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Neurofilament alterations in axonopathies of unknown aetiology and necrotizing angeitis

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ABSTRACT

To describe the ultrastructural characteristics of axon cytoskeleton alterations occurring during axonopathies of unknown aetiology (AUE), and necrotizing angeitis (NA), which are partly unknown. We have analyzed by transmission electron microscopy nerve biopsies from 8 and 6 cases of AUE and NA, respectively, in which previous studies by optic microscopy had shown decreased fibre immunostaining for neurofilament (NF), contrasting with increased labelling for α tubulin. Morphometry was performed in fibres devoid of obvious degeneration signs. NF and microtubule (μT) density, as well as minimal inter-NF distance were measured in 10 myelinated fibres per class of diameter, and compared to controls (without neurologic involvement). In most cases of AUE and NA a severe decrease in NF density was observed, it associated with an increase in μT density in AUE only. Variability of cytoskeleton alterations could be extreme, i.e. NF decreased by 5-fold in some cases, or NF density could be nearly normal. In AUE μT increased by 5 to 10-fold, whereas they decreased in NA in one case excepted. These results confirm those of immunostaining for NF in AUE and in NA, and demonstrate that decreased NF immunostaining corresponds to a decrease in NF density in residual fibres and could not be ascribed simply to fibre loss. Abnormal NF synthesis (AUE), or increased degradation (NA), could account for this pattern. Since this study was performed on apparently non-degenerating fibres, it highlights also an unsuspected extent of axonal damage in these neuropathies.

KEYWORDS: axonal cytoskeleton, microtubules, morphometry, neurofilament, tubulin

ABBREVIATIONS

AUE, axonopathies of unknown aetiology; CIDP, chronic inflammatory demyelinating polyneuropathy; CONT, control; NA, necrotizing angeitis; mdNF, minimal inter-NF distance; NCV, nerve conduction velocities; NF, neurofilaments; μT, microtubules; TEM, Transmission Electron microscopy; TUB, tubulin

INTRODUCTION

The integrity of the axonal cytoskeleton, and the surrounding myelin, is essential for proper nerve conduction, and alterations of neurofilaments (NF) and microtubules (μT) have been reported in experimental neuropathies such as axotomy [1, 2], intoxications with hydrocarbons [3], or aluminium [4], as well as in spreptozotocin-induced diabetes [5]. Nevertheless, the extent of neuronal disorganization is still poorly documented in human neuropathies. Moreover, despite cautious clinical examination and extensive laboratory tests, a number of axonopathies (around 20-30%) remain without aetiology (AUE) even after nerve biopsy [6]. A decrease in NF immunoreactivity has been observed in axonopathies [7]. Also, in severe Guillain-Barré syndromes [8] and in

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Charcot-Marie-Tooth (CMT) disease type 1a [9] decreased NF and/or tubulin (TUB) have been reported. Several mutations in the NFL gene induce various phenotypes of CMT type 2 with giant axons and focal accumulation of NF [10], or lack of NF and increased μT [11]. Finally, in chronic inflammatory demyelinating neuropathy (CIDP) frequent shrinking of axons and increased NF density were reported [12].

We have observed a decreased NF and an increased TUB immunostaining in AUE and in vasculitic neuropathy of systemic necrotizing angeitis (NA), parallel to fibre density decrease [13, 14]. Since these diseases are characterized primarily by axonal involvement, we thought it important to determine whether decreased NF immunostaining could result from a decrease in NF density per residual fibre, or if it could be ascribed simply to fibre loss, and to determine the ultrastructural alterations of axon cytoskelelton.

MATERIALS AND METHODS

Patient selection and optic microscopy findings

Samples from a bank of biopsies performed for clinical diagnosis [15] were compared to 5 controls ((CONT), autopsy cases without peripheral nerve involvement, aged 54 ± 2 years). Two groups previously analysed by morphometry and immunohistochemistry of the axon cytoskeleton (see below) [13, 14, 16] were analyzed: AUE (8 cases, Table 1), and NA (6 patients, Table 2).

