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Research Article

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Serum from Patients with Type 2 Diabetes with Neuropathy Induces Complement-independent, Calcium-dependent Apoptosis in Cultured Neuronal Cells

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Abstract

We hypothesized that sera from type 2 diabetic patients with neuropathy contains an autoimmune immunoglobulin that promotes complement-independent, calcium-dependent apoptosis in neuronal cell lines. Neuronal cells were cultured in the presence of complement-inactivated sera obtained from patients with type 2 diabetes with and without neuropathy and healthy adult control patients. Serum from diabetic patients with neuropathy was associated with a significantly greater induction of apoptosis, compared to serum from diabetic patients without neuropathy and controls. In the presence of calcium channel antagonists, induction of apoptosis was reduced by ~ 50%. Pretreatment of neuronal cells with serum from diabetic patients with neuropathy was associated with a significant increase in elevated K^+-evoked cytosolic calcium concentration. Serum-induced enhancement in cytosolic calcium and calcium current density was blocked by treatment with trypsin and filtration of the serum using a 100,000-kd molecular weight filter. Treatment with an anti–human IgG antibody was associated with intense fluorescence on the surface of neuronal cells exposed to sera from patients with type 2 diabetes mellitus with neuropathy. We conclude that sera from type 2 diabetic patients with neuropathy contains an autoimmune immunoglobulin that induces complement-independent, calcium-dependent apoptosis in neuronal cells. (*J. Clin. Invest.* 1998. 102:1454–1462.) Key words: programmed cell death • serum factors • cell injury • autoimmune • cytosolic calcium

Introduction

Diabetic neuropathy is a common symptomatic complication of diabetes mellitus and encompasses a group of clinical syndromes with manifestations involving somatic and autonomic peripheral nerves. Diabetic neuropathy occurs with the same frequency in type 1 and 2 diabetic patients (1). Peripheral nerve abnormalities in animal and human diabetic patients, such as decreased conduction velocity, axonal swelling, and nerve fiber loss (2–5) have been linked to metabolic alterations (6), including altered calcium signaling (7). Studies involving human and animal models of diabetes indicate that altered cytosolic calcium homeostasis is a common defect in both insulin-dependent and non-insulin–dependent diabetes, resulting in increased cytosolic calcium levels (7–11). Elevation in cytosolic calcium concentration has been linked to apoptosis in a number of experimental models (12–15).

Recent evidence suggests that a serum factor (or factors) may play a role in the pathogenesis of diabetic neuropathy in patients with type 1 diabetes mellitus. Treatment of cultured pancreatic β-cells with serum from patients with type 1 diabetes mellitus was associated with enhanced L-type calcium currents (16). Exposure of neuroblastoma cells to complement-activated sera from patients with type 1 diabetes with neuropathy was associated with decreased neurite outgrowth, enhancement of calcium influx, and induction of apoptosis (17–19). However, the linkage of enhanced calcium influx and induction of apoptosis was not proven in previous studies (16, 19). Furthermore, the potential contribution of autoimmune immunoglobulin(s) and altered calcium signaling in the pathogenesis of neuropathy in type 2 diabetes has not been examined. We observed the presence of an autoimmune immunoglobulin in sera from type 2 diabetic patients with neuropathy that was associated with complement-independent, calcium-dependent induction of apoptosis in vitro in neuronal cell lines.

Methods

Patient information

Prior approval for these studies was obtained from the University of Michigan Institutional Review Board. After informed consent was obtained, sera were collected from patients with type 2 diabetes with (DN^+)[1] and without (DN^-) neuropathy and healthy adult controls. All patients were recruited from the Michigan Diabetes Research and Training Center Clinical Core at the University of Michigan (Ann Arbor, MI). Data on the sex, weight, hemoglobin A_{1C} (HbA_{1C}), serum glucose, serum creatinine, duration of diabetes, and neuropathy is presented in Table I. None of the patients had evidence of any other autoimmune diseases. Control patients were free of diabetes, autoimmune disease, and neuropathy. All control studies were performed on the same day as with diabetic patients. The control and diabetic samples were age matched and both stored at −70°C for the same duration of time.

Clinical neuropathy was defined as an abnormal neurological examination that was consistent with the presence of peripheral sensorimotor neuropathy plus either abnormal nerve conduction in at least two peripheral nerves or unequivocally abnormal autonomic nerve testing. Abnormal sensorimotor exam was determined by

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecules; DN^+, diabetic patients with neuropathy; DN^-, diabetic patients without neuropathy; DRG, dorsal root ganglion; NGF, nerve growth factor; sBP, systolic blood pressure; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

Table I. Demographics and Patient Characteristics of Study Patients

	DN^+	DN^-
Patients (*n*)	10	7
Age (yr)	65±2.5	67±6
Sex (% female)	15	15
Weight (lbs)	207±10	200±17
Duration of diabetes (yr)	21±3	2.75±0.5
Duration of neuropathy (yr)*	7.7±1.6	N/A
HbA_{1C} (%)	8.4±0.5	7.4±0.4
Serum glucose (mg/dl)	222±2.5	178±24
Serum creatinine (mg/dl)	1.2±0.2	1.1±0.2

*Neuropathy defined by the presence of symptoms and signs of neuropathy and the presence of delayed nerve conduction velocity by electromyogram (Methods, patient data), and/or autonomic neuropathy. N/A: not applicable. All values are x±standard error. Adult control patients had no evidence of diabetes, autoimmune disease, or neuropathy. No significant difference was noted in age, sex, weight, HbA_{1C}, or creatinine in DN^+ versus DN^- patients. The duration of diabetes was significantly lower in DN^- patients compared to DN^+. $P < 0.05$.

screening for symptoms of numbness, burning, pain or cramps in legs or feet, and signs of abnormal sensation (light touch, pain, and vibration), muscle strength, and tendon reflexes in the extremities. Patients with nephropathy and proliferative retinopathy were excluded from the study. Nerve conduction velocities were performed on the nondominant side with the lower limb maintained at 32°C and the upper limb at 33°C. Sural, median, and ulnar sensory–evoked potential amplitudes, and distal and peak latencies were evaluated. The amplitudes of the compound muscle action potentials for the peroneal and median motor nerves and their respective distal latencies and conduction velocities were performed. A nerve was considered abnormal if any attribute (amplitude, distal latency, or conduction velocity) was not within the normal limits, defined as values between the first and 99th percentiles (20). When two or more nerves were abnormal, nerve conduction was considered abnormal.

