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DIABETIC NEUROPATHY-IMMUNOPATHOGENESIS

Abstract

Quantitative immunocytochemical analysis of complement proteins (CP) was performed on sural nerve biopsies from 15 patients with diabetic neuropathy (DN) and 18 nondiabetic patients with other forms of chronic neuropathy (ON). The mean age of the patients and the pathological severity of the neuropathy were similar in both groups. The percentage of patients that expressed strongly immunoreactive CP in the walls of endoneurial microvessels was significantly greater in DN than in ON for all proteins tested. C3d neoantigen was expressed in 100% of DN cases compared with 17% of ON; and membrane attack complex (MAC), C5b-9 neoantigen, in 93% of DN and 17% of ON. In the cases with DN, 81% of endoneurial microvessels, as identified by the endothelial marker, *Ulex europaeus*, contained C5b-9 neoantigen deposits, compared with 22% in those of ON, and the staining in DN was significantly more intense. Expression of the neoantigens of C3d and C5b-9 in nerve implies local activation of the complement system. In DN, activation of the complement pathway and formation of the MAC could injure blood vessels and adversely affect the circulation in the endoneurium.

Key words: Diabetes mellitus, Diabetic neuropathy, Complement components, Membrane attack complex, Microvessels

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Introduction

Neuropathy is common in patients with diabetes mellitus. The cause of diabetic neuropathy (DN) is not well understood, although metabolic and vascular abnormalities are suspected. Recent studies suggest that immunological mechanisms contribute to the pathogenesis of the disorder.

The metabolic defects are thought to be caused by hyperglycemia and altered metabolism of glucose, resulting in increased sorbitol and fructose, depletion of myoinositol, impaired turnover of phosphoinositides and reduced activation of protein kinase C. These metabolic alterations in nerve are thought to result in diminished (Na, K)-ATPase activity, osmotic perturbations, disturbance of cellular oxidation and reduction mechanisms, and abnormal metabolism of fatty acids and prostaglandins. These, in turn, could be responsible for the nerve conduction abnormalities [9, 11,14, 31, 35]. Accelerated nonenzymatic glycation of proteins by glucose or its metabolites in diabetes could also alter protein structure and interfere with normal cellular function [6, 7, 23, 30, 32, 36, 38]. The metabolic disorder may directly injure the axons and Schwann cells or it may act indirectly by altering interstitial tissue or the microenvironment of nerve fibers.

There is increasing evidence that DN may be caused by abnormal function of the small blood vessels in the endoneurium [10]. Early pathological changes of microangiopathy are found in the walls of the capillaries, arterioles and venules of nerves. The structural abnormalities are postulated to cause decreased perfusion and eventual ischemic injury of nerve fibers. Microangiopathy appears to precede the onset of neuropathy in diabetic patients [12] and the quantified abnormalities in vessels increase in parallel with the severity of the neuropathy [12, 39]. Accordingly, both reduced blood flow and reduced oxygen have been documented in the peripheral nerve, and similar abnormalities have been found in animal models of the disease [22]. Microangiopathic changes are also strongly implicated in the pathogenesis of retinopathy and nephropathy, which are important complications of diabetes mellitus.

The contribution of autoimmune mechanisms to the pathogenesis of DN was first suggested by Duchen et al. [8], who found inflammatory infiltrates in autonomic ganglia from affected patients. Other investigators observed similar mononuclear cell infiltrates in peripheral nerves [3, 18, 21,

28, 29, 37, 40]. Deposits of immunoglobulins and complement components, C3 and C4, in diabetic nerves were first described by Graham and Johnson [13]. In our own studies of diabetic nerves, epineurial microvasculitis was associated with deposits of activated complement proteins located chiefly within the walls of the endoneurial microvessels [40]. Diabetes itself is an autoimmune disease that targets the pancreatic islets [1, 17] and is associated with other autoimmune phenomena, such as the anti-glutamate dehydrogenase antibodies of stiff man syndrome [15] and antibodies to sympathetic ganglia [5].

In this study, we used quantitative immunohistochemistry to evaluate complement deposition in the endoneurial microvessels of patients with DN, and compared the abnormalities with those found in nerves from patients with other types of neuropathy (ON). We used the Endoneurial cell marker, *Ulex europaeus* agglutinin (UEA-I) to identify blood vessels, and measured the activated forms of C4, C3 and C5b-9, and the complement regulatory proteins, S-protein and SP40,40. The findings indicate local activation of the complement pathway in microvessels of DN, leading to formation of membrane attack complex (MAC).