Mean age of patients with AUE was 60 ± 15 years [13]. Disease duration varied from a few months up to 24 years, evolution was progressive except in case 97017 (with a subacute post-surgery course). In most cases clinical signs were severe, symetric, restricted to lower limbs or affecting the 4 limbs, motor impairment was noticed except in cases 0012 and 99017. The following laboratory tests were normal: cell blood count and erythrocyte sedimentation

Table 1. Axonopathies of unknown etiology: Clinical and morphometric features.

	Patients							
	0012	98043	97031	99032	99046	99017	97017	0014
Age (years)	58	63	69	71	53	22	69	68
Sex	F	F	M	M	M	F	M	M
Duration	9 months	10 years	24 years	6 years	8 years	3 years	2 months	15 years
Density/mm^2	8535	7233	6040	4738	4304	3713	3327	976
Morphometry	Large fibres atrophy	Large fibres atrophy	Large fibres atrophy and degeneration	Large fibres degeneration	all fibres degeneration	large and medium fibres degeneration	Large and medium fibres degeneration	all fibres degeneration

Controls (5 cases): mean age = 54 ± 2 years, density of myelinated fibres/mm^2 = 7595 ± 362.

Table 2. Necrotizing angeitis: Clinical and morphometric features.

	Patients					
	98054	97048	96065	97023	98034	96040
Age (years)	53	70	55	56	61	63
Sex	F	F	F	M	M	F
Etiology	RA	RA	Purpura	Wegener	RA	PAN
Duration	1 month	2 months	4 months	1 month	6 months	3 months
Density/mm^2	3240	3010	1970	1557	1140	0
Morphometry	large fibres degeneration	large and medium fibres degeneration	all fibres degeneration	all fibres degeneration	all fibres degeneration	all fibres complete degeneration

rate, serum glucose and creatinine, liver function tests, serum proteins with immunofixation electrophoresis, vitamin B12 and folic acid levels, anti-nuclear antibodies and rheumatoid factor, thyroid function tests. Cerebrospinal fluid analyses were normal. Other laboratory investigations were also negative (serology for Lyme's disease and HIV, Sjögren's syndrome antibodies, screening for toxic compounds, occult malignancy...etc). Electrophysiology demonstrated mild reduction of nerve conduction velocities (NCV), with reduced amplitude potentials, indicative of primary axonal involvement [13, 14]. In AUE (Table 1) the density of myelinated fibres varied from normal (2 cases) to moderate and severe (976/mm^2) fibre loss (6 cases). The g ratio was always normal. There was a constant decrease in the density of axons immunolabelled for the light chain of NF (NFL) from 92 to 6% of CONT. Contrary to NF, the number of fibres labelled for TUB per optic field increased significantly in all except 2 cases (99046 and 0014) from 52 to 102% over control values. Thus the density of myelinated fibres was inversely correlated with the TUB+/NFL+ fibre ratio [13].

Patients with NA were aged 59.6 ± 5.7 years in mean [14] (Table 2). Mean clinical duration was 2.8 ± 1.7 months. Multineuritis was observed in 4 cases (case 97048 and 96040 excepted), and sensori-motor signs were present in 5 cases. Evolution was characterized by relapses in 2 cases (98054, 98034), sequelae in 3 others, and death in one case (96040). Rheumatoid Arthritis (RA) was the main aetiology (3 cases). In all cases electrophysiology results were suggestive of a primary axonal type of neuropathy. Necrotizing vasculitis of the epineurium was constant. Acute axon-myelin degeneration features were noticed in 4 cases. Fibre loss was constant (and reached >70% in 4 cases). The g ratio was elevated (0.75) in case 96065. In case 96040 a complete lack of myelinated fibres precluded further analysis. Fibres immunolabelled for NFL were always decreased by 30-95%, contrary to fibres expressing TUB which were increased by 40-140%, and the ratio of the number of fibres expressing TUB to NFL+ fibres was increased by 3 to 33-fold [14].

