Increase in albumin, IgG, and IgM blood–nerve barrier indices in human diabetic neuropathy

(sural nerve biopsy/endoneurial compartments/capillary macromolecular transport/endothelial–perineurial barrier/antibody capture assay–streptavidin-biotin amplification)

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ABSTRACT The albumin (Alb), IgG, and IgM concentrations in the endoneurium of fascicular sural nerve biopsy samples were evaluated in controls (n = 9 or 10), diabetic patients without neuropathy (n = 6), and diabetic patients with polyneuropathy (n = 17 or 18). These values were significantly increased in diabetic patients with and without neuropathy when expressed both per endoneurial dry weight or endoneurial total protein compared to biopsy samples from healthy controls. When these concentrations, expressed per endoneurial total protein, were related to plasma concentrations similarly expressed, the resulting blood–nerve barrier (BNB) indices were significantly increased for Alb (6.1 times; P < 0.00001), IgG (4.9 times; P = 0.00037), and IgM (2.7 times; P = 0.015). The diabetic patients without neuropathy (defined as having an index of pathology of >0.65; a measure of the severity of the pathological abnormality based on morphological criteria) also had significant increases in two of these BNB indices that were intermediate between the diabetic neuropathy patients and controls (Alb, 3.9 times controls; P = 0.00002; IgG, 4.6 times controls; P = 0.00016; IgM, 1.8 times controls; not significant). No correlations were observed between the endoneurial concentrations of these plasma proteins or the BNB indices and the index of pathology, suggesting that these increases in endoneurial plasma proteins precede the pathological alterations. The increased values for the diabetics in the absence of pathological abnormalities may prove useful in predicting neuropathic complications. The ratio of the IgG-BNB index to the Alb-BNB index was decreased 19%, and the ratio of the IgM-BNB index to the Alb-BNB index was decreased 56% in diabetic neuropathy patients compared to controls. Although the IgG and IgM concentrations are increased in the diabetic endoneurium, the Alb increase is greater and a mechanism other than size indiscriminate extravasation of plasma proteins, therefore, is suggested. Morphometric assessment of the endoneurial compartments, which would be expected to contain these plasma proteins, suggests that they are not altered in diabetic neuropathy; hence, it is hypothesized that the observed increase in endoneurial concentration of these plasma proteins results from altered transport through the endothelial or perineurial barrier, which supports an underlying vascular mechanism in the development of diabetic polyneuropathy.

The mechanisms of macromolecular transport across capillaries of human peripheral nerve under normal and pathological conditions are poorly understood. Peripheral nerve fibers are protected within a specialized endoneurial compartment, which is separated from the blood by the blood–nerve barrier (BNB) (1, 2). The anatomical basis of the BNB is the high resistance, tight junctions, and the absence of fenestrations and vesicular channels (pores) in the endoneurial capillary endothelium. Another important component of the BNB is the perineurial barrier, which separates the endoneurial compartment from epineurial/perineurial extracellular space.

Previous investigations from this laboratory have demonstrated that albumin (Alb) is the major protein component of the endoneurial fluid, as well as the epineurial/perineurial fluid (3). It was demonstrated that in lead neuropathy (4, 5) and in Wallerian degeneration (5), where predictable changes in BNB are observed, an alteration of the relative Alb concentration between the endoneurium and serum provides an indication of the integrity of the BNB. Poduslo et al. (4) postulated that an increase in proteins derived from serum and not produced locally within the endoneurium would permit an assessment of potential BNB impairments in fascicular sural nerve biopsy samples from patients with neuropathy. An increase in endoneurial Alb concentration and the Alb–BNB index was observed in sural nerve biopsy samples from diabetic patients with polyneuropathy (6, 7), which suggested barrier alterations in diabetic neuropathy. Because of the lack of both fenestrations and vesicular channels, restrictive tight junctions (which limit the entry of small peptides, such as microporoxidase (Mf, 2000) (8), and the integrity of the plasma membrane, transmural passage of macromolecules across the capillary endothelium into the normal nerve endoneurium likely involves vesicular transcytosis associated with pinocytosis or receptor- or absorptive-mediated endocytosis. This is supported by the investigations of Arvidson (9), who provided evidence of vesicular transport of horseradish peroxidase (Mf, 40,000) across normal endoneurial vessels of mouse sciatic nerve. Although an Alb receptor on brain capillaries is yet to be unambiguously demonstrated (10), more recent data infer that cationized Alb is rapidly taken up through the blood–brain barrier by a receptor-mediated (11) or absorptive-mediated (12) transport process. The implication of this finding would be that cationic ligand-modified Alb or the highly protonated B isomer of Alb (13) is selectively transported. Such receptor-mediated transport would be consistent with other tissues, such as lung, heart, and diaphragm, where transport occurs in transcytotic vesicles (14, 15).

