ACCUMULATION OF DIABETIC RAT PERIPHERAL NERVE
MYELIN BY MACROPHAGES INCREASES WITH THE
PRESENCE OF ADVANCED GLYCOSYLATION
ENDPRODUCTS

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Diabetic peripheral neuropathy, the most common long-term complication of
diabetes mellitus, is characterized morphologically by segmental demyelination.
The extent and degree of this pathological change correlates with the severity
and duration of hyperglycemia (1).

We have previously shown that increased nonenzymatic glycosylation (NEG),
a process known to occur in a number of body proteins (2–4), occurs in peripheral
and central nervous tissue of diabetic humans and animals (5). More recently,
we have demonstrated that most of the NEG occurs specifically on the structurally
and quantitatively major myelin proteins: PO-protein in peripheral nerve myelin
and proteolipid and basic protein in central nervous system myelin (6). The
pathophysiologic mechanism by which this biochemical alteration might lead to
myelin breakdown and removal is not as yet understood.

Short-term incubation of proteins with glucose results in the formation of
ketoamine adducts (Amadori product) with protein amino groups. These adducts
are reversible and reach equilibrium after several weeks. In addition to detaching
from the protein, these glyco-adducts can undergo very slowly further rearrange-
ments, dehydrations, and reactions to form a number of advanced glycosylation
end-products (AGE), which are characteristically yellow-brown fluorescent chromophores that can cross-link proteins. In contrast to the initial adduct, these
AGE adducts are irreversible and can therefore accumulate on long-lived proteins
such as collagen, lens crystallins, basement membrane, and myelin (7).

The recent isolation of and assignment of a structure to an AGE protein
adduct has provided crucial information about how two NEG proteins can

1 Abbreviations used in this paper: AGE, advanced glycosylation end products; BSA, bovine serum albumin; FBS, fetal bovine serum; FFI, 2-furoyl-4(5)-(2-furanyl)-1H-imidazole; NEG, nonenzymatic
glycosylation; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.
condense to form a specific yellow fluorescent product, 2-furoyl-4(5)-(2-furanyl)-1H-imidazole (FFI) (8).

We postulated that perhaps more extensive modification of myelin protein structure by these AGE adducts might be necessary to stimulate mobilization of tissue macrophages against myelin. Such a stimulus could induce myelin protein uptake and degradation through receptor-mediated phagocytosis, proteolytic enzyme secretion (9), or complement activation in the absence of myelin-specific antibodies (10).

In this study, we report that AGE-myelin is modified so that it becomes recognizable by macrophages. We have demonstrated that elicited macrophages accumulate 125I-labeled peripheral myelin that has been extensively glycosylated in vitro and in vivo. This response correlates well with the extent and duration of myelin glycosylation.

**Materials and Methods**

*Myelin Preparation from Normal and Diabetic Rats.* Peripheral myelin was isolated from sciatic nerves of alloxan-induced diabetic (mean blood glucose above 250 mg/dl), andagematched normal Sprague Dawley rats by standard ultracentrifugation procedures as described previously (6). Myelin was isolated from young animals with early diabetes (4-5 wk) and from older rats with diabetes of long duration (1.5-2.0 years). Aliquots of myelin (10 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) for verification of preparation purity (11). The pattern of protein bands from all diabetic myelin samples was identical to that observed with identically treated normal myelin.

*In Vitro Glycosylation of Peripheral Nerve Myelin.* Normal peripheral myelin was incubated separately in 50 mM glucose, 50 mM glucose-6-phosphate (G-6-P) or 0.1 M phosphate-buffered saline (PBS) at 37°C for 8 wk, in the presence of protease inhibitors (PMSF 1.5 mM, EDTA 0.5 mM) and antibiotics (penicillin 100 U/ml, gentamicin 40 mg/ml).