Transmission electron microscopy (TEM)

Specimens were superficial fibular nerve (nervum peroneus superficialis) biopsies at the level of the inferior third of the calf, previously analysed by optic microscopy [13, 14, 16]. Fixation and embedding procedures were standardized. Ultrathin sections were performed as described [17], and observed with a Jeol JEM-2010 electron microscope (Service Commun d'Imagerie et d'Analyses Microscopiques of the Faculty of Medicine of Angers).

Microphotographs were taken at low magnification (x 10 000 - 20 000) to measure the axon diameter, and myelin thickness. Myelination was assessed by calculating the g ratio (axonal diameter/fibre diameter) [18]. Myelin compaction and the axonal cytoskeleton were studied at high magnification (x 80 000). For each section microphotographs of 10 fibres per diameter group (Aδ fibre diameter ≤ 6 µm, 6 µm < Aβ fibre diameter ≤ 10 µm, and Aα fibre diameter > 10 µm) were taken, and the number of NF and µT per area were counted using a millimetric grid [17]. To obtain an accurate description of the consequences of the pathological process on the axonal cytoskeleton, fibres presenting obvious features of degeneration (e. g. vacuoles, shrunk axoplasm...) were excluded from counting, and fibres having a normal appearance were chosen for this purpose. The minimal inter-NF distance [17, 19] was measured using millimetric lens (x 10 magnification). Results for one case were averaged by axon diameter class. Since there was no significant difference in the number of NF between large and medium diameter fibres [20], we have then grouped results for these axons on one hand, and for Aδ fibres (which have classically a higher µT density) on the other.

Statistical analyses used the Student's *t* test adapted to small samples, ANOVA, and linear regression coefficient.

RESULTS

Alterations of fibres in AUE and NA

TEM did not reveal abnormalities that could have argued against the diagnosis (e.g. widespread hypomyelination, abnormal inclusions...etc [21]). Major fibre alterations such as acute degeneration were observed in most cases of AUE and NA, they generally involved relatively few fibres. In AUE the most noticeable degeneration features were various aspects of vacuolization of some

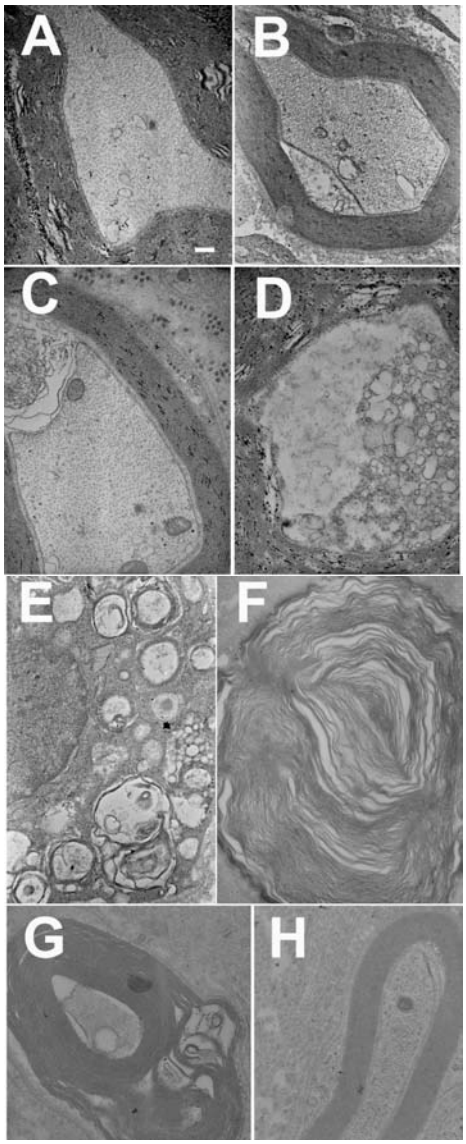

Fig. 1. Microphotographs of ultrathin sections of control (A), AUE (B-D) demonstrating different types of fibre and axonal vacuolisation. In necrotizing angeitis cases (E-H) several fibre abnormalities were encountered such as macrophage infiltration (E), complete decompaction of myelin (F, G), and hypomyelinated axon with increased g ratio (H). Bar = 200 nm.

