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Immunocytochemical Studies of Serum Proteins and Immunoglobulins in Human Sural Nerve Biopsies

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Summary. Post-embedding immunocytochemical studies on immunoglobulins (Ig) and other serum proteins were carried out on 38 human sural nerve biopsies using the PAP method. In addition to toxic, hereditary, metabolic, dysproteinemic, and vasculitic-neuritic neuropathies, morphologically normal sural nerves were included as controls. The intensity of the immunocytochemical reactions was strong for proteins, such as IgG, the light chains of Igs, and albumin, but weak or absent for others like complement component C3, IgA, ceruloplasmin, and alpha-1-antitrypsin (AAT) in normal nerve biopsies and in all pathologic groups. IgG, the light chains of immunoglobulins, and albumin could readily be detected in perineurium, endoneurial interstitium, and blood vessel walls. IgM, C3, and beta-lipoprotein (BLP) were largely confined to the walls of blood vessels and perineurium, thus indicating that they do not penetrate the blood nerve barrier. Only in a few cases, in vasculitic-neuritic and dysproteinemic neuropathies, staining of the endoneurial interstitium for IgM and C3 was observed. Increased staining for the corresponding heavy or light chains was not detected in the endoneurium in any of the neuropathies associated with gammopathy.

The results stress that PAP immunocytochemistry is suitable for studying the blood-nerve barrier (BNB) and provides new aspects to the concept of the BNB with respect to the steady state of serum proteins between endoneurial and vascular spaces. It is suggested that, in addition to serum concentration and molecular weight of serum proteins, the permeability of the BNB is influenced by other yet undefined factors.

Key words: PAP immunocytochemistry – Immunoglobulins – Serum proteins – Sural nerve biopsies – Blood-nerve barrier (BNB)

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Introduction

The endoneurial space of peripheral nerves is generally thought to constitute a special biologic compartment. This compartment is anatomically defined and delineated by the perineurium and the endoneurial endothelial cells which lack fenestrae but exhibit tight junctions (Olsson 1975). Its fluid, electrolyte, and protein composition is determined by both the blood nerve barrier (BNB) and the selective permeability of the axonal membranes (Myers et al. 1983). In this context, the term barrier describes the dynamic steady-state equilibrium of hydrophilic substances between intravascular and endoneurial fluid compartments under the condition of constant permeability and flow (Felgenhauer 1980). The endoneurial fluid plays a vital role in nerve conduction as a sink for axonal electrolyte exchange necessary for the propagation of action potentials (Myers et al. 1983). Therefore, disturbance of the BNB that lead to deviations of the normal protein composition may alter the physicochemical properties of the endoneurial fluid and thus affect the function of peripheral nerves. Little is known, however, about the role of the BNB in human peripheral nerve disorders. The presence and distribution of serum proteins in human sural nerve biopsies was studied with immunofluorescence techniques (Van Lis and Jennekens 1977). All proteins were demonstrated in the nerves. There seemed to be a correlation between the intensity of the endoneurial fluorescence and the molecular weight of the corresponding proteins. In pathologically altered nerves proteins of higher molecular weight (MW) tended to accumulate at the site of the perineurium (Van Lis and Jennekens 1977). In peripheral neuropathies that are believed to be immunologically mediated, i.e., Guillain-Barré syndrome (Luijten et al. 1972), chronic relapsing polyneuropathy (Dalakas and Engel 1980), and neuropathies associated with macroglobulinemia (Chazot et al. 1976; Iwashita et al. 1974; Propp et al. 1975; Swash et al. 1979; Smith et al. 1983) or