The present investigation focuses on the fascicular sural nerve biopsy from patients with diabetic neuropathy. We ask (i) whether the previously described increase in endoneurial Alb concentration is selective or whether it extends to other plasma macromolecules of increasing hydrodynamic radius (IgG and IgM); (ii) whether the increased concentration of these plasma macromolecules can be attributed to altered endoneurial compartments in sural nerve biopsy samples.

Abbreviations: BNB, blood–nerve barrier; Alb, albumin; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.
from patients with diabetic neuropathy or to alterations in capillary transport; and (iii) whether the ratios of the BNB indices from patients compared to controls are indicative of general extravasation of plasma proteins in the diabetic neuropathy patient.

MATERIALS AND METHODS

Patient Selection. The standard ankle level fascicular sural nerve biopsy was performed on 10 healthy subjects who were recruited by advertisement in the institution's employee magazine. These individuals (mean age, 42 years; median age, 35.5 years; 6 females and 4 males) were interviewed and examined to be sure they did not have a neuropathy or a disease that may cause neuropathy. The controls underwent the same evaluation for neuropathy as did diabetics. All subjects gave written informed consent. Sural nerve biopsy samples were obtained from 24 diabetics (mean age, 47 years; median age, 49 years; 13 females and 11 males). Six of these diabetics did not have neuropathy as determined by an index of pathology of >0.65 (16) and included 3 females with insulin-dependent diabetes mellitus (IDDM) (mean age, 44.3 years; median age, 43.5 years) and 1 female and 2 males with non-insulin-dependent diabetes mellitus (NIDDM) (mean age, 55.3 years; median age, 56.5 years). The diabetic neuropathy patients had a distal symmetric sensory polyneuropathy with variable autonomic dysfunction. These included 4 females and 2 males with IDDM (mean age, 33 years; median age, 36 years) and 5 females and 7 males with NIDDM (mean age, 51.7 years; median age, 50.5 years). The diagnosis of neuropathy was obtained by using the minimal criteria as described (16). None of the nerves of the healthy subjects had morphometric and teased fiber evidence of neuropathy.

Sural Nerve Biopsy/Plasma Preparations. Immediately after obtaining the fascicular sural nerve biopsy sample, wet weight was measured. The fascicular biopsy sample was desheathed (endoneurium stripped of perineurium and epineurium). Previously, Poduslo et al. (3) used the term endoneurium to describe the desheathed nerve, which consists of not only the interstitial connective tissue (collagen) but also the neural elements (including myelinated and nonmyelinated axons), Schwann cell elements (including Schwann cells, myelin, and extracellular matrix consisting of basement membrane, collagen fibrils, etc.), other cell types (including fibroblasts, macrophages, capillary endothelial cells, and pericytes), and the fluid that bathes these components. The fascicular sural nerve biopsy evaluated in the present study was stripped of the epineurium and perineurium; hence, the BNB sites are confined to the capillary endothelial cells of the endoneurium. The increase in plasma macromolecules found in the endoneurium of diabetic neuropathy patients, however, could also be attributed to changes in the perineurial barrier. The endoneurium was lyophilized to measure dry weight. The dried endoneurium was then homogenized with a ground glass homogenizer (100 μl), and aliquots were taken from the homogenized endoneurium for protein analysis, as well as for analysis of Alb, IgG, and IgM. Protein determinations were made according to the procedure of Lowry et al. (17) as modified by Hess and Lewin (18). Monomer standard bovine serum Alb (Miles) was used for the protein determination in a range of 2.5–10 μg by least-squares linear regression analysis. Aliquots of the homogenized endoneurium were diluted 0.5–1.0 × 10^4 for IgG, 1.5–3.0 × 10^5 for Alb, and were left undiluted for IgM. Plasma, obtained in the operating room at the time of nerve biopsy and after fasting, was aliquotted and stored at −70°C until analysis. Plasma dilutions of 1.5 × 10^3–1.1 × 10^6 for IgG, 1.2 × 10^5–1.4 × 10^4 for IgM, and 1.2 × 10^3–1.4 × 10^5 for Alb were used.

