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Amyloid neuropathy: immunocytochemical localization of intra- and extracellular immunoglobulin light chains*

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Summary. Sural nerve specimens from ten patients with amyloidosis (hereditary, associated with lymphoproliferative disorders, or of unknown origin) and peripheral neuropathy were investigated by immunohistochemistry at the light and electron microscopic level. Peroxidase-antiperoxidase and immunogold techniques were applied to glutaraldehyde-fixed, osmicated and epoxy-embedded tissue. In five cases, four of which associated with lymphoproliferative disorders, amyloid deposits strongly and exclusively reacted with antibodies to kappa or lambda light chains, respectively. By electron microscopy, bundles of immunogold-labelled amyloid fibrils could be identified in coated and uncoated single membrane-bound vesicles of endoneurial macrophages. Schwann cells did not contain intracellular amyloid but their processes were entangled in amyloid fibrils and their basement membranes were sometimes fused with the fibrillar masses. It is concluded that immunoglobulin light chains in AL (amyloid of immunoglobulin light chain origin) amyloidosis precipitate, forming amyloid fibrils, in the presence of, and presumably with the assistence of, endoneurial cells. Inefficiency of phagocytosis appears to be one of the major causes for the deleterious effects of amyloid.

Key words: Amyloidosis – Neuropathy – Immunoelectron microscopy – Immunoglobulin light chains – Peripheral nerve

The presence of intracellular amyloid fibrils has long been a matter of debate [3, 38]. In amyloid neuropathy, several authors observed amyloid fibrils in Schwann cells [4, 25, 29], histiocytes and fibroblasts [39]. It has been argued, however, that these fibrils may not have been amyloid fibrils, since their morphological appearance is indistinguishable from intracellular cytoplasmic, i.e., intermediate filaments [3]. More definite evidence of intracellular amyloid was obtained from tissue in experimental amyloidosis, in cell cultures, in human lymph nodes, and bone marrow. Hypotheses about amyloid formation and degradation were derived from these morphological findings ([6–9, 12, 14, 19, 24, 26, 30–34, 39, 45] cf. Table 2). Although immunohistochemical methods have been used to identify and classify amyloid in various tissues [5, 15, 18, 23, 27, 34, 37], including peripheral nerve [20, 22], no immunoelectron microscopic studies have been carried out in human amyloid neuropathy thus far. In the present study, sural nerve specimens from ten patients with peripheral neuropathy and amyloidosis of different types were investigated by immunohistochemistry and immunocytochemistry at the light and electron microscopic level, respectively. With immunogold labelling of immunoglobulin light chains amyloid could be detected in different types of vesicles in endoneurial macrophages. Also, the morphological relationship between immunolabelled amyloid fibrils and Schwann cells could be studied at the electron microscopic level.

Materials and methods

Patients

Clinical data of ten patients with polyneuropathy and amyloidosis are summarized in Table 1. Sural nerve biopsies were performed for diagnostic purposes in patients 1–8 and 10, specimens from case 9 were obtained at autopsy. Specific clinical diagnoses in cases 6–8 were based on bone marrow biopsies and in case 9 on serum immune electrophoresis. Case 1–3 had a positive familiy history and were, therefore, regarded as hereditary amyloidosis.

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Table 1. Clinical data and fine structural findings in the cases studied

Case	Age	Sex	Symptoms and clinical features	Etiology	Neuropathy		$KMnO_4$	Immuno-histo-chemistry
					Axonal	demyelination		
1	32	M	Chronic progressive sensorimotor neuropathy, hypohidrosis, impotence, vomiting	Hereditary	++	–	Resistant	–
2	71	M	Sensorimotor neuropathy, stomach atony, weight loss, vomiting	Hereditary	+++	–	Resistant	–
3	64	M	Chronic progressive sensorimotor neuropathy, reflux esophagitis, gastritis, hypotension	Hereditary	+++	–	Resistant	–
4	51	M	Chronic progressive sensorimotor neuropathy with vegetative disorders	Unknown	+++	–	Resistant	–
5	73	M	Chronic progressive sensorimotor neuropathy, vertigo, syncopes, hypohidrosis	Unknown	++	–	Resistant	–
6	63	M	Chronic progressive sensorimotor neuropathy, macroglossia, cardiomyopathy	Low malignancy lymphoma	++	–	Resistant	–
7	65	M	Predominantly sensory neuropathy, diarrhea, hypotension, syncopes, nocturnal apnoea	Plasmacytoma	+++	–	Resistant	Lambda++
8	61	F	Progressive sensorimotor neuropathy, distally accentuated	Non-Hodgkin lymphoma	++	–	Resistant	Lambda++
9	64	F	Renal failure, cardiomyopathy, diarrhea	IgG-gammopathy	+	–	Resistant	Lambda++
10	67	M	Chronic progressive sensorimotor neuropathy	Unknown	++	–	Resistant	Kappa ++

Standard histological techniques and electron microscopy

Nerve tissue was fixed in 3.9% buffered glutaraldehyde, pH 7.4, embedded in paraffin, sectioned and stained with hematoxylin and eosin and Congo red with and without potassium permanganate pretreatment [40]. Part of each specimen was postfixed in 2% phosphate-buffered osmium tetroxide, and embedded in epoxy resin. Semithin sections were stained with paraphenylenediamine and toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Philips EM 400 T electron microscope.

