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Neuronal Toxicity of Humoral Factors From Patients With Diabetic Neuropathy and Mechanism of Neuronal Death on Cultured Adrenergic Neurons: Studies of Autoimmune Pathogenesis in Diabetic Neuropathy

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NEURONAL TOXICITY OF HUMORAL FACTORS FROM PATIENTS WITH DIABETIC NEUROPATHY AND MECHANISM OF NEURONAL DEATH ON CULTURED ADRENERGIC NEURON
STUDIES OF AUTOIMMUNE PATHOGENESIS IN DIABETIC NEUROPATHY

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A Dissertation submitted to the Faculty of Eastern Virginia Medical School and Old Dominion University in Partial Fulfillment of the Requirement for the Degree of
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ABSTRACT

NEURONAL TOXICITY OF HUMORAL FACTORS FROM PATIENTS WITH DIABETIC NEUROPATHY AND MECHANISM OF NEURONAL DEATH ON CULTURED ADRENERGIC NEURONS

STUDIES OF AUTOIMMUNE PATHOGENESIS IN DIABETIC NEUROPATHY

Dong Liu
Eastern Virginia Medical School/Old Dominion University, 1996
Director: Dr. Gary L. Pittenger

Diabetic neuropathy is an extremely common medical problem because of the high frequency of diabetes and its complications. Diabetic neuropathy consists of not one, but many patterns of nerve injury, each potentially with its own discrete pathogenic mechanisms. Evidence is accumulating that indicates autoimmunity plays a role in diabetic peripheral neuropathy. However, the significance of immunopathogenesis of diabetic neuropathy is as yet unclear.

Using the N1E-115 mouse neuroblastoma cell line (NB) as a model of the adrenergic autonomic neuron, we found that sera of IDDM patients with neuropathy inhibit growth and differentiation of adrenergic neurons in culture (Pittenger et al, 1993; 1995). Some sera were cytotoxic and caused neuronal death. Further characterization of the inhibiting, or toxic, factor in IDDM patient serum indicated that it had the properties of an immunoglobulin. The toxic factor was present in the immunoglobulin fraction, and was pre-absorbed by neuroblastoma cells and protein A beads. Immunocytochemistry revealed a specific cell-surface binding pattern of immunoglobulin IgG which suggested the presence of an antibody recognizing antigens on the neuroblastoma cell surface. Moreover, the inhibiting effect of IDDM serum could be reversed by heat inactivation, indicating the possible involvement of complement.

Further investigation revealed that the neuronal death induced by IDDM serum occurred through the programmed cell death mechanism, apoptosis, which was
characterized by cell shrinkage, cytoplasm condensation and DNA fragmentation. A sustained increase of cytosolic calcium concentration was detected by Fura-2, a fluorescence indicator, at an early stage after exposure to IDDM serum. Apoptosis-related protein expression was also examined in our model. Fas, a cell-surface receptor, seemed to be expressed at the N1E-115 cell surface and recognized by IDDM serum. Whether it was the antigen involved in apoptotic cell death or whether other antigens were also involved remains to be further determined.

In conclusion, autoimmune-related humoral factors in the serum from IDDM patients with neuropathy had an ability to inhibit neuronal cell growth and differentiation, in some cases inducing cell death by apoptosis in cultured adrenergic neurons. However, because N1E-115 cell was a murine tumor cell line, the significance of the findings to diabetic neuropathy and clinical correlation needs to be further evaluated. These studies indicated that immunopathogenesis may play an important role in the development of diabetic neuropathy in IDDM patients.
To my parents, for their endless love for science and people. Their speculation is the great encouragement and inspiration through the past years.