In order to determine the amount of nonenzymatic glycosylation present in these preparations, an affinity chromatography system that selectively retains glycosylated adducts was utilized. Portions of myelin preparations were first subjected to reduction with NaB3H4 (specific activity 80 µCi/µmol) and then chromatographed on immobilized m-aminophenyl-boronic acid as described previously (12). Glycosylated myelin averaged 12,000 ± 2,000 cpm/µmol of total amino acid, while control samples averaged only 2,000 ± 000 cpm/µmol of total amino acid. Aliquots of myelin (10 µg) were subjected to SDS–PAGE for verification of preparation purity (11). The pattern of protein bands from all glycosylated myelin samples was identical to that observed with identically treated normal myelin. 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 (13). However, brown pigments were observed in the in vitro and the long-term diabetic samples.

*125I-Iodination of Myelin Proteins.* Portions of each myelin preparation were radioiodinated by the Iodogen method of Fraker and Speck (14). Samples were dialyzed against a 1,000-fold excess of 0.01 M PBS (pH 7.0) until >95% of the radioactivity was TCA precipitable. Protein concentration was determined by the Bradford method (15). Specific activity varied from 45,000-50,000 cpm/µg.

*Isolation of Mouse Peritoneal Macrophages.* Female NCS mice (25-30 g) were obtained from the LARC facility of The Rockefeller University. Resident peritoneal macrophages were harvested in PBS as described by Edelson and Cohn (16). Inflammatory macrophages were obtained 6 d after the intraperitoneal injection of 3.0 ml of Brewer’s thioglycolate broth (Difco Inc., Detroit, MI). The fluid from 15-20 mice (6.8 × 10⁶ cells/mouse) was pooled and centrifuged at 500 g for 10 min, washed once with 20 ml of Dulbecco’s
Modified Eagle's Medium (Gibco Laboratories, Grand Island, NY) and resuspended in medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Aliquots were plated in Linbro plastic petri dishes (1.0 × 3.5 cm) (Linbro Scientific, Inc., Hamden, CT), and incubated at 37°C in 5% CO2 for 2 h. After three washes with medium, each well received 1.0 ml of fresh medium, containing varying amounts of 125I-labeled rat peripheral nerve myelin preparations. Each dish at that point contained ~60% of the total number of cells originally plated (4–5 × 10^5 cells/well).

**Myelin Uptake and Degradation Experiments.** To evaluate the accumulation of myelin by macrophages as a function of time, aliquots containing 80–100 μg of normal, short-term diabetic, long-term diabetic, and in vitro glycosylated myelin protein were added to each macrophage culture dish and incubated for various periods of time. At selected intervals, the culture medium was removed, cell monolayers were washed three times and then lysed by either addition of 1.0 ml distilled H2O or by rapid freeze-thawing. The lysate in each well was counted in a Packard liquid scintillation counter in order to determine the amount of cell-associated 125I-radioactivity.

In order to establish that cell-associated radioactivity represents internalization rather than surface binding of protein, the 125I-myelin-containing medium was removed at the end of a 5-h incubation, and trypsin (Sigma Chemical Co., St. Louis, MO) was added (0.05% final concentration) to the wells. After incubation at 37°C for 5 min, the cell monolayers were washed four times with media containing 20% FBS and processed as described above.

Further evidence that internalization had occurred was sought by incubating identical concentrations (5 to 100 μg) of AGE- and control-myelin at 37°C and 4°C for 4 h (17).

The specificity of the AGE-myelin interaction with macrophages was assessed by incubating 25 μg of 125I-AGE-myelin with varying amounts (25 to 2,500 μg) of potentially competing unlabeled compounds as previously described (17). Substances evaluated were AGE-myelin, AGE-BSA, yeast mannan (Sigma), control myelin, and control BSA.

To evaluate rates of cellular myelin degradation, 125I-myelin (50 μg/well) from diabetic and age-matched normal tissue was incubated with cells for 6 h. The myelin-containing medium was removed and the cells were washed three times with 1.0 ml serum-free medium. Following this, the amount of 125I-labeled material remaining in the cells was measured at 0, 2, 4, 6, and 12 h.