Immunohistochemical methods

Semithin sections. The unlabelled peroxidase-antiperoxidase (PAP) method [36] was employed on deplastified, deosmicated semithin sections (10% sodium methoxide 20 min, 5% aqueous hydrogen peroxidase 1 min). The following reagents were applied sequentially: (1) Fetal calf serum (FCS, Serva, Heidelberg, FRG) 1:10 in Tris-buffered saline (TBS) for 20 min, (2) primary antibody (rabbit to human IgA, IgE, IgG, IgM, kappa and lamba light chains, complement components C1 and C3, Dako, Hamburg, FRG) 1:500 for 1 h, (3) swine anti-rabbit serum (Dako) 1:50 for 30 min, and (4) rabbit PAP complex (Dako) 1:100 for 30 min. Dilutions of the sera (2) to (4) were made in Tris-HCl saline buffer (0.05 M Tris HCl, 150 mM NaCl) with 2.5% FCS at pH 7.4. For staining, 0.05% 3,3′diaminobenzidine (Sigma, St. Louis, Mo) with 0.01% H_2O_2 was used, sections were counterstained in hemalaun, controls were performed by omission of the primary antibodies.

Ultrathin sections. These were collected on uncoated nickel grids and the indirect immunogold technique was applied following a modification of the methods of Bendayan and Zollinger [1] and Viale et al. [41]: Grids were floated on drops of freshly prepared 4% sodium metaperiodate (Merck, Darmstadt, FRG) for 1 h, rinsed in distilled water and placed on drops of FCS 1:10 in TBS for 30 min. For antibody dilutions and washes, TBS-HCl saline buffer containing 0.5% FCS and 0.02% Tween (Merck), pH 7.4, was used. Sections were incubated in primary antibody (1:500, see above) for 2 h at room temperature or for 20 h at 8°C, then thoroughly rinsed in buffer and placed on drops of a 1:10 solution of gold-labelled goat anti-rabbit serum (particle size 10 nm, Janssen, Olen, Belgium) for 1 h at room temperature. Grids were finally rinsed in TBS and distilled water, dried, and counterstained with uranyl acetate and lead citrate. Control sections were prepared by omission of the primary antibodies.

Results

Standard light and electron microscopy

Amyloid deposits, identified by green birefringence in polarized light, were present in variable amounts in