axons (Fig. 1A-D). In NA abnormalities encountered comprised macrophage infiltration in one sample (Fig. 1E), and complete decompaction of myelin with degenerative profiles (Fig. 1F-G). In case 96065, hypomyelinated axons with increased g ratio (Fig. 1H) were observed, as already noticed on semithin sections. There were no other myelin

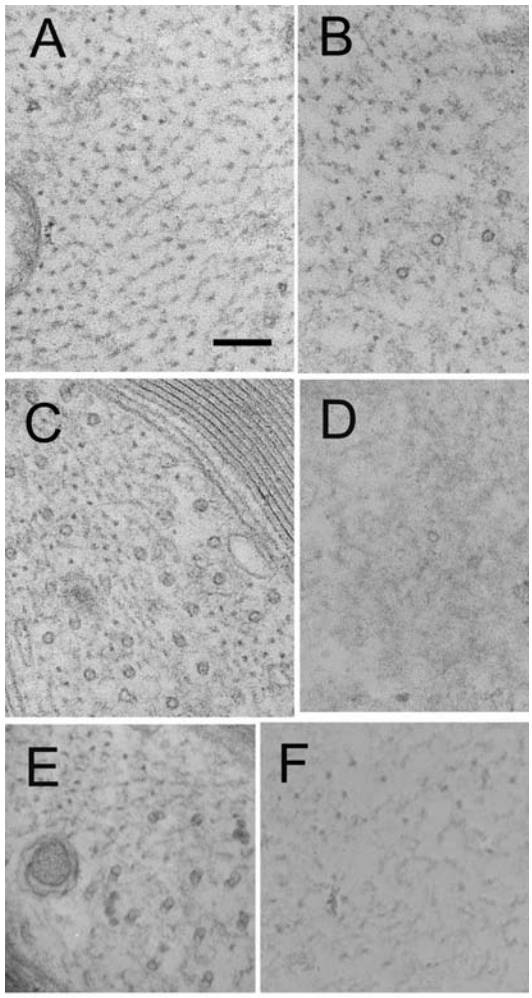

Fig. 2. Microphotographs of ultrathin sections of control (A), AUE (B, C), and necrotizing angeitis (D-F) demonstrating similar axon cytoskeleton alterations in both types of diseases: NF density decrease and increased inter-NF spacing (B, D, F), as well as increased microtubule density (C, E). Note the peculiar association of microtubules by pairs in (E). Bar = 100 nm.

alterations, and myelin compaction was normal in the other cases of AUE and of NA (mean myelin periodicity similar to CONT). These fibres with obvious alterations were excluded from counting and morphometric analysis of the cytoskeleton.

In the fibres devoid of degeneration features axonal lesions were restricted to the cytoskeleton (Fig. 2-4). Alterations were partly similar in AUE and in NA (Fig. 2), compared to CONT. They were characterized by decreased NF density, and increased inter-NF spacing (Fig. 2). This was