Table 1. Clinical and neuropathologic diagnosis: Synopsis

NP-No.	Age (yr)	Sex	Clinical diagnosis	Principal neuropathologic findings	Pathology[a]
Controls					
562/81	50	f	Muscular dystrophy	Normal	
563/81	60	f	Motor neuron disease	Normal	
118/82	31	m	Motor neuron disease	Normal	
230/82	13	m	Progressive degenerative CNS disease of unknown type	Normal	
326/82	41	m	Motor neuron disease	Normal	
404/82	32	f	Motor neuron disease	Normal	
1/83	38	f	Motor neuron disease	Normal	
72/83	42	m	Progressive tetraparesis	Normal	
S174/81	20	m	Road traffic accident	Normal	
S123/83	59	m	Road traffic accident	Normal	
Dysproteinemic neuropathies					
484/80	38	f	IgG dysproteinemia accompanying polyneuropathy	Demyelination	2
325/82	60	m	IgA-lambda gammopathy accompanying polyneuropathy	Demyelination	3
424/82	65	f	IgA-kappa monoclonal gammopathy accompanying polyneuropathy (plasmocytoma)	Demyelination, fibrosis, remyelination	2
78/83	65	m	IgM-lambda paraproteinemia (plasmocytoma), polyneuropathy	Chronic degenerative amyloid neuropathy	3
Neuritic-vasculitic neuropathies					
340/82	69	f	Polyneuropathy	Vasculitic neuropathy	2
373/82	19	m	Tibialis anterior syndrome	Vasculitic neuropathy	1
413/82	43	f	Relapsing (axonal) polyneuropathy	Vasculitic neuropathy	1
464/82	67	f	Polyneuropathy or motor neuron disease	Neuritis and vasculitis	3
628/82	74	f	Hypothyroidism, cryoglobulinemia	Vascular neuropathy	2
Toxic, hereditary and metabolic neuropathies					
487/81	18	m	Polyneuropathy or spinal muscular atrophy	Axonal degeneration	1
504/81	24	m	Polyneuropathy	Axonal degeneration	1
512/81	47	m	Polyneuropathy	Endstage degenerative neuropathy	3
15/82	28	m	HSMN II	Axonal degeneration	2
23/82	62	m	Polyneuropathy	Chronic demyelination, remyelination, fibrosis	3
24/82	17	f	Polyneuropathy	Hypertrophic neuropathy	3
82/82	40	m	HSMN I	Axonal degeneration	3
111/82	69	m	System degeneration of CNS and PNS of unknown type	Axonal degeneration and demyelination	1
119/82	60	m	Motor polyneuropathy	Progressive axonal degeneration and demyelination	1
175/82	26	m	Recurrent plexus brachialis paresis	Tomacolous neuropathy	
177/82	16	f	Metachromatic leukodystrophy	Metachromatic leukodystrophy	2
198/82	22	f	HSMN I	Hypertrophic neuropathy	3
224/82	46	f	HSMN I	Hypertrophic neuropathy	3
339/82	43	m	Relapsing polyneuropathy	Chronic progressive demyelination	3
341/82	39	m	Adrenomyeloneuropathy	Adrenomyeloneuropathy	1
402/82	76	m	Motor polyneuropathy	Progressive axonal degeneration and demyelination	2
426/82	42	m	HSMN II	Demyelination	2
648/82	62	m	Toxic myopathy and neuropathy	Axonal degeneration	1
77/83	51	f	Multiple sclerosis	Axonal degeneration and demyelination	1

[a] Degree of neuropathologic changes: 1 slight; 2 moderate; 3 strong

monoclonal gammopathies (Dalakas and Engel 1981), endoneurial immunoglobulin depositon were demonstrated using conventional immunofluorescence techniques. More recently, unlabeled antibody enzyme methods were successfully applied for the identification of IgM in sural nerves from patients with monoclonal gammopathies or paraproteinemias associated with neuropathy (Abrams et al. 1982; Meier et al.

Table 2. Investigated plasma proteins

	Molecular weight (MW)	Hydrodynamic radius (Å)	Serum concentration (g/l)	Working dilutions of primary antibodies
$LC_{K/L}$	22,000	12.2	–	1:50,000
AAT	45,000	22–27	2.4–2.9	1: 5,000
Albumin	66,000	35.8	35–55	1:50,000
IgG	140,000	53.4	7.5–24	1:20,000
IgA	160,000	56.8	0.5–2.2	1: 5,000
Ceruloplasmin	160,000	46.8	0.15–0.6	1: 5,000
C3	180,000		1.4–1.8	1: 1,000
IgM	900,000	121	0.6–2.8	1:10,000
BLP	2,500,000	124	2.5–3.0	1: 1,000

$LC_{K/L}$: kappa- and lambda-type light chains
AAT: alpha-1-antitrypsin
C3: complement component C3
BLP: beta-lipoprotein

1983). Yet, the pathogenic implications of immunoglobulin demonstration in peripheral nerves are still unclear.