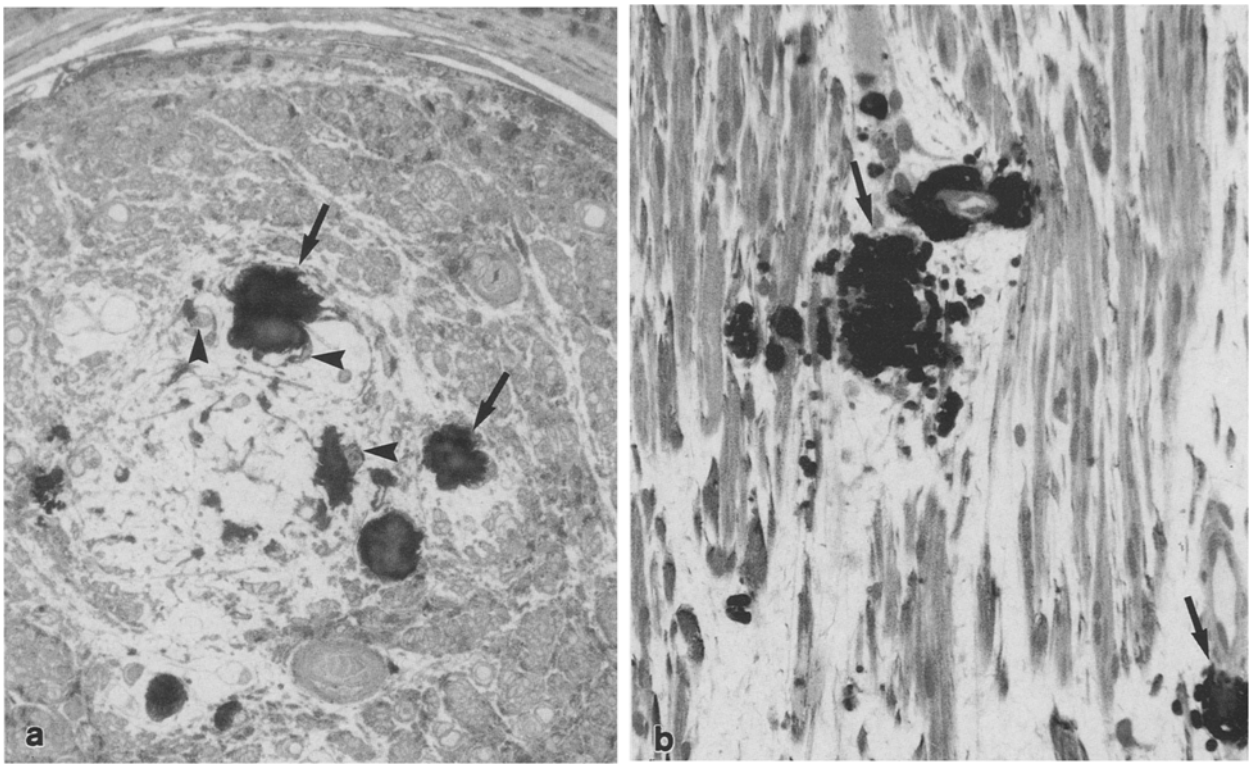

Fig. 1. a Case 7. Amyloid deposits strongly and exclusively reacted with antibodies to lambda light chains (AL amyloid) (*arrows*). Endoneurial cells are in close contact with amyloid plaques (*arrowheads*). **b** Case 10. Amyloid reacted with anti-kappa antibodies (*arrows*). **a** ×430, **b** ×800

the endo-, peri- and epineurium of cases 1–8 and 10, and in the epineurium of case 9. They were located in plaques with or without evidence of a neighboring blood vessel in cross or longitudinal sections (Fig. 1). Amyloid was especially abundant in cases 1, 4, 7 and 10. There was marked (cases 1–8, 10) to moderate (case 9) axonal loss with no evidence of segmental demyelination (Table 1). Single amyloid plaques were located in close vicinity to neighboring nerve fibers. In longitudinal sections, these were seen to be slightly displaced and distorted. Areas with several amyloid plaques were surrounded by a space devoid of nerve fibers, where single endoneurial cells were seen, frequently in close contact with the periphery of the plaques (Fig. 1a).

Affinity for Congo red was not lost after incubation with potassium permanganate, so amyloid proved to be potassium permanganate resistant in all cases.

Electron microscopy revealed reduction of myelinated as well as of unmyelinated fibers. Amyloid deposits, consisting of characteristic 7–10 nm filaments, were of varying morphological appearance, which comprised small aggregates of fibrils, compact fibrillar masses involving collagen fibrils, and bundles of fibrils orientated in parallel, sometimes arranged in a characteristic sunflower shape. Cells in contact with the amyloid plaques were Schwann cells, fibroblasts, perineurial cells and macrophages. Rarely, structures derived from degenerating axons were seen associated with the plaques.

Light microscopic immunohistochemistry

In cases 6 to 9, amyloid deposits strongly and exclusively reacted with antibodies to lambda light chains (Fig. 1a) and could thus be classified as AL-lambda amyloid (AL: amyloid of immunoglobulin light chain origin). In case 10, amyloid was of the AL-kappa type and reacted only with anti-kappa antibodies (Fig. 1b). No reaction occurred with antibodies to the immunoglobulin heavy chains, or to the complement components C 1 and C 3 (not illustrated). In the familial cases (AF-amyloid, case 1–3) (AF: familial amyloidosis) and in two of the cases of unknown etiology (4 and 5), amyloid did not react with any of the antibodies to immunoglobulins.

Electron microscopic immunocytochemistry

The same pattern of reaction as described above was observed on ultrathin sections. There were no im-