Materials and methods

Subjects

After obtaining informed consent, biopsies of the whole sural nerve were performed just above the lateral malleolus in 15 patients with progressive DN and in 18 with other forms of chronic neuropathy. The 15 patients with DN were included in a previous immunohistochemical study of lymphocytes, immunoglobulins, and the complement proteins, C3 and C5b-9 [40]. The sural nerves were submitted for routine histology (paraffin sections of formalin-fixed tissue), resin histology (semithin sections of epoxy resin-embedded tissue), and immunohistochemical analysis (cryosections of unfixed, frozen tissue). The patients who served as disease controls were chosen so as to have a similar range of age and pathological severity of neuropathy as the diabetic group. Of the 15 patients with DN, 9 had non-insulin-dependent diabetes mellitus (NIDDM), and 6 had an insulin-dependent form (IDDM). Seven of the patients had proximal DN (PDN), 6 had

distal symmetrical polyneuropathy (DSPN), and 2 had mononeuropathy multiplex (MNM) (see Table 2). The controls included 13 patients with a chronic axonopathy, 3 with chronic inflammatory demyelinating polyneuropathy and 2 with amyotrophic lateral sclerosis (ALS) (see Table 3). The sural nerves of the 2 ALS patients had minor "neuropathic" features, a common finding in the disease.

Histology and immunohistochemistry

Serial cryosections of sural nerves were cut 6 μm in thickness and stained with UEA-I and with antibodies to C4d, C4d neoantigen, C3d, C3d neoantigen, C5b-9 neoantigen, S-protein/vitronectin and SP40,40/clusterin. The antigens, antibodies, dilutions and commercial sources are indicated in Table 1. The primary antibodies that recognize C4d and C3d bind to the whole complement protein as well as the proteolytic fragments that are formed upon activation of the complement system and subsequent degradation to inactive peptides. Hence, these antibodies do not distinguish the whole protein from the activated state or the inactive split peptides. However, three of the monoclonal antibodies recognize neoantigens of C4d, C3d and C5b-9, and these epitopes are expressed only upon activation of the complement system. Two monoclonal antibodies have specificity for two regulatory proteins that inhibit activity of the terminal components of the complement system. One of the monoclonal antibodies binds to the S-protein (Quidel) either as a single protein or as a component of the SC5b-9 complex, an inactive form of the MAC. The second monoclonal antibody reacts with human SP40,40 (Quidel), which is another protein that also becomes a component of the SC5b-9 complex. UEA-I, a lectin that binds to a-linked fucose residues of glycoproteins and glycolipids, is a marker for human endothelial cells and was used to determine the location of blood vessels in the nerves. The avidin-biotinylated peroxidase (ABC) method employed the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.) and diaminobenzidine (DAB) as chromogen and detected the binding of these antibodies or the lectin to tissue sections.

On the UEA-I-stained sections, endoneurial vascular profiles (microvessels) were counted manually. (To test whether UEA-I staining was quantitatively reproducible, we localized and counted the lectin-stained blood vessels on nine serial 6-μm transverse and longitudinal sections of normal nerve. The number and localization were identical on all nine sections.) The total endoneurial area and UEA-I -stained area were determined by computerized image analysis. The immunostaining intensity of complement com-

ponents and regulatory proteins was measured semiquantitatively on a scale of 0 (none) to 3 (intense). The number of vascular profiles that express neoantigens of C4d, C3d and C5b-9 on each section was counted by hand. The area and optical density of the same three immunoreactive neoantigens in blood vessels were determined by computerized image analysis (see below). In 22 cases (10 DN, 12 ON) Epon-embedded tissue was available to quantify the histological severity of the neuropathies. Transverse semithin sections of these specimens were stained with *p*-phenylenediamine, and the number of myelinated fibers was counted in each nerve fascicle and expressed as the number per mm of cross-sectional area of endoneurium (myelinated fiber density or MFD).

