Recognition and Uptake of Human Diabetic Peripheral Nerve Myelin by Macrophages

HELEN VLASSARA, MICHAEL BROWNLEE, AND ANTHONY CERAMI

SUMMARY

Macrophages recognize and ingest several human proteins whose amino groups have been modified in vitro by specific chemical reagents. Because amino groups of human peripheral nerve myelin proteins become covalently modified in vivo by products of nonenzymatic glycosylation, we examined myelin/macrophage interactions using peripheral nerve myelin prepared from diabetic and nondiabetic patients.

Intracellular accumulation of diabetic myelin increased with concentration in an apparently saturable fashion, reaching levels 3–4 times higher than those of age-matched nondiabetic samples. Low-temperature inhibition of cellular myelin accumulation further suggested that diabetic myelin uptake was associated with adsorptive endocytosis. Macrophage recognition and accumulation of nondiabetic myelin glycosylated in vitro increased with duration of sugar incubation, to a level nearly nine times that of the same sample incubated in buffer alone. Data from competition experiments with albumin and myelin glycosylated in vitro showed that recognition of human peripheral nerve myelin proteins by macrophages is specific for protein-bound products of nonenzymatic glycosylation.

In vivo, such macrophage recognition of and interaction with nonenzymatic glycosylation products on diabetic peripheral nerve myelin could contribute to the pathogenesis of segmental demyelination. DIABETES 1985; 34:553–57.

Unique structural features of specific macromolecules render them recognizable by macrophages. Known examples include β-VLDL, LDL that has been modified in vitro by chemical reagents or in vivo by endothelial cells,[1] and immune complex-associated IgG.[2] Binding of these ligands to sites on the cell membrane stimulates interiorization and intracellular accumulation. Macrophage interactions with such specific macromolecules are thought to have an important role both in the maintenance of normal homeostasis and in the pathogenesis of several disease states.[3,4] We have recently provided evidence that protein modification by products formed during nonenzymatic glycosylation results in specific recognition and uptake by macrophages.[5] These data, obtained both in vitro and in vivo, suggest that advanced glycosylation endproducts (AGE), rather than the initial Amadori glycosylation products from which they slowly form, function as the primary recognition signal. In the present article, we have extended these investigations by examining the effects of in vitro and diabetes-induced nonenzymatic glycosylation on macrophage recognition and uptake of human peripheral nerve myelin.

MATERIALS AND METHODS

Human tissue. The samples of peripheral nerve used in this study were provided by the National Diabetes Research Interchange, Philadelphia, Pennsylvania. Autopsy tissue was obtained from seven subjects with type II diabetes and from eight nondiabetic subjects who had no clinical or pathologic evidence of peripheral neuropathy. Clinical information about these patients is summarized in Table 1.

Myelin preparation. Peripheral nerve myelin was isolated from normal and diabetic autopsy specimens by standard ultracentrifugation procedures described previously.[6] Preparation purity was documented by SDS-polyacrylamide gel electrophoresis.[7] Myelin prepared from normal and diabetic individuals had identical electrophoretic patterns.

In vitro glycosylation of nondiabetic myelin. Peripheral nerve myelin obtained at autopsy from a 44-yr-old nondiabetic individual (subject no. 11) was incubated in either 50 mM glucose-6-phosphate or 0.1 M phosphate-buffered saline for 8 wk at 37°C. Protease inhibitors (PMSF 1.5 mM, EDTA 0.5 mM) and antibiotics (penicillin 100 U/ml, gentamycin 40 mg/ml) were added to both. These incubation conditions

From the Laboratory of Medical Biochemistry, The Rockefeller University, New York, New York.

Address reprint requests to Helen Vlassara, M.D., Laboratory of Medical Biochemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021.

Received for publication 4 August 1984 and in revised form 20 November 1984.