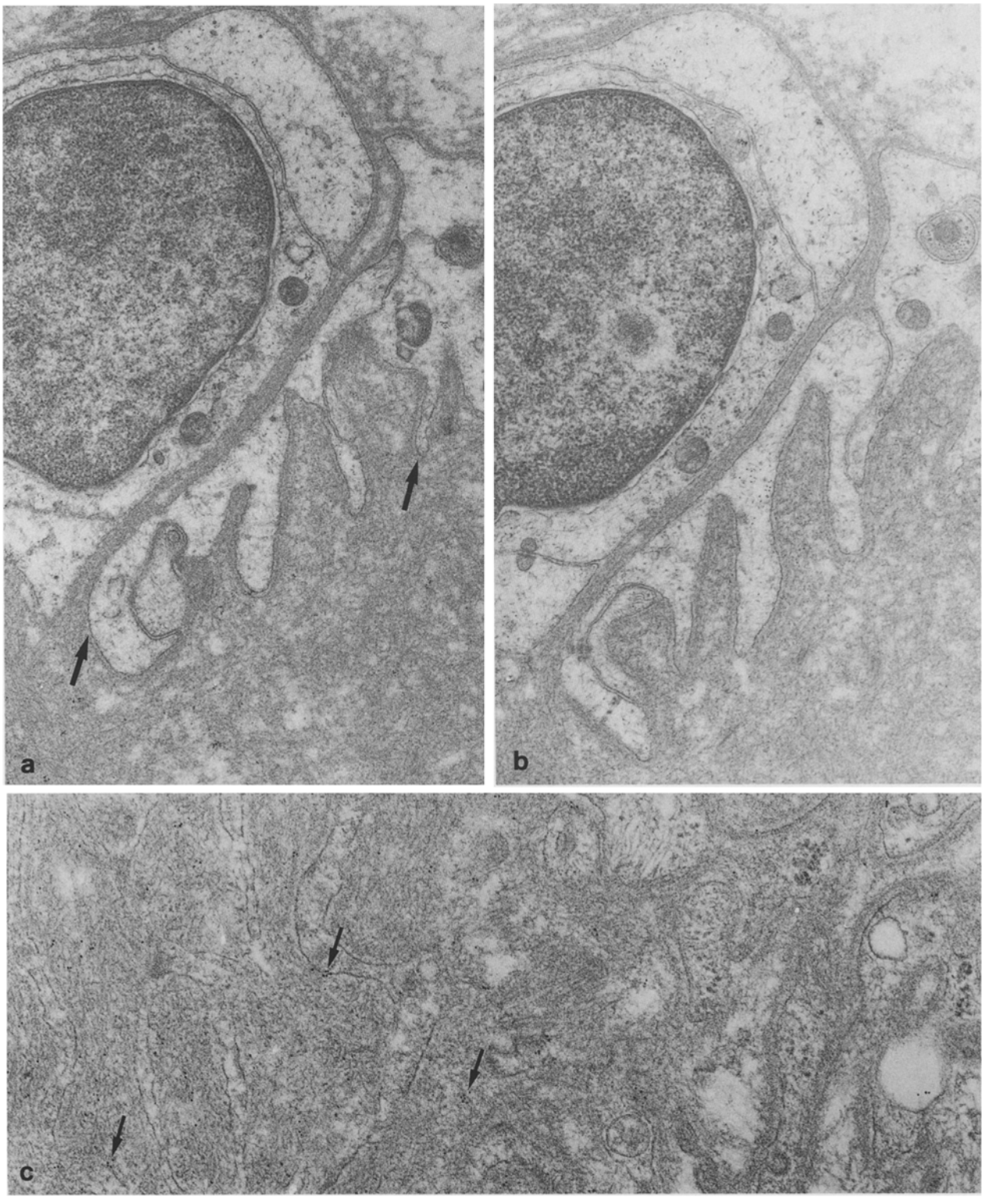

Fig. 2. Processes of Schwann cells presumably from unmyelinated axons are entangled in immunogold-stained amyloid fibrils (AL amyloid), and their basement membranes are fused with the fibrillar masses (*arrows* in **a**). No intracellular amyloid could be detected in Schwann cells. **a, c** Positive immunogold staining (*arrows* in **c**) after incubation with anti-lambda antibodies. **b** Section adjacent to **a**. No immunogold decoration after incubation with anti-kappa antibodies. **a, b** ×21000, **c** ×37000