To my husband and my son, for their understanding and standing by me.
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CHAPTER I. BACKGROUND AND SIGNIFICANCE

1.1. Diabetic Neuropathy and its pathogenesis

Diabetic neuropathy develops as a significant complication in patients with all forms of diabetes mellitus. Estimates of the prevalence of neuropathy in the population of patients with diabetes range from 10-50% (Boulton et al., 1985; Newrick et al., 1986; Harati, 1992), dependent on the criteria used to define neuropathy. Diabetic neuropathy is an insidious degenerative disease of peripheral nerves. The most common form, distal symmetrical sensorimotor neuropathy, affects sensory, motor and autonomic nerves. It has a predilection for neurons with the longest axons and shows fiber loss in the more remote nerve trunks. The loss of sensation predisposes the patients to lesions, especially of the foot, potentially resulting in gangrene and amputation. In other cases, focal and multifocal neuropathy are asymmetrical, and might affect cranial, trunk or limb innervation. Many of the diabetic neuropathic syndromes are characterized by painful symptoms with a sensation of burning and associated with troublesome hyperesthesia (Vinik et al., 1988; 1992; 1994; Brewster et al., 1994; Boulton et al., 1986).

Autonomic neuropathy is a serious component of diabetic neuropathy, heralding mortality rates of 25-50% within 2-3 years of diagnosis of autonomic neuropathy (Vinik et al., 1988, 1992; Ewing et al., 1976; 1982; O'Brien et al., 1986). Diabetic autonomic neuropathy may manifest as dysfunction of several different organ systems: cardiovascular, gastrointestinal, genitourinary, sudomotor, and ocular. The symptoms are important in individual patients, but difficult to evaluate and quantitate, because they are often nonspecific (Julius, 1991). Symptoms can occur as early as the first year after diagnosis, presenting as a combination of the following: orthostatic hypotension, diminished sweating, diarrhea or constipation, hypoglycemic unawareness, urinary dysfunction, impotence, delayed gastric emptying, alternating bouts of diarrhea and constipation,
bladder atony and/or exercise intolerance (Pfeifer et al., 1984; O'Brien et al., 1986). Cardiac conduction abnormalities may lead to cardiorespiratory arrest and resting tachycardia. Autonomic neuropathy is associated with an increased mortality from renal failure and an increased frequency of retinopathy and peripheral neuropathy (O'Brien et al., 1991).

The most important pathological changes in diabetic neuropathy are loss of myelinated and unmyelinated axons, demyelination and axonal degeneration (Dyck et al., 1986). Distal nerves are more affected than proximal nerves, as determined by postmortem nerve fiber density (Chopra et al., 1971). Subtle changes at the nodes of Ranvier along diabetic human nerve, including paranodal swelling and alterations of Schwann cell attachments, termed axo-glial dysjunction are described (Sima et al., 1988).

In contrast to other peripheral neuropathy, autonomic nerve damage is confined to small, predominantly unmyelinated nerve fibers. Pathologic changes in the autonomic nervous system studied from postmortem samples of diabetic patients with severe autonomic neuropathy reflect severe loss of myelinated fibers and degeneration. Adrenal medullary fibrosis was found in a retrospective pathology study in IDDM patients with long duration (Brown et al., 1989). Infiltration of lymphocytes and macrophages is seen in autonomic ganglia and in or around autonomic nerve bundles of unmyelinated nerve fibers, suggesting that a different pathogenesis may be involved in the development of autonomic neuropathy in diabetes (Duchen et al., 1980).

Most discouraging, the cause of diabetic neuropathy is uncertain and an effective treatment has not been identified. Several independent modifying factors and multiple pathogenetic mechanisms, some of which have been researched extensively, may interact to produce neuropathy (Ward, 1989). The current hypotheses include:

1. Chronic hyperglycemia and/or insulin deficiency are the primary factors in the causation of diabetic neuropathy (Winegrad et al., 1977). There is evidence that the incidence and severity of complications secondary to diabetes correlate with the degree of
metabolic control (DCCT, 1993). Hyperglycemia results in intracellular accumulation of toxic metabolic products such as sorbitol, fructose, galactitol and advanced glycosylation end products. Increased polyol-pathway activity and decreased sodium dependent myo-inositol uptake content, reduced energy use and defective function of (Na, K)-ATPase have been described (Greene et al., 1988; 1989; Stevens et al., 1993; 1994), all of which may be responsible for osmotic stress and diminished nerve conduction velocity. Impaired Na-K-ATPase activity also appears to be responsible for intracellular Na⁺ accumulation and resultant localized axonal paranodal swelling that characterizes diabetic neuropathy in both humans and laboratory animals (Dyck et al., 1988).

