Experimental Diabetic Neuropathy

Effect of Ganglioside Treatment on Axonal Transport of Cytoskeletal Proteins

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Abnormalities in axonal transport of proteins are thought to play an important role in the pathogenesis of diabetic neuropathy. Gangliosides exert a positive action on numerous alterations in biochemistry and physiology of diabetic nerves. This study was undertaken to assess the effects of exogenous gangliosides on the axonal transport of structural proteins such as actin and tubulin in the sensory fibers of short-term (9-wk) and long-term (6-mo) diabetic rats. Adult Sprague-Dawley rats were made diabetic with a single injection of 70 mg/kg streptozocin i.p. Subgroups were injected daily with either highly purified ganglioside mixture (10 mg/kg i.p.) or saline for 1 mo, beginning either 2 or 17 wk after streptozocin injection. Age-matched rats were used as controls. Axonal transport was studied by the pulse-labeling technique. Three weeks after labeling, sciatic nerves were dissected out and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. In diabetic rats of both experimental designs, the transport rate of tubulin and actin was decreased by ~30% compared with control rats. Ganglioside treatment counteracted such alterations in both 9-wk and 6-mo diabetic rats. These data suggest a pharmacological effect that could be correlated with molecular interactions between integral membrane glycolipids and cytoskeletal elements.
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Experimental diabetic neuropathy, the pathogenesis of which is far from being completely understood (1,2), is characterized by dysmetabolic events leading, in the chronic state, to functional and structural derangements of the nerve fibers, such as reduction in nerve conduction velocity, axonal atrophy, and degeneration (3,4).

Many reports indicate that the transport rate of some cytoskeletal proteins is affected in the nerve fibers of diabetic animals (5–7). Axonal transport of molecules and organelles either synthesized or assembled in the perikaryon is crucial to the maintenance of the structural and functional integrity of axons and synaptic terminals (8). Therefore, it is conceivable that any defect in the continuous delivery of materials travelling down the axon entails neuropathological changes, leading eventually to marked dysfunction of peripheral nerves (9), such as that observed in experimental diabetes.

In addition, several studies emphasize that the axonal transport is affected more precociously and to a greater extent in sensory fibers of peripheral nerves than in motor fibers (7,10,11). These experimental findings correlate well with the clinical symptoms and signs in human diabetic polyneuropathy, in which sensory deficits occur earlier in the course of the disease.

Within this framework, numerous preclinical studies have addressed the issue of pharmacological approaches that, by normalizing the axonal transport, could be capable of treating the neuropathic complication as a whole. Among the drugs tested (such as insulin and aldose reductase inhibitors; 12,13), we have focused our attention on gangliosides (GA), sialic acid–containing glycosphingolipids particularly abundant in the neuronal plasma membrane (14). GA facilitate structural and functional regeneration of peripheral diabetic nerves, an effect correlated with prevention and/or recovery of axonal metabolic activities (15,16), increase in the rate of axonal maturation (17,18), and reestablishment of functionally and morphologically normal neuromuscular junctions (19). In addition, GA treatment is efficacious in restoring the axonal transport of several enzymes, such as acetylcholinesterase (20), 6-phosphofructokinase (21), and Na^+,K^+-ATPase (22) in either alloxan- or streptozocin (STZ)-induced diabetic rats. Clinically, these ex-

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perimental data support the increasing number of reports that demonstrate the efficacy of ganglioside therapy in improving several sensory parameters in diabetic patients with either borderline or clearcut neuropathic signs and symptoms (23,24).

On these grounds, this study was undertaken to ascertain whether the multifaceted ganglioside effects could also be attributed to the maintenance of normal axonal transport of structurally important molecules, such as the cytoskeletal proteins actin and tubulin, the delivery of which along sensory fibers should subserve a correct relationship between axolemma and intra-axonal compartments.

RESEARCH DESIGN AND METHODS

Induction and monitoring of diabetes. This study used male Sprague-Dawley rats (Charles River Italia, Calco, BG, Italy) weighing ~250–300 g. One group of rats was made diabetic with a single dose of 70 mg/kg STZ i.p. (Sigma, St. Louis, MO) dissolved to a concn of 70 mg/ml in 10 mM citrate buffer (pH 4.5). Two weeks later, glycosuria was determined by Glucur-test strips (Boehringer Mannheim, Mannheim, Germany); nonfasting blood glucose levels were measured at the beginning of labeling experiments and at the killing time by Glucoscot (Menarini, Firenze, Italy). STZ-treated rats with blood glucose levels <16.8 mM were excluded from the study. At killing time, diabetic state was further assessed by evaluating HbA_{1c} (Glycohemoglobin-HbA_1 kit, Sigma).