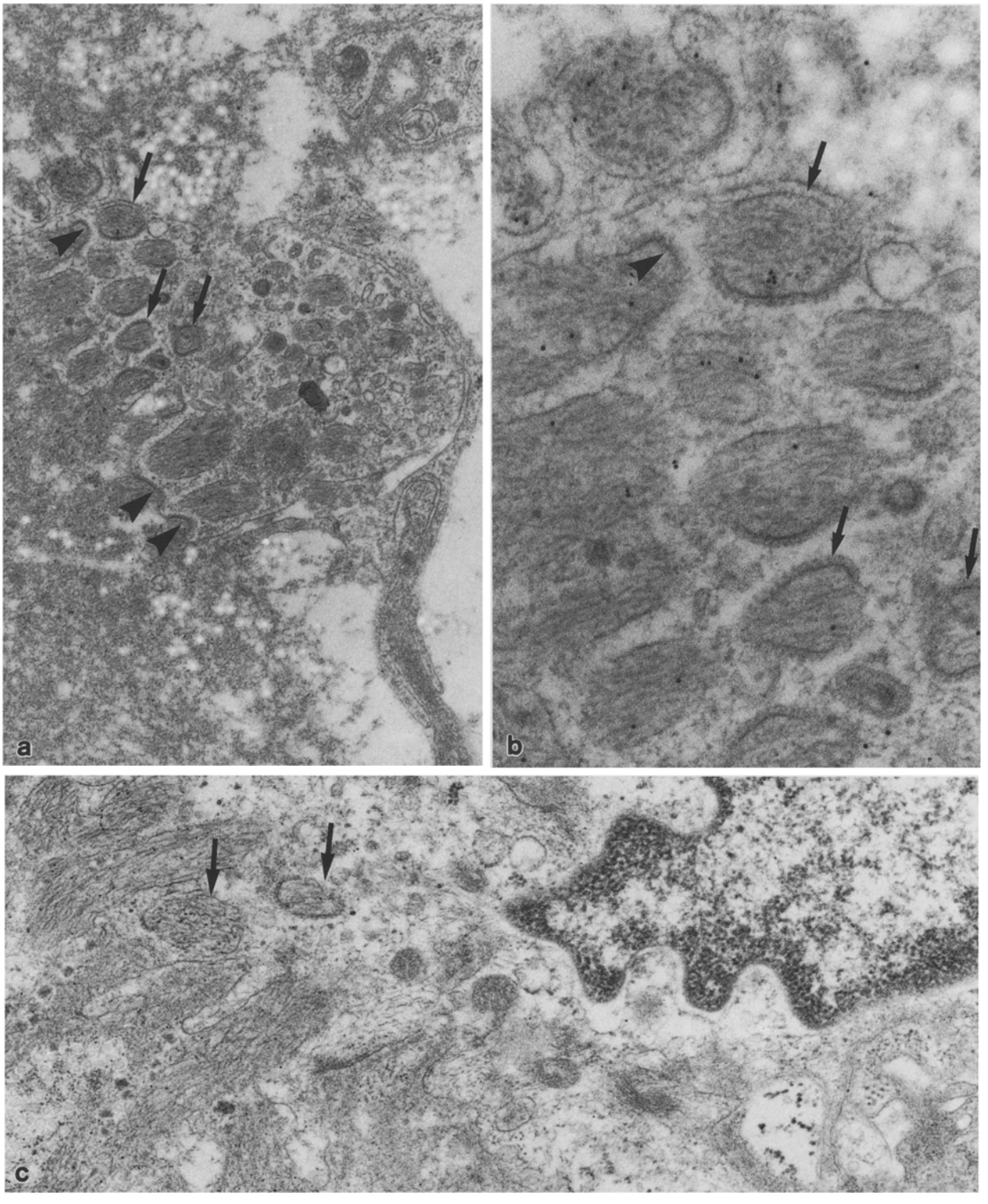

Fig. 3a, b. Macrophages in contact with amyloid show hemidesmosome-like structures (*arrowheads*) as well as coated vesicles (*arrows*), containing bundles of immunogold-stained amyloid fibrils. **a** ×23000, **b** ×77000. **c** Perineurial cells tightly packed with coated and uncoated membrane-bound vesicles containing immunogold-labelled amyloid (*arrows*) are also apparent. ×32000

