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Corpora amylacea from multiple sclerosis brain tissue consists of aggregated neuronal cells

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In this report, we describe proteomic analysis of corpora amylacea collected by postmortem laser microdissection from multiple sclerosis (MS) brain lesions. Using low level protein loads (about 30 μg), a combination of two-dimensional electrophoresis with matrix-assisted laser desorption/ionization-time of flight mass spectrometry and database interrogations we identified 24 proteins of suspected neuronal origin. In addition to major cytoskeletal proteins like actin, tubulin, and vimentin, we identified a variety of proteins implicated specifically in cellular motility and plasticity (F-actin capping protein), regulation of apoptosis and senescence (tumor rejection antigen-1, heat shock proteins, valosin-containing protein, and ubiquitin-activating enzyme E1), and enzymatic pathways (glyceraldehyde-3-dehydrogenase, protein disulfide isomerase, protein disulfide isomerase related protein 5, lactate dehydrogenase). Samples taken from regions in the vicinity of corpora amylacea showed only traces of cellular proteins suggesting that these bodies may represent remnants of neuronal aggregates with highly polymerized cytoskeletal material. Our data provide evidence supporting the concept that biogenesis of corpora amylacea involves degeneration and aggregation of cells of neuronal origin.

Keywords: corpora amylacea, multiple sclerosis, proteomics, neuronal aggregates

INTRODUCTION

Corpora amylacea are globular basophilic bodies, 10–50 μm in diameter, which may stain deeply with iodine. They are commonly seen in subpial regions of brains of elderly subjects. Corpora amylacea develop in astrocytic processes and are associated with neurodegeneration (Singhrao *et al.,* 1993). They were originally described by Purkinje (1837) but have long been disregarded by neuropathologists as being of limited pathologic relevance (Buzzard *et al.,* 1921). Application of new methods has shown that these polyglucosan bodies contain substances that have their origin in CNS cells (Loeffler *et al.,* 1993; Cissé *et al.,* 1993; Singhrao *et al.,* 1994). Several immunocytochemical reports have suggested that corpora amylacea contain proteins and other materials principally found in neurons. For example, positive staining was reported for tau (Nolan & Brown, 1989; Loeffler *et al.,* 1993), extracytoplasmic domain of amyloid precursor protein (Tate-Ostroff *et al.,* 1989), hemoxygenase-1 (Iwaki *et al.,* 1996), serum carnosinase, a neuronal GABA releasing enzyme (Jackson *et al.*, 1994), heat shock protein like Hsp-72 (Martin *et al.,* 1991), Hsp-60 (Gati & Leel-Ossy, 2001), and Hsp27 (Erdamar *et al.,* 2000), S100 protein (Hoyaux *et al.,* 2000), ubiquitin (Cissé *et al.,* 1993), myelin basic protein, proteolipid protein, oligodendrocyte

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Abbreviations: CNS, central nervous system; MS, multiple sclerosis; CA, corpora amylacea; DTT, dithiothreitol; IEF, isoelectrofocusing; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; LRP-1, LDL receptor-related protein 1.

glycoprotein, ferritin (Singhrao *et al*., 1994), nestin (Buervenich *et al*., 2001), Bcl-2, C-Jun and Bax (Botez *et al*., 2001). These observations support the concept that in the development of corpora amylacea, the incipient process is most probably degenerative in nature, which later on may be accompanied by stress protein synthesis.

Interestingly, recent data show that corpora amylacea have an affinity to nucleic acids as shown by *in situ* hybridization. The binding affinity varied with the nucleotide sequence, the most intense signal being produced by adenosine-2A receptor antisense probe. Since proteinase K pretreatment caused a complete loss of staining, the binding moiety appeares to be partially proteinaceous (Balea *et al*., 2006).