TABLE 1
Clinical data

Patients	Subject no.	Age (yr)	Race/sex	Duration of diabetes (yr)	Treatment	Complications	Macrophage myelin uptake (μg/mg cell protein)
Diabetic	1	44	Black/male	15	Unknown	Atherosclerosis	4.7
	2	55	White/male	20	Insulin	Atherosclerosis hypertension	4.2
	3	61	White/female	23	Chlorpropamide	Atherosclerosis hypertension	5.3
	4	73	White/male	25	Tolazamide	Atherosclerosis heart failure	3.5
	5	75	White/female	14	Diet	Atherosclerosis hypertension	4.8
	6	79	White/male	2	Diet	Atherosclerosis hypertension	3.7
	7	72	Black/male	8	Chlorpropamide	Atherosclerosis hypertension	3.1

Patients	Subject no.	Age (yr)	Race/sex	Diagnosis	Macrophage myelin uptake (μg/mg cell protein)
Nondiabetic	8	10	White/male	Bronchial asthma, COPD	0.8
	9	12	White/male	Obstructive hydrocephalus	1.3
	10	35	Black/female	Unknown	0.8
	11	44	Black/male	Unknown	0.6
	12	52	Black/male	Metastatic squamous cell cancer	1.6
	13	56	White/male	Myocardial infarct	2.0
	14	64	Black/female	Cerebrovascular accident	1.26
	15	78	Black/female	Coronary artery disease	1.2

have been shown to produce a nearly sixfold increase in the extent of myelin nonenzymatic glycosylation.[8] The development of brown pigment on myelin incubated with glucose-6-phosphate suggests that significant accumulation of post-Amadori advanced glycosylation endproducts (AGE) occurs under these conditions as well.[9] After incubation, aliquots of the myelin samples (10 μg) were again subjected to SDS-polyacrylamide gel electrophoresis to establish that significant degradation of myelin proteins had not occurred.

It should be noted that only Amadori products are detected by current methods used to quantitate the extent of nonenzymatic glycosylation. At present, there is no specific quantitative assay for AGE proteins. However, brown pigments were observed in the in vitro and the long-term diabetic samples.

Radioiodination of myelin proteins. The method of Fraker and Speck was used to label human peripheral nerve myelin preparations with $Na^{125}I$.[10] The in vitro glycosylated preparations were radioiodinated after the completion of incubation with sugars. In the myelin used for macrophage experiments, >95% of the radioactivity was TCA precipitable. The specific activity was 5–10 × 10^3 cpm/μg and was not consistently different between glycosylated and nonglycosylated samples.

Isolation of mouse peritoneal macrophages. Inflammatory macrophages were obtained from thioglycolate-treated female NCS mice as described previously in detail.[5] Briefly, cells were obtained 6 days after injection of thioglycolate broth and plated in Linbro plastic Petri dishes (1.0 × 3.5 cm) (Linbro Scientific Inc., Hamden, Connecticut). Cells were incubated at 37°C in 5% CO_2. Each well contained 2 × 10^6 cells/well. Resident mouse peritoneal macrophages were incubated with both normal myelin and myelin glycosylated in vitro. The cellular accumulation of both normal and glycosylated myelin samples was low and not significantly different.[5] Similar experiments using thioglycolate-elicited macrophages instead resulted in marked differences in cell accumulation of radioactivity between normal and glycosylated myelin. For this reason, only elicited macrophages were used in these studies.

Myelin uptake experiments. Macrophage accumulation of human peripheral nerve myelin was evaluated by incubating aliquots of myelin with cells (25 μg/well) for selected periods of time up to 24 h. At each time point, the cell monolayers were lysed by addition of distilled H_2O after the culture medium had been removed and the cell monolayer had been washed three times. The amount of cell-associated ^{125}I-radioactivity was determined by counting the lysate in each well. The radioactivity in the cell lysate was 95% protein bound as assessed by TCA precipitation. In these experiments, macrophage accumulation of nondiabetic myelin glycosylated in vitro was compared with uptake of nonglycosylated myelin from the same preparation (subject no. 11), and accumulation of human peripheral nerve myelin from a diabetic subject (no. 6) was compared with uptake of myelin from an age-matched nondiabetic subject (no. 15).