Autonomic function testing. The resting heart rate was calculated after a period of 20-min supine rest. The heart rate variability response to six deep breaths/min (5 s in and 5 s out) was recorded for 1 min on a continuous electrocardiogram trace. The maximum and minimum R–R intervals during each breathing cycle were measured and converted to beats/min, and a mean value was calculated for the six measured cycles. The heart rate response to the Valsalva maneuver (expiration against a pressure of 40 mm Hg for a period of 15 s) was performed three times, and a mean value was calculated for the ratio of the longest R–R interval after the maneuver to the shortest R–R interval during the maneuver. The patient then rested supine for 20 min. After a mean supine systolic blood pressure (sBP) was measured, the patient stood erect, and the sBP was recorded immediately and at 1-min intervals, thereafter, for a further 5-min period. The lowest standing sBP was recorded, and the sBP fall calculated. If two of four tests were outside published normal values (21), the patient was considered to have abnormal autonomic function.

Reagents

DME, Ham's F-12, FBS, penicillin, streptomycin, goat serum, and trypsin were obtained from GIBCO BRL Laboratories (Gaithersburg, MD). 7S-murine nerve growth factor (NGF), aphidocolin, collagenase, trypsin, trypan blue, neural-buffered formalin, RNase, *cis*-platin, FITC-linked anti–human IgG antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Terminal transferase enzyme, biotin-conjugated dUTP and FITC-avidin, were obtained from Boehringer Mannheim Biochemical (Indianapolis, IN). Fura-2 acetylmethyl ester and slowfade were obtained from Molecular Probes (Eugene, OR).

Cell cultures

Studies were performed on two neuronal cell lines, as well as primary sensory neurons (dorsal root ganglion neurons [DRG]), to corroborate our observations in more than one type of neuronal preparation.

SH-SY5Y. The human neuroblastoma cell SH-SY5Y clone was provided by Dr. Martin Stevens, Department of Endocrinology, University of Michigan. The neuronal cells were maintained in 150-cm^2 Corning T-150 flasks in a 1:1 mixture of DME and Ham's F-12 containing 15% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 15-mm Hepes buffer at 37°C with a 10% CO_2 atmosphere. Neuronal cells were detached using Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) for 2 min at 37°C and replated at 10^6 cells/150-cm^2 flask. Passage number never exceeded 30.

Cell differentiation. SY5Y cells were exposed to 1 μg/ml 7S-murine NGF in the above growth medium, immediately after replating, and the medium was changed every two days for the duration of the culture. Aphidocolin, an inhibitor of a DNA polymerase, was added to the medium in the second week of treatment at 10 μg/ml. After 4 wk of differentiation with NGF, the neurons were used in the experiments (22). Differentiated SY5Y neuronal cells were plated at 0.3 million cells per 22-mm coverslip in a 35-mm culture dish. Neuronal cells were cultured in DME/F-12 containing 15% FCS for 24 h. The medium was removed and washed with PBS and the neuronal cells cultured for 48 h in DME/F-12 containing 15% of either control or test serum.

F-11 neuronal cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2/95% air in Ham's F-12 media supplemented with 15% FCS and penicillin (100 IU/ml) and streptomycin (100 μg/ml) (23). Media were changed three times per week.

DRG neurons. Isolated, acutely dissociated DRG neurons were aseptically prepared from 3–6-wk Sprague-Dawley rats (Indianapolis, IN). Rats were killed by inhalation of 100% carbon dioxide, the spinal column removed, and thoracic and lumbar DRGs extracted. DRGs were trimmed, minced, incubated with 0.3% collagenase and 0.1% trypsin, and then triturated and centrifuged. Enzymes and incubating media were composed of sterile MEM supplemented with 16 mM $NaHCO_2$ and 28 mM D-glucose (320 mosm), and filtered (0.2 μm). Isolated DRGs were resuspended in supplemented MEM containing 10% sera from DN^+ patients with neuropathy and non-diabetic controls, and plated onto collagen-coated tissue culture dishes. No other sera or growth factors were added to the culture media. Cells were incubated in 93% air + 7% CO_2 at 37°C for 24 h (24).

Cell viability

After exposure of SY5Y neuronal cells to medium containing 5–15% of either control or test serum, cell viability was assessed. Cells were exposed to 0.2% trypan blue for 10 min and the number of trypan blue positive cells per 200 cells were counted in a blinded manner (25).

Determination and quantitation of apoptosis

Apoptosis was detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method, as described below. The morphological characteristics of apoptosis induced by serum was confirmed using electron microscopy.

TUNEL method. SY5Y neuronal cells were cultured for 24–72 h in DME containing 5–15% of either control or test serum. On a single experimental day, each subject's serum was applied on neurons, and this was performed in duplicate. Neuronal cells were fixed in 4% neural-buffered formalin for 15 min followed by methanol for 10 min. Neurons were stored at −20°C for 24 h. Apoptosis was detected using the TUNEL method (26). The test is based on the principle that terminaldeoxynucleotidyl transferase catalyzes a template-independent addition of deoxynucleotides to free 3′OH ends present in DNA

breaks. This tailing reaction is especially sensitive to the type of DNA fragmentation occurring in apoptotic rather than necrotic cell death (27). Coverslips were washed with cold PBS and incubated with biotin-conjugated dUTP and terminal transferase enzyme for 1 h at 37°C. After washing, the neurons were incubated with FITC-avidin for 1 h at room temperature (light protected). The specimens were mounted in slowfade containing 5 μg/ml propidium iodide and 0.05 mg/ml Dnase-free Rnase. The FITC-labeled DNA fragments in the apoptotic cells can be visualized using fluorescent microscope. 200 neuronal cells were counted blindly and the positive cells expressed as a percentage of total neurons counted. The percentage of neurons undergoing apoptosis was standardized to the number of neurons attached to the coverslip after exposure to serum for 48 h, followed by fixation and immunohistochemistry using the TUNEL method. This includes adherent early necrotic cells that will stain with propidium iodide but not with the FITC. The number of necrotic neurons was not subtracted from each group before calculating the percent of apoptotic neurons. Negative control received only the label solution without terminal transferase and the positive control had Cisplatin (2 μg/ml) in the medium (28). The reliability of the TUNEL method for detection of apoptosis was established in our laboratory using *cis*-platinum as a positive control for the induction of apoptosis. The sensitivity and specificity of the TUNEL method was confirmed in our system using measurement of neuronal DNA content in conjunction with flow cytometry, and electron microscopy.