2. Depletion or alteration of neurotrophic growth factors may cause neuronal dysfunction in diabetes. A variety of neurotrophic factors are necessary for normal growth, development, maintenance and regeneration of the peripheral nervous system. Physiological concentrations of insulin, IGF-I or IGF-II reversibly enhance neurite outgrowth and increase the contents of neurofilament, alpha-tubulin, and beta-tubulin mRNAs in cultured human neuroblastoma SH-SY5Y cells (Wang et al., 1992; Recio et al., 1984). Neurotrophic factor accessibility and action may be diminished in diabetes due to defective transport and post-receptor signal transduction or due to reduced levels of circulating growth factors (Faradji et al., 1990; Schmidt et al., 1993).

3. Microvascular insufficiency may contribute to diabetic neuropathy. Endothelial cell swelling and proliferation, as well as platelet aggregation resulting in vessel occlusion have been noted in the pathogenesis of diabetic neuropathy. The diabetic nerve is also more susceptible to ischemia and death (Tuck et al., 1984; Dyck et al., 1986). Endoneurial capillary density is significantly reduced in severely neuropathic diabetic patients when compared with control subjects (Malik et al., 1989). It is possible that altered peripheral blood flow leads to increased spontaneous activity in nociceptive afferent fibers that are present in the axonal sprouts that characterize small fiber neuropathy (Boulton et al., 1986).

4. Autoimmunity may play an important role in the development of diabetic
neuropathy. The awareness of the immunological process as one of the possible pathogenetic mechanisms in diabetic neuropathy has increased, especially in IDDM patients. Although it has been questioned, immunological processes are strongly associated with diabetic autonomic neuropathy, indicated by clinical, morphological, serological and cellular observations (Sundkvist et al., 1994). A role for autoimmunity in the pathogenesis of diabetic neuropathy is as described below.

1.2. The role of autoimmunity in diabetic neuropathy

IDDM is a chronic autoimmune disease with a long prodromal phase, during which antibodies that react with islet cells are often present (Bottazzo et al., 1986). Autoimmunity is a known factor in the pathogenesis of islet cell destruction (Herold et al., 1992; Boitard, 1992), but little is known of its role in the pathogenesis of the neuropathy complications of diabetes.

Because diabetic neuropathy in IDDM patients is a common complication of the autoimmune disease, they may share some common immunological defects or genetic background. There are many similarities between islet cells and neurons. Some neuronal cell-surface markers, such as tyrosine hydroxylase for neurons, are also present on pancreatic islets, suggesting a possible common origin of pancreatic islet cells and neurons (Pearse, 1982). Neurons release neurotransmitters in much the same manner as pancreatic islets release hormone in response to stimuli. There are antigens shared between islet cells and neurons such as gangliosides (Nayak et al, 1985) and glutamic acid decarboxylase (GAD) (Clare-Salzler et al., 1992; Kaufman et al., 1992; Esclapez et al., 1994). Because of these similarities between islet cells and neurons, it is thought that the immunological process directed against islet cells could also attack neurons. Thus, a role for the immunopathological process in pathogenesis of diabetic neuropathy should be considered.

The humoral immunity in diabetic neuropathy
The capacity to produce autoantibodies is an inherent property of the normal immune system. The vast immunoglobulin repertoire gives B cells the potential to produce autoantibodies against a list of autoantigens. Nevertheless, autoantibodies, including natural autoantibodies, never appear against autoantigens in normal conditions, probably because of deletion or anergy of particular autoreactive T-cell and B-cell clones early in ontogeny. However, the traditional theory about self-nonself being distinguished and tolerance established in the neonatal period has been questioned recently (Pennisi, 1996). It has been suggested that the dendritic cells and B cells of the immune system may regulate immune activation and immune tolerance. If B cells fail to inactivate the dendritic cells presenting autoantigens, autoimmunity may result (Ridge et al., 1996).