In this study, the presence and distribution of immunoglobulins and other serum proteins in sural nerve biopsies of patients with various neuropathies were investigated using the unlabeled antibody enzyme peroxidase-antiperoxidase (PAP) method (Sternberger 1979). This technique provides higher sensitivity and specificity than immunofluorescence techniques; it can be applied to formalin-fixed, paraffin-embedded tissue and may therefore be performed in biopsies that were taken many years previously, and preparations are permanent.

Material and Methods

Sural nerve biopsies from 36 patients with different neurologic diseases were investigated (Table 1). The nerves were neuropathologically classified on the basis of conventional light- and electron-microscopic evaluation. Included were 19 toxic, metabolic, and hereditary neuropathies, four neuropathies associated with gammopathy, and five neuritic-vasculitic neuropathies. Eight normal sural nerves taken from patients with motor neuron disease (five cases), cerebral disorders (two cases), and muscular dystrophy (one case), and two sural nerves from patients with lethal road traffic accidents served as controls. For the immunocytochemical studies the nerves were immediately fixed with 4% buffered formaldehyde for 24–48 h and embedded in paraffin. Serial sections, 3 µm thick, were prepared with glass knives and stained for immunoglobulins and other serum proteins with a modified PAP technique (Mepham et al. 1979). As primary reagents, commercially available polyvalent rabbit-antibodies against human IgG, IgA, IgM, kappa, and lambda type light chains, albumin, ceruloplasmin, alpha-1-antitrypsin, beta-lipoprotein (Dakopatts), and complement component C3 (Cappel Labs., antibody from goat) were used. The optimal dilutions employed are depicted in Table 2. As secondary antibodies, swine anti-rabbit or rabbit-anti goat immunoglobulins (Dakopatts) diluted 1:50 were used followed by PAP complexes from rabbit or goat (Dakopatts), diluted 1:200.

For immunocytochemical controls the primary antibodies against human IgG, IgM, and albumin were preabsorbed with purified human IgG, IgM, and albumin, respectively. In addition, in some cases $F(ab')_2$ fragments of rabbit antibodies to human IgG, IgM, and albumin (Cappel Labs.) were used to prevent non-specific binding by Fc-receptors. Before mounting, sections were counterstained with hematoxylin.

Table 3. Number of sural nerve biopsies with positive immunocytochemical staining

Primary reagent against	Perineurium	Vessel walls	Endoneurial interstitium
A. controls (10 cases)			
$LC_{K/L}$	9	10	9
AAT	0	2	0
Albumin	10	10	10
IgG	9	10	8
IgA	3	6	0
Ceruloplasmin	3	6	1
C3	6	10	2
IgM	7	10	3
BLP	9	10	4
B. Toxic, metabolic, and hereditary neuropathies (19 cases)			
$LC_{K/L}$	19	19	19
AAT	0	4	0
Albumin	19	19	19
IgG	19	19	19
IgA	5	13	0
Ceruloplasmin	9	14	0
C3	15	19	4
IgM	17	19	4
BLP	18	18	6
C. Dysproteinemic neuropathies (4 cases)			
$LC_{K/L}$	4	4	4
AAT	0	3	0
Albumin	4	4	4
IgG	4	4	4
IgA	1	4	1
Ceruloplasmin	2	3	0
C3	3	4	2
IgM	4	4	3
BLP	4	4	3
D. Vasculitic and neuritic neuropathies (5 cases)			
$LC_{K/L}$	5	5	5
AAT	0	3	0
Albumin	5	5	5
IgG	5	5	5
IgA	1	3	2
Ceruloplasmin	1	4	0
C3	5	5	2
IgM	5	5	2
BLP	4	5	2

$LC_{K/L}$: kappa- and lambda-type light chains
AAT: alpha-1-antitrypsin
C3: complement component C3
BLP: beta-lipoprotein