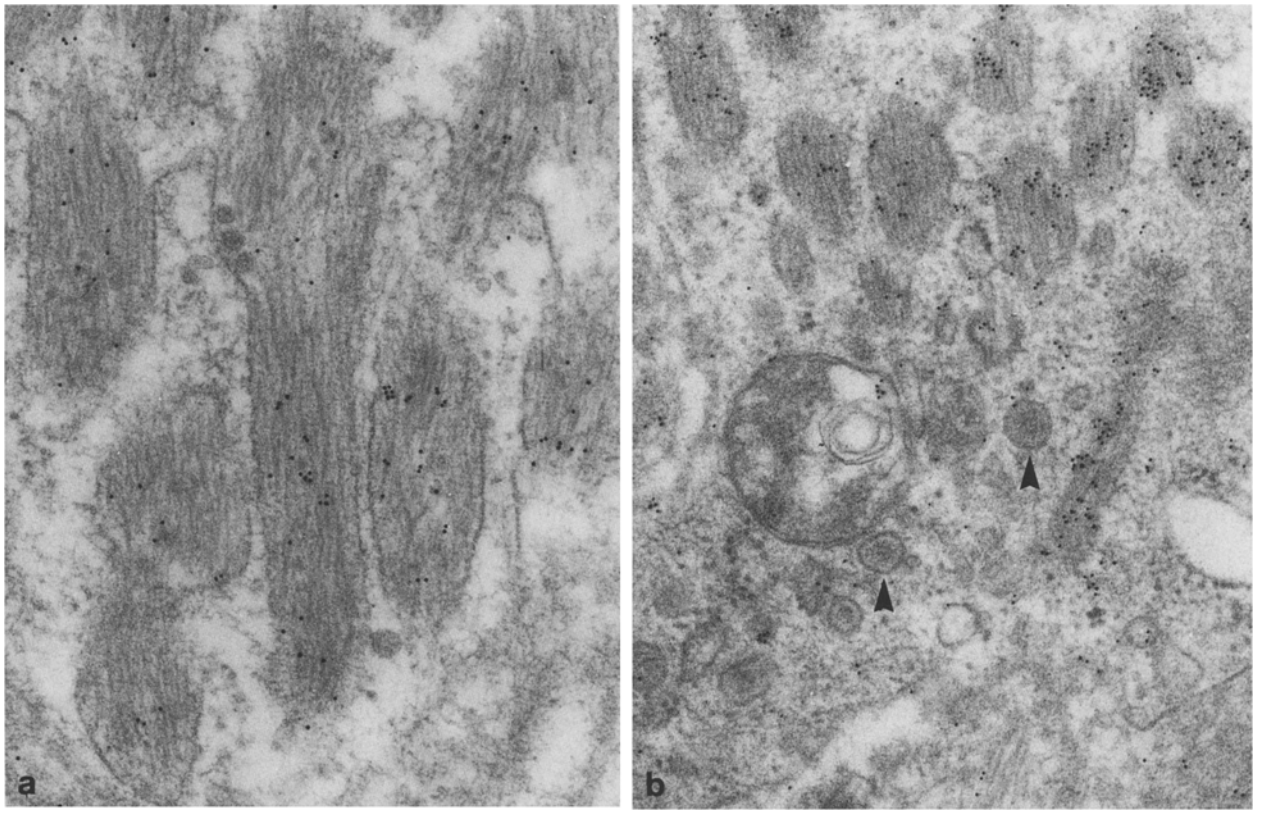

Fig. 4a. In a macrophage bundles of immunolabelled amyloid fibrils are seen deeply indenting the cell membrane. They are enclosed by an uncoated membrane. **b** Occasional smaller, lysosome-like structures (*arrowheads*) occur in the vicinity of the larger vesicles. **a** ×55000, **b** ×44000

munogold deposits on the amyloid plaques of cases 1–5, but intense immunogold decoration of plaques occurred in cases 6–9 after reaction with anti-lambda antibodies and in case 10 after incubation with anti-kappa antibodies.

Adjacent cells were seen in contact with the larger amyloid plaques in four major arrangements:
1. Most frequently, Schwann cell processes were intermingled with amyloid fibrils. The Schwann cell basal lamina was sometimes incorporated into the fibrillar masses (Fig. 2a–c). No intracellular staining of AL-amyloid occurred in Schwann cells. 2. In areas with either very large, or numerous smaller amyloid plaques, cell processes from macrophages (Fig. 3a, b) and modified perineurial cells (Fig. 3c) showed a submembranous electron-dense layer at contact sites with bundles of amyloid fibrils. These fibrils were arranged in parallel, perpendicularly to the cell surface membrane. In the same cells, coated vesicles containing bundles of amyloid fibrils were also seen (Fig. 3a–c). Occasionally, coated and uncoated vesicles, both containing amyloid fibrils, were apparent in one cell. Mostly, cells were so tightly packed with these compartments, that only a thin rim of cytoplasm was remaining in between (Fig. 3a, b). 3. In other cells, bundles of amyloid fibrils were seen, deeply indenting the cell surface membrane. Large vesicles containing amyloid fibrils and enclosed by an uncoated membrane occupied a major portion of the cytoplasm of these cells (Fig. 4a). Occasionally, smaller, lysosome-like structures occurred in the vicinity of the larger vesicles (Fig. 4b). 4. Long, slender processes from modified perineurial cells and fibroblasts, incompletely covered by a basal lamina, partially surrounded amyloid plaques.

Discussion

The present study revealed that kappa and lambda light chains in AL (amyloid of immunoglobulin light chain origin) amyloidosis can be immunostained at the light and electron microscopic level in glutaraldehyde-fixed, osmicated, epoxy-embedded peripheral nerve tissue. While amyloid in five out of ten investigated cases, i.e., in four cases with lymphoproliferative dis-

orders and in one case of unknown etiology proved to be of the AL type, in the three familial cases, as expected, no reaction with antibodies to immunoglobulins occurred. Two of three cases of unknown origin also reacted negatively. These may either represent sporadic cases of AF familial amyloid (AF) amyloidosis, or, although belonging to the AL type, amyloid did not react with the antibodies to light chains employed here. AL amyloid is known to consist of the variable region of light chains (VL), mostly lambda VI and kappa I [23]. Standard antibodies to kappa or lambda light chains may not recognize all possible VL-regions in amyloidosis. Since amyloid was $KMnO_4$ resistent in all cases, secondary amyloidosis (AA = amyloid A), which in a low percentage also causes neuropathy [16], is unlikely to exist in any of the present cases with unknown etiology.