The effect of degree of nonenzymatic glycosylation on macrophage myelin accumulation was assessed by experiments in which 25 μg aliquots of the in vitro myelin incubations described above were removed after 2–45 days of glycosylation and assayed. Identical aliquots of the same sample of myelin incubated in PBS without glucose-6-phosphate were used in control assays.

The effect of temperature on macrophage myelin accu-

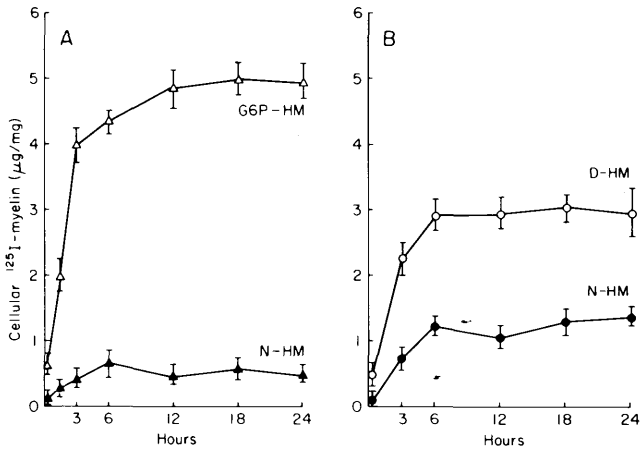

FIGURE 1. Accumulation of ^{125}I-human myelin by mouse peritoneal macrophages as a function of time (h). (A) Normal human peripheral nerve myelin previously incubated either in 50 mM glucose (△) or PBS (▲) at 37°C for 6 wk. (B) Diabetic human peripheral myelin from subject no. 6 (Table 1) (○) compared with normal myelin from age-matched subject no. 15 (Table 1) (●). Each well received 25 μg of labeled myelin. The data are expressed as the mean of four independent measurements.

mulation was evaluated by incubating identical concentrations (5–100 μg) of myelin from a diabetic subject (no. 3) and an age-matched nondiabetic subject (no. 14) at 37°C and 4°C for 4 h to confirm that accumulation of cell-associated radioactivity involved a temperature-dependent process of endocytosis.[11]

To demonstrate that the specificity of macrophage recognition was for the advanced nonenzymatic glycosylation products on diabetic myelin, competition experiments were performed[11] using myelin prepared from a 12-yr-old, nondiabetic subject (no. 9). A portion of this preparation was glycosylated in vitro and iodinated as described above. Varying amounts (25–2500 μg) of potentially competing unlabeled compounds (AGE-myelin, AGE-bovine serum albumin [BSA], nonglycosylated myelin from the same subject, and nonglycosylated BSA) were incubated with 25 μg of ^{125}I-AGE-human myelin, and the degree of uptake inhibition was determined.

In all experiments, myelin adsorption to plastic was ruled out by demonstrating that, in control wells containing ^{125}I-myelin alone, radioactivity did not exceed 0.5% of that observed in association with cells.

RESULTS

Macrophage accumulation of nondiabetic human peripheral nerve myelin glycosylated in vitro was nearly nine times greater than accumulation of the same sample incubated in phosphate-buffered saline (4.75 μg/mg cell protein versus 0.55 μg/mg, Figure 1A), and almost twice that of a representative diabetic myelin sample (3.7 μg/mg cell protein, Figure 1B). Intracellular accumulation of diabetic myelin from this patient (subject no. 6) was 2.6 times greater than accumulation of nondiabetic myelin (1.2 μg/mg) from an age-matched control (subject no. 15).