Table 1
List of antibodies (*ABC* avidin biotin complex, *IF* immunofluorescence, *FITC* fluorescein isothiocyanate-conjugated)

Antibody description	Dilution	Catalog no	Source	Method
Murine monoclonal anti-human C4d	1:300	A 213	Quidel	ABC
Murine monoclonal anti-human C3d	1:300	CLSIF 33001	Quidel	ABC
Murine monoclonal anti-human C4d neo	1:400	CLSIF 25701	Quidel	ABC
Murine monoclonal anti-human C3d neo	1:300	CLSIF 35501	Quidel	ABC
Murine monoclonal anti-human C5b-9	1:100	M 777	Dako	ABC
Murine monoclonal anti-human S-protein	1:200	A237 F29101	Quidel	ABC
Murine monoclonal anti-human SP40,40	1:200	A241 F35201	Quidel	ABC
Ulex europaeus agglutinin I	1:50	L-1060	Vector	ABC
Rabbit anti-human albumin-FITC	1:50	F 117	Dako	IF
Rabbit anti-human fibrinogen-FITC	1:50	F 111	Dako	IF
Goat anti-human IgG-FITC	1:100	F 1641	Sigma	IF
Goat anti-human IgM-FITC	1:20	F 5384	Sigma	IF
Goat anti-human IgA-FITC	1:20	F 5259	Sigma	IF

Computerized image analysis

To quantify immunoreactivity, digitized images were acquired by a video camera mounted on a microscope and attached to a Macintosh IIfx computer using the public domain NIH Image program. Using standardized illumination, optical density (OD) values were calibrated from a photographic gray scale. A series of images acquired with a ×4 objective comprised the endoneurium of the entire nerve in each section. A threshold set by the operator distinguished “stained” from “unstained” objects. Once a threshold

was chosen for a given component of complement, the same threshold was used for all cases.

Analysis of results

The following measurements were performed on the 33 nerve biopsies (15 patients with DN and 18 with ON): (1) the intensity of immunoreactivity of each complement neoantigen and regulatory proteins measured semiquantitatively in endoneurial microvessel walls; (2) the proportion of immunoreactive proteins in vascular profiles to total number of UEA I-labeled endoneurial microvessels; (3) the mean OD of suprathreshold immunoreactivity for each neoantigens in the microvessels; (4) the integrated complement neoantigens immunoreactivity normalized for UEA I-stained area (neoantigens OD x neoantigen area of immunoreactivity/UEA I-labeled area); (5) the number of UEA I-labeled vascular profiles per mm; and (6) the fraction of endoneurial area occupied by UEAI-labeling. The effects of diabetes on semiquantitative intensity of immunoreactivity were evaluated by Mantel-Haenzel test. Other comparisons between DN and ON were performed by ANOVA. These were repeated with MFD as an additional covariate for the 22 cases on which Epon-embedded tissue was available with age as covariate. For evaluation of the number of UEA I-labeled blood vessels per mm, orientation of the section was included as a categorical covariate. Linear regression was used to evaluate the relationship of MFD with complement deposits and vascular density. The above analyses revealed deposits of activated complement in the thickened blood vessels of DN biopsy samples. To rule out the possibility that these were related to thickening of blood vessels alone, we subsequently stained a series of nerve biopsy specimens from four patients with Charcot-Marie-Tooth disease, type 1 (younger than the DN cases but matched to them by semiquantitative assessment of myelinated fiber loss) and five patients with neuropathy and an IgM paraprotein reactive to myelin-associated glycoprotein (anti-MAG neuropathy) (matched to the DN cases by age and semiquantitative assessment of myelinated fiber loss). Complement deposits in these cases were assessed visually.

Results

Severity of neuropathy

Epon-embedded material was available for 10 cases of DN and 12 cases of ON (Tables 2, 3). The spatial density of myelinated fibers in DN (2427 ± 1489 fibers/mm^2) and ON (1840 ± 718 fibers/mm^2) did not differ significantly (t = 1.21, df = 20, P = 0.24). In addition, microscopic semiquantitative assessment of cryosections from all 33 subjects demonstrated a similar loss of myelinated fibers in both groups.

Immunohistochemistry

Immunoreactivity of complement proteins

Semiquantitative assessment revealed more C4d, C3d, and C5b-9 immunoreactivity in the walls of endoneurial blood vessels in the 15 patients with DN than 18 with ON using monoclonal antibodies that recognize neoantigens (see Fig. 1 C–1F). The differences between the diabetic patients and the disease controls were statistically significant (C4d neoantigen, Mantel-Haenzel χ^2 = 7.1, df = 1, P = 0.008; C3d neoantigen, Mantel-Haenzel χ^2 = 22.4, df = 1, $P < 10^{-5}$; C5b-9 neoantigen, Mantel-Haenzel χ^2 = 20, df = 1, $P = 10^{-5}$) (Tables 2, 3). Semiquantitative ratings of 2 or 3 were obtained for C4d neoantigen in 67% of DN and 28% of ON, for C3d neoantigen in 100% of DN and 17% of ON; and for C5b-9 neoantigen in 93% of DN and 17% of ON. These neoantigens are only expressed upon activation of the complement components. Expression of C4 and C3 was also tested using two other monoclonal antibodies, which each recognize as a corresponding epitope of the whole protein (C4 or C3) and split fragment (C4d or C3d). Semiquantitative assessment of these components revealed more C3/C3d immunoreactivity in the endoneurial microvessels of DN than ON (Mantel-Haenzel χ^2 = 13.6, df = 1, P = 0.0002) but similar levels of C4/C4d (Mantel-Haenzel χ^2 = 0.98, df = 1, P = 0.32).

