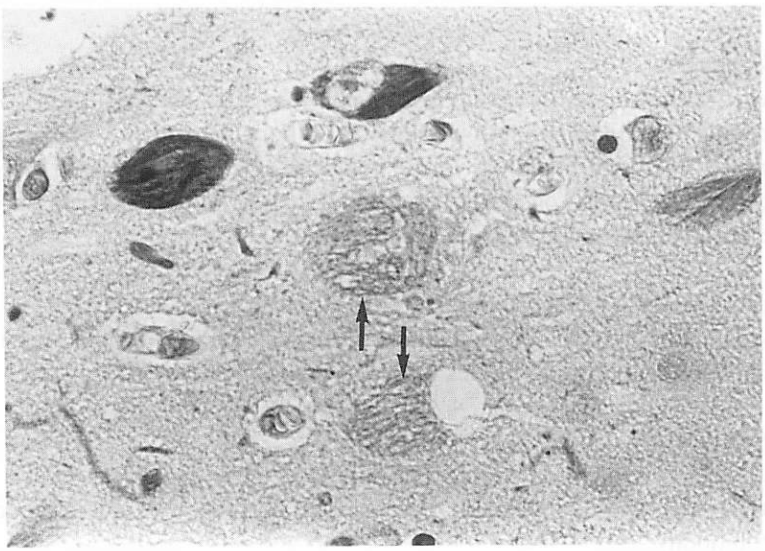


Fig. 1. Intracellular and extracellular neurofibrillary tangles within pyramidal cells of area CA1 of the hippocampus of patient 19. In anti-tau (a) and anti-ubiquitin (c) staining intracellular tangles are clearly resolved. However, using anti-tau (b) other tangled pyramidal cells (probably extracellular) (arrowed) show partial loss of immunoreactivity and using anti-ubiquitin (d) some intracellular pyramidal cells, although





containing tangle material (arrowed), remain unreactive. Extracellular tangles (arrowed) however, are unstained with silver (e) and anti-tau (b) and less strongly stained with anti-PHF (f). Using anti-ubiquitin (g) the tangle material itself is also unstained, though small granular deposits (arrowed) of immunoreactive material are present around the tangle. a–d, f, g, immunoperoxidase-haematoxylin; e, silver. All × 475.

of an even and granular nature and was clearly distinct in both quantity and distribution from that clearly associated with the NFT, in NFT containing cells (Fig. 2). These weakly stained neurones were seen in all patients over 50 years of age and also in those patients under 50 years in whom a moderate number of NFT containing cells were already present (i.e., in patients 6, 11 and 13).

g

Fig. 1g. For legend, see page 252.

(b) Senile plaques

Degenerating nerve processes (neurites) were demonstrable within SP using silver, anti-PHF, anti-tau and anti-ubiquitin. Additionally, separate clumped areas of immunoreactivity within plaque periphery could be localized using both anti-tau and anti-ubiquitin. The "cores" of senile plaques were strongly fluorescent with thioflavin S, but were not immunolabelled with any antibody.

(c) Granulovacuolar degeneration and Hirano bodies

Pyramidal nerve cells showing granulovacuolar degeneration were not immunolabelled, nor were the Hirano bodies present within the neuropil.

(d) Cerebral vessels

Although in most patients, meningeal and some intracortical vessels showed strong fluorescence with thioflavin S, these amyloid deposits were not immunolabelled with any antibody.

(iii) Quantitative results

The number of silver-positive and immunopositive neuronal perikaryal profiles, within areas CA1 to CA5 and subiculum, were counted for each patient directly under the microscope at a magnification of $\times 200$ (Table 2). The weakly positive anti-tau staining neurones, referred to previously, were not interpreted as definite NFT containing cells and are therefore not reflected in the total anti-tau profile counts. The number of positive profiles revealed by each method was compared, within each patient group, using a non-parametric matched-pairs (Wilcoxon) test (Siegel 1956).