In all cases, accumulation increased to a steady-state level over the first 6 h. After this, levels remained constant for the duration of the experiment (24 h), indicating that the rate of degradation was equal to the rate of continuing uptake. These findings are similar to those previously reported for acetyl-LDL.[11] The different steady-state levels observed with different myelin preparations reflect differences in concentration of the modification being recognized by macrophages. Macrophage myelin uptake data for each of the other diabetic and nondiabetic subjects are presented in Table 1.

Since the rate and extent of nonenzymatic glycosylation is determined by time of exposure to glucose at any given glucose concentration,[9] macrophage recognition and accumulation of nondiabetic myelin was measured after increasing periods of in vitro nonenzymatic glycosylation. As shown in Figure 2, macrophage accumulation of nondiabetic myelin increased with increasing extent of nonenzymatic glycosylation over a period of 15 days. Beyond this time, no further increase in accumulation was observed over the next 25 days. No change was observed in uptake of identical samples incubated without sugar over the same period of time.

The effect of myelin protein concentration on macrophage accumulation is shown in Figure 3. When macrophages were incubated with increasing concentrations of ^{125}I-myelin from diabetic subject no. 3 and from an age-matched nondiabetic control (no. 14), the intracellular accumulation of the diabetic myelin preparation increased in an apparently saturable fashion, reaching levels 3–4 times higher than the nondiabetic samples. When identical experiments were carried out at both 37°C and 4°C, a temperature-dependent inhibition of cellular accumulation of radioactivity was observed (Figure 3). Together, these phenomena operationally define the process of adsorptive endocytosis.[11]

To assess the specificity of diabetic myelin accumulation by macrophages, competition experiments were performed using radioiodinated, in vitro-glycosylated peripheral nerve myelin from a 12-yr-old nondiabetic subject (no. 9). Increasing concentrations (25–500 μg) of unlabeled glycosylated

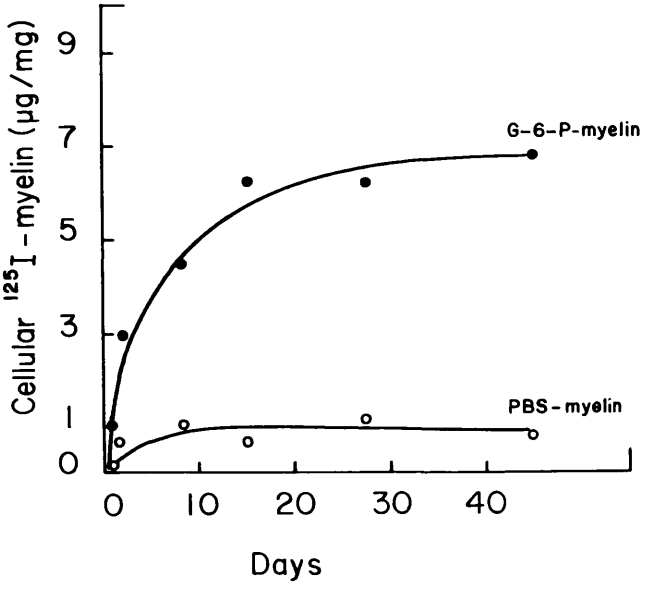

FIGURE 2. Effect of increasing in vitro nonenzymatic glycosylation on intracellular myelin accumulation. Identical concentrations of normal peripheral nerve myelin from subject no. 9 (Table 1) were incubated in the presence of 50 mM G-6-P (●) or PBS (○) for 2–45 days. Aliquots (25 μg) were assayed in the presence of macrophages at 37°C at the indicated time intervals.