Table 2

Summary of diabetic neuropathy cases. Immunostaining intensity of complement components and regulatory proteins is measured semiquantitatively on a scale of 0 (none) to 3 (intense) [*MFD* myelinated fiber density (number of myelinated fibers/mm), *DSPN* distal symmetrical polyneuropathy, *PDN* proximal diabetic neuropathy, *MNM* mononeuropathy multiplex, *IDDM* insulin-dependent diabetes mellitus, *NIDDM* non-insulin-dependent diabetes mellitus, *N/A* not available]

Case no	C3d neo	C4d neo	C5b-9 neo	S-protein	SP40,40	MFD	Age	Sex	Diabetic type	Clinical diagnosis
1	3	2	3	3	2	548	65	F	IDDM	PDN
2	3	3	3	3	2	N/A	59	F	IDDM	DSPN
3	3	3	3	2	3	N/A	62	M	NIDDM	DSPN
4	2	1	3	2	2	203	89	M	IDDM	PDN
5	2	0	1	2	2	2550	43	M	NIDDM	PDN
6	3	1	2	2	2	N/A	52	M	IDDM	PDN
7	3	3	3	3	3	3869	49	M	IDDM	PDN
8	2	1	3	2	2	1580	67	M	NIDDM	DSPN
9	3	2	3	2	2	N/A	58	M	IDDM	MNM
10	3	3	3	3	2	N/A	64	M	NIDDM	MNM
11	3	2	3	2	2	3141	74	M	NIDDM	DSPN
12	3	3	3	2	2	4279	63	F	IDDM	DSPN
13	3	1	3	2	2	2648	83	F	NIDDM	PDN
14	3	3	3	3	3	1229	59	F	IDDM	DSPN
15	3	3	3	3	2	4223	84	F	NIDDM	PDN

Table 3

Other neuropathies. Immunostaining intensity of complement components and regulatory proteins is measured on a scale of 0 (none) to 3 (intense) (*PN* peripheral neuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, *ALS* amyotrophic lateral sclerosis, *N/A* not available)

Case no.	C3d neo	C4d neo	C5b-9 neo	S-protein	SP40,40	MFD	Age	Sex	Clinical diagnosis
16	0	1	0	1	1	3198	51	F	PN
17	0	1	1	1	1	1298	63	M	CIDP
18	3	3	3	2	2	1237	75	M	CIDP
19	1	2	1	1	1	1343	72	F	PN
20	0	0	0	N/A	N/A	2525	74	F	PN
21	0	0	0	1	1	N/A	48	F	PN
22	0	0	0	1	1	1519	76	F	PN
23	0	0	0	1	1	N/A	64	F	ALS
24	0	0	0	1	1	1283	51	F	PN
25	2	3	2	2	2	N/A	62	M	CIDP
26	0	1	0	1	1	N/A	45	F	PN
27	1	2	1	1	1	2183	75	M	PN
28	0	1	1	1	1	N/A	78	M	PN
29	2	2	2	1	1	1339	65	M	PN
30	1	0	0	1	0	N/A	59	M	ALS
31	0	0	0	1	1	1668	66	M	PN
32	1	0	1	1	1	3061	39	M	PN
33	0	0	0	N/A	N/A	1436	67	M	PN

$P = 0.008$; C3d neoantigen, Mantel-Haenzel $\chi^2 = 22.4$, $df = 1$, $P < 10^{-5}$; C5b-9 neoantigen, Mantel-Haenzel $\chi^2 = 20$, $df = 1$, $P = 10$) (Tables 2, 3). Semiquantitative ratings of 2 or 3 were obtained for C4d neoantigen in 67% of DN and 28% of ON, for C3d neoantigen in 100% of DN and 17% of ON; and for C5b-9 neoantigen in 93% of DN and 17% of ON. These neoantigens are only expressed upon activation of the complement components. Expression of C4 and C3 was also tested using two other monoclonal antibodies, which each recognize as a corresponding epitope of the whole protein (C4 or C3) and split fragment (C4d or C3d). Semiquantitative assessment of these components revealed more C3/C3d immunoreactivity in the endoneurial microvessels of DN than ON (Mantel-Haenzel $\chi^2 = 13.6$, $df = 1$, $P = 0.0002$) but similar levels of C4/C4d (Mantel-Haenzel $\chi^2 = 0.98$, $df = 1$, $P = 0.32$).