Although there is no link between corpora amylacea and multiple sclerosis (MS), corpora amylacea have been frequently described in MS brain tissue (Raine, 1994), however, their occurrence was not attributed any pathologic significance. In a recent analysis of multiple sclerosis brain tissue material we detected many old lesions containing abundant of corpora amylacea, while the adjacent tissue was entirely unaffected. These observations suggested that the generation of corpora amylacea was probably a secondary phenomenon in the pathology of MS. In an attempt to analyze the mechanism underlying the generation of corpora amylacea, we assessed their protein composition**.** For this purpose, corpora amylacea were laser microdissected from MS lesions, solubilized and their major protein components identified according to a generally accepted proteomic protocol. This technique identified a number of cellular proteins that indicated that corpora amylacea are derived from degenerated and aggregated cells of neuronal origin.

MATERIALS AND METHODS

Immunohistochemistry. Frozen sections were stained with the avidin–biotin–peroxidase complex (ABC) technique. Tissue sections were air dried, fixed in acetone, quenched with 0.03% hydrogen peroxide and blocked with normal serum. They were then incubated with primary antibodies followed by biotinylated anti-mouse IgG and the ABC reagent. The same lesions (serial sections) were stained with antibodies detecting demyelinated axons, SMI 32, interacting with abnormally phosphorylated neurofilament H, as well as normal axons, SMI 31, interacting with phosphorylated neurofilament H (both from Sternberger Monoclonals). Serial sections were also stained with anti-galactocerebroside (GC) and anti-myelin basic protein (MBP) antibodies to detect oligodendrocytes, and anti-glial fibrillary acid protein (GFAP) antibodies, to detect astrocytes.

Collection of corpora amylacea. Early autopsy freshly frozen brain tissue from cases of multiple sclerosis was used for this study. Tissue blocks containing chronic active lesions with corpora amylacea as defined by H&E staining were cut into semithin sections. Adjacent unstained sections were also used for laser-capture microdissection by Leica Microsystems (Wetzlar GmbH, Wetzlar, Germany). Corpora amylacea were visible as dense bodies scattered throughout the lesions, often in the gliotic margins of blood vessels. One thousand and seven hundred corpora amylacea were dissected from several lesions and catapulted into collecting tubes. The same volume of tissue from the lesions left after corpora amylacea dissection was used as a control. In addition, we dissected and catapulted fragments of non-affected tissue from regions beyond the lesions.

Sample preparation. Lysates of corpora amylacea were prepared using 150 μl of lysis buffer (9 M urea, 4% Chaps, 1% DTT, 2% Pharmalyte, and Complete Inhibitors (Boeringer)). A buffer volume approximately equal to the packed tissue volume was used. To improve resolution and recovery of proteins in 2D electrophoresis, corpora amylacea and tissue extracts were treated with PlusOne 2-D Clean-Up Kit (Amersham-Biosciences). Protein concentration in the supernatant fraction was determined by PlusOne 2-D Quant Kit (Amersham Biosciences), using bovine serum albumin as a standard and tissue lysates were stored at –80ºC until analyzed.

Two-dimensional polyacrylamide gel electrophoresis. Proteins were separated by two-dimensional electrophoresis, using ready-made gels with immobilized pH gradients (Amersham-Biosciences). For the first dimension, samples containing approx. 30 μg of soluble protein in lysis buffer were mixed with IPG Reswelling Solution (8 M urea, 1% Chaps, 0.4% DTT, 0.5% Pharmalyte) to obtain a final volume of 450 μl. They were then loaded onto 24 cm immobilized pH linear gradient strip gels (IPG, pH 3–10). IEF strips were allowed to rehydrate for 5 h, and isoelectric focusing was performed according to the manufacturer's protocol by gradual increase of voltage (30 V for 5 h, 500 V for 1 h, 1 000 V for 1 h, followed by 70 kVh at 8 000 V), using an IPGphor system (Amersham-Biosciences). SDS electrophoresis was performed on 12.5% polyacrylamide gels using the Ettan Dalt vertical system. Protein spots were visualized by silver-staining according to the method compatible with analysis of proteins by mass spectrometry (Shevchenko *et al*., 1996). Silver-stained gels were digitized using a laser Image Scanner (Amersham-Biosciences). Computerized 2D gel analysis was performed with the Image Master 2D software package Version 3.0. Protein spots were

excised from the gel and subjected to in-gel digestion with trypsin.