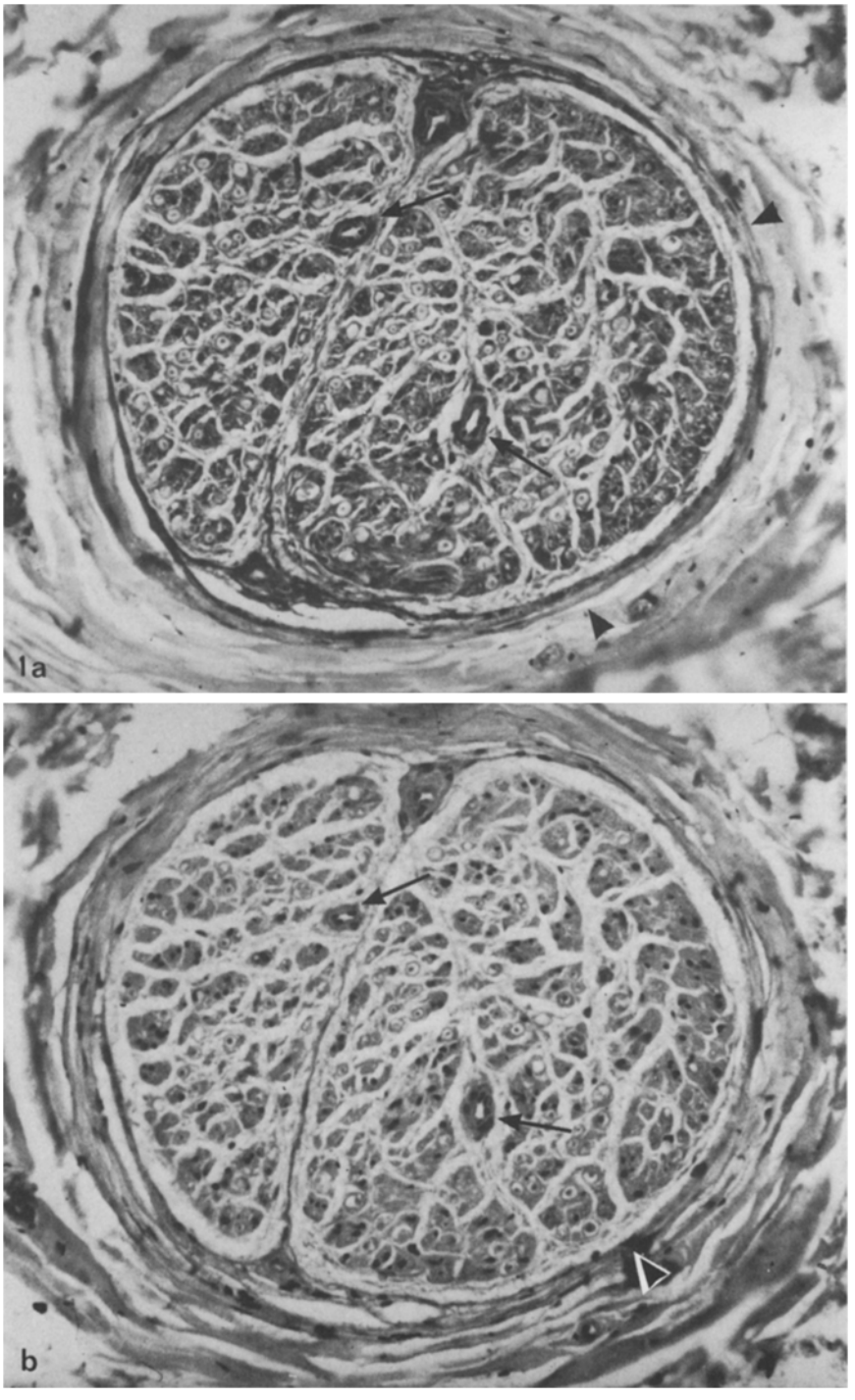

Fig. 1 a, b. Dysproteinemic neuropathy (NP 78/83). Endoneurial vessels (*arrow*), perineurium (*arrowhead*), and endoneurial interstitium stain strongly for IgG (**a**). Reaction for IgA is identified in walls of endoneurial vessels (**b**)

Results

The immunocytochemical staining was evaluated according to intensity and location and graded subjectively. The pattern of the immunocytochemical reaction varied for each protein and between the different groups of biopsies, but the patterns were not related to the degree of the neuropathologic changes within each group (Table 3). The intensity of the immunocytochemical reaction was increased in pathologic nerves. Strong staining was achieved for IgG, albumin, and the light chains of Igs, whereas the reaction for IgM, IgA, and the other proteins was weak or absent (Fig. 1). The most intense staining of all proteins was consistently seen in the lumina and walls of endo- and epineurial vessels (Fig. 2). The epineural