Electron microscopy. Differentiated SY5Y neuronal cells were plated on 35-mm dishes and cultured for 1 d (28). Neuronal cells were exposed to 15% sera from control or a type 2 diabetic patient with neuropathy for 48 h. The neuronal cells were fixed for 10 min in 3% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4, postfixed in 1% OsO4 in 0.18 M phosphate buffer for 10 min at 40°C, dehydrated in graded alcohols, and embedded in epoxy resin. Thin sections were cut perpendicular to the bottom of the culture dish and stained with uranyl acetate and lead citrate. Sections were examined with a Phillips CM100 transmission electron microscope (Mahwah, NJ) and digitized images were obtained using a Kodak Mega Plus 11.6 camera (Rochester, NY).

Effect of calcium channel antagonists on serum-induced apoptosis

SY5Y neuronal cells and F-11 neuronal cells were exposed to control or diabetic serum for 24–48 h in the presence of calcium channel antagonists, ω-conotoxin GVIA (*N*-type channel antagonist, 100 nM), and nifedipine (L-type channel antagonist, 10 mM) at 37°C. Experiments were performed in the dark to avoid inactivation of nifedepine. After 24–48 h, neuronal cells were fixed and stained for apoptosis by the TUNEL method described above. The number of TUNEL(+) neuronal cells per 200 neurons were counted under fluorescent microscopy. Counting was performed in a blinded manner.

Measurement of calcium

Calcium measurements were performed using two different techniques: the calcium-sensitive dye Fura-2 to measure cytosolic calcium concentration, and patch-clamp electrophysiology to measure calcium current density.

Cytosolic calcium. Using the Fura-2 technique (29), cytosolic calcium ($[Ca^{2+}]_i$) concentration was measured in differentiated SY5Y neuronal cells exposed to control, DN^-, or DN^+ serum for 48 h. Coverslips were washed in PBS, and then incubated for 40 min in culture medium containing 2.5-mM Fura-2 acetylmethyl ester and free acid at 37°C in an incubator, and then placed into a perfusion chamber on the stage of an inverted microscope (Nikon Inc., Melville, NY) and superfused continuously with oxygenated Krebs buffer at 37°C. To avoid any bias due to decreased responsiveness to depolarization in neurons previously depolarized, only one set of neuronal cells was studied on each slip. Fluorescence was elicited by illumination with light wavelengths alternating between 340 and 380 nm at 0.5-s intervals. Fluorescence was recorded using a calcium-imaging system (Zeiss Axiovert; Attaflor Ratiovision, Rockville, MD). The ratio of the signals generated at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ (nM) by the method of Grynkiewicz et al. (29). Calibration of $[Ca^{2+}]_i$ was performed according to Gelperin et al. (30). The intracellular calcium is calculated using the formula $[Ca^{2+}]_i = K_d\,[R - R(Lo)/R(Hi) - R]*Den(Lo)/Den(Hi)$ where R: measured 340:380 ratio, K_d: dissociation constant for the dye, R(Lo): ratio for low standard, R(Hi): ratio for high standard, Den(Lo): denominator intensity for low standard, and Den(Hi): denominator intensity for high standard. The SY5Y neuronal cells were depolarized with 15-s applications of elevated K^+ buffer (60 mM KCl). The basal $[Ca^{2+}]_i$ and amplitude of peak $[Ca^{2+}]_i$ response to depolarization was measured. Acute serum application studies were performed by exposure of SY5Y or F-11 neuronal cells to 1/40 dilution of serum in buffer for 30 s, followed by depolarization with elevated K^+ buffer for 15 s. Studies done with each subject's sera were repeated twice on two separate experiment days.

Whole-cell voltage-clamp recordings. 24 h after exposure to control or diabetic sera, whole-cell voltage-clamp recordings were performed on DRG neurons. Isolated, phase-bright DRG neurons 20–40 mm in diameter were identified and whole-cell voltage-clamp recordings using the whole-cell variant of the patch-clamp technique (31) were made at room temperature. Glass recording patch pipettes (Fisher Microhematocrit tubes; Fisher Scientific Co., Pittsburgh, PA) with electrode resistance 1–2 MΩ, and seal resistances > 1 GV were used. Recordings were made in culture dishes containing nonperfused external bath solution consisting of: 5 mM $CaCl_2$, 67 mM choline Cl, 100 mM TEA Cl, 5.6 mM glucose, 5.3 mM KCl, 10 mM Hepes, and 0.8 mM Mg^{2+} Cl (pH 7.4, 320–330 mosmol). Recording electrodes were filled with 140 mM CsCl, 10 mM Hepes, 10 mM EGTA, 5 mM Mg^{2+} ATP, and 0.1 mM Li GTP (pH 7.3–7.4, 280–290 mosm). DRGs were depolarized with voltage steps generated by the program CLAMPEX (pCLAMP; Axon Instruments, Foster City, CA). Calcium currents were recorded using an Axopatch 200 A amplifier with an input resistance of 1–3 MΩ, filtered with a Bessel filter 10 kHz, sampled at 20 kHz, and stored on hard disk. High-threshold currents were evoked in neurons held at −80 mV by 100 ms duration depolarizations to +10 mV every 20 s. Current-voltage (IV) curves were generated by depolarizing neurons from a holding potential of −80 mV in 10 mV steps for 100 ms from command potentials of −110 to 180 mV. This protocol allowed for examination of the range of activation (from low- to high-threshold) calcium currents.

Analysis of current components. The effect of diabetic sera on high-threshold calcium currents was evaluated. High-threshold currents (*N*-, L-, P-, and Q-type) are activated by depolarizing the neuron from holding potentials of −80 or −90 mV to clamp potentials of +10 mV. To normalize currents to size of cell body, whole cell currents were divided by whole cell capacitance. Capacitance traces were elicited by small depolarizing calibration voltage pulses (+5 mV for 12 s) from a holding potential of −80 mV. Whole cell capacitance (proportional to surface area) was calculated from the formula $C = A/V$, where C = capacitance (pF), A = area under the capacitance current curve from the peak inward current to the point at which $I_{Ca} = 0$ (pF/mV), and V = calibration voltage step (mV). Peak inward currents were divided by cell capacitance, and normalized current density expressed in units of pA/pF.

Serum and drug preparation

20 ml of serum obtained from patients were stored in aliquots at −70°C. Complement was heat inactivated by heating serum in a water bath at 56°C for 40 min. Ω-Conotoxin (ω-conotoxin GVIA, ω-cgTx) 1 mM stock solution (Sigma Chemical Co.) was prepared with filtered distilled water and lyophilized in 10 ml aliquots, and then stored at −20°C. On the experimental day, 100 nM ω-cgTx was prepared in the culture media. Nifedipine (100 mM) solution in DMSO was prepared fresh on the experimental day and diluted 1:10,000 in culture medium. The solution was protected from light throughout the experiment.