Mass spectrometry, data base search and data processing. Proteins in each gel slice were subjected to reduction with 10 mM dithiothreitol, alkylation with 55 mM iodoacetamide, and tryptic digestion with 12.5 μg/ml modified trypsin (Roche Molecular Biochemicals) at 37°C for 14 h. After in-gel digestion, the product peptides were extracted with 5% formic acid and acetonitrile, vacuum-dried, and dissolved in 5% formic acid. Proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry and by partial peptide sequencing with ion trap electrospray mass spectrometry. Multiple peptides from each protein were detected, adding confidence to the protein identification. Trypsin autodigestion peaks were excluded from the database searching. Multiple digested peptides were obtained from a single run of each gel slice sample by microcapillary C18 reverse phase chromatography (200 μm × 5 cm capillary; Michrom BioResources, Inc.) and directly applied to the spectrometer. The ion trap was programmed to carry out two successive scans consisting of the first full scan MS over the range 300–2000 *m*/*z* and the second data-dependent scan of the most abundant ion in the first scan. Automatic MS/MS spectra were obtained from the highest peak in each full scan by setting a relative collision energy of 30% and an exclusion time of 5 min for molecules of the same *m*/*z* value range. We searched the NCBI nonredundant protein data base with the Mascot program (Matrix Science) for high fits with the ion spectrum data generated by LC/MS/MS. The raw results identified the top 20 candidates for each spectrum. We examined their automatic ordering manually in terms of their reliability scores and MS spectrum profiles to pick up only highly reliable peptide data (sorted data).

RESULTS

Corpora amylacea presence in MS lesions

In this report we attempted to assess the protein composition of corpora amylacea to test the concept that corpora amylacea may arise from injured axons or glial cells. For this purpose, we used samples from chronic MS lesions in which numerous corpora amylacea were present (Fig. 1A). Such profiles are frequently encountered in MS pathology. Corpora amylacea were spread throughout the lesion area with an average density of 6 per mm^2. Sometimes they were more numerous in close vicinity of blood vessels. Away from lesions, corpora amylacea were absent. The morphology of corpora amylacea was characterized as dense, rounded bodies sometimes with a ring-like structure (starch-grain). Corpora amylacea were removed from the tissue by laser microdissection. In parallel, samples of control white matter (Fig. 1B), normal and degenerated, were taken in a similar fashion and analyzed. Samples of degenerated white matter were collected from areas in close vicinity of corpora amylacea.

2D gel electrophoresis of corpora amylacea lysates

In order to identify the major protein components, the same volumes of isolated corpora amylacea and control white matter were sonicated in lysis buffer (Amersham), the extracted proteins further purified by Clean-Up Kit (Amersham), and separated by two-dimensional gel electrophoresis (Fig. 2A, B, C). After staining with silver, 34 proteins of interest were excised from the gels, digested with trypsin, and their identities established by matrix-assisted laser desorption/ionization mass spectrometry. The