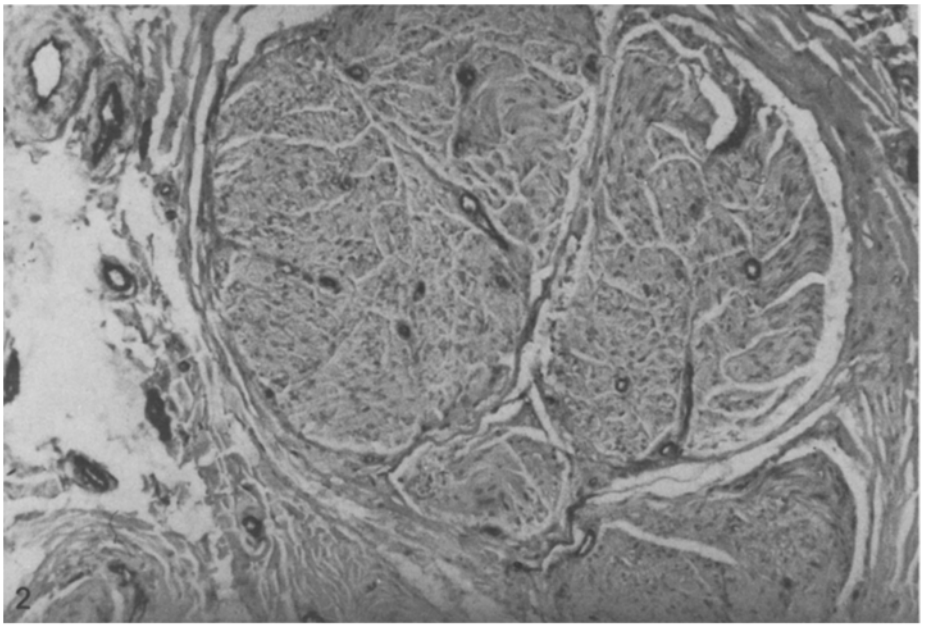

Fig. 2. Demyelinating neuropathy (NP 426/82). Anti-beta-lipoprotein as primary antibody. Reaction is positive in the walls of endoneurial and epineurial vessels. Epineurial connective tissue and endoneurial interstitium remain unstained

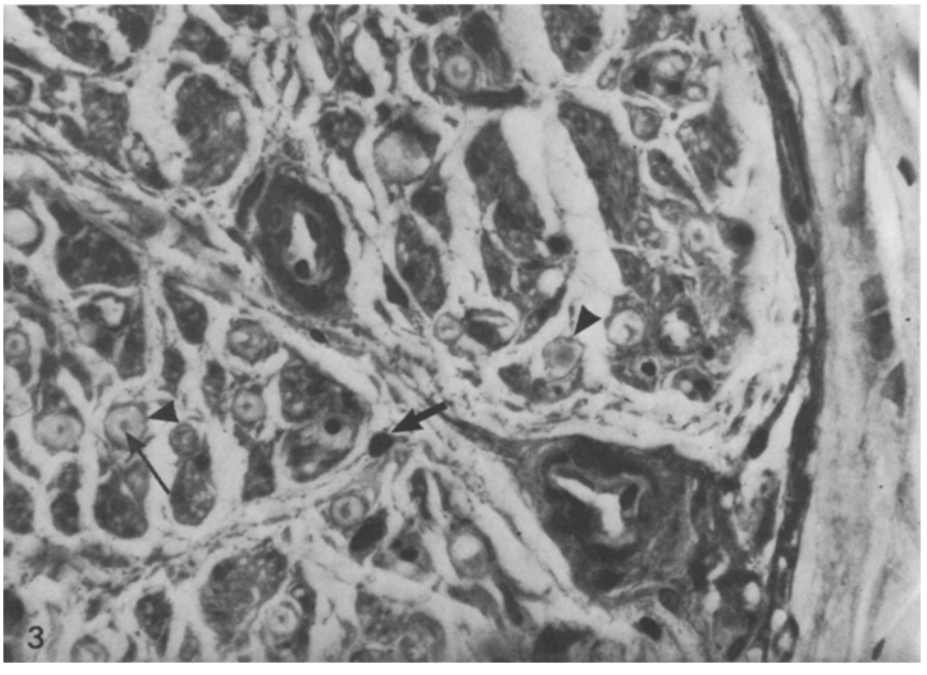

Fig. 3. Dysproteinemic neuropathy (NP 78/83). Myelin sheaths (*arrowhead*) exhibit no positive immunocytochemical reaction for IgG in contrast to the endoneurial interstitium. Shrunken axons (*small arrow*) and cellular nuclei (*large arrow*) appear dark due to counterstaining with hematoxylin