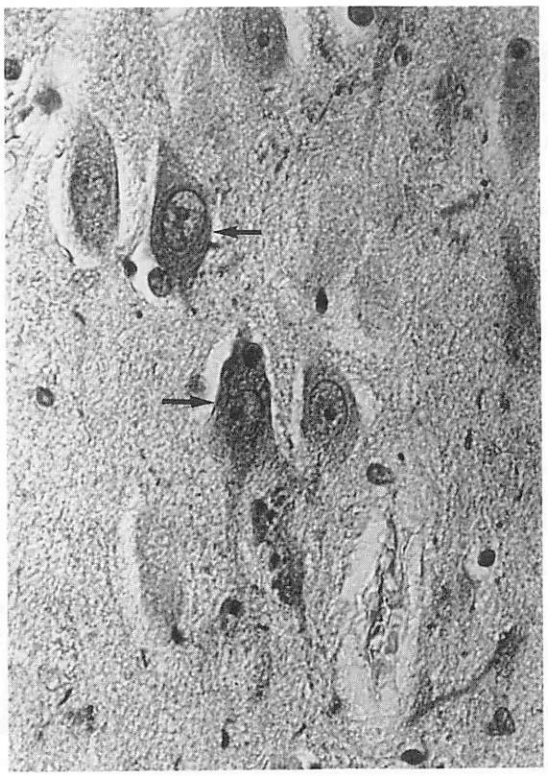


Fig. 2. Pyramidal cells of area CA3 of the hippocampus of patient 19 showing some pyramidal cells (arrowed) to be diffusely and evenly immunostained with anti-tau. Such cells were not stained with silver, anti-PHF or anti-ubiquitin. Immunoperoxidase-haematoxylin; × 475.

Over all 24 patients, no significant differences ($P > 0.05$) were obtained between the numbers of positive profiles visualized when comparisons using either of the 2 anti-PHF antisera, either of the 2 anti-tau antisera, or the silver and either of the 2 anti-PHF antisera were made.

In group 2 patients, the number of immunopositive profiles visualized with anti-ubiquitin was less ($P < 0.05$) than those demonstrated by silver staining; no other significant differences involving other comparisons were noted in this group.

In group 3 patients, the number of PHF (both antisera) positive profiles did not differ from the number of silver positive profiles. However, the number of profiles visualized with one of the anti-human tau, (Ihara et al. 1988) was significantly less than that visualized with either silver ($P < 0.05$), or (both) anti-PHF ($P < 0.01$) antisera. The number of profiles demonstrated by the other anti-human tau (Défossez et al. 1988), although less, did not significantly differ in these ways. The number of profiles visualized with anti-ubiquitin differed significantly from that number visualized with either silver ($P < 0.001$), (both) anti-PHF ($P < 0.001$) and (both) anti-tau ($P < 0.01$) antisera.

TABLE 2

NUMBER OF STAINED PROFILES AS VISUALIZED BY EACH METHOD IN THE 24 PATIENTS WITH DOWN'S SYNDROME

Group 2 and group 3 mean (± SD) values are also given. Anti-PHF_1 from Ihara et al. 1986; anti-PHF_2 from Persuy et al. (1985). Anti human tau_1 from Ihara (1988); anti-human tau_2 from Défossez et al. (1988).

Patient No.	Age	Silver	Anti-PHF_1	Anti-PHF_2	Anti-human tau_1	Anti-human tau_2	Anti-ubiquitin
1	13	0	0	0	0	0	0
2	31	0	0	0	0	0	0
7	42	0	0	0	0	0	0
3	37	2	1	5	1	0	3
4	38	0	0	0	0	1	0
5	40	6	0	4	4	0	2
6	41	44	47	100	48	53	10
9	43	11	5	6	3	3	4
11	48	179	188	206	243	204	53
12	49	6	3	2	12	15	2
13	50	146	152	152	143	123	30
Mean ± SD		49.3 ± 71.8	49.5 ± 76.6	59.4 ± 82.3	56.8 ± 89.6	49.9 ± 75.5	13.0 ± 18.9
8	42	472	436	422	386	347	219
10	47	304	297	305	235	412	159
14	52	624	594	766	161	166	191
15	53	562	584	640	813	887	172
16	58	626	670	455	497	488	232
17	58	706	676	683	617	661	227
18	59	456	359	529	306	282	291
19	59	588	629	605	477	510	279
20	60	585	642	548	367	476	269
21	60	702	648	589	540	560	250
22	64	694	636	679	659	716	317
23	65	575	518	520	349	608	354
24	71	350	626	583	332	404	217
Mean ± SD		557.3 ± 128.3	562.7 ± 123.6	563.4 ± 122.2	441.5 ± 182.4	501.3 ± 191.0	244.3 ± 56.7