Pharmacological treatment of rats. STZ-induced diabetic rats were divided into two subgroups and treated immediately as follows: one group (untreated diabetic rats) received daily intraperitoneal injections of saline, whereas the second group (GA-treated diabetic rats) was injected daily with 10 mg/kg of highly purified GA mixture i.p. (20% GM_1, 42% GD_{1a}, 15% GD_{1b}, 19% GT_{1b}, 2% GD_3, and 2% GQ_{1b}) for 4 wk. Age-matched rats, injected with either saline or GA, were used as controls.

The remaining diabetic rats were subdivided into two additional groups and treated 17 wk after STZ injection with daily injections of either saline or GA (10 mg/kg i.p.) for 4 wk. Two more groups of age-matched rats were used as controls. All rat procedures were carried out as described previously (24a).

Axonal transport studies. Axonal transport of cytoskeletal proteins was studied at the end of the pharmacological treatments, 6 and 21 wk after STZ injection, respectively, with the pulse-labeling technique. To label sensory axons of the sciatic nerve, the fourth lumbar dorsal root ganglion (DRG) was exposed by laminectomy under deep halothane anesthesia; 50 μCi [^{35}S]methionine (Amity, Milano, Italy; sp act 1200 Ci/mM) in 0.5 μl were injected unilaterally, with a glass capillary micropipette (tip internal diam 20–40 μm) over a 5-min period. Rats were killed by decapitation 21 days after intraganglionic injection; the sciatic nerves were dissected out, frozen in liquid N_2, and stored at −70°C until processed. The nerves were then cut into 3-mm–long consecutive segments, homogenized in 50 μl sample buffer (65 mM Tris-HCl, 2% [wt/vol] sodium dodecyl sulfate [SDS], 10% glycerol, 2 mM EGTA, 5% [wt/vol] 2-mercaptoethanol [pH 6.8]), and heated at 100°C for 5 min. The homogenates were spun for 3 min in an Eppendorf centrifuge, and the supernatants were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in a 5–15% gradient acrylamide slab gel, according to Laemmli (25). Coomassie Brilliant Blue–stained gels were dried and exposed to Kodak XOmat films. Fluorograms of the gels were scanned by a LKB-Ultroscan laser densitometer and analyzed through the LKB 2190 Gelscan program. The analysis focused on polypeptides identified as tubulin and actin by virtue of their molecular weight (56,000 and 43,000 M_r, respectively).

Quantification of axonal transport. The amount of radioactivity associated with tubulin and actin along the sciatic nerve was calculated from the densitometric reading of fluorograms. The radioactivity of each nerve segment was expressed as the percentage of total radioactivity, according to the following equation:

$$\text{relative radioactivity of the } i\text{th segment} = (ri/\Sigma ri) \times 100$$

where ri is the densitometric reading corresponding to the radioactivity of the ith segment (i = any nerve segment between 1 and 15). The mean distribution was obtained by averaging the relative radioactivity values in each segment. The transport rate of slow component b (SCb) for tubulin and actin was calculated according to the method described by Jakobsen and Sidenius (26), by determining the position of the center of mass in which most radioactivity was concentrated in each nerve, omitting the L_4 DRG (site of injection) and the four most proximal nerve segments (12 mm) that describe SCa. The transport rate was then expressed as the average distance (mm) traveled by the radioactivity 21 days after labeling. Data from each group were statistically analyzed by Duncan's test.

RESULTS

Body weight, blood glucose levels, and HbA_{1c} percentages are shown in Table 1. In both experimental paradigms (short- and long-term diabetes), untreated and GA-treated diabetic rats showed a similar weight loss compared with controls. Hyperglycemia and high HbA_{1c} were found in both 9-wk and 6-mo diabetic rats; GA treatment did not modify these parameters in either group.