connective tissue rendered only faint specific immunocytochemical staining. In the perineurium the immunocytochemical reaction for IgG, albumin, and the light chains of the Igs was equal or slightly more intense than in the endoneurium. Albumin and the light chains were identified in the full thickness of the perineurium of all but one biopsy in which the light chains were not detected in the perineurium (Table 3). IgG and the complement component C3 were localized either in all layers of the perineurium or predominantly on its inner aspects. Proteins of higher molecular weight, such as IgM and beta-lipoprotein (BLP) were visualized primarily at the inner site of the perineurium (Fig. 4). The endoneurial interstitium was stained in most biopsies for the kappa and lambda type light chains, IgG, and albumin (Table 3) often displaying distinct circles of increased intensity around myelinated nerve fibers (Fig. 3). Myelin sheaths were not stained. The unmyelinated nerve fibres were difficult to assess. Some axons appeared unstained, while other apparently shrunken axons were densely stained. IgA, ceruloplasmin, and alpha-1-antitrypsin

Table 4. Distribution of serum proteins in sural nerve biopsies

Diagnosis	Location	$LC_{K/L}$	Albumin	AAT	IgG	IgA	Cerulo-plasmin	C3	IgM	BLP
Controls	PN	2	2	0	2	0	0	1	1	1
	V	3	3	1	3	1	1	2	2	2
	EN	1	1	0	1	0	0	0	0	0
Toxic, hereditary, and metabolic neuropathy	PN	2	2	0	2	0	0	1	1	1
	V	3	2	1	3	1	1	2	2	2
	EN	2	2	0[a]	2	0	0	0	0	0
Dysproteinemic neuropathy	PN	2	2	0	2	0	1	2	2	1
	V	3	3	1	3	1	1	2	2	2
	EN	2	2	0[a]	2	0	0	1	1	0
Vasculitic-neuritic neuropathy	PN	2	2	0	2	0	0	1	2	1
	V	3	3	1	3	1	1	2	2	2
	EN	3	2	0[a]	3	1	0	1	2	1

PN, perineurium; V, walls of endoneurial and epineurial vessels; EN, endoneurial interstitium; [a] staining of interstitial phagocytes; $LC_{K/L}$, kappa- and lambda-type light chains; AAT, alpha-1-antitrypsin; C3, complement component C3; BLP, beta lipoprotein
Intensity of immunocytochemical staining: 0 negative; 1 weak; 2 moderate; 3 strong

(AAT) were preferentially localized at endoneurial and epineurial blood vessels (Table 4); occasionally, faint reaction products were detected at the perineurium on the inner or outer layers. AAT was additionally localized in phagocytes which also stained for IgG and the light chains of immunoglobulins in serial sections.

In the three cases of neuropathies associated with IgA or IgM gammopathy no increased staining for the heavy chain of the corresponding immunoglobulin was visualized in the endoneurial interstitium. Especially, the myelin sheaths remained unstained. More pronounced interstitial staining and even slight interfascicular differences in the intensity of immunocytochemical reactions were observed in neuritic-vasculitic neuropathies (Table 4). Some fascicles revealed positive reaction products for IgA, IgM, and BLP, while adjoining fascicles displayed no staining for these proteins (Fig. 5). This observation was not correlated wih the extent of the inflammatory change. Single cells constituting the cellular infiltrates were reactive for IgG.

The described immunocytochemical staining patterns for IgG, IgM, and albumin were substantially identical when $F(ab')_2$ fragments of the respective antibodies were used. The various immunocytochemical controls with preabsorbed antibodies rendered no specific reaction.

Discussion

The high specificity and sensitivity of the unlabeled enzyme-antibody (peroxidase-antiperoxidase, PAP) method has opened a new approach for identification of various agents in formalin-fixed, paraffin-embedded tissue (Sternberger 1979). With immunohistological methods the distribution of serum proteins and immunoglobulins was visualized in various organs, such as skeletal muscle (Seitz et al. 1983) and in perfusion-fixed peripheral nerves from mice and monkeys (Liebert et al. 1983; Heininger et al. 1984).