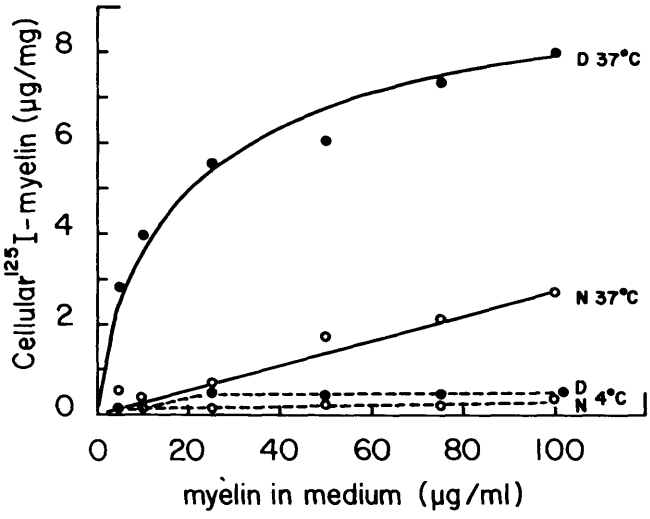

FIGURE 3. Effect of concentration and temperature on cellular accumulation of ^{125}I-diabetic myelin from subject no. 3 (Table 1) (●) and ^{125}I-control myelin from age-matched subject no. 14 (Table 1) (○). Identical concentrations of each ligand (5–100 µg/ml) were incubated at either 37°C or 4°C.

human peripheral nerve myelin and, similarly, glycosylated bovine serum albumin (BSA) competed effectively with ^{125}I-advanced glycosylation endproduct (AGE)-myelin accumulation. Nonglycosylated nondiabetic myelin and nonglycosylated BSA, on the other hand, failed to compete even at maximum concentrations (Figure 4).

DISCUSSION

The proteins of peripheral nerve myelin have been shown to undergo nonenzymatic glycosylation both in vitro and in vivo.[5,6,8] The hyperglycemia of diabetes produces elevated levels of the initial Amadori glycosylation products on these long-lived structural proteins. In addition, subsequent reactions of the Amadori product slowly give rise to pigmented advanced glycosylation endproducts (AGE), which accumulate to a greater extent on diabetic nerve as well. The observations described in this report suggest that such excessive nonenzymatic glycosylation could contribute to the development of long-term diabetic peripheral nerve pathology by rendering myelin proteins recognizable by macrophages. Recognition of human diabetic peripheral nerve myelin proteins by macrophages is specific for protein-bound products of nonenzymatic glycosylation, as demonstrated by competition experiments, and is associated with adsorptive endocytosis, as demonstrated by low-temperature inhibition of cellular myelin accumulation and by saturation of myelin accumulation with increasing ligand concentration. These data are similar to those reported for macrophage recognition and endocytosis of proteins such as LDL after covalent modification.[3,4] Products of nonenzymatic glycosylation formed both in vitro and in vivo on human peripheral nerve myelin initiate recognition and uptake by macrophages. The greater degree of accumulation observed with in vitro glycosylated myelin most likely reflects more extensive formation of recognizable products of nonenzymatic glycosylation achieved by incubation with high concentrations of glucose-6-phosphate.

Caution must be used in extrapolating from in vitro experiments to the in vivo situation, since isolated myelin may provide a nonphysiologic access to phagocytes. However, in vivo studies of peripheral nerve morphology in chronic galactose neuropathy suggest that this is not the case.[12] In this diabetic-like model, electron microscopy of peripheral nerve revealed numerous macrophages encircling and penetrating damaged fibers, stripping away myelin.[12] Macrophage-like cells containing axonal or myelin debris have also been observed in peripheral nerves of diabetic mice.[13]

The importance of macrophage interactions with AGE-myelin proteins may vary from patient to patient. Segmental demyelination, which does not appear to be a secondary response to axonal atrophy, is prominent in some cases of polyneuropathy, while axonal degeneration is prominent in others.[14,15] Since nonenzymatic glycosylation has been shown to be increased in the axonal component of rat diabetic nerve as well,[6] an interaction of axonal proteins with macrophages may also occur. This possibility has not yet been evaluated, however.