The most important finding of the present study, however, appears to be intracellular amyloid immunoreactive with antibodies to lambda and kappa light chains. In peripheral nerves, only uncoated vesicles and cell membrane indentations containing amyloid have been described before [42, 43]. Similar indentations and vesicles have also been observed in other tissues following experimental amyloidosis in mice and in cell cultures to which amyloid had been added ([12, 14, 24, 33, 45] cf. Table 2). Such intracellular compartments containing amyloid have usually been regarded as the site of amyloid formation [6, 12, 19, 24, 26, 32–34, 39], but sometimes as morphological evidence for amyloid uptake or phagocytosis [44, 45]. An electron-dense layer at the cytoplasmic side of the cell surface membrane and of coated vesicles in cells in contact with amyloid have only been described by Sirahama and Cohen [30] in macrophage cultures. They regarded them as evidence for phagocytosis of amyloid. In contrast to small coated vesicles (50–60 nm in diameter) that are associated with the Golgi complex, these large coated vesicles (about 100 nm in diameter) are known to contain no hydrolases. They have been shown to originate from the cell surface by a process of invagination or pinocytosis [10, 28] and have usually been regarded as phagocytotic and not as excretory structures [10, 30, 44]. Since we observed single membrane-bound uncoated and coated vesicles as well as intermediate types (partly coated vesicles) in close vicinity within the same cell, it appears likely, that they have similar functional properties.

The formation of long cell processes from macrophages and perineurial cells, partly surrounding even larger plaques, may also represent a reaction of the cells to the deposition of amyloid. Morphologically, there is an analogy to filopodia of synovial cells wrapping around extracellular fibrin, followed by endocytosis [10]. Whereas phagocytosed fibrin, however, is effectively degraded, amyloid fibrils appear to be resistant to phagocytosis. This obvious resistance may be one of the reasons why amyloid has such deleterious effects in so many tissues.

Kirkpatrick et al. [17] reported a case of systemic light chain disease where typical light chain deposits occurred in some organs and amyloid deposits in others. They postulate a factor in the extracellular matrix determining the mode in which light chains are deposited. Polymerization of the precursor protein to amyloid in the extracellular space is also assumed by Coimbra and Andrade [4], who emphasize the influence of collagen fibrils and acid mucosubstances, and by Beyreuther et al. [2], who suggest extracellular self-assembly of an enzymatically cleaved presurcor protein to senile amyloid. Fuks and Zucker-Franklin [9] assumed polymerization of AA peptide into amyloid on the surface membrane of Kupffer cells. In vitro studies showed, that partial enzymatic degradation of Bence-Jones-protein is necessary to form amyloid fibrils [11]. One can thus conclude that the presence of cells possessing such enzymes facilitates amyloid formation. The frequent association of cells with small as well as large amyloid plaques noted in the present study supports this hypothesis.

Concerning the pathogenesis of amyloid neuropathy, it was apparent that the neuropathy was always of the neuronal type and severe to very severe in all cases with endoneurial amyloid deposits. Mild axonal alterations only occurred in a case showing exclusively perivascular amyloid in the epineurium. Hanyu et al. [13] in a recent study also observed axonal degeneration in sural nerves from three cases of familial amyloidosis, whereas segmental demyelination occurred in more proximal nerves. The distortion of nerve fibers observed in longitudinal sections, compatible with earlier findings of Dyck and Lambert [7] and Said et al. [29], and the absence of nerve fibers in a considerable area around large plaques appear to imply an injurious influence of amyloid on nerve fibers. Coimbra and Andrade [4] found severe neuropathy in their cases of early amyloidosis, i.e., in cases with only scarce endoneurial amyloid deposits. They conclude that damage to nerve fibers takes place prior to amyloid deposition. However, Krücke [21] considers amyloid deposits in spinal ganglia as the main cause of amyloid polyneuropathy. This is consistant with the present observation that there was virtually no regeneration of nerve fibers. Hanyu et al. [13], however, found spinal nerve roots unaffected in their cases of familial amyloid neuropathy.

The absence of complement components in and around endoneurial amyloid plaques, noted in the present study, appears to exclude the involvement of antigen-antibody reactions. Endoneurial blood ves-

Table 2. Review of literature on interactions between amyloid and neighboring cells