Axonal transport. Gel electrophoresis and the relevant fluorograms showed many bands that, by comparison with peptidic markers of known M_r (Fig. 1*A*), were recognizable as neurofilament (NF) subunits (200,000, 145,000, and 68,000 M_r, respectively), tubulin, and actin (Fig. 1*B*). The analysis of the distribution of radioactivity associated with tubulin and actin indicated that the portion of the polypeptides carried by SCa was not affected in diabetic rats (data not shown; 10). Reconstruction of densitometric scans (Fig. 2) gave rise to curve profiles describing the proteins belonging to SCb; these curves appeared shifted toward more proximal segments in the diabetic nerve regardless of the duration

TABLE 1
Metabolic parameters in 9-wk and 6-mo diabetic rats

	n	Weight (g)	Blood glucose (mM)	HbA_{1c} (%)
Nine wk				
Untreated control	6	502 ± 15	7.72 ± 1.23	3.53 ± 0.3
GA-treated control	6	501 ± 24	5.52 ± 1.56	3.72 ± 0.45
Untreated diabetic	5	272 ± 49	29.9 ± 5.82	6.95 ± 0.83
GA-treated diabetic	5	283 ± 61	29.5 ± 4.2	6.75 ± 0.68
Six mo				
Untreated control	6	538 ± 61	5.04 ± 0.56	3.18 ± 0.85
GA-treated control	6	551 ± 53	5.28 ± 1.62	3.2 ± 0.71
Untreated diabetic	5	250 ± 39	30.8 ± 5.2	6.92 ± 1.1
GA-treated diabetic	5	249 ± 50	29.4 ± 7.8	7.15 ± 1.3

Values are means ± SD. GA, gangliosides.

of diabetes, thereby indicating a delay in the transport (Fig. 3, *A–D*).

The decrease in transport rate of tubulin was ~30% in both experimental designs (Table 2). In diabetic rats treated with GA for 1 mo, this decrease was limited to only 10%, corresponding to a 60% recovery; interestingly, in long-term (6-mo) diabetes, the tubulin transport rate recovered by ~90%. In addition, a similar impairment was observed in the transport rate of actin in both 9-wk and 6-mo diabetic rats, the decrease being 35 and 25%, respectively, when compared with the transport rate in control rats. GA treatment induced a recovery of 32% in short-term diabetes and 65% in long-term diabetes (Table 2). Note that GA did not affect the axonal transport of cytoskeletal proteins in controls; the transport rate of tubulin and actin in GA-treated controls being not significantly different from that of untreated controls (Table 2).

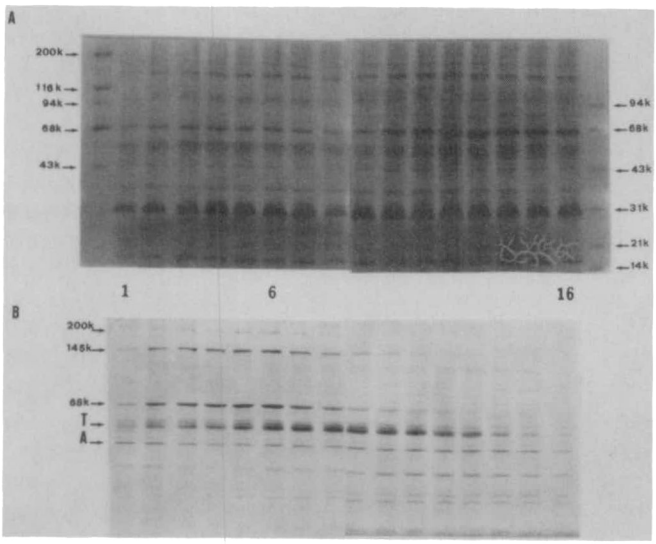

FIG. 1. Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis slab gel (*A*) and relative fluorogram (*B*) of consecutive segments inclusive of L_4 dorsal root ganglion (*lane 1*) and sciatic nerve (*lanes 2–16*) 21 days after intraganglionic injection of ^{35}S-methionine. *Lanes* on extreme *left* and *right* (*A*) display M_r markers (proteins with known M_r used as reference). Neurofilament subunits are 200,000, 145,000, and 68,000 M_r; tubulin (T) and actin (A) are indicated (*B*).

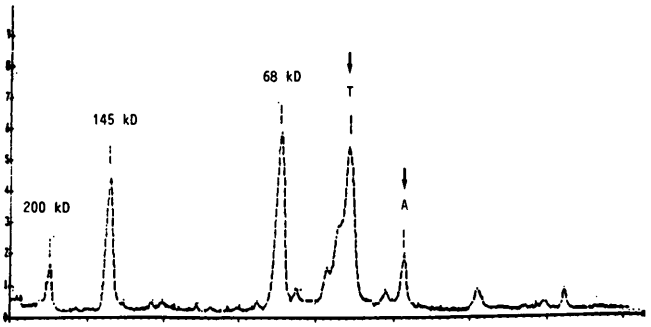

FIG. 2. Densitometric scan of *lane 6* in the fluorogram of Fig. 1*B*. *Arrows*, peaks of proteins tubulin and actin.