Multivariate ANOVA of the densitometric measurements revealed significant effects of diabetes on neoantigens deposits, whether expressed as a fractional number of UEA-I-stained vessels, OD of staining, or integrated immunoreactivity (see Methods). The proportion of Endoneurial microvessels that displayed each complement protein was greater in the DN group than in the ON group. By univariate ANOVA on these 33 subjects, with age as a covariate, the differences were statistically significant for all three neoantigens (Fig. 2). The OD of the immunostained vessels was also greater in DN than in ON. The differences were significant for C3d and C5b-9 neoantigens, but not for C4d (Fig. 3). Likewise, integrated immunoreactivity, normalized for the area of UEA-I labeling, provided significant differences for C3d and C5b-9 neoantigens, but not for C4d (Fig. 4). Similar results were obtained with fiber density as an additional covariate, for the 22 subjects with Epon-embedded specimens.

All complement proteins including the neoantigens, but not UEA-I, were expressed in the perineurium of all nerves of the DN and ON patients (Fig. 1). We have routinely immunostained C3d and C5b-9 of over 1000 nerve biopsies as part of the diagnostic evaluation, and these complement proteins are expressed in the perineurium of most normal and diseased nerves. Hence, we attach no significance to the apparent immunoreactivity of the perineurium in DN.

All 15 cases of DN but one exhibited prominent hyaline thickening of the blood vessel walls within the endoneurium. Sural nerves with similar vessel thickening were present in 4 patients with Charcot-Marie-Tooth disease, type 1 (CMT), and 5 patients with anti-MAG neuropathy. No detectable C3d neoantigen was found in the blood vessel walls of any of the 9 patients. C5b-9 was absent in the vessels of the CMT cases, but expressed weakly immunoreactive MAC in the vessel walls of anti-MAG neuropathy. All of the anti-MAG nerves displayed C3d on the surface of myelin sheaths, but no C5b-9.