Authors	Tissue	Observations	Interpretation
Gueft and Ghidoni [12]	Mouse spleen casein amyloidosis	Amyloid in deep cell identations, in contact with nuclear membrane	Production of amyloid by macrophages arranged in a circle, excreting amyloid to their center, thus forming an "amyloid star"
Shirahama and Cohen [30]	Cell culture: macrophages and amyloid	Hemidesmosome-like structures, amiloid in coated vesicles	Phagocytosis of amyloid in coated vesicles
Zucker-Franklin [45]	Cell culture: leucocytes and amyloid	Amyloid in single membrane-bound vesicles in leucocytes only after addition of antiserum	Phagocytosis of amyloid in leucocytes only after opsonization
Shirahama and Cohen [31]	Cell culture: macrophages and amyloid	Large vesicles in contact with lysosomes, amyloid fibrils in different stages of organization	Degradation of amyloid in macrophages with lysosomal enzymes
Coimbra and Andrade [4]	Human peripheral nerve	Pericollagenous amyloid, invading Schwann cell basal laminae and cytoplasm	Amyloid formation in a matrix of collagen fibrils and acid mucosubstances
Franklin and Zucker-Franklin [8]	Human lymph node	Bundles of amyloid fibrils in plasma cells	"Fatigue phenomenon": intracellular fibrils only occur in heavily infiltrated organs
Shirahama and Cohen [32]	Mouse liver, casein amyloidosis	Macrophages with amyloid in vesicles in contact with lysosomes	Amyloid formation in macrophages
Shirahama and Cohen [33]	Mouse liver, casein amyloidosis	Amyloid in cell invaginations and in vesicles in contact with lysosomes	Intralysosomal formation of amyloid fibrils
Kjeldsberg et al. [19]	Human amyloidotic tissue	Amyloid fibrils in the Golgi complex of plasma cells, histiocytes, tubulus cells, hepatocytes	Amyloid formation in the Golgi complex and lysosomes
Neundörfer et al. [25]	Human peripheral nerve	Amyloid fibrils in Schwann cell cytoplasm	Damage to Schwann cells leading to demyelination and axonal degeneration
Durie et al. [6]	Myeloma cell culture	Amyloid in indentations of macrophages	Partial degradation of light chains in macrophages, subsequent formation of amyloid
Numura et al. [26]	Human myeloma	Amyloid fibrils in myeloma cells in vicinity of lysosomes	Formation of amyloid fibrils
Nakagawa [24]	Human amyloidotic tissue	Bundles of amyloid fibrils in membrane indentations, three types of cell membrane changes	Various cell types are able to produce amyloid
Said et al. [29]	Human peripheral nerve	Amyloid plaques distorting myelin sheaths, fibrils invading Schwann cell basal lamina and cytoplasm, degenerative changes in fibroblasts	Mechanical and metabolic or toxic effect of amyloid to nerve fibers
Fuks and Zucker-Franklin [9]	Mouse liver casein amyloidosis	1. Amyloid fibrils in Kupffer cell vacuoles. 2. Amyloid fibrils in Kupffer cell membrane indentations	1. Phagocytosis of amyloid fibrils. 2. Possible formation of amyloid from precursors
Hikita et al. [14]	Brain of Creutzfeld-Jakob mice	Amyloid plaques surrounded by macrophages, amyloid fibrils in indentations of macrophages	Amyloid precursor is taken up by macrophages, which produce amyloid and whose cytoplasm breaks when too much amyloid is produced
Shirahama et al. [35]	Mouse liver casein amyloidosis	AA reactive substance in the rough endoplasmic reticulum (RER), Golgi apparatus, cell membrane and microvilli of hepatocytes	Amyloid precursor is produced in hepatocytes and follows the common routes of synthesis established for other proteins
Uchino et al. [39]	Mouse liver, casein amyloidosis	Amyloid precursor in the RER, Golgi complex and secretory granules of hepatocytes and amyloid fibrils in invaginations of Kupffer cells	Production of amyloid precursor in hepatocytes and formation of amyloid fibrils in Kupffer cells by lysosomal enzymes

(continued overleaf)

Table 2 (continued)

Authors	Tissue	Observations	Interpretation
Westermark [44]	Human islets of Langerhans	Bundles of fibrils in indentations of β-cells	Amyloid fibrils penetrating cell
Vital and Vallat [43]	Human peripheral nerve	Amyloid fibrils invading Schwann cell basal laminae, occasionally cytoplasm, bundles of amyloid fibrils in the ER of hystiocytes	Secretion of amyloid

sels, even when surrounded by amyloid, were not obstructed, which is considered as evidence against an ischemic etiology of the neuropathy. Nevertheless, direct mechanical compression remains to be considered, as well as nutritional or toxic effects of amyloid in the nerve trunk or spinal ganglia. The occurrence of amyloid fibrils in close association to Schwann cells, invading their basal lamina, favors the latter hypothesis. It is of interest in this context that the P-component of amyloid is also frequently seen in basement membranes [35].

In conclusion, this study has shown intracellular amyloid by electron microscopic imunocytochemistry in cases of human amyloid neuropathy. It is suggested that endoneurial and perineurial cells influence amyloid formation by enzymatic cleavage of light chains while amyloid degradation by phagocytotic uptake remains inefficient. This inefficiency of phagocytosis appears to be one of the major causes for the deleterious effects of amyloid on various tissues.

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