Evidence indicates that in diabetic patients with autonomic neuropathy, there are complement-fixing autoantibodies against autonomic nerve tissue components in sympathetic ganglia, adrenal medulla and vagus nerve (Brown et al., 1989; Rabinowe et al., 1990; Zanone et al., 1993.). Anti-ganglioside GT1b IgG, associated with both sympathetic ganglia and adrenal medulla antibodies, has been reported in IDDM patients with neuropathy and is inversely related to the change in orthostatic blood pressure (Rabinowe et al., 1991). In one study, sera of 120 IDDM patients were examined for the presence of complement-fixing anti-sciatic nerve antibodies and 22% had fluorescent scores exceeding normal control subjects (MacLaren et al., 1989). If these antibodies attack neuronal tissue, they should be detectable in nerve tissues as they are attacked. Immunoglobulin deposition in the perineurial regions and axons has been noted in nerve biopsies from diabetic patients with neuropathy (Graham et al., 1985; Schenone et al., 1988; Milicevic et al., 1995).

Clinical evidence suggests that immune therapy is effective in diabetic neuropathy, further supporting the notion that immune processes may be involved in the pathogenesis of autoimmune-mediated neuropathy. Plasmapheresis alleviates neuropathy associated with anti-myelin IgM (Haas et al., 1988). Plasma exchange in neuropathy patients with
IgA and IgG gammopathies, effectively attenuates neuropathy based on disability score, muscle weakness and compound motor nerve action potentials (Dyck et al., 1991). High dose intravenous immunoglobulin (IVIg) treatment is effective in patients with a variety of neuropathies associated with autoimmunity, including diabetic neuropathy (Nobile et al., 1993; Karlson et al., 1994; Krendel et al, 1995). However, the mechanism by which IVIg acts is uncertain.

In many studies (Brown et al., 1989; Rabinowe et al., 1990; Zanone et al., 1993), autoantibodies against autonomic nerve tissue components tend to appear more frequently in the neuropathic patients than in the diabetic control subjects with disease of similar duration, suggesting that neuronal tissue autoantibodies may have a role in the development of symptomatic diabetic neuropathy. However, it is not understood whether these autoantibodies are primary causes (directly causing neuronal tissue damage and playing a role in pathogenesis of neuropathy), or secondary effects of the neuropathy due to exposure of autoantigens after neuronal tissue damage. It is also possible that autoantibodies raised secondarily to neuronal damage may accelerate the neuronal loss, thus still playing an important role in the development of neuropathy. Populations studies have noted the presence of complement-fixing antibody against adrenal medullary in 30% of IDDM patients, which decrease in patients with diabetes for greater than 16 years (Brown et al., 1989), suggesting that antibody may play an early causative role in the development of neuropathy. Autoantibody against sympathetic ganglia is associated with a diminished catecholamine response to changes in posture and orthostasis in IDDM patients (Brown et al, 1989), supporting the idea that the adrenergic nervous system is a target for autoimmune destruction.

Autoantibodies detected in sera of patients with neurological disease have been reported in chronic inflammatory demyelinating polyneuropathy (Weng et al., 1992), myasthenia gravis (Rowland et al., 1991), Stiff-man syndrome (Pugliese et al., 1993; Solimena et al., 1988; 1990), Guillain-Barré syndrome (Quarles et al., 1990), lower motor
neuron disease (Freddo et al., 1986), multiple sclerosis (McFarland et al. 1989), and acute post-infectious disseminated encephalomyelitis (Segal, 1983). Circulating antibodies may also play a pathogenetic role in neuropathies associated with amyloidosis, myeloma, macroglobulinemia, lymphoma, and certain leukemias (Dyck et al., 1991). It appears that the location of the presumptive target antigen critically influences the pathogenetic potential of autoantibodies. Autoantibodies directed against cell-surface targets are often pathogenetic, such as in myasthenia gravis (characterized by autoantibodies against the acetylcholine receptor in membranes of skeletal muscle fibers) (Schonbeck et al., 1990). Those directed against extracellular matrix components may or may not cause damage. Antigens may be both cytoplasmic or exposed on the cell surface, such as 64-kDa islet cell antigen or GAD (glutamic acid decarboxylase), an enzyme involved in the synthetic pathway of the inhibitory neurotransmitter, gamma-aminobutyric acid (Kaufman et al., 1992).