In this immunocytochemical study, the influence of the BNB of human sural nerve on the access of serum proteins to the endoneurial space was investigated. Igs and other serum proteins are suitable endogenous markers of blood tissue barriers (Felgenhauer 1980). IgG, kappa, and lambda type light chains of immunoglobulins and albumin have readily been demonstrated in the perineurial and the endoneurial connective tissue of normal and pathologic sural nerves, while proteins of high molecular weight like IgM and beta-lipoprotein were restricted primarily to the inner site of the perineurium. In inflammatory neuropathies, however, IgM, BLP, IgA, and C3 were detected in moderate amounts in the endoneurium in addition to IgG and albumin. In the neuropathies associated with gammopathies that are believed to be at least in part mediated by humoral immune mechanisms the dysglobulins were not localized within the myelin sheaths or in the endoneurium. Stefansson et al. (1983) obtained similar results when using the avidin biotin complex (ABC) method which is even more sensitive than the PAP technique (Hsu et al. 1981), while by immunofluorescence the dysglobulin was readily identified within the endoneurium of a nerve from a patient with neuropathy accompanying IgM-lambda monoclonal gammopathy.

The distinct patterns of immunocytochemical staining in the present study are evidently not due to non-specific binding of immunoglobulin molecules to

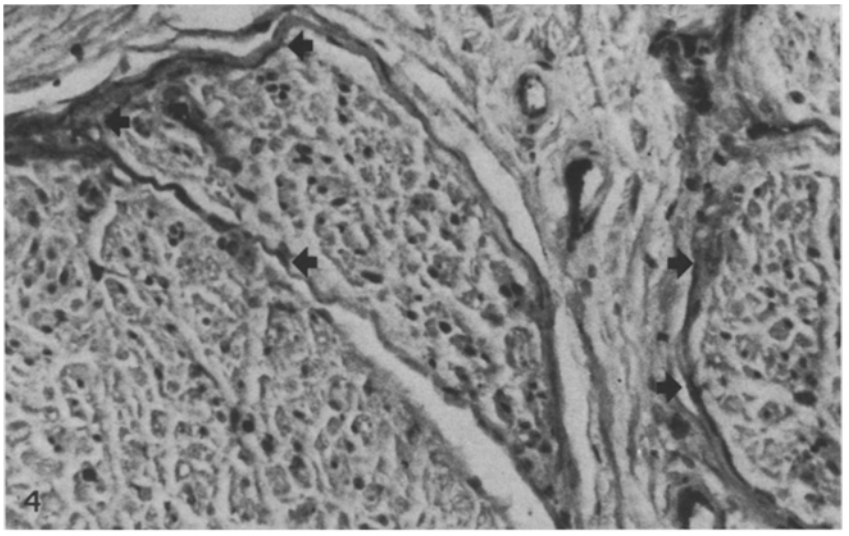

Fig. 4. Vasculitic neuropathy (NP 628/82). Restriction of positive reaction products for IgM to the perineurium (*arrow*) and small endoneurial vessels

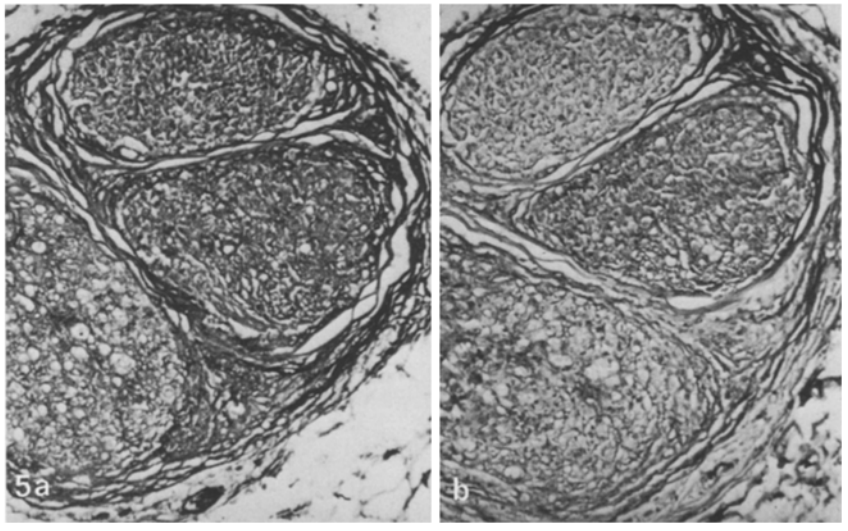

Fig. 5a, b. Vasculitic neuropathy (NP 340/82). Immunocytochemical reaction with anti-IgG (**a**) and anti-IgM (**b**) antibodies. The three fascicles stain with anti-IgG antibodies but to a varying extent. Staining with anti-IgM is much weaker