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

**Results**

**Uptake of Myelin Proteins Glycosylated In Vitro.** 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 (mean 0.4 ± 0.2 μg/well). 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 subsequent studies.

The specificity of AGE-myelin accumulation by thioglycolate-elicited macrophages was assessed by experiments in which macrophages were incubated with 125I-AGE-myelin in the presence of increasing concentrations (25–2,500 μg) of cold AGE-myelin, AGE-BSA, yeast mannan, control myelin, and control BSA (Fig. 1). Unlabeled AGE-myelin and AGE-BSA were both effective competitors of 125I-AGE-myelin accumulation. Similar experiments in which 25 μg of 125I-control myelin was incubated with increasing amounts (25–2,500 μg) of unlabeled control myelin showed a concentration-dependent inhibition of 125I-control mye-
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FIGURE 1. Intracellular $^{125}$I-AGE-myelin accumulation in the presence of increasing concentrations of potentially competing unlabeled compounds. Substances tested were AGE-myelin (O), AGE-BSA (△), yeast mannan (O), control myelin (●), and control BSA (X). Each dish received $25 \mu g$ of $^{125}$I-AGE-myelin and the indicated amounts of unlabeled competitor and was incubated for 5 h at $37°C$. Values are expressed as percent of control. The 100% value was $5.9 \mu g$.

FIGURE 2. Effect of temperature on cellular accumulation of $^{125}$I-AGE-myelin (△) and $^{125}$I-control myelin (●). Identical concentrations of each labeled ligand ($25 \mu g$ to $100 \mu g/ml$) were incubated at either $37°C$ or $4°C$.

lin (data not shown). In contrast, neither unlabeled control nor unlabeled control BSA competed with $^{125}$I-AGE-myelin accumulation. Yeast mannan also failed to compete.

In these experiments 75–80% of cell-associated radioactivity remained after incubation with trypsin. A temperature-dependent inhibition of cellular accumulation of radioactivity was observed when identical experiments were carried out at both $4°C$ and $37°C$ (Fig. 2).

Macrophage accumulation of $^{125}$I-myelin previously incubated in 50 mM glucose or G-6-P for 8 wk at $37°C$ was compared with the accumulation of myelin incubated in 0.1 M phosphate-buffered saline (PBS) for the same period of time. Fig. 3 displays the uptake of the various myelin preparations. Accumulation increased in a linear fashion for the first 6 h, and remained constant
thereafter (up to 24 h). Maximal rates of intracellular accumulation of G-6-P glycosylated myelin (mean 10.8 ± 0.4 μg/well) and glucosyl-myelin (mean 6.4 ± 0.4 μg/well) were 10 times and 5 times greater, respectively, than myelin samples incubated in buffer alone (mean 1.2 ± 0.2 μg/well). The difference between the rate observed with glucose and G-6-P probably reflects differences in the rate of nonenzymatic protein glycosylation, which increases significantly when G-6-P is substituted for glucose in equimolar concentrations (18).

**Uptake of Myelin Proteins from Short- and Long-term Diabetic Animals.** Experiments comparing uptake of 125I-myelin isolated from short-term diabetic animals (4–6 wk) with uptake of myelin from age-matched controls showed similar low rates of intracellular accumulation of labeled material in both groups (normal: 1.2 ± 0.2 μg/well; diabetic: 2.0 ± 0.4 μg/well) (Fig. 4). In contrast, accumulation of 125I-myelin isolated from rats with long-term diabetes (1.5–2.0 years) was three to four times greater than that of age-matched normals (normal: 2.4 ± 0.5
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μg/well; diabetic: 9.0 ± 0.5 μg/well) (Fig. 5) and nine times greater than that of the 4–6-wk old normal myelin shown in Fig. 4.

Effect of AGE on Intracellular Myelin Degradation. After incubating ¹²⁵I-myelin (50 μg/well) from both long-term diabetic and normal tissue with macrophages for 6 h, the myelin-containing medium was removed, the cell monolayers were washed thoroughly and fresh medium was added. At specific time intervals, trichloroacetic acid (TCA)-soluble radioactivity in the fresh media and TCA-insoluble radioactivity within the cells were measured separately.