The interaction between human diabetic peripheral nerve myelin and macrophages initiated by accumulated products of nonenzymatic glycosylation could contribute to the segmental demyelination associated with diabetes by a variety of mechanisms. One possibility is that demyelination may result from augmented secretion of proteolytic enzymes triggered by interaction of AGE-myelin with its receptor. Macrophage secretion of such neutral proteases as plasminogen activator has already been reported in response to maleylated albumin binding.[16] Similar plasminogen activator secretion induced by covalently modified myelin would probably lead to segmental demyelination resembling that associated with diabetes, since the major protein components of peripheral nerve myelin are particularly susceptible to degradation by plasmin.[17]

Intracellular glycosylated protein accumulation similar to that described here (Figure 1A) has been observed using

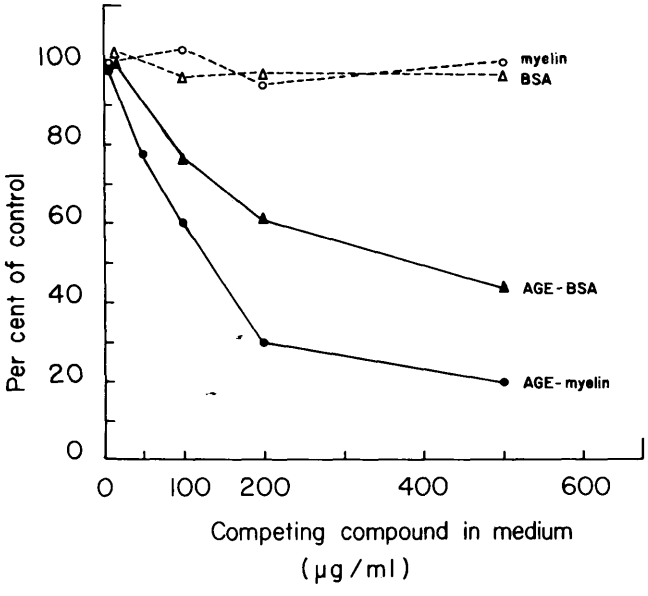

FIGURE 4. Intracellular accumulation of ^{125}I-AGE-myelin from subject no. 9 (Table 1) in the presence of increasing concentrations of unlabeled normal (○) or AGE (●) myelin from the same subject and normal (△) or AGE-BSA (▲). Each well received 25 µg of ^{125}I-AGE-myelin and the indicated amounts of unlabeled competitor. Values are expressed as percent of control. The 100% value is 5.4 µg.

serum albumin and isolated microvessels.[18] Unmodified albumin was excluded from ingestion by microvessel endothelial cells, while albumin that had been nonenzymatically glycosylated was taken up rapidly by endocytosis. This recognition and endocytosis of nonenzymatically glycosylated plasma albumin by capillary endothelial cells could be involved in the blood-retinal barrier dysfunction that occurs soon after the onset of diabetes. More precise definition of the biologic consequences of both macrophage and endothelial cell interactions with glycosylated body proteins will clarify the role of recognition and endocytosis in the pathogenesis of diabetic complications.

Excessive formation of advanced glycosylation endproducts on myelin protein most likely acts in synergy with other pathogenetic mechanisms known to occur in diabetic peripheral nerve. Diabetes-induced increases in polyol pathway activity[19] and decreases in nerve *myo*-inositol content in conjunction with reduced Na^+/K^+ ATP-ase activity[20] also appear to contribute to the progressive deterioration of peripheral nerve function. The rate at which peripheral neuropathy develops in a given diabetic individual may reflect independent contributions from each of these causal factors.

ACKNOWLEDGMENTS

We thank Celia Curran for excellent technical assistance. The expert secretarial assistance of Alice Striegel is also greatly appreciated.

This work was supported by NIH grants 1-RO1-AM-19655 and 1-KO1-AG-00148. H. V. is the recipient of a Research Scientist Development Award (1-KO1-AG-00148) from the National Institute of Arthritis, Metabolism, and Digestive Diseases and the National Institute of Aging. M. B. is the recipient of a Special Emphasis Research Career Award from the National Heart, Lung, and Blood Institute and the National Institute of Arthritis, Metabolism and Digestive Diseases and the American Diabetes Association, New York Diabetes Affiliate.