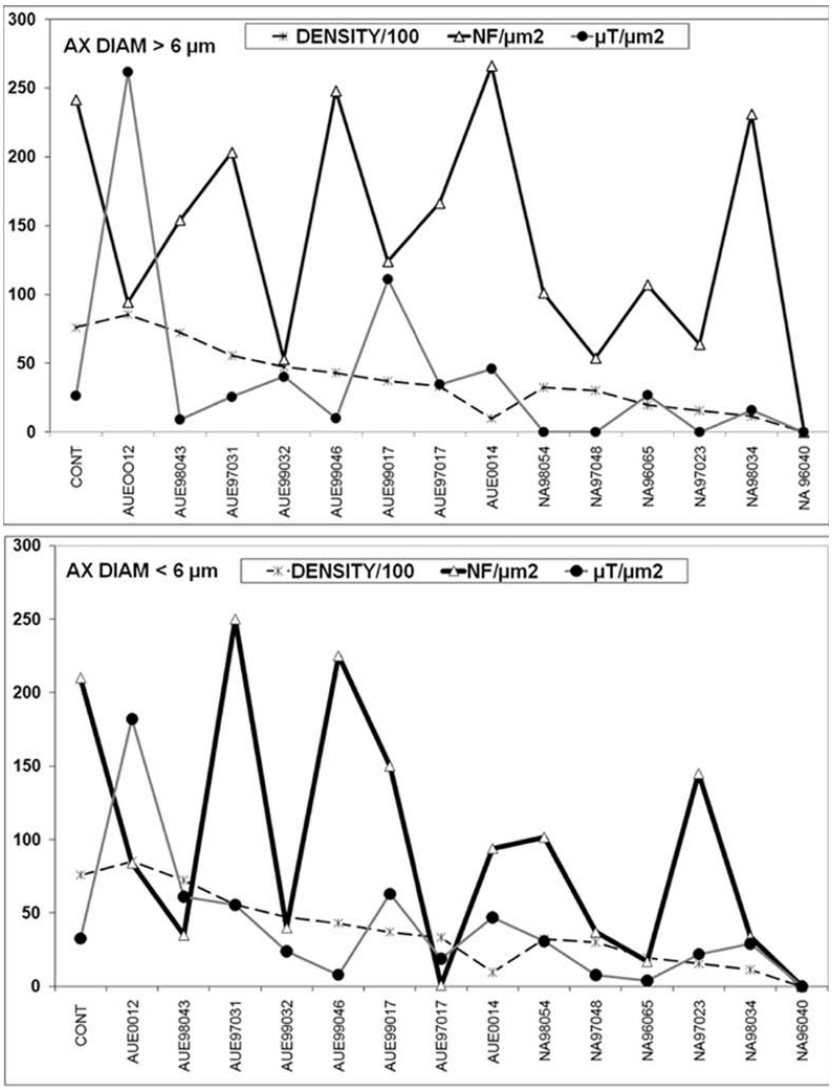

Fig. 3. Neurofilament (open triangles) and microtubule (black circles) density in each of the cases of axonopathies (AUE) and necrotizing angeitis (NA). Mean values for controls (CONT, n = 5) are presented on the left. Cases are in order of increasing fibre loss (density, dotted line). Values for large and medium diameter are grouped (upper panel); values for small diameter fibres correspond to the lower panel. Each result is the mean of counts in 10 fibres per diameter class.

associated, in AUE only, to increased µT density (Fig. 3). For one given sample alterations were generally in the same range for large and small diameter fibres (Fig. 3), except in 2 cases: AUE0014 and NA98034, which had normal NF density in large diameter fibres, contrasting with a severe decrease in NF in small diameter ones. These variations (decrease in NF density, increase in µT density) were not correlated with fibre density recorded on semithin sections, contrary to what had been observed for NF labelling (Fig. 4A).

In AUE and NA the decrease in NF was more pronounced in small fibres, whereas alterations of µT predominated in large fibres.

Cytoskeleton alterations in AUE

The most frequent alteration in AUE was a decrease in NF density for large and/or small diameter fibres (Fig. 3), observed in all but one cases (99046). A slight increase in NF density was observed in case 99046, 0014 and 97031 (non significant) either in large and/or in small