Despite the several-fold greater amount of diabetic myelin accumulated within the cells at the end of 6 h, the relative rate of disappearance of TCA-insoluble myelin from within the cells (Fig. 6), and the rate of appearance of soluble degradation products in the fresh media (data not shown) was the same for both diabetic and normal after 12 h (Fig. 6).

Discussion

The results presented in this report show that the NEG products formed by long-term exposure of peripheral nerve myelin proteins to glucose in vitro and in vivo markedly alter the way in which myelin interacts with activated macrophages. In this interaction, macrophages appear to specifically recognize Advanced Glycosylation Endproducts (AGE) on myelin since AGE-BSA competes nearly as effectively as AGE-myelin, while neither unmodified BSA nor unmodified myelin compete. The failure of yeast mannan to interfere with macrophage recognition of AGE-myelin suggests that the mannose/fucose receptor does not mediate this process.

Recognition of AGE-protein by macrophages is associated with endocytosis, as demonstrated by resistance of cell-associated radioactivity to removal by trypsin action, and by low-temperature inhibition of ligand accumulation in the cellular fraction. The rate and extent of NEG of proteins are functions of both glucose concentration and time of exposure to glucose (19). Thus, the high sugar concentrations used (900 mg/dl) in the in vitro glycosylation experiments resulted in a significant increase in myelin accumulation, while peripheral nerve myelin

![Figure 5. Accumulation of ¹²⁵I-myelin from long-term diabetic (●) and age-matched normal rats (○) by mouse peritoneal macrophages as a function of time (h). Each well (4.0 × 10⁵ cells) received 100 μg of labeled myelin. The data are expressed as a mean of six independent measurements.](image)
from rats having diabetes for a similar length of time (4–6 wk, blood glucose range 250–350 mg/dl), was taken up by macrophages almost to the same low degree as myelin from age-matched normals. Accumulation of myelin from diabetic rats exposed to similar levels of hyperglycemia for two years, however, was almost 9 times greater than that of myelin from young normal and 3.5 times more than that of myelin from age-matched controls. These data suggest that short-term hyperglycemia in vivo results in fewer glucose-induced alterations "recognizable" by macrophages than does long-term hyperglycemia. Different degrees of myelin accumulation by macrophages were also observed between the two normal myelin preparations from rats differing only in age. This most probably reflects a similar, although much slower, NEG process occurring in normal myelin as a function of age.

Intracellular myelin accumulation reflects an equilibrium between uptake, degradation, and secretion by the macrophages. In order to dissect out the main components associated with the observed intracellular difference, the disappear-
ance of acid-insoluble radioactivity from within the cells and the appearance of acid-soluble radioactivity released into the medium were monitored independently over an extended period of time (12 h). The observed rate of intracellular degradation recorded was very similar for the two groups (Fig. 6). Thus, increased uptake of long-term diabetic myelin appeared to be the main contributor to the striking difference in accumulation seen between the two groups.

Endocytosis of myelin with advanced glycosylation is consistent with previously described increases in receptor-mediated phagocytosis associated with macrophage stimulation (20). The mechanism by which this activation occurs in vivo is not known.

Macrophage endocytosis of a number of covalently modified proteins appears to be mediated by scavenger receptors that recognize induced changes in charge and/or conformation (17, 21). Proteins modified using formaldehyde, glutaraldehyde, malondialdehyde, acetylation, acetoacetylation, carbamylation, and maleylation have all been shown to stimulate uptake via these scavenger receptor pathways (17, 22–24).

The observations presented in this report suggest that covalent protein modification by AGE results in a similar recognition and uptake by a scavenger pathway. Witzum et al. (25) have reported no scavenger pathway uptake of glycosylated low density lipoprotein by peritoneal macrophages following short-term glucose incubation. However, Haberland et al. (21) have recently demonstrated that protein uptake by a scavenger receptor does not occur until a threshold level of modified residues is reached (21). The differences observed by us between macrophage uptake of long-term and short-term diabetic myelin are most likely a reflection of this phenomenon.