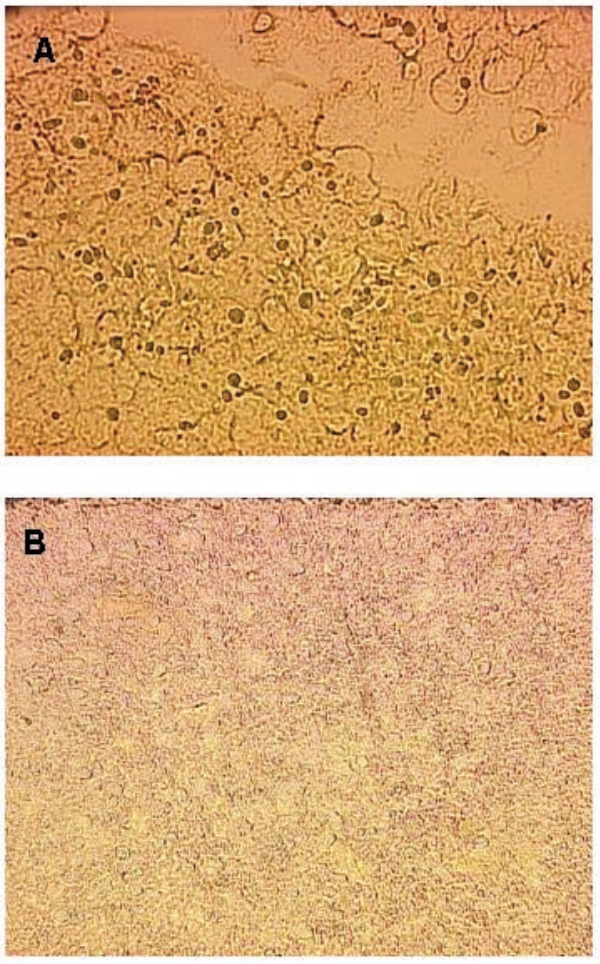

Figure 1. Corpora amylacea in brain tissue.
MS lesion with corpora amylacea (A) and adjacent unaffected white matter without corpora amylacea (B). Magnification × 400.