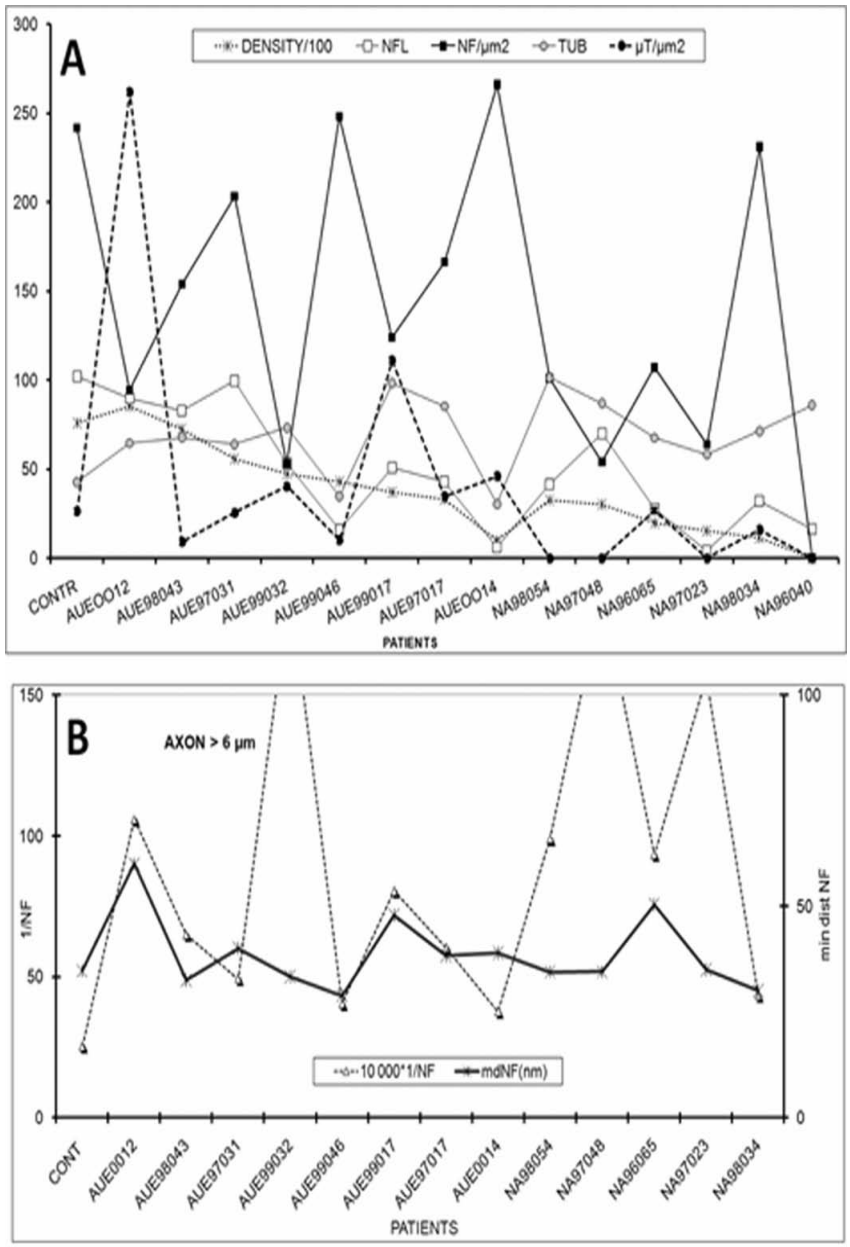

Fig. 4. (**A**) Neurofilament (black squares) and microtubule (black circles, dotted line) density plotted against NF (open squares) and tubulin (grey circles) immunostaining. Mean values for controls (CONT, n = 5) are presented on the left. Cases are in order of increasing fibre loss (density/100, crosses, dotted line). (**B**) Minimal inter-neurofilament distance (mdNF) for large diameter fibres (mean of counting in 10 fibres) (black line and crosses) plotted against 1/neurofilament density (open triangles, dotted line). Mean values for controls (CONT, n = 5) are presented on the left. Cases are in order of increasing fibre loss. NA 96040 with complete myelinated fibre loss is not represented.

diameter fibres. In mean NF density decreased by 32% versus CONT for large and medium diameter fibres, and by 48% for small diameter fibres. This decrease in NF density, ranging from -78% (case 99032) to -31% (case 97017), was pronounced in 5 out of 8 cases. Roughly the extent in NF decrease was in the same range in small and large diameter axons.