Characterization of serum factor

Studies were performed to determine whether the serum factor associated with increased calcium influx was trypsin sensitive and possessed a molecular weight > 100,000 kd. Complement-inactivated serum samples from controls and diabetic patients were treated with trypsin (0.005%) and, subsequently, evaluated for their effect on elevated K^+-evoked cytosolic calcium concentration. In separate studies, sera from control and diabetic patients were passed through molecular sizing filters (Amicon, Beverly, MA) that trap substances with a molecular weight > 100,000 kd. Subsequently, the effect of the residue (> 100,000 kd) and filtrate (< 100,000 kd) on elevated K^+-evoked cytosolic calcium concentration was evaluated.

To confirm the presence of an autoimmune immunoglobulin in diabetic serum, sera from control and diabetic patients were tested for binding to the surface membrane of SY5Y neuronal cells by immunocytochemistry (18). SY5Y neuronal cells were plated on coverslips coated with Matrigel and cultured for 24 h. The cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. Cells were exposed to 10% vol/vol goat serum in PBS for 30 min, and then incubated with complement-inactivated serum obtained from control, DN^+, or DN^- patients (10% vol/vol) for 90 min. Finally, neuronal cells were incubated with FITC-linked anti–human IgG antibody (1:64 dilution), (Sigma Chemical Co.) for 60 min. The coverslips were washed with PBS for 5 min, three times after each step. All incubations were performed at room temperature. Coverslips were mounted on a glass slide using a glycerol-based mounting medium. Images were obtained on a MRC-600 Biorad Confocal microscope (Hercules, CA) using a 60× oil immersion lens. The fluorescent intensity of the microscope was calibrated before each experiment to minimize interrun variability. Calibration of the confocal microscope was done using the InSpecK fluorescent microscope image intensity calibration kit (Molecular Probes). The microscope is calibrated with fluorescent bead standards that do not vary in intensity.

Statistical analysis

The data are summarized as the mean±standard error. Statistical analysis was performed using one-way ANOVA with appropriate adjustment for nonparametric statistics. All statistics were done using the InSTAT software program. Statistical significance was accepted at the $P < 0.05$ level.

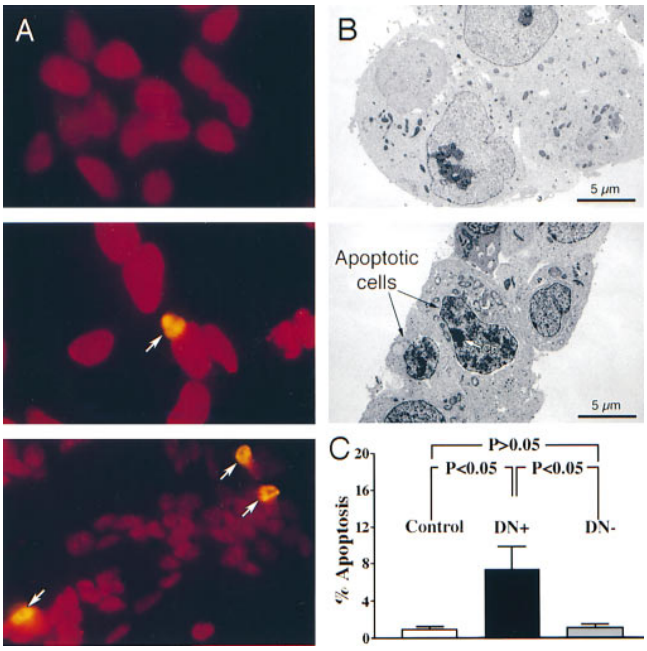

Figure 1. Apoptosis in SY5Y neuronal cells. (*A*) Representative photographs of SY5Y neuronal cells stained for apoptosis using the TUNEL method. (*Top*) Neuronal cells grown in culture medium supplemented with non-diabetic control serum (15%, 48 h). (*Center*) Neuronal cells grown in culture medium supplemented with serum obtained from a DN^+ patient (15%, 48 h). (*Bottom*) Positive control: neuronal cells cultured in the presence of media containing FCS and *cis*-platin (2 μg/ml, 48 h). 60×. *Arrows* point to TUNEL + apoptotic cells. (*B*) Transmission electron micrographs of differentiated SY5Y neuronal cells cultured in the presence of (*upper*) non-diabetic control serum and (*lower*) serum obtained from a DN^+ patient (15%, 48 h). *Arrows* point to the apoptotic cells demonstrating chromatin condensation (*Ch*), preservation of mitochondria, and plasma membrane. (*C*) Neuronal cells were cultured in the presence of human serum obtained from DN^+ and DN^- patients and non-diabetic control patients. The mean±SEM percent of apoptosis in neuronal cells in the presence of sera from non-diabetic controls, DN^+ and DN^- patients is shown.

Results

Patient profile. Adult control patients had no evidence of diabetes, autoimmune disease, or neuropathy. 7 non-diabetic adult controls, 10 DN^+ patients, and 7 DN^- patients were studied. No significant difference was present in age, sex, weight, HbA_{1C}, or creatinine in DN^+ versus DN^- patients ($P > 0.05$). The duration of diabetes was significantly lower in DN^- patients compared to DN^+ patients, $P < 0.05$ (Table I).

Exposure of SY5Y neuronal cells to sera from DN^+ patients was associated with induction of apoptosis. Sera from DN^+ patients were associated with a significantly greater induction of apoptosis (7.37±2.4%, $n = 10$ patients) compared to DN^- (1.02±0.3%, $n = 7$ patients, $P < 0.05$) and non-diabetic control sera (0.8±0.25%, $n = 7$ patients, $P < 0.05$) (Fig. 1, *A* and *C*) patients. Statistical analysis was performed using non-parametric one-way ANOVA. Experiments using sera from each subject were repeated twice on two separate experimental days. On each experimental day, a subject's serum was evaluated in duplicate cultures. For each subject, the percentage of apoptosis represents the mean of apoptosis on different experimental days.

Electron microscopy. Serum from one DN^+ patient known to induce apoptosis by the TUNEL method was used to confirm apoptosis by electron microscopy based on the morphological characteristics. SY5Y neuronal cells, cultured in the presence of serum from the DN^+ patient but not control serum, demonstrated the characteristic chromatin condensation and preservation of mitochondria and plasma membrane observed in apoptosis (32). In neuronal cells exposed to non-diabetic control sera, 0/52 neuronal cells demonstrated apoptosis, whereas 4/38 (10.5%) demonstrated apoptosis after exposure to serum from the DN^+ patient (Fig. 1 *B*).