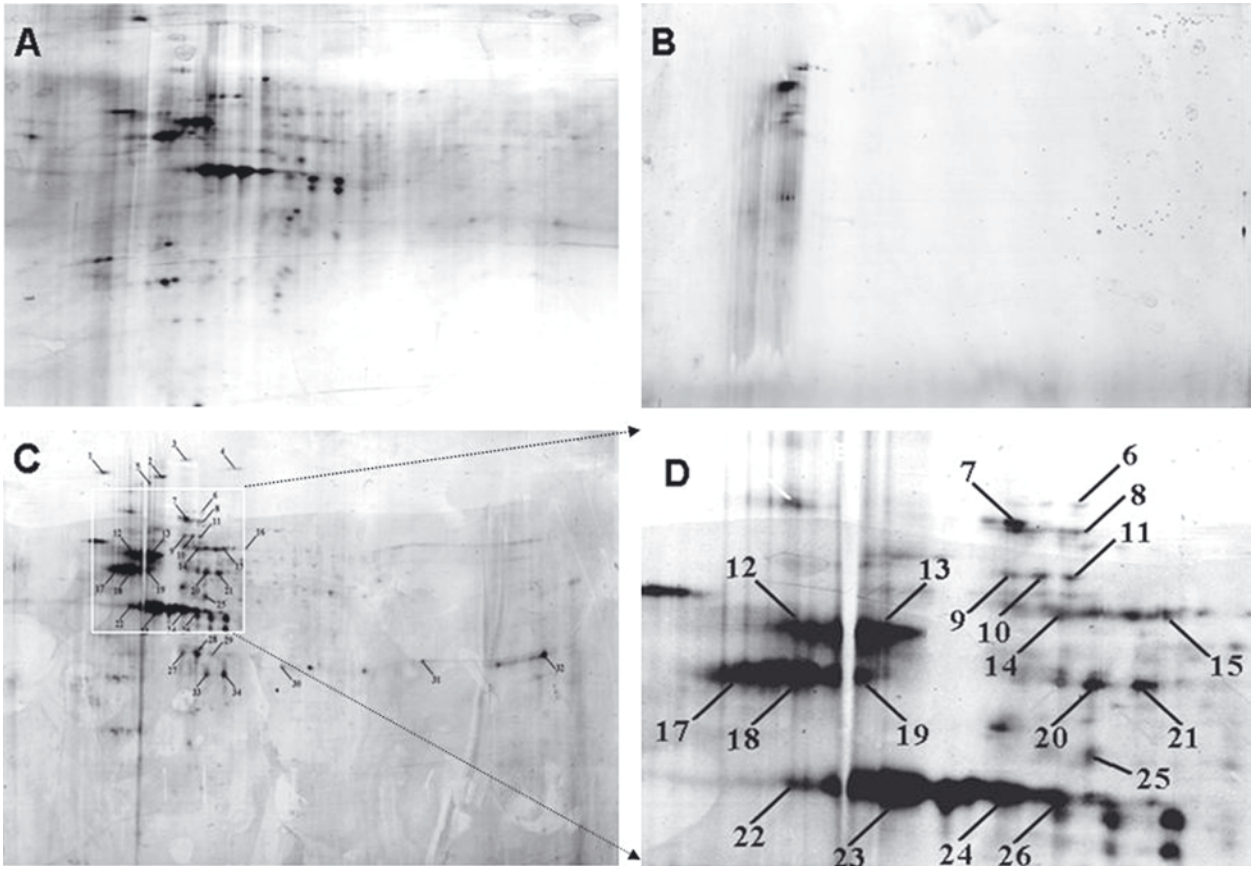

Figure 2. Typical silver-stained 2D gel electrophoregram of corpora amylacea laser microdissected from multiple sclerosis lesions.
Proteins present in extracts of the same volume of unaffected tissue (panel A), tissue from the lesions left after corpora amylacea dissection (panel B) and 1700 corpora amylacea bodies (panel C) obtained by laser-capture microdissection, were separated by 2D electrophoresis, stained with silver and identified by sequencing according to a proteomic protocol. Panel D shows a magnified region of the gel with the highest number of cytoskeletal proteins.

resulting spectra were used to identify the proteins using the MS-FIT search program. Of the 34 spots excised, 24 contained a single protein while 10 spots consisted of more than one protein but they could be identified by the presence of two or more tryptic peptide fragments. Since some proteins were present in several different spots (n = 34) showing similar or different molecular masses, in fact, there were only 24 different proteins.

MALDI analysis

The major proteins identified were members of the cytoskeletal system (Fig. 2C, D). Slices taken from different areas of major spots A and B showed the presence of α and β subunits of tubulin, particularly their α2, β1 and β5 variants. Interestingly, despite its much higher molecular mass, vimentin comigrated with both tubulin subunits. Both major groups of spots, A and B, showed a wide pI in the range of 4.5–5.0 and 4.3–4.9, respectively. Similarly the C group of spots, containing β actin and its β' mutant, had pI which ranged from 4.6 to 5.3. Such wide pI ranges indicate that these proteins exist in several forms differing due to posttranslational modifications. Table 1 shows that in addition to cytoskeletal proteins, corpora amylacea also contained proteins specific for different cellular locations, including: (a) the cell membrane (LDL receptor-related protein 1, LRP-1), (b) the endoplasmatic reticulum (thiol oxidoreductase ER60, heat shock protein gp96), (c) mitochondria (ATP synthase), (d) cytosol (valosin-containing protein p97, alanyl-t-RNA synthetase, protein disulfide isomerase, 60S acidic ribosomal protein), and (e) the nucleus (RuvB-like 2 protein). Obviously, due to the limited number of laser-microdissected corpora amylacea, low amounts of extracted proteins were loaded. Therefore, only the most abundant intracellular proteins could be detected after separation by electrophoresis. Comparison of proteins found in corpora amylacea with those of white matter extracts showed strong and significant similarities (not shown). However, there were some proteins missing from corpora bodies suggest-

Table 1. Proteins identified in corpora amylacea.

The corpora amylacea were solubilized and separated by 2D electrophoresis. Slices from CBB-stained gels illustrated in Fig. 2 were subjected to sequencing. Proteins were identified by peptide mass fingerprinting using MALDI-TOF MS and by partial peptide sequencing with ion trap electrospray MS. *Indicates the presence of more than one protein in the analyzed gel slice.