DISCUSSION

Previous reports have shown that in AD, NFT within nerve cells are immunoreactive to, not only those antisera raised against extracted PHF protein (e.g., Ihara et al. 1986; Delacourte and Défossez 1986), but also with antisera directed against tau proteins isolated either from human (Ihara et al. 1986; Kosik et al. 1986; Kowall and Kosik 1987; Défossez et al. 1988; Ihara 1988) or non-human (Delacourte and Défossez 1986; Kosik et al. 1986; Iqbal et al. 1986; Wood et al. 1986; Kowall and Kosik 1987; Love et al. 1988) tissues. Other work (Mori et al. 1987; Perry et al. 1987; Lennox et al. 1988; Love et al. 1988; Lowe et al. 1988) indicates NFT to be immunoreactive with

anti-ubiquitin antisera. In this study, we have shown that NFT within pyramidal neurones of the hippocampus (and indeed NFT within other neuronal types; Mann, D. M. A., unpublished observations) in DS are also immunoreactive to the same kinds of antisera. Such data strengthen the suggestion (Mann 1988b) that persons with DS do indeed acquire the (same) neuropathological changes of AD in later life and, importantly, indicate that DS can act as a useful model for studies on the pathogenesis and progression of the pathological changes of AD itself.

The origins of NFT are still controversial. From immunohistochemical studies of AD, it is concluded that tau protein is a major antigenic determinant of NFT (Delacourte and Défossez 1986; Grundke-Iqbal et al. 1986; Ihara et al. 1986; Kosik et al. 1986; Wood et al. 1986; Kowall and Kosik 1987; Défossez et al. 1988; Ihara 1988). Indeed, it is possible that NFT are produced entirely from an abnormal phosphorylation of tau protein. The appearance of epitopes for tau in intracellular NFT, of younger patients with DS, at the same time as such NFT become visible with silver and anti-PHF antisera, would be consistent with such a suggestion. The few cells showing a diffuse anti-tau immunoreactivity in the absence of definite NFT may even be cells destined to contain NFT (see also Hyman et al. 1988). However, because in older patients there is an overall reduction in the number of tau-immunoreactive neurones compared to the number of silver or anti-PHF stained cells (the apparent weaker anti-PHF staining of extracellular tangles reflects a dispersion of PHF fibres within neuropil rather than loss of affinity), it becomes possible that tau proteins (and ubiquitin) only decorate a basic PHF skeleton which is composed of an, as yet, unidentified protein. Tau and ubiquitin might then be removed by macrophagic protease activity following nerve cell death. Alternatively it is possible that the loss of anti-tau, or anti-ubiquitin in the extracellular NFT reflects a change in configuration that might make the epitope inaccessible to antibodies to native or synthetic tau. Such cytochemical changes could also be brought about by macrophagic/microglial activity following nerve cell death and loss of the limiting outer cell membrane. It is not currently possible, using such immunochemical methods, to distinguish between these various options.

Current direct protein chemical analyses of NFT do not resolve this issue either. Kondo et al. (1988) while confirming that tau is indeed incorporated into NFT, via its carboxyl third, were of the opinion that this material represents only a fraction of the total protein within the NFT. Similarly, Wischik and colleagues (Goedert et al. 1988; Wischik et al. 1988a, b) have isolated a tau derived peptide from pronase-treated NFT. This material seems to relate to that which can be "stripped off" the inner core region of the NFT, but this latter (unidentified) component may still account for as much as 90% of the tangle mass.

Hence, although at present it seems clear that tau (either the whole molecule or a partially degraded C-terminus fragment) represents a significant component of the NFT both in AD and DS, the question as to whether NFT are derived *entirely* from tau still remains open.

The presence of anti-ubiquitin immunoreactivity within NFT and the relative paucity of anti-ubiquitin stained NFT, as compared with anti-tau and anti-PHF antisera, deserves mention. Ubiquitin is a 76 amino acid residue protein which is found

throughout the animal and plant kingdom (hence its name) (Rechsteiner 1987); its production is increased in response to stress (Bond and Schlesinger 1985). The ATP-dependent formation of ubiquitin-protein conjugates is a signal for the selective degradation of abnormal and short lived proteins (Hershko et al. 1980; Ciechanover et al. 1984). Ubiquitin may thus have a role in mediating cellular responses to many stresses, which include heat shock, viral infection and nutritional deficiencies and may be involved in response to damaging stimuli in general. The lower level of detection of cells with *intracellular* NFT, with anti-ubiquitin (than with anti-tau or anti-PHF antisera) does not seem to reflect a consequence of different staining sensitivities since a similar level of detection was noted using a streptavidin–biotin system at an antibody dilution of 1/500 (Mann, D. M. A., unpublished observations). Hence, it is possible that anti-ubiquitin immunoreactivity of NFT may be a transient phenomenon, commencing perhaps after the initial formation of NFT, and lasting only as long as the normal protein degradation processes are viable within the diseased and metabolically failing neurone. Loss of anti-ubiquitin reactivity may occur at, or around the time of cell death.

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