Alb RIA. An antigen–antibody precipitation assay was used for measurement of Alb as described by Poduslo et al. (19) Briefly, this procedure involved radioiodinating chromatographically purified human Alb (Cappel Laboratories, Cochranville, PA) by the chloramine-T procedure. Rabbit anti-human Alb IgG fraction was obtained from Cappel. The immune complex was precipitated with excess bovine immunoglobulin and 20% polyethylene glycol. Radioactivity was determined in a Beckman 8000 γ-counter, and data were analyzed by the unweighted log-log method of Rodbard and Lewald (20).

IgG/IgM Antibody Capture RIA. A solid-phase antibody capture assay with amplification using biotin/125I-labeled streptavidin was used for assessing IgM and IgG in both plasma and homogenized endoneurium according to the procedure described (19). Data were analyzed by direct counting of the 125I-labeled streptavidin–biotin immune complex in a γ-counter with subsequent analysis using an unweighted log-log method.

BNB Indices. The BNB index was determined for Alb, IgG, and IgM by evaluating the ratio of the individual protein to the total protein in the homogenized endoneurial fraction and in plasma as described by Poduslo et al. (19). Data are presented as the mean ± SD. Statistical analysis is by the two-sample t test (two-tailed) with criterion for statistical significance being P < 0.05. Upper limits from normal values were defined as the mean + 2 SD.

RESULTS

A comparison of the Alb, IgG, and IgM-BNB indices is shown in Fig. 1. A value of 5.40 ± 2.53 was obtained for Alb in the control nerves. This was 1.4–1.9 times less than previously published values (6) and results from a change in the homogenization procedure. Previously, a Polytron homogenizer was used with a PT-20ST probe generator. Subsequent studies revealed loss of connective tissue by this procedure because of presumed loss of connective tissue to the generator probe. We have demonstrated in previous studies (3, 21) that a small volume ground glass homogenizer readily provides a uniform homogenate of the endoneurium from the fascicular sural nerve biopsy from which both connective tissue and non-connective tissue protein are uniformly sampled. The IgG-BNB index for normal nerve was 2.07 ± 1.10, and the IgM-BNB index was 4.09 ± 1.95.

All of the diabetic neuropathy patients (n = 18) showed an increase in the Alb-BNB index that ranged from 13.9 to 62.1, with a mean of 32.74 ± 15.39, which was 6.1 times that of control values. The IgG-BNB index was increased in 16 of 18 diabetic neuropathy patients, with a range for the increased values from 4.6 to 29.8 and a mean for all patients of 10.18 ±
6.59, which was 4.9 times that of controls. Eight of 17 diabetic neuropathy patients showed an increase in the IgM-BNB index, which ranged from 11.8 to 33.9 with an apparent bimodal distribution. A mean value of 10.93 ± 8.73 was obtained for the 17 patients, which was 2.7 times that of control values.

Also shown in Fig. 1 are the BNB indices for the diabetic patients without neuropathy. In general, the values are intermediate between the diabetic neuropathy patients and controls. For example, all of the diabetic patients showed an increase in the Alb-BNB index, with a mean of 21.00 ± 7.9, which is 3.9 times that of control values. Five of six diabetic patients showed an increase in the IgG-BNB index, with a mean of 9.49 ± 4.84, which is 4.6-fold greater than control values. In contrast, only one of the six diabetic patients showed an increase in the IgM-BNB index. A mean of 7.16 ± 6.38 was obtained, which was 1.8 times the control values.

These data are further summarized in Table 1, which shows the concentration of Alb, IgG, and IgM in the endoneurium expressed per mg of dry weight and per mg of total protein. Values for plasma are expressed per ml of plasma and per µg of total plasma protein and BNB index is given as a ratio. The Alb concentration was significantly increased in the endoneurium of all diabetic neuropathy patients compared with controls when expressed per dry weight of endoneurium or per total endoneurial protein (P < 0.00001). The diabetic patients without neuropathy also had a highly significant increase in endoneurial Alb when expressed per dry weight (P = 0.00001) or total protein (P = 0.0002). No significant differences were found in the plasma concentration of Alb. A highly significant difference was observed in the Alb-BNB index for both the diabetic neuropathy patients (P < 0.00001) and the diabetic patients without neuropathy (P = 0.00002).