basic groups of collagen by F_c-receptors (Weston and Poole 1973; Bosman et al. 1977) since the epineurial connective tissue was not stained, and the pattern of endoneurial and vascular immunocytochemical reaction was not substantially changed by using $F(ab')_2$ fragments of antibodies against Igs and albumin.

In the present series, C3 was inconsistently demonstrated in the endoneurial space and the perineurium. The reaction, however, was weak. This is comparable to the finding that, when using the PAP method, C3 is easily visualized in formalin-fixed sections of kidney from NZB mice but in much weaker intensity than would be anticipated from the immunofluorescence findings on cryostat sections (Liebert and Schwendemann, unpublished data). Tissue fixation and processing conceivably render the antigenic determinants of this protein liable to some alteration so that just small amounts remain detectable even to most sensitive techniques. Therefore, the demonstration of C3 within the endoneurium remains of uncertain significance.

Obviously, the immunocytochemical pattern for serum proteins and immunoglobulins found in the control group of nerve biopsies reflects their physiologic distribution. Albumin and IgG constitute the predominant proteins in the interstitial fluid of the nerve at the moment of fixation and embedding (Burns et al. 1978). Recently, the protein content of endoneurially enriched fluid was determined, but its composition was not identical to that of blood serum. The molecular weight ranged from 10^4 to 2×10^5 (Low et al. 1982); thus primarily comprising albumin and IgG. The immunocytochemical demonstration of these two proteins in the endoneurial space and the relative absence of IgM, IgA, and other proteins parallel these data and provide further evidence that

albumin and IgG can pass the barrier system and reach the endoneurial space in both pathologic and physiologic conditions. A partial permeability of the BNB for IgG and albumin was demonstrated in normal mice and marmoset monkeys under physiologic conditions and after injections of syngenic and xenogenic IgG (Liebert et al. 1983; Heininger et al. 1984; Seitz et al. 1985). Within 24 h biotinylated tracer IgG was detected in various organic tissues, such as kidney, liver, skeletal muscle, and perineurium, while the endoneurium was spared. At 4 days, syngenic and xenogenic IgG was present in the endoneurial space for the first time.

Therefore, endoneurial vessels function apparently as a barrier with graded permeability. In pathologic conditions, however, protein leakage is increased. The proteins, especially those of higher molecular weight, extravasated in the endoneurium may accumulate at the perineurial sheath. The perineurium is functioning as a metabolically active diffusion barrier maintaining a constant ionic composition of the endoneurial fluid and thus the microenvironment of the peripheral nerves (de la Motte et al. 1975). The barrier function has been assigned to its inner site (Shinowara et al. 1982). Proteins are either trapped in the perineurium or pass through depending on their physico-chemical properties, such as molecular weight and hydrodynamic radius. They are removed from the extravascular spaces in the epineurium via lymph and blood vessels (Olsson 1971; Söderfeldt et al. 1973).

The factors which determine the permeability for proteins into the endoneurial space remains unclear. The serum concentration appears to play an important role. The ubiquitous staining for IgG and albumin in the endoneurium reflects high serum concentrations (Table 2). The molecular weight of proteins may also play part in the permeability properties. In vasculitic and neuritic processes larger serum proteins penetrate the BNB and appear in the endoneurium together with increased amounts of smaller tracers, e.g., IgG and albumin. Molecular weight and serum concentrations may, however, not be the only determining factors. Unexpectedly, no increased endoneurial staining for IgA or IgM was observed in IgA and IgM gammopathy despite grossly elevated serum concentrations for IgA and IgM. Felgenhauer and et al. (1976) found an exponential correlation between protein content in the cerebrospinal fluid and hydrodynamic radius. A comparable correlation may conceivably be postulated for the endoneurial interstitial fluid. From the present study it can be assumed that fluid barriers of the peripheral nervous system represent a complex and differentiated system whose biochemical functions can only be a matter of speculation of this stage.

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