Necrosis. The percentage of necrotic neurons was similar after exposure to sera from DN^+ (21±4% in SY5Y neuronal cells, 6.25±1.7 in F-11 neuronal cells, $n = 6$ patients) compared to sera from controls (16±3.8% in SY5Y neuronal cells, 6±0.03 in F-11 neurons, $n = 6$ patients, $P = 0.4$ in SY5Y neuronal cells and $P = 0.9$ in F-11 neuronal cells).

Exposure of neurons to sera from DN^+ patients enhanced calcium influx. Experiments were performed using separate neuronal preparations exposed to control, DN^+, and DN^- sera on a particular experimental day. Repeated measurements in

neuronal cells on the same day exposed to one serum sample revealed that the data were highly reproducible. Because the day to day variability in neuronal cytosolic calcium responses was relatively high, (109±40 nM), compared to the variability recorded on the same day (39±5 nM), the responses to DN^+ and DN^- sera were expressed as a percentage increase over the response to control serum, recorded on the same day. Basal and peak cytosolic calcium response for DN^+ and DN^- serum was expressed as the percentage increase over basal and peak responses to control serum. Statistical analysis of the effect of control, DN^+, and DN^- sera on cytosolic calcium levels was performed using one-way ANOVA and (n) as the number of subjects tested. Sera from DN^+ patients caused a significant enhancement (143±10% increase, $n = 5$ patients, average 23 neurons with each serum, $P = 0.01$) in elevated K^+ (60 mM, 15 s)-evoked cytosolic calcium levels compared to the calcium levels observed after exposure to control sera ($n = 5$ subjects, average 16 neurons each). Serum from DN^- patients was not associated with calcium enhancement compared to controls (73±16% of control serum, $n = 3$ patients, 5 neurons evaluated with each serum, $P = 0.3$) (Fig. 2, *A–C*). Short-term (30 s) exposure of SY5Y neuronal cells and F-11 neuronal cells to sera from DN^+ ($n = 2$, 1:40 dilution) before depolarization with elevated K^+ (60 mM, 15 s) significantly enhanced cytosolic calcium (680±5% increase compared to neuronal cells exposed to non-diabetic control sera, $n = 60$ neuronal cells, $P = 0.0001$). The effect of exposure to sera from diabetics with neuropathy was concentration-dependent, as enhancement in

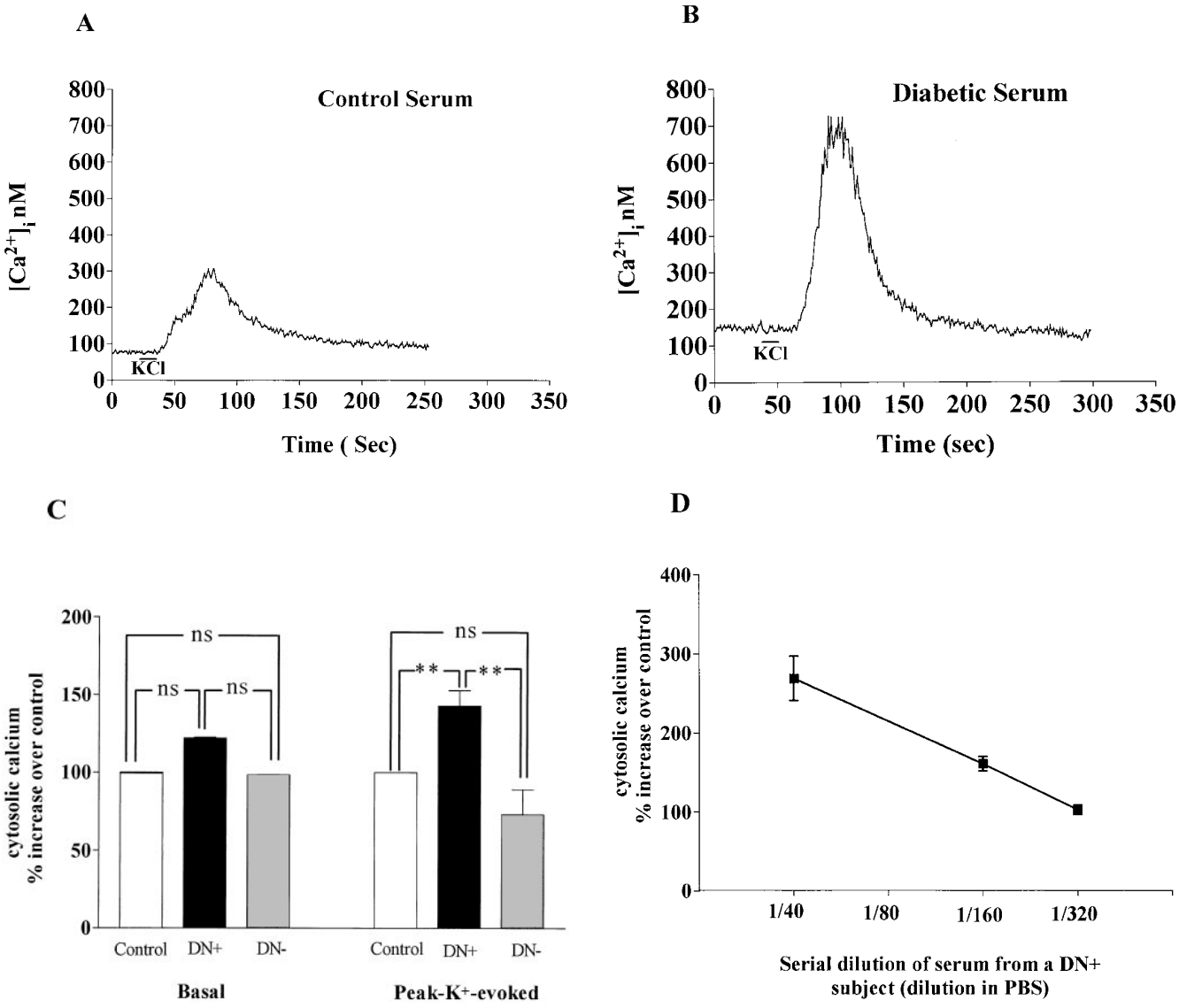

Figure 2. (*A* and *B*) Cytosolic calcium ($[Ca^{2+}]_i$) response in differentiated SY5Y neuronal cells. Representative tracings of elevated K^+ (60 mM)-evoked cytosolic calcium ($[Ca^{2+}]_i$) response in differentiated SY5Y neuronal cells, cultured in the presence of serum for 48 h, from either control subjects (*A*) or DN^+ patients (*B*). (*C*) Basal and elevated K^+ (60 mM, 15 s)-evoked cytosolic calcium recorded in differentiated SY5Y neuronal cells in the presence of serum from non-diabetic controls, DN^+ patients, and DN^- patients. Results expressed as a percentage of calcium level recorded after exposure to non-diabetic control serum. $^{**}P = 0.01$. (*D*) Dilution-response curve of $[Ca^{2+}]_i$ response in F-11 neurons exposed to serum from a DN^+ patient applied for 30 s. Results are expressed as a percentage increase in cytosolic calcium concentration compared to control serum. Serial dilution attenuated the cytosolic calcium response to the DN^+ serum in PBS.

cytosolic calcium was observed at dilutions up to 1 in 1,000, $n = 3$ (Fig. 2 *D*).