Gel slice number	gi	Mass	Total score	Peptides matches	Annotation
1	4507677	92696	786	15	Tumor rejection antigen-1 (gp96)
2	6005942	89950	600	14	Valosin-containing protein
3*	23510338	118858	533	13	Ubiquitin-activating enzyme E1
	1362855	100135	281	8	lrp protein
	4501841	107476	226	4	Alanyl-tRNA synthetase
4	2274968	106900	384	10	Glucosidase II
5	340219	53738	1076	25	Vimentin
6	292059	74019	284	5	MTHSP75
7	5729877	70898	668	16	Heat shock cognate 71 kDa protein
8	4502109	75904	410	10	Protein p68, calcium binding p68 (annexin VI)
9	24307939	59671	82	2	Chaperonin containing TCP-1
10	24307939	59671	253	5	Chaperonin containing TCP-1
11	24307939	59671	253	5	Chaperonin containing TCP-1
12*	2119204	53676	802	19	Vimentin
	17986283	50136	164	4	Tubulin α brain specific
13*	2119204	53676	802	19	Vimentin
	7986283	50136	164	4	Tubulin α brain specific
14	1085373	57043	308	6	Protein disulfide-isomerase ER60 precursor
15	1085373	57043	308	6	Protein disulfide-isomerase ER60 precursor
16	135538	60819	109	2	T-complex protein 1, α subunit
17*	2119204	53676	802	19	Vimentin
	20809886	49808	153	3	Tubulin β2
	28557150	48797	57	1	Hornerin
18*	20809886	49808	610	17	Tubulin β2
	114549	56525	231	6	ATP synthase, (mitochondrial F1 complex)
19*	16198437	30625	104	3	Protein similar to tubulin, β5
	1710248	46512	71	2	Protein disulfide isomerase-related protein 5
20*	5031573	47797	190	5	ARP3 actin-related protein 3 homolog
	5730023	51296	189	4	RuvB-like 2 protein
21	5031573	47797	97	3	ARP3 actin-related protein 3 homolog
22	4501887	42128	254	7	Actin gamma 1
23	4501885	42052	543	19	β actin
24	4501885	42052	543	19	β actin
25	28336	42128	330	10	β actin mutant (β' actin)
26	28336	42128	220	7	β actin mutant (β' actin)
27	28336	42128	45	3	β actin mutant (β' actin)
28	28336	42128	194	6	β actin mutant (β' actin)
29*	4506667	34423	111	2	60S acidic ribosomal protein P0
	5453597	33073	57	1	F-actin capping protein α1 subunit
30	442631	35246	51	1	Annexin I
31	18645167	38780	347	8	Annexin A2
32	31645	36202	89	2	Glyceraldehyde-3–phosphate dehydrogenase
33	4557032	36900	279	6	Lactate dehydrogenase B
34	4557032	36900	316	10	Lactate dehydrogenase B

ing that only those directly associated with the actin, cytoskeleton or tubular systems were protected from proteolysis in the neuronal cell aggregates.

Occurrence of corpora amylacea correlates with lack of axonal staining

Using antibodies interacting with nonphosphorylated and phosphorylated neurofilament H, we immuno-stained the lesions with and without corpora amylacea for the presence of demyelinating and non-demyelinating axons and found that in the lesions in which large numbers of corpora were present axonal staining was minimal (Fig. 3A). On the other hand, lesions with preserved axons showed no corpora amylacea (Fig. 3B).

DISCUSSION

In this report we provide for the first time a direct evidence that corpora amylacea represent

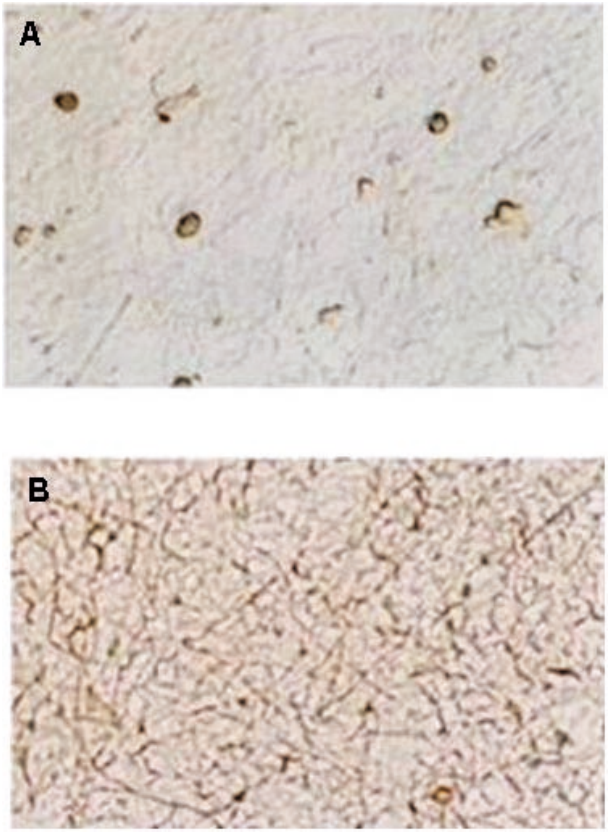

Figure 3. Frozen MS tissue stained with SMI 32 Ab.
A) MS lesion with numerous corpora amylacea, B) MS lesion without corpora amylacea. Note prominent SMI 32 axonal staining in (B), but not in (A). Magnification × 300.