DISCUSSION

STZ administration to rats induces a pathological condition characterized by high levels of blood glucose, high percentage of HbA_{1c}, ketoacidosis, and body weight loss, thereby providing an appreciable model for human insulin-dependent diabetes mellitus (27). STZ-treated rats also develop a neuropathy with decrease in nerve conduction velocity and reduction of distal axonal caliber (5). This study, although confirming that a derangement in the transport rate of cytoskeletal proteins occurs early in the course of experimental diabetes and persists for at least several months after the induction of the disease, demonstrates that this alteration is relatively steady with time and affects mainly the SCb of tubulin and actin (6).

Data relevant to axonal transport in experimental diabetes have been mainly obtained with either radioactive isotope pulse-labeling techniques or measurement of accumulated endogenous substances at ligatures (28). Because both methods provide only indirect evaluation of axonal transport (29,30), conflicting results have been reported especially regarding the effects of diabetes on the fast component. However, despite these methodological constraints, most of the previous studies have shown clearcut modifications of slow axonal transport in diabetic neuropathy, therefore suggesting the existence of an intrinsic relationship between disease processes and axonal transport (31).

Our data demonstrate that GA treatment counteracts the alterations in the transport of cytoskeletal proteins. With different methods, GA had already proved to be efficacious in restoring the axonal flow of soluble enzymes (20,21) and the membrane-bound Na^+,K^+-ATPase (22). Although these results coincide in demonstrating the capability of GA to partially reestablish this particular function in diabetic nerves, the mechanism or mechanisms by which GA could exert their action remains to be fully elucidated.

Several investigations pointed out that GA are bidirectionally transported with the fast (~360 mm/day) component of axonal transport in both motor and sensory fibers of sciatic nerve (32,33). There is no evidence that such an antero- and retrograde transport may be involved in the action of exogenous GA. Published data also indicate a selective effect of GA on the induction of cytoskeletal proteins, such as MAP-2 and tubulin, and suggest a possible GA-mediated regulation of their gene