Axon cytoskeleton alterations were also characterized, although inconstantly, by an increase in µT density (5 out of 8 cases: 0012, 99032, 99017, 97017, and 0014). It reached 155% in mean over CONT values in large diameter fibres, and increased less (75%) in small diameter axons. These variations were extreme for cases 0012 (+895%) and 99017 (+322%). On the contrary, µT decreased strongly by 66 and 62% in cases 98043 and 99046, respectively.

For one given sample correlations for NF and µT density with previous immunocytochemical quantifications were weak (Fig. 4A).

Cytoskeleton alterations in NA

In NA, fibre loss was far more pronounced than in AUE (76% compared to CONT) (Fig. 3). NF density decreased in mean by 62% in large and medium diameter axons versus CONT. In case 98034 excepted, it ranged between 55-100% in the 5 other cases. This decrease was also constant in small diameter fibres and reached 73% in mean versus CONT.

Contrary to AUE, µT decreased in mean by 73% and 52% (ranging from 31 to 100%), in large and small diameter fibres, respectively. In small diameter fibres µT were constantly decreased. Case 96065 was the only case with a normal µT density for large diameter fibres, and a strong decrease in small ones (88%). Correlations with previous immunocytochemical quantifications could not be established (Fig. 4A).

Neurofilament spacing in AUE and NA

In most AUE cases (5 out of 8 cases) minimal inter-NF distance (mdNF) (Fig. 4B) was moderately increased (+15% in mean above CONT). It increased only slightly in NA (+6% above CONT). In 3 cases, mdNF was strikingly increased: this was observed in 2 cases of AUE (0012 and 99017, with mdNF increased by 73 and 38% respectively compared to CONT), and one case of NA (96065) in which mdNF increased by 45%.

On the contrary, in 3 cases of AUE mdNF decreased (98043, 99032, 99046); this was more pronounced only in case 99046 (-17%). A moderate decrease in mdNF (-13%) was also observed in NA case 98034 (which had the most pronounced fibre loss). These alterations did not correlate linearly with NF density (AUE : $\rho = 0.1236$, NA: $\rho = 0.0012$). Nevertheless, mdNF varied in proportion to 1/NF density, except in AUE 99032 (AUE: F test = 3.10^{-4}, NA: F = 2.10^{-3}).

DISCUSSION

Autopsy samples are widely used as controls [22, 23], and morphometric data from our controls were in the ranges previously described [20, 23, 24]. So, an artificial decrease in NF and µT in our cases is unlikely since procedures were standardized, and the ultrastructure of the nerves was otherwise preserved [17].

Since NF were counted per axon area, their decrease in pathological cases corresponds to a reduced NF density in residual axons. This demonstrates that the decrease in NF immuno-labelling previously observed by optic microscopy in AUE and in NA [13, 14] did not result from decreased fibre density only, or from a modification of NF antigenicity. Moreover, TEM revealed that NF alterations were observed in apparently normal fibres (without degeneration features, and normally myelinated) demonstrating that the pathological process had spread more than evidenced by optic microscopy. Thus, as could be expected, TEM is more accurate than immunohistochemistry to detect the extent of axon cytoskeleton alterations. Fibres characterized only by an altered ultrastructure of the cytoskeleton could correspond either to fibres that have not degenerated yet, or to fibres previously altered and undergoing regeneration. Although NF density determined by TEM and the number of fibres immunostained for NFL were not correlated, NF decrease was observed for both techniques, and was nearly constant in AUE as well as in NA. The differences observed in some cases between NF density and immunocytochemistry possibly result from our deliberate choice to count cytoskeleton elements only in fibres with a normal appearance, whereas immunocytochemistry was a global analysis of the section. Obviously, decrease in NF density would have been greater if degenerating fibres had been taken into account.