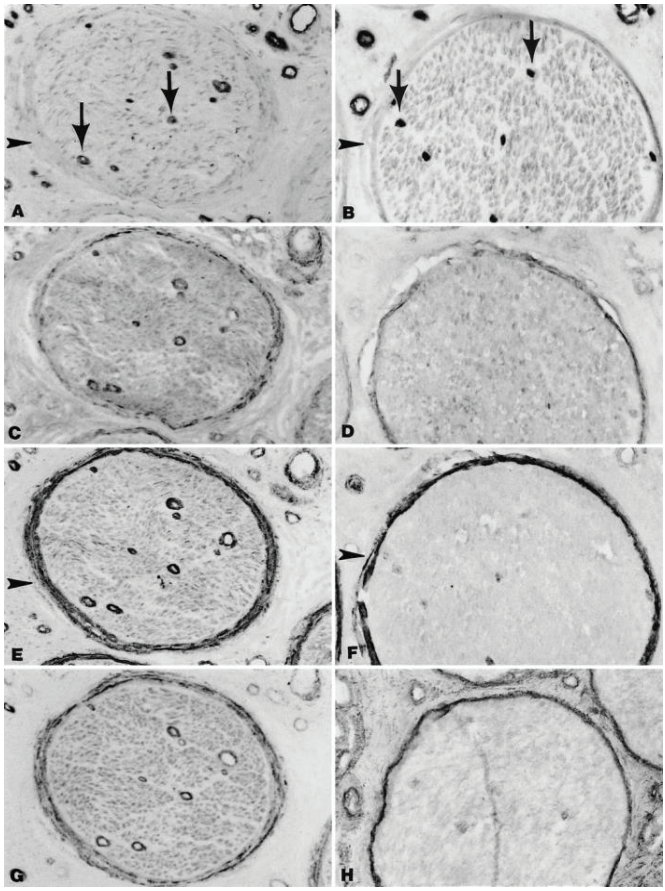

Fig. 1 – **A–H** Immunoperoxidase stains of the endoneurial microvessels using the lectin, UEA-I and monoclonal antibodies to complement proteins in sural nerve biopsies of DN and a disease control (ON). The nerve in DN is depicted in the same field of four semiserial cryosections (**A**, **C**, **E**, **G**). The endoneurium contains nine microvessels, as indicated by the endothelial marker, UEA-I (**A**, *arrows*). The C3d neoantigen (**C**), C5b-9 (**E**) and S-protein (**G**) are expressed in the same nine blood vessels. The nerve in ON shows similar semiserial sections (**B**, **D**, **F**, **H**) except that **H** is a different field. The nerve fascicle encloses six blood vessels marked by UEA-I (**B**, *arrows*), but there is little or no immunoreactive C3d neoantigen (**D**), C5b-9 (**F**) or S-protein (**H**). Note that the same complement proteins (**C–H**), but not UEA-I (**A**, **B**), are also located in the perineurium (*arrowheads*), a common finding in many different types of neuropathies (*UEA-I Ulex europaeus*-I, *DN* diabetic neuropathy, *ON* other neuropathy). **A–H** × 120

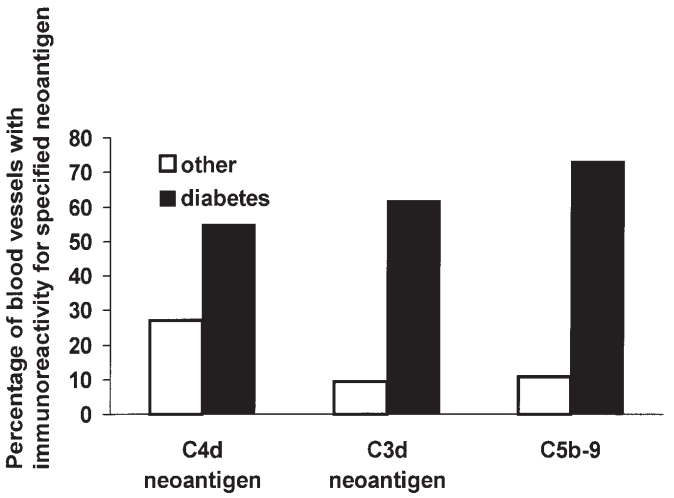

Fig. 2 – Age-adjusted mean percentage of blood vessels displaying immunoreactivity for each neoantigen. ANOVA (*df* = 30,1,1); C4d: $F = 5.49$, $P = 0.026$; C3d: $F = 32.79$, $P = < 0.0005$; C5b-9: $F = 47.11$, $P = < 0.0005$

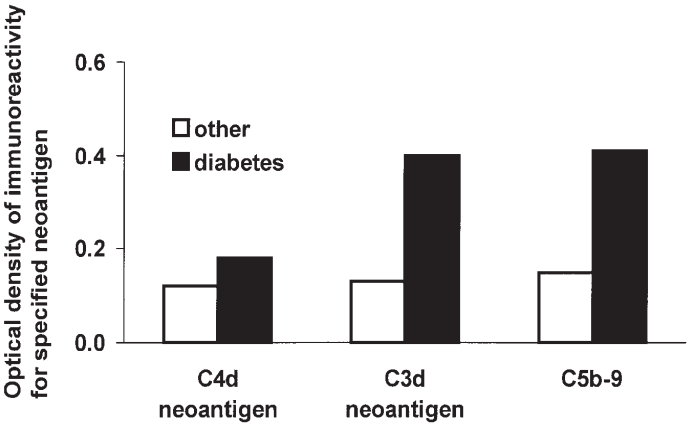

Fig. 3 – Age-adjusted optical density of immunoreactive vessel walls for each neoantigen. ANOVA (*df* = 30,1,1); C4d: $F = 2.71$, $P = 0.11$; C3d: $F = 29.48$, $P = < 0.0005$; C5b-9: $F = 31.08$, $P = < 0.0005$