Exposure of neurons to serum from DN$^+$ patients was associated with enhanced calcium current density

We confirmed DN$^+$ serum–mediated enhancement in calcium influx electrophysiologically. Peak calcium current density recorded from DRG neurons exposed to serum from a DN$^+$ patient known to enhance cytosolic calcium was significantly higher (687±121 pA/pF, $n = 3$ neurons) compared to treatment of neurons with sera from non-diabetic controls (227±42, pA/pF, n = total of 9 neurons using sera from three controls). The calcium current density in neurons exposed to serum from a DN$^+$ patient was > 95% confidence intervals for the non-diabetic controls. Statistical analysis was performed using one-way ANOVA, using n as the number of neuronal cells.

Combination of N- and L-type calcium channel antagonists attenuated the effect of sera from DN$^+$ patients on apoptosis

We next evaluated the effect of sera from DN$^+$ patients on apoptosis in the presence and absence of calcium channel antagonists. In the presence of N- and L-type calcium channel antagonists, induction of apoptosis was significantly decreased (50±17% reduction, $n = 4$ patients, $P = 0.03$) (Fig. 3).

Characterization of serum factor

The sera from two DN$^+$ patients known to enhance cytosolic calcium and two controls were used to study the effect of trypsin and filtration on serum-induced cytosolic calcium enhancement. Our initial studies indicated that the diabetic serum–mediated enhancement in calcium influx was abolished after treatment with trypsin. The peak in elevated K^+ (60 mM)-evoked $[Ca^{2+}]_i$ in neuronal cells exposed to sera from DN$^+$ patients was 618±23 nM. After treatment with trypsin, the $[Ca^{2+}]_i$ response decreased to 298±22 nM ($n = 67$ neuronal cells, $n = 2$ patients, $P < 0.002$) (Fig. 4 *A*). Exposure of neuronal cells to trypsin alone did not affect serial depolarizations with elevated K^+. Passing the sera through a molecular weight sizing filter revealed that the serum factor associated with enhanced calcium influx was located predominately in the residue, e.g., > 100,000 kd. Elevated K^+-evoked $[Ca^{2+}]_i$ response in the presence of reconstituted residue (1:40 dilution) from control sera was 924±143 nM, whereas the residue from DN$^+$ sera was 1263±78 nM, $n = 15$ neuronal cells, $P = 0.04$. The $[Ca^{2+}]_i$ response to control filtrate (MW < 100,000 kd) was 473±35 nM and DN$^+$ filtrate 488±36 nM, ($n = 15$ neuronal cells, not significant) (Fig. 4 *B*). The calcium current density in DRG neurons exposed to the DN$^+$ residue (retained 85% of original activity) was significantly larger than the current density observed in neurons exposed to the DN$^+$ filtrate (retained 51% of activity) (Fig. 4 *C*). Statistical analysis was performed using one-way ANOVA, using n as the neuronal cells. These studies suggested that the serum factor was a large molecular weight protein, possibly an immunoglobulin.

We next examined whether exposure of SY5Y neuronal cells to DN$^+$ serum known to induce apoptosis and enhance neuronal calcium was associated with the presence of autoimmune immunoglobulin(s) directed against autoantigens on the surface membrane. Minimal fluorescence was observed in SY5Y neuronal cells exposed to control sera and, subsequently, evaluated for the presence of immunoglobulins in the IgG class. In contrast, neuronal cells exposed to the serum from the DN$^+$ patient demonstrated intense fluorescence on the cell surface membrane (Fig. 5). The average pixel intensity in neuronal cells exposed to the serum from the DN$^+$ patient was significantly larger compared to sera from DN$^-$ and control patients, (controls: 108±6 pixels/mm^2, $n = 32$ neuronal cells using sera from two subjects; DN$^-$ patients: 134±6 pixels/mm^2, $n = 36$ neuronal cells using sera from two patients; DN$^+$ patient: 173±7 pixels/mm^2, $n = 23$ neuronal cells, $P < 0.001$) (Fig. 5). Statistical analysis was performed using one-way ANOVA.

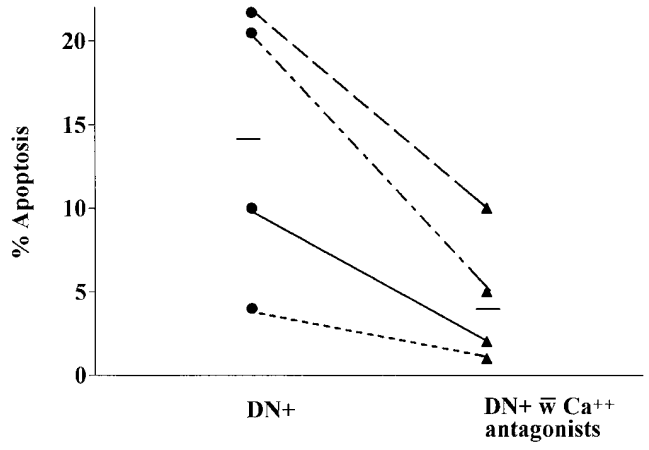

Figure 3. Effect of calcium channel antagonists on diabetic serum–induced apoptosis. Differentiated SY5Y neuronal cells were exposed to non-diabetic control or DN$^+$ sera for 48 h in the presence of calcium channel antagonists, ω-conotoxin GVIA (100 nM, N-type calcium channel antagonist), and nifedipine (10 mM L-type calcium channel antagonist). In the presence of N- and L- type calcium channel antagonists, induction of apoptosis by DN$^+$ serum was significantly reduced. Each line represents apoptosis induced by serum from a DN$^+$ patient in the absence (*solid circle*) and presence (*solid triangle*) of calcium channel antagonist. The mean is denoted by a horizontal bar.