The decreased NF density, associated with an increase in µT, were the main alterations in AUE. Roughly the extent of axon cytoskeleton damage appeared similar by TEM between small and large

diameter fibres, whereas in some cases morphometry of semithin sections identified the involvement of large fibres only (atrophy, or degeneration). The involvement of both small and large diameter fibres, in most of our cases, suggests that pathological processes have a common final pathway which could alter all types of myelinated fibres. Enhanced destruction of NF, or alternately reduced synthesis due either to energy metabolism failure, or to a lack of trophic support for example, might be hypothesized. The first hypothesis is coherent with the report of intraaxonal granular debris immunostained by NF-antibodies after experimental nerve section, and in human axonopathies [7]. The second one is supported by the decreased immunolabelling of fibres for Nerve Growth Factor (NGF) and Insulin-like Growth Factor 1 (IGF-I) in these cases of AUE. This suggests that these growth factors, known to promote NF synthesis, might be involved in the pathological process [16].

Correlations between NF density and axon atrophy established on semithin sections appear likely. NF are major determinants of axon calibre [25, 26], and decreased NF immunoreactivity associates with fibre atrophy after axon injury [27]. Thus, NF density decrease is likely responsible for isolated large fibre atrophy (without fibre loss) observed in some of our AUE cases. We hypothesize that secondarily, atrophy could be compensated, at least partly, by increased inter-NF spacing, which is present in most of our cases.

The pattern of NF and µT alterations in AUE shares similarities with that of the mutant quail Quv, which is characterized by lack of NFL, axon atrophy, and increased µT density [28]. Apart from this mutant, and from NFH-lacZ transgenic mice, in which all NF accumulate in the perikarya [25], neuropathies with increased TUB and decreased axonal NF have not been described to our knowledge. Interestingly, low levels of NF and increased TUB expression are observed during development and regeneration [26]. It appears plausible that in our cases increased µT density might reflect regeneration also. This is supported by the observation of a similar pattern of increased µT density in 3 cases of CIDP, associated with an increase in growth associated protein-43 (GAP-43) [17], which is known also to increase during regeneration.

Variations in minimal inter-NF spacing were less pronounced than that of NF density, and mainly characterized by a slight increase. On the contrary, in some demyelinating neuropathies, narrowing of NF could result from NF dephosphorylation due to anti-myelin associated glycoprotein (MAG) antibodies [22]. Abnormal NF distribution has been reported in leprosy also [29]. Roughly mdNF increased in parallel to NF loss in our cases. This is not specific since we have observed also a mild increase in mdNF associated with decreased NF density in CIDP [17]. Despite profound decrease in NF density in some cases, mdNF was modified only moderately, in accordance with experimental data with β, β'-iminodipropionitrile, showing that mdNF remains relatively constant despite significative changes in NF densities [30].

Correlations between immunocytochemistry and TEM for microtubules were weaker, especially in NA. In AUE, and in NA, increased TUB immunoreactivity was a hallmark [13, 14]. Nevertheless, TEM disclosed inconstantly an increased µT density in AUE. In NA, and contrary to TUB immunostaining, TEM revealed a decreased µT density in all except one cases. This discrepancy might correspond to increased TUB synthesis - as detected by immunolabelling - without µT assembly precluding its detection by TEM. Since µT increase during regeneration [26], our cases might correspond to different periods along this process, with decreased NF reflecting degeneration, and increased TUB synthesis resulting from attempts of repair. Different and complex alterations of NF and TUB transcription have been described during experimental neuropathies [1, 4, 5], or in CMT disease type 1 [9] and type 2 [10].

In conclusion, TEM demonstrates that decreased NF immunostaining in AUE and NA corresponds to a decrease in NF per residual fibre, and could not be only ascribed to fibre loss, or to a modification of NF antigenicity. It evidences a more pronounced involvement of myelinated fibres than suspected by immunocytochemitry analyzed by optic microscopy. The cytoskeleton of apparently normal fibres, both small and large,

was constantly altered in our cases, independently of clinical signs and evolution. Variability among cases might reflect different mechanisms of nerve insults, especially in AUE, as well as various periods in the process of degeneration-regeneration. Although involvement of small fibres and NF decrease were shared features, increase in µT appeared restricted to AUE. These results have to be confirmed in order to gain insight in the pathophysiology of these disabling diseases.

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