The interaction of maleylated albumin with its receptor has recently been reported to trigger secretion of neutral proteases such as plasminogen activator (26). Since the major protein components of peripheral nervous myelin are particularly susceptible to degradation by plasmin (27), similar plasminogen activator secretion induced by covalently modified myelin could lead to the segmental demyelination associated with diabetes.

AGE-adducts attached to long-lived proteins such as collagen or myelin have been shown to trap covalently plasma proteins such as IgG leading to in situ formation of immune complexes (28). This could also contribute to macrophage-associated destruction of diabetic tissues. Indeed, deposition of IgM and complement (C3) has recently been shown to occur on peripheral nerves of diabetic patients having clinical peripheral neuropathy (29).

The observations described in this report provide a pathophysiological link between excessive myelin protein nonenzymatic glycosylation induced by hyperglycemia and segmental demyelination in diabetes. Over long periods of time, diabetes can induce chemical modification of long-lived myelin proteins, which results in the formation of advanced glycosylation endproducts (10, 15). Formation of these irreversible products appears to modify the properties of myelin proteins in a way "recognizable" by macrophages, thus, potentially initiating or contributing to the segmental demyelination associated with diabetes and the normal aging of peripheral nerve. The early, more reversible steps of nonenzymatic glycosylation do not appear capable of inducing such a response. The
irreversible nature of AGE adducts formed on long-lived proteins such as myelin suggests that clinical efforts at controlling hyperglycemia in diabetic patients must be initiated early in the course of the disease in order to be effective.

**Summary**

We have previously shown that increased nonenzymatic glycosylation occurs in peripheral nervous tissue of diabetic humans and animals, primarily on the PO-protein of peripheral never myelin. The pathophysiologic mechanism by which this biochemical alteration leads to myelin breakdown and removal is not as yet understood.

In the present study we show that advanced glycosylation end-product (AGE) adducts that form during long-term exposure of peripheral nerve myelin proteins to glucose in vitro and in vivo markedly alter the way in which myelin interacts with elicited macrophages. In this interaction, macrophages appear to specifically recognize AGEs on myelin, since AGE-BSA competes nearly as effectively as AGE-myelin, while neither unmodified BSA nor unmodified myelin compete. The failure of yeast mannan to interfere with macrophage recognition of AGE-myelin suggests that the mannose/fucose receptor does not mediate this process.

Recognition of AGE-protein by macrophages is associated with endocytosis, as demonstrated by resistance of cell-associated radioactivity to removal by trypsin action, and by low temperature inhibition of ligand accumulation in the cellular fraction. $^{125}$I-labeled myelin that had been incubated in vitro with 50 mM glucose for 8 wk reached a steady state accumulation within thioglycolate-elicited macrophages that was five times greater than that of myelin incubated without glucose. Similarly, myelin isolated from rats having diabetes for 1.5–2.0 years duration had a steady state level that was 9 times greater than that of myelin from young rats, and 3.5 times greater than that of myelin from age-matched controls. In contrast, myelin isolated from rats having diabetes for 4–5 wk had the same degree of accumulation observed with myelin of age-matched normal rats. These data suggest that the amount of increased nonenzymatic glycosylation observed in the myelin of short-term diabetic rats had not yet resulted in the significant accumulation of AGE-myelin present both in vitro and in the long-term diabetic rats.

The disappearance of acid-insoluble radioactivity from within the cells and the appearance of acid-soluble radioactivity released into the medium were very similar for the two groups, suggesting that the striking difference in accumulation seen between normal myelin and AGE-myelin is due primarily to increased uptake.

Formation of irreversible AGE-adducts on myelin appears to promote the recognition and uptake of the modified myelin by macrophages. This interaction between AGE-myelin and macrophages may initiate or contribute to the segmental demyelination associated with diabetes and the normal aging of peripheral nerve.

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