A highly significant increase in IgG values in the endoneurium, whether expressed on a dry weight or total protein basis, was observed for both diabetic neuropathy patients (P = 0.0036 and P = 0.0011, respectively) and the diabetic patients without neuropathy (P = 0.0004 and P = 0.00005, respectively). No significant differences were found in the plasma IgG concentrations. A highly significant P value was observed in the IgG-BNB index for the diabetic neuropathy patients (P = 0.00037) and the diabetics without neuropathy (P = 0.00016).

The IgM values in the endoneurium had a P value of 0.024 when expressed per dry weight and a P value of 0.032 when expressed per total protein for the diabetic neuropathy patients and P values of 0.049 and 0.03, respectively, for diabetic patients without neuropathy. No significant differences were observed for the IgMs with neuropathy in the IgM values in plasma compared to controls, although the mean IgM values were increased. In contrast, the diabetic patients without neuropathy had a significant increase in plasma IgM levels, which resulted in a nonsignificant increase in the IgM-BNB index. The diabetic neuropathy patients, however, had a significant increase in this index (P = 0.015).

No correlations were found between the index of pathology and any of the BNB indices. In addition, no correlations were observed between IDDM and NIDDM patients with or without diabetic neuropathy. An exception to this was found for diabetic patients without neuropathy where endoneurial IgG per dry weight was significantly increased in IDDM patients (3.51 ± 0.48) compared to NIDDM patients (1.76 ± 0.96) (P = 0.024) and where plasma IgM concentrations per ml were significantly increased for NIDDM patients (1.35 ± 0.37) compared to IDDM patients (0.6 ± 0.37) (P = 0.048).

**DISCUSSION**

As illustrated in Fig. 1 and elaborated in Table 1, the increased concentration of Alb found in the endoneurium of diabetic neuropathy patients is, therefore, not specific for Alb and can be extended to other larger plasma proteins, specifically IgG and IgM. The dramatic increase of these plasma macromolecules observed in the sural nerve biopsy of diabetic patients with and without neuropathy is suggestive of BNB disturbance, which in turn could result in an alteration in the nerve microenvironment and subsequent development.

Table 1. Alb, IgG, and IgM measurements in plasma and the endoneurial compartment of controls and diabetics with or without neuropathy

<table>
<thead>
<tr>
<th></th>
<th>Endoneurium</th>
<th>Plasma</th>
<th>BNB index</th>
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<tr>
<td></td>
<td>a (µg/ml)</td>
<td>b (µg/mg)</td>
<td>b/d, x 10^2</td>
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<tr>
<td>Alb</td>
<td></td>
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<tr>
<td>Controls (n = 10)</td>
<td>8.71 ± 3.72</td>
<td>2.48 ± 1.07</td>
<td>33.05 ± 6.20</td>
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<td>Diabetics (n = 6)</td>
<td>21.85 ± 4.60</td>
<td>10.60 ± 5.52</td>
<td>38.92 ± 6.80</td>
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<td>0.00001</td>
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<tr>
<td>Diabetic neuropathy (n = 18)</td>
<td>36.35 ± 12.20</td>
<td>14.12 ± 6.34</td>
<td>33.94 ± 13.38</td>
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<td>&lt;0.00001</td>
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<td>P, C vs. DN</td>
<td>0.005</td>
<td>NS</td>
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<tr>
<td>P, D vs. DN</td>
<td>NS</td>
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<tr>
<td>IgG</td>
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<tr>
<td>Controls (n = 10)</td>
<td>0.91 ± 0.45</td>
<td>0.27 ± 0.15</td>
<td>9.25 ± 2.32</td>
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<td>Diabetics (n = 6)</td>
<td>2.64 ± 1.17</td>
<td>1.30 ± 0.79</td>
<td>12.06 ± 5.16</td>
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<tr>
<td></td>
<td>0.0004</td>
<td>NS</td>
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<tr>
<td>Diabetic neuropathy (n = 18)</td>
<td>3.51 ± 2.78</td>
<td>1.24 ± 0.70</td>
<td>10.05 ± 4.41</td>
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<td>0.0036</td>
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<td>P, C vs. DN</td>
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<td>P, D vs. DN</td>
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<td>IgM</td>
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<tr>
<td>Controls (n = 9)</td>
<td>0.08 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>0.46 ± 0.17</td>
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<td>Diabetics (n = 6)</td>
<td>0.17 ± 0.14</td>
<td>0.10 ± 0.10</td>
<td>1.02 ± 0.49</td>
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<td>0.049</td>
<td>0.03</td>
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<tr>
<td>Diabetic neuropathy (n = 17)</td>
<td>0.20 ± 0.16</td>
<td>0.07 ± 0.07</td>
<td>0.78 ± 0.89</td>
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<td>0.024</td>
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<td>P, C vs. DN</td>
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Values shown represent mean ± SD. Where x is total Alb, IgG, or IgM, a = µg of x per mg (dry weight) of endoneurium; b = µg of x per µg of total protein of endoneurium (×10^2); c = µg of x per ml of plasma; d = µg of x per µg of total protein of plasma (×10^2); P values are derived from a two-sample t test; NS, not significant (P > 0.05). C, control; D, diabetic; DN, diabetic neuropathy.
of neuropathy. Although an underlying vascular mechanism is suggested, clearly more information is needed concerning the validity of assessing plasma macromolecules in the endoneurium of sural nerve biopsy samples and the usefulness of the BNP indices.