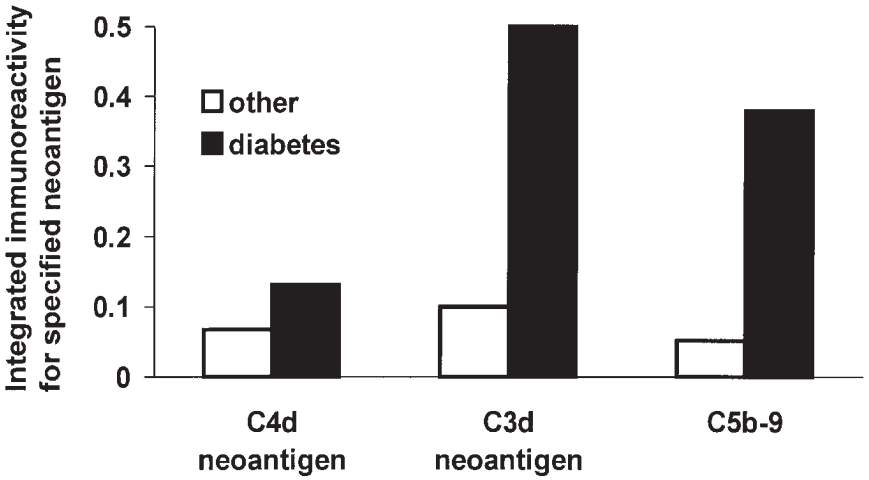

Fig. 4 – Age-adjusted integrated immunoreactivity for each neoantigen. ANOVA (df = 30,1,1); C4d: $F = 1.87$, $P = 0.182$; C3d: $F = 13.42$, $P = < 0.0005$; C5b-9: $F = 28.48$, $P = < 0.0005$

Immunoreactivity of S protein (vitronectin) and SP40,40 (clusterin)

The expression of S-protein and SP40,40 (Fig. 1 G, H; Tables 2, 3) in the walls of endoneurial microvessels of DN exceeded that of ON based on semiquantitative analysis (S-protein, Mantel-Haenzel $\chi^2 = 22.0$, $df = 1$, $P < 10^{-5}$; SP40,40, Mantel-Haenzel $\chi^2 = 20.0$, $df = 1$, $P = 10^{-5}$). S protein and SP40,40 were generally colocalized with all three neoantigens within the walls of the endoneurial microvessels in DN (Fig. 1).

Immunoreactivity of immunoglobulins, fibrinogen and albumin

Immunoreactivity for IgG, IgM, and IgA was previously demonstrated in DN and normal nerves [40], and in this study, these immunoglobulins were also present in ON. Albumin was expressed diffusely in the endoneurium in both DN and ON. No deposits of fibrinogen were observed in the tissue of any of the biopsy specimens.

Correlation between severity of neuropathy, the age of the patient and complement deposits

The extent of complement neoantigen immunoreactivity, measured by any of the above parameters, was not correlated with severity of neuropathy or age, neither among the whole group of subjects, nor within either group separately ($r^2 < 0.1$ for all correlations).

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Density of endoneurial blood vessels

The number of UEA-I-labeled vascular profiles was expressed per unit of endoneurial area. The density of these microvessels was significantly greater in the DN group than in the ON group, (means adjusted for age, MFD, and orientation of section: DN 34.3/mm ; ON 14.1/mm^2 ; F =12.13, df = 17, 3, 1, P = 0.003; all cases, covarying for age and orientation only: DN 29.2, ON 15.9, F = 10.61, df = 29, 2, 1, P = 0.003). Likewise, when the fraction of the endoneurial area occupied by UEA-I staining was considered, the difference was statistically significant (means adjusted for age and MFD: DN 1.67%, ON 0.61%, F = 21.13, df = 18,2,1, $P < 0.0005$; all cases, covarying for age only: DN 1.45, ON 0.8, F = 8.41, df = 30, 1, 1, P = 0.007). There was no statistically significant correlation between the age and the number or area of UEA-I-labeled vascular profiles.

Discussion

The findings described here indicate an activation of the complement system in DN, as was found by expression of the neoantigens C3d and C5b-9 in sural nerve biopsy specimens. These complement deposits were located in the thickened walls of endoneurial microvessels, and they were more

intensely immunoreactive and more extensive than in other types of neuropathy. The quantitative differences were significant and imply that the abnormalities are not simply a by-product of an injury of nerve fibers. The results are also not a general response to vascular thickening, as similar abnormalities were present in other chronic neuropathies in this study.

Widespread pathological alterations occur in microvessels of diabetes mellitus, and they have been postulated to cause major complications of the disease, namely, neuropathy, retinopathy, and nephropathy. In a diabetic nerve, the endoneurial microvessels exhibit thickening and proliferation or reduplication of the basal lamina, injury with loss of pericytes and hyperplasia of endothelial cells [10, 12, 39]. This microangiopathy has been linked to increased permeability of the vessels, edema, and hypoxemia of the nerve [22, 26] and the development of neuropathy. The vascular abnormalities have previously been attributed to metabolic abnormalities and advanced glycated end products, but the pathogenesis of the microangiopathy is not understood.