Discussion

Autoimmune immunoglobulins have been identified in type 2 diabetes mellitus (33, 34), but their involvement in the pathophysiology of diabetic neuropathy has not been examined. Our studies support the presence of immunoglobulins in sera from DN$^+$ patients that was associated with complement-independent, calcium-dependent induction of apoptosis. We believe that these are the first studies to demonstrate calcium-dependent apoptosis induced by serum from type 2 diabetic patients. Sera from DN$^+$ patients caused a ninefold increase in apoptosis compared to non-diabetic control sera. The induction of apoptosis by sera from DN$^+$ patients may have clinical significance, as long-term exposure of nerves to serum in vivo could contribute to the development of neuropathy. Under our culture conditions, SY5Y neuronal cells had a reproducibly low rate of baseline apoptosis (> 1%), which allowed us to readily detect differences in apoptosis in response to exposure to sera from controls and diabetics. The range of induction of apoptosis by sera from DN$^+$ patients varied from 0–21%. Of the 10 patients who demonstrated a significant increase in apoptosis, cytosolic calcium responses were recorded using sera from five patients. Sera that were the most potent in inducing apoptosis were used to examine the effect of calcium channel antagonists and

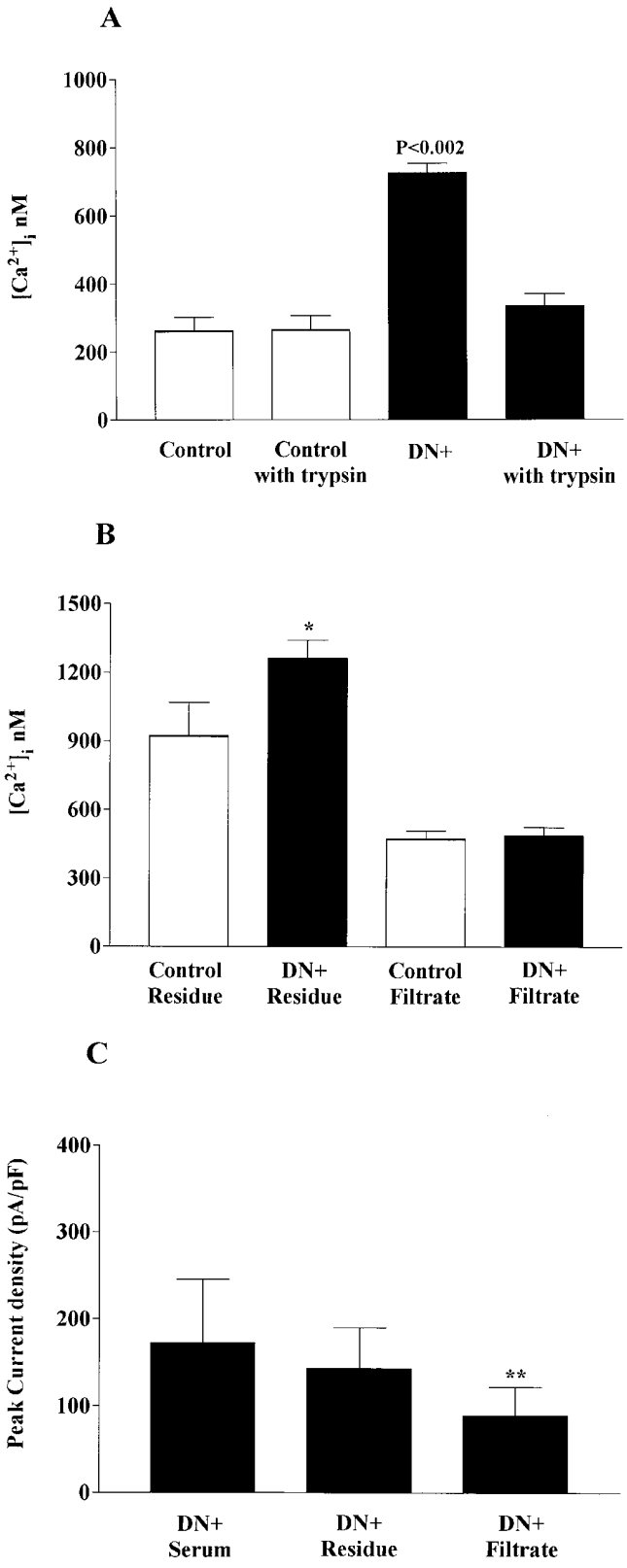

Figure 4. Characterization of serum factors. (*A*) Elevated K^+-evoked cytosolic calcium response in neuronal cells exposed to DN^+ serum was 618±23 nM. After treating the DN^+ sera with trypsin, the K^+-evoked $[Ca^{2+}]_i$ response decreased to 298±22 nM, $n = 67$ neurons, $P < 0.002$. Numbers 1 and 2 represent sera from two DN^+ patients. The peak elevated K^+-evoked $[Ca^{2+}]_i$ response is depicted before and after treatment with trypsin. (*B*) Calcium current density in DRG neurons exposed to serum from a DN^+ patient passed through a 100,000 molecular weight filter. The residue (> 100,000 kd) retained the ability to enhance calcium currents, whereas the filtrate (< 100,000 kd) lost this ability. (C) Elevated K^+-evoked cytosolic calcium in the presence of reconstituted serum residue (> 100,000 kd, 1:40) from a control and DN^+ patient. The K^+-evoked $[Ca^{2+}]_i$ response was significantly greater in the presence of the residue from a DN^+ patient compared to the residue from a non-diabetic control. The filtrate (< 100,000 kd) from the control and DN^+ patient did not cause enhancement of the elevated K^+-evoked $[Ca^{2+}]_i$ response.

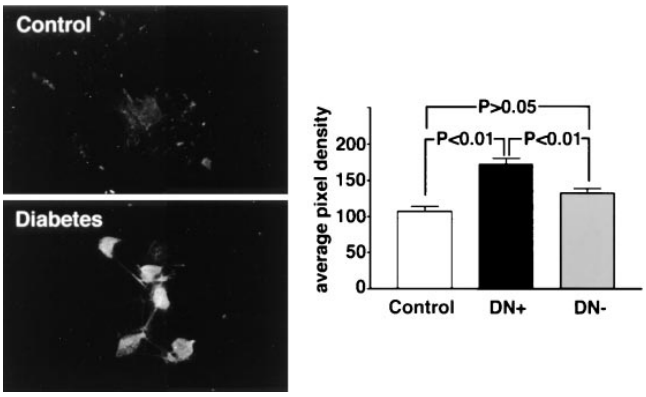

Figure 5. Immunofluorescence images of SY5Y neuronal cells using confocal microscopy. (*Left*) Representative immunofluorescence images of SY5Y neuronal cells exposed to sera from controls and DN^+ patients and, subsequently, evaluated for the presence of immunoglobulins (IgG class) by indirect immunofluorescence using confocal microscopy (60× lens). Neuronal cells exposed to serum from DN^+ patients demonstrated intense fluorescence on the surface membrane that was not observed in neurons exposed to sera from controls. (*Right*) SY5Y neuronal cells exposed to sera from controls, DN^+, and DN^- patients and, subsequently, evaluated for the presence of immunoglobulins (IgG class) by indirect immunofluorescence using confocal microscopy. The average pixel intensity in neuronal cells exposed to DN^+ serum was significantly higher compared to sera from DN^- and control subjects.

to further characterize serum factors. We used a variety of methods using two neuronal cell lines, as well as primary sensory neurons, to corroborate our observations on calcium signaling and induction of apoptosis.