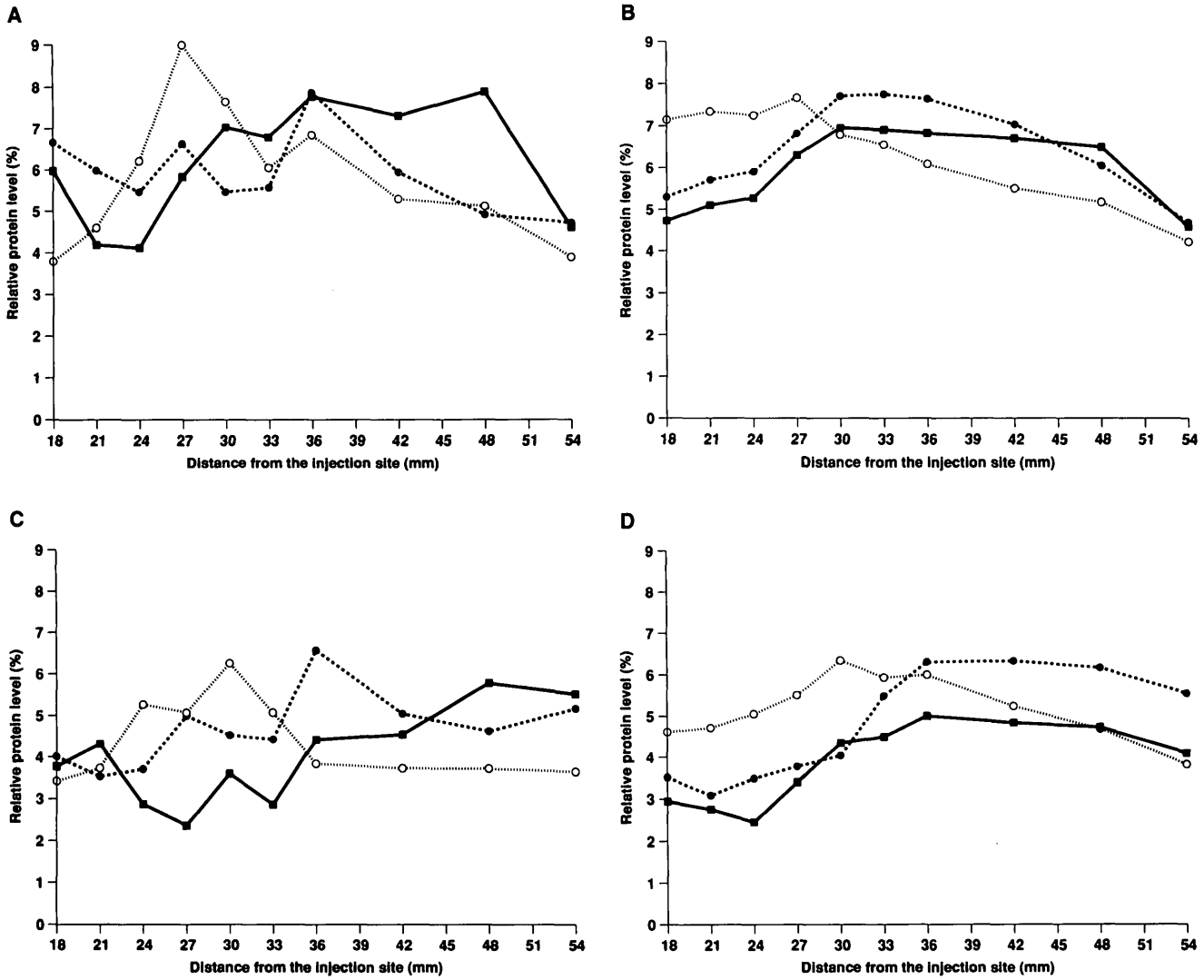

FIG. 3. Radioactivity profiles associated with tubulin (*A* and *B*) and actin (*C* and *D*) slow component b (between 18 and 54 mm from the injection site L4 dorsal root ganglion) in the sensory fibers of sciatic nerve from 9-wk (*A* and *C*) and 6-mo (*B* and *D*) diabetic rats. ■, Control; ○, untreated diabetic; ●, ganglioside-treated diabetic.

expression (34,35). However, in diabetes, and in other neuropathic conditions, it remains to be clearly assessed whether the decrease in the transport rate of cytoskeletal proteins entails or depends on a correspondent reduction of their gene expression and synthesis (36,37).

An alternative hypothesis envisages a molecular interaction between membrane-inserted GA and cytoskeletal elements, mediated through some proteins of the so-called subaxolemmal cytoskeleton (38–40). The role of these proteins, such as ankyrin and spectrin, has been investigated recently to a great extent. In particular, several data suggest a direct link between some integral membrane proteins (Na^+,K^+-ATPase, Na^+ channels) related to the conduction of action potential, and ankyrin, which in turn appears to be associated either directly to microtubules or indirectly to actin microfilaments through fodrinlike molecules (41–43). Because GA are highly efficacious in correcting the defects of Na^+,K^+-ATPase activity in diabetic nerve (15,16,44), it is likely that the recovery of derangements in the axonal transport elicited by gangliosides could be mediated by these integral membrane proteins. On the other hand, some of them are definitely involved in ionic pumps or in the regulation of voltage-activated ionic channels. It is also well known that a correct intra-axonal concentration of some ions, such as Ca^{2+} and Mg^{2+}, is required to maintain axoplasmic transport (45) and to regulate cytoskeletal dynamics (46); in fact, these ions are involved in the enzymatic activities (ATPases) of both membrane- and microtubule-associated proteins. Therefore, exogenous GA, once inserted into the neuronal plasma membrane, might exert an indirect effect on the axonal transport by modulating the mechanisms that control intra-axonal ionic concentration (47).

In conclusion, the data provided herein demonstrate that GA treatment improves the defects in the axonal transport of tubulin and actin in both short- and long-term diabetic rats. Therefore the GA-induced normalization of the axonal flow can subserve to better understand the

TABLE 2
Transport rate (mm/21 days) of tubulin and actin in the sciatic sensory fibers of 9-wk and 6-mo diabetic rats

	Tubulin	Actin
Nine wk		
Untreated control	40.5 ± 1.5	49.5 ± 2.8
GA-treated control	39.0 ± 3.0	45.0 ± 3.5
Untreated diabetic	30.6 ± 1.1*	31.8 ± 0.7*
GA-treated diabetic	36.0 ± 2.4†	38.0 ± 2.0*†
Six mo		
Untreated control	38.4 ± 2.2	44.4 ± 4.0
GA-treated control	37.5 ± 4.5	39.0 ± 3.0
Untreated diabetic	27.5 ± 1.4*	33.0 ± 1.0‡
GA-treated diabetic	37.5 ± 2.8§	40.7 ± 1.7†

Values are means ± SE. GA, gangliosides.
*$P < 0.01$, ‡$P < 0.05$, vs. control.
†$P < 0.05$, §$P < 0.01$, vs. untreated diabetic (Duncan's test).

beneficial effects of GA in both experimental and human diabetic neuropathy.

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