MAC has the potential to induce cell injury or death, and it could be responsible, in part, for the microangiopathy seen in diabetic nerves. MAC acts by becoming inserted into the surface membrane of host cells, where it creates abnormal pores. The alteration disrupts the integrity of the membrane and results in leakage of the cell membrane with dysfunction and death of cells [19, 24, 25]. The interaction of the MAC with endothelial cell (EC) membrane could also result in the release of soluble macromolecules including fibroblast and platelet-derived growth factors, which have been shown to be potent mitogens for mesangial cells or pericytes in the kidney [2]. In addition, activation of the complement system releases C3a and C5a into the extracellular fluid, where they act to increase permeability of blood vessels and serve as chemokines to promote influx of polymorphonuclear leukocytes and recruitment of monocytes. Diabetic nerves exhibit chronic inflammation [3, 18, 21, 28, 29, 37, 40], numerous histiocytes and macrophages in the endoneurium [40] and endoneurial edema [26], and these findings are consistent with a chronic low-level state of complement activation.

The mechanisms responsible for activation of the complement system in DN are not clear. In general, the classical pathway is activated by antibody-antigen interactions, whereas carbohydrates initiate the alternative pathway [25]. In addition, the mannose binding protein (MBP) activates the

classical pathway by binding to carbohydrate groups, a process described recently as the MBP pathway [34]. These three pathways converge to generate C3b as a result of sequential proteolytic cleavage of the complement components [25]. The active fragment, C3b, binds covalently to macromolecules on the surface membrane of cells, where it amplifies the action of the alternative pathway to yield abundant C3b. It acts, in turn, to split C5 yielding C5b, and this fragment assembles the terminal components, C6, C7, C8 and C9 in sequence to form the C5b-9 or MAC. In this study, analysis of the early complement pathways was limited to the C4d neoantigen, an activation marker of the classical and MBP pathways. Expression of this complement component was inconstant in different diabetic nerves and OD of the protein was not significantly greater than disease controls. Hence, further investigation of the activation of the complement system is necessary.

Whether C3b proceeds to activate the terminal components with assembly of C5b-9 depends critically on regulatory proteins. These molecules are located in extracellular fluid and cell membranes where they tightly control complement function. Some of these proteins effectively restrict the action of C3b to the immediate vicinity of the bound fragment, and others degrade the peptide to inactive fragments. Still other proteins inhibit the terminal pathway and limit formation of C5b-9. In this study, the diabetic nerves expressed two strongly immunoreactive complement regulatory proteins, S-protein and SP40,40, in the walls of the endoneurial microvessels of DN. The intensity and extent of immunostaining exceeded that of ON, and these regulatory proteins were generally colocalized with the neoantigens of C4d, C3d and C5b-9. S-protein, also known as vitronectin, associates with the terminal components of complement as they are assembled to form the inactive complex, SC5b-9 [27, 33]. This complex is soluble and cannot be inserted into target cell membranes [19]. SP40,40 (clusterin) inhibits the function of MAC and is also an integral component of the inactive soluble complex, SC5b-9 [25]. The increased immunoexpression of the neoantigen, C5b-9, in diabetic nerves implies that S-protein, clusterin, and other regulatory proteins, such as CD 59, have failed to suppress the terminal pathway, thereby, permitting formation of MAC. Possible mechanisms of this pathological state include an overwhelming acceleration of complement activation or impaired activity of regulatory proteins, possibly rendered by advanced glycation, as has previously been shown for S-protein (vitronectin) in the diabetic state [16].

The expression of active complement components in microvessel walls of DN was found in all 15 cases, but the abnormality is not unique as 3 of the 18 controls expressed a similar pattern of complement proteins. Two of the three control patients had chronic inflammatory demyelinating polyneuropathy, a disorder that is thought to be autoimmune [4]. The trigger for complement local activation in vessel walls in diabetic nerves is unknown. Possible triggers include anti-endothelial cell or pericyte antibodies, interaction of the complement pathways with abnormally glycated proteins, or exposure of normal cellular molecules, such as sulfatide [20]. Loss or impaired function of regulatory proteins may also promote deposition of lytic C5b-9. Identification of the mechanisms responsible for activation and dysregulation of the complement system could lead to a better understanding of the disease.

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