The observed increase in these plasma macromolecules within the endoneurium of diabetic patients without neuropathy is important since it suggests that these changes may precede the development of neuropathy. In support of this is the lack of a correlation between the BNP indices and the index of pathology for the diabetic neuropathy patients. The BNP indices may prove useful, therefore, in predicting when neuropathy may develop.

The endoneurial compartments containing the plasma proteins include the endoneurial fluid (space) compartment, the vascular compartment, and the cellular compartment. The assessment of these compartments in human sural nerve is currently dependent on morphometric methods of evaluation since dynamic permeability tracer studies have not been done. As a result, the following discussion addresses what is known about these compartments, particularly as it relates to the diabetic neuropathy patient.

The endoneurial cellular compartment is likely responsible for the local catabolism of these plasma macromolecules. Little information is available concerning the ultimate fate of the plasma proteins in the endoneurium. Previously, it was demonstrated that Alb was the major protein of the endoneurial fluid, which was collected by an elution technique, as identified by immunoblot analysis. We have hypothesized that the principal role of Alb in the endoneurial fluid is to maintain the osmotic pressure of the fluid, with secondary roles involving ligand transport into the endoneurium and cellular catabolism, which provides a nutritional source of amino acids. Immunoblot analysis of a soluble endoneurial fraction probed with Alb antibody indicated no lower molecular weight Alb immunoreactive peptides, suggesting that the cellular catabolism of Alb is likely to be either very low or very efficient and not a significant contribution to total endoneurial Alb concentration. This is supported by the immunocytochemical localization studies of Mata et al. (22), who have demonstrated Alb localization only to the endoneurial space and not to cells within the normal endoneurium. In addition, Seitz et al. (23) demonstrated IgG present in the endoneurial connective tissue of peripheral nerves 4 days after intraperitoneal injection of biotinylated IgG. Further immunocytochemical studies on sural nerve biopsy samples from diabetic neuropathy patients are clearly warranted, particularly since Schwann cells and axons have been demonstrated to show Alb immunoreactivity shortly after crush injury, which disappears as Schwann cell-axon contact is made during reinnervation (24). In addition, trapped immunoglobulins have been demonstrated on peripheral nerve myelin from diabetic patients, where the amount of trapped IgM was substantially greater than IgG. Deposition of immunoglobulins in peripheral nerve of diabetic neuropathy patients has also been observed by direct immunofluorescence.

With regard to the vascular compartment, studies comparing capillary number in human diabetic sural nerve showed no difference compared with controls (controls, 86.1 capillaries per mm²; diabetics, 53.8 capillaries per mm²; not significant; ref. 16). In addition, Yasaki and Dyck (27) demonstrated that there was no significant difference in the endothelial lumen area between diabetic neuropathy patients and controls (controls, 17.9 ± 16.0 µm²; diabetics, 21.0 ± 35.4; not significant). As a result, the increased endoneurial Alb found in diabetic neuropathy patients is not likely the result of a change in vascular space.