REFERENCES

[1] Steinberg, D.: Lipoproteins and atherosclerosis—a look back and a look ahead. Arteriosclerosis 1983; 3:283–301.

[2] Silverstein, S. C., Steinman, R. M., and Cohn, Z. A.: Endocytosis. Annu. Rev. Biochem. 1977; 46:669–722.

[3] Goldstein, J. L., and Brown, M. S.: The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 1977; 46:897–930.

[4] Brown, M. S., and Goldstein, J. L.: Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu. Rev. Biochem. 1983; 52:223–61.

[5] Vlassara, H., Brownlee, M., and Cerami, A.: Accumulation of diabetic rat peripheral nerve myelin by macrophages increases with the presence of advanced glycosylation endproducts. J. Exp. Med. 1984; 160:197–207.

[6] Vlassara, H., Brownlee, M., and Cerami, A.: Excessive nonenzymatic glycosylation of peripheral and central nervous system myelin components in diabetic rats. Diabetes 1983; 32:670–74.

[7] Spritz, N., Singh, H., and Marinan, B.: Metabolism of peripheral nerve myelin in experimental diabetes. J. Clin. Invest. 1975; 55:1049–56.

[8] Vlassara, H., Brownlee, M., and Cerami, A.: Nonenzymatic glycosylation of peripheral nerve protein in diabetes mellitus. Proc. Natl. Acad. Sci. USA 1981; 78:5190–92.

[9] Brownlee, M., Vlassara, H., and Cerami, A.: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann. Intern. Med. 1984; 101:527–37.

[10] Fraker, P. J., and Speck, J. C.: Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. 1978; 80:849–57.

[11] Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S.: Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Natl. Acad. Sci. USA 1979; 1:333–37.

[12] Powell, H. C., and Myers, R. R.: Schwann cell changes and demyelination in chronic galactose neuropathy. Muscle Nerve 1983; 6:218–27.

[13] Carson, K. A., Bossen, E. H., and Hanker, J. S.: Peripheral neuropathy in mouse hereditary diabetes mellitus. II. Ultrastructural correlates of degenerative and regenerative changes. Neuropathol. Appl. Neurobiol. 1980; 6:361–74.

[14] Thomas, P., and Eliasson, S.: Diabetic neuropathy, *In* Peripheral Neuropathy. Vol. 2. Dyck, P. J., Thomas, P. K., and Lambert, E. H., Eds. Philadelphia, W. B. Saunders, 1975:956–76.

[15] Sidenius, P.: The axonopathy of diabetic neuropathy. Diabetes 1982; 31:356–63.

[16] Johnson, W. J., Pizzo, S. V., Imber, M. J., and Adams, D. O.: Receptors for maleylated proteins regulate secretion of neutral proteases by murine macrophages. Science 1982; 218:574–76.

[17] Cammer, W., Brosnan, C. F., Bloom, B. R., and Norton, W. T.: Degradation of the P_0, P_1, and P_2 proteins in peripheral nervous system myelin by plasmin: implications regarding the role of macrophages in demyelinating diseases. J. Neurochem. 1981; 36:1506–14.

[18] Williams, S. K., Devenny, J. J., and Bitensky, M. W.: Micropinocytic ingestion of glycosylated albumin by isolated microvessels: possible role in pathogenesis of diabetic microangiopathy. Proc. Natl. Acad. Sci. USA 1981; 78:2393–97.

[19] Clements, R. S., Jr.: The role of abnormal polyol metabolism in diabetic complications. *In* Diabetes Mellitus and Obesity. Brodoff, B. N., and Bleicher, S. J., Eds. Baltimore, Williams and Wilkins, 1982:117–28.

[20] Greene, D. A.: Metabolic abnormalities in diabetic peripheral nerve: relation to impaired function. Metabolism 1983; 7 (Suppl. 1):118–23.