remnants of degenerated and aggregated neuronal cells. This concept is supported by proteomic analysis of precisely collected corpora amylacea that were laser microdissected from multiple sclerosis brains. Sequencing of the major protein spots revealed that they were mostly composed of cytoskelatal proteins that constituted the three major filament systems in neurons, i.e. microtubules, intermediate filaments, and actin microfilaments, forming interwoven scaffolding throughout the cytoplasmic space of the cell. Microtubules support the assembly of cytoskeletal structures of diverse morphology and function. The basic building blocks of all microtubules are heterodimers composed of α- and β-tubulins encoded by multiple gene families. The bulk of cellular tubulin is cytoplasmic, but a significant fraction is embedded in, or firmly associated with, the plasma membrane and other membranes (Kelly *et al.*, 1983). Microtubules are often seen in contact with mitochondria (Smith *et al.*, 1977; Heggeness *et al.*, 1978; Bernier-Valentin *et al.*, 1983), synaptic vesicles (Gray, 1975), endoplasmic reticulum (Vogl, 1996), lysosomes (Mithieux, 1988), cilia (Dentler, 1981), and plasma membranes (Hardham & Gunning, 1978). These features are highly ordered and have a uniformly polarized organization providing great stability. Therefore, in such a state, cytoskeletal components and cellular proteins associated with the cytoskeleton are resistant to proteolytic cleavage, which may explain why several of the proteins found in corpora amylacea were not degraded. Our results are consistent with an earlier concept suggesting that the function of corpora amylacea could be to prevent the recognition of immunogenic proteins (such as classical pathway-specific components, the activation products C3d and the terminal complement complex, the C3 convertase regulator membrane cofactor protein (MCP) and the fluid phase regulators S-protein and clusterin) by lymphocytes and microglia and thus to protect the CNS from further injury (Singhrao *et al.*, 1995).

There was a surprisingly low level of protein within the degenerated white matter surrounding corpora amylacea indicating perhaps that most cellular proteins had accumulated within corpora amylacea leaving only some outside. Furthermore, due to different amounts of α and β tubulin in the degenerated white matter, one may conclude that there was extensive proteolytic degradation around corpora amylacea.

Since in multiple sclerosis tissue both oligodendrocytes and axons are lost (Lassmann *et al.*, 1998), the striking result of the present study is a lack in corpora amylacea of myelin/oligodendrocyte-specific proteins such as myelin basic protein, proteolipid protein, and myelin/oligodendrocyte glycoprotein. This may indicate either that oligodendrocyte proteins were not incorporated into corpora amylacea, or that oligodendrocyte proteins are highly sensitive to early proteolysis. The latter explanation seems plausible since in the tissue surrounding corpora amylacea, myelin/oligodendrocyte proteins were also not detected. The prominent presence of microtubule-related proteins might rather suggest that dying axons provided a major component of corpora amylacea. Loss of axons, although known for many years, was only recently reemphasized in studies on multiple sclerosis (Trapp *et al.*, 1998). If corpora amylacea were to be the tombstones of damaged axons, an important question arises, why do they only accumulate in some MS lesions? This might be related to the degree of axonal loss and/or the mechanisms leading to the axonal injury. Interestingly, when lesions containing different amounts of corpora amylacea were stained for the presence of demyelinated and non-demyelinated axons, there was decreased axonal staining with increased prevalence of corpora amylacea. This observation might support the relationship between corpora amylacea and degenerated axons. The mechanism of axonal transection in MS is not known but several immune pathways could lead to axonal injury. For example,

cytotoxic T cells have been identified in close vicinity to transected axons (Giuliani *et al*., 2003) but other immune mechanisms and glutamate excitotoxicity have also been implicated in axonal loss in multiple sclerosis (Neumann, 2003). Future studies might answer whether corpora amylacea occur in association with any particular pattern of axonal damage.

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