Morphometric studies have demonstrated that the endoneurial fluid compartment is much larger than the vascular compartment; hence, the primary compartment occupied by plasma proteins is clearly the endoneurial fluid. Although it has been hypothesized that the spinal fluid acts as a sink in the elimination of endoneurial fluid (28), studies with 125I-labeled Alb injected endoneurally in rat sciatic nerve show no direction of flow other than what would be expected from diffusion (J.F.P. and P.A. Low, unpublished data), although early work by Weiss et al. (29) suggested a slow proximodistal fluid convection in the endoneurial spaces.

Since the endoneurial fluid compartment is the major compartment in which Alb (3) and presumably other plasma proteins are found, this compartment in diabetic neuropathy patients requires careful evaluation. It has been demonstrated that the median intercapillary distance is not significantly different between patients and controls (controls, 0.08 mm; diabetics, 0.13 mm) (16), suggesting that endoneurial edema is not prominent in diabetic neuropathy. Since multifocal fiber loss is characteristic of diabetic neuropathy (30, 31), an increase in endoneurial space might be expected; however, this could be occupied by increased production of extracellular matrix. In fact, it has been suggested that improved return of function after nerve injury or recurrent demyelination might result if excess collagen synthesis could be minimized (32). In addition, an increase in endoneurial space would be expected to result in unchanged or decreased steady-state concentrations of endoneurial plasma proteins. Instead, increased endoneurial plasma proteins are found, suggesting that the endoneurial fluid compartment is not altered. It is clear that a more rigorous morphometric assessment of the endoneurial fluid compartment is needed in diabetic neuropathy patients.

From this discussion, it is reasonable to hypothesize that the endoneurial compartments containing plasma proteins in the diabetic neuropathy patients are similar to controls. If this is true, then alterations in macromolecular transport through the capillary endothelial barrier could explain the observed increases in endoneurial Alb, IgG, and IgM. This could result from an increase in selective transport in diabetics due to the aforementioned specific ligand modification of plasma proteins or, alternatively, from an alteration in the transport process across the capillary endothelium. Increased routes of entry might involve leaky tight junctions or plasma membranes; however, inspection of endoneurial microvessels at the electron microscope level does not indicate such abnormalities. Also, no fenestrations were observed in the diabetic nerve, although there have been separate reports of fenestrated endoneurial capillary endothelium in one case (33, 34).

An increase in intracellular vesicle transport could explain the present data. Interestingly, Yasuda et al. reported a significant increase in number of total cytoplasmic vesicles of endoneurial endothelial cells between control and diabetic subjects (controls, 225.8 ± 43.9; diabetics, 261.9 ± 64.0; P < 0.05); however, when expressed as a total number of vesicles per µm², no significant difference was found (H. Yasuda, J.F.P., and P.J.D., unpublished data). In addition, the density of free vesicles and the ratio of free/attached vesicles increased significantly with endoneurial Alb content for diabetic nerve. This might be related to the endothelial hyperplasia observed in sural nerves from diabetics (16). It has also been demonstrated in heart capillaries that there is an increase in cytoplasmic vesicles in alloxan diabetics (35).

Regardless of the altered route of entry, it is possible to address whether there is increased specific transport of plasma macromolecules or a general extravasation due to leaky tight junctions or plasma membranes or increased pinocytic uptake. If the ratio of BNP indices were the same in the diabetic endoneurium as that found in controls, then a general extravasation of plasma proteins would be expected based on their increasing hydrodynamic radii. As determined from Table 1, the ratio of the IgG/Alb-BNB indices was
decreased 19%, and the ratio of the IgM/Alb-BNB indices was decreased 56% in diabetic neuropathy patients compared to controls. Although the IgG and IgM concentrations are increased in the diabetic endoneurium, the Alb increase is greater, and a mechanism other than size indiscriminate extravasation of plasma proteins is, therefore, suggested.

Further research efforts are directed at defining the route of entry of these plasma macromolecules into the endoneurium, the mechanism of their transport, and their distribution within the endoneurial compartments in health and disease.

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