The relative inability of sera from DN^- patients to either induce apoptosis or enhance cytosolic calcium response to depolarization supports the hypothesis that serum factors play a pathophysiological role in neuropathy independent of hyperglycemia. We believe that serum-induced apoptosis was not due to hyperglycemia, per se, as the glucose concentration of the medium (20 mM) precluded a significant effect of the serum glucose concentration after a fivefold dilution. Other studies have demonstrated that physiological concentrations of glucose had no effect on free intracellular calcium in PC12 cells and a fourfold elevation of glucose above physiological levels reduced calcium influx (35). The data obtained using sera from patients with diabetes without neuropathy provide some insight regarding the pathophysiology of the autoimmune mechanisms in type 2 patients. These individuals had diabetes mellitus for shorter duration (2.75±0.5 compared to 21±3 yr), demonstrated an intensity of anti–IgG immunofluorescence

that was intermediate to non-diabetic controls and diabetics with neuropathy, and did not demonstrate a significant increase in either cytosolic calcium levels or apoptosis compared to non-diabetic controls. These results suggest that the development of autoantibodies in diabetes may precede abnormalities in calcium signaling and apoptosis. The range of apoptosis observed in the DN^+ group is consistent with heterogeneous expression of the proposed mediator(s) of this process. Future studies will be required to identify, quantify, and correlate potential relationships between the duration of diabetes, titer of autoimmune immunoglobulin, enhancement in cytosolic calcium, and induction in apoptosis. The effect appeared to be selective for apoptosis as necrosis rates were not different. Both short-term (30 s) and long-term (48 h) exposure of neurons to serum from DN^+ patients enhanced elevated K^+-evoked cytosolic calcium response. Evaluation of whether distinct signal transduction pathways are involved in the calcium elevation in these two models will be the subject of future studies.

Juntti-Berggren et al. (16) demonstrated enhanced calcium currents (L-type) in pancreatic β-cells exposed to sera from newly diagnosed patients with type 1 diabetes. They postulated that the factor in diabetic sera was an immunoglobulin. Pittinger et al. (17–19) reported that exposure of neuroblastoma cultures to complement-activated serum from type 1 diabetics with neuropathy was associated with neuronal injury, apoptosis, and enhanced calcium influx. The cause–effect relationship between increased cytosolic calcium and apoptosis was not tested in previous studies. Pittinger et al. (17–19) suggested that the serum factor was an autoimmune immunoglobulin (most likely a complement-fixing IgG). Our study design used heat-inactivated sera to eliminate the effect of complement-induced cell death.

We used a combination of N- and L-type calcium channel antagonists to inhibit influx of calcium into the neurons. SY5Y neuronal cells have multiple voltage-gated calcium channels, and the majority ($> 70\%$) of calcium influx is via N- and L-type channels. Therefore, we used a combination of N- and L-type calcium antagonists (36). We observed $\sim 50\%$ reduction in apoptosis, indicating that the pathway underlying DN^+ serum–induced apoptosis is most likely calcium dependent. Enhancement of multiple neuronal calcium currents in diabetes (8) provides an explanation for the limited improvement in nerve conduction velocity observed in diabetic rats after treatment with a selective L-type calcium channel antagonist (11, 37).

Considerable evidence suggests that metabolic overstimulation by cytosolic calcium may contribute to neuronal cell injury and apoptosis (12–14). Internucleosomal DNA cleavage by a Ca^{2+} and Mg^{2+}-dependent endonuclease, activated by intranuclear Ca^{2+} uptake, is a characteristic of programmed cell death. Furthermore, the cellular threshold for apoptosis is highly regulated, especially by the members of the Bcl-2 family of proteins, including Bcl-2, Bax, and Bcl-x (38). Serum from type 2 diabetic patients may induce apoptosis via modulating expression of the Bcl family of proteins (39). In mammalian cells, the cell surface receptor Fas (APO-1, CD-95) induces apoptosis when multimerized with Fas ligand (40). Therefore, activation of Fas may contribute to diabetic serum–induced apoptosis.

The target of the immunoglobulins is not known, although studies with type 1 serum suggest Fas. Several autoantigens have been proposed as possible sites for the targeting of autoimmune immunoglobulins in diabetes mellitus, including phospholipids (41), gangliosides (42), and glutamic acid decarboxylase (43). Other potential targets are cell adhesion molecules (CAMs) of the immunoglobulin superfamily. CAMs (including neural CAM and myelin-associated glycoprotein) have immunoglobulin folds in their extracellular domains (44) and are important in the myelination of axons (45, 46). The autoantigen(s) in human type 2 diabetes have not been characterized. It remains to be resolved whether autoimmune immunoglobulin(s) are directly or indirectly involved in the pathophysiology of diabetic neuropathy.

In summary, our studies suggest that patients with type 2 diabetes with neuropathy are at risk for generating an autoimmune immunoglobulin associated with enhanced calcium influx and induction of apoptosis in neurons. Attenuation of serum-mediated apoptosis by calcium channel antagonists suggests that the mechanism is calcium dependent. Initial characterization indicates that the serum factor is trypsin sensitive, has a molecular weight $> 100{,}000$ kd, and enhances calcium influx at dilutions up to 1:1,000, suggesting that the substance is most likely an immunoglobulin. Immunofluorescence studies demonstrated the presence of an (IgG class) immunoglobulin in sera from type 2 diabetic patients with neuropathy. Because sera were heat inactivated, neither the action of the immunoglobulin(s) to increase calcium influx nor the ability to bind neuronal surface membrane autoantigenic sites required complement-fixation. These studies do not rule out the possibility that additional immunoglobulins will be identified in sera from type 2 diabetic patients with neuropathy. The presence of autoimmune immunoglobulin(s) in the serum of type 2 diabetic patients may represent a new risk factor for the development of diabetic neuropathy.

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