The Cellular immunity in diabetic neuropathy

The cellular immune system is the engine of all immunity, including antibody production. A role for cell-mediated immunity to nervous system antigens in the pathogenesis of diabetic neuropathy has also been suggested (Segal et al., 1983; Gilbey et al., 1988). In tissue samples from diabetic patients with severe autonomic neuropathy, inflammatory infiltrates composed of lymphocytes, macrophages and occasional plasma cells are seen in autonomic ganglia and in or around autonomic nerve bundles of unmyelinated nerve fibers (Duchen et al., 1980). In a group of similar patients, an increased level of circulating activated T lymphocytes was observed, suggesting a role of the cellular immune system in the pathogenesis of neuropathy (Gilbey et al., 1988). Levels of activated T lymphocytes are increased in newly diagnosed Type 1 diabetic patients, but tend to return to normal within 2-3 years. Their persistence or, possibly, reappearance in diabetic neuropathy may represent continuing immune activation against neuronal targets.
(Zanone et al., 1993). It is unknown whether the generation of antibody preceded this immune infiltration.

There is evidence using the lymphocyte transformation technique, that lymphocytes from some patients with diabetic neuropathy undergo significant stimulation when cultured in vitro in the presence of both CNS and PNS basic protein antigen (encephalitogenic protein and P2) (Segal et al., 1983). Cell-mediated immunity to neural antigens has also been reported in myeloradiculitis, chronic relapsing polyneuritis (Abramsky et al., 1975) and transverse myelopathy (Abramsky et al., 1977).

**Autoantigen Identification**

Identifying and characterizing the relevant autoantigens is still a challenge in destructive organ-specific autoimmunity, but it might lead to advances in diagnosis, treatment and even prevention of autoimmune diseases.

Data from studies of animal diabetes and autoantibodies in IDDM suggest that many epitopes or antigen structures become autoantigens during disease pathogenesis. Previous studies on antibodies to autonomic nervous structures indicated a higher prevalence of these antibodies in recent-onset Type 1 diabetic patients, who were ICA (islet cell cytoplasmic antigen) positive, than in those with long-term diabetes. This suggests that antibodies to nerve tissue and ICA may have target antigens in common (Brown et al., 1989). However, in other studies no significant correlation between anti-nerve tissue autoantibodies and ICA or other conventional tissue autoantibodies was found. Therefore, antibodies to nerve tissue may occur independently and the target antigens are tissue specific (Zanone et al., 1993).

**a. Gangliosides**

Studies suggest that the autoantigens of pancreatic tissues have the properties of a glycolipid containing sialic acid (Nayak et al., 1985; Powers et al., 1984; Dotta et al., 1989). This may account for the failure to detect islet-cell surface antibodies with
Chapter I. Background and Significance

processes utilizing organic solutions (Vives et al., 1992). Monoclonal anti-ganglioside antibodies produced by immunization with fetal rat brain react specifically with all cell surfaces of pancreatic islets of human, rat, mouse (Ariga et al., 1987). It is generally thought that in organ-specific autoimmune diseases, the autoantibodies are specific for each organ and do not cross-react (Bottazzo et al., 1986). However, some islet cell antigens (e.g., gangliosides) are present in significant amounts in other organs besides pancreatic islets, such as neurons, renal glomerular cells, retinal microvascular pericytes and adrenal medulla. Thus, it is possible that the common antigens may be involved in multiple organ damage and may cause diabetic complication, such as neuropathy, retinopathy and nephropathy (Nayak, et al., 1985; Eisenbarth et al., 1988).

Anti-ganglioside antibodies have been found in Guillain-Barré syndrome (Quarles et al., 1990; Ilyas, 1988), lower motor neuron disease (Freddo et al., 1986), chronic inflammatory polyneuropathy (Baba, 1989), multiple sclerosis (Endo et al., 1984), and amyotrophic lateral sclerosis (Pestronk, 1988b), neuromuscular disease (Adama et al., 1991), as well as neuropathy in type 1 diabetes (Rabinowe et al., 1991). Indeed, ganglioside GM1 treatment with formation of anti-ganglioside antibodies is associated with an increased incidence of Guillain-Barré syndrome (Landi et al., 1993), suggesting a causative relationship for GM1 autoantibodies.

In neuroblastoma cells, gangliosides are abundant on cell surface membranes (Wu, 1991). The composition of gangliosides in different cell types or cell lines varies. IgM antibody that recognizes gangliosides GM1, GD1b and asialo-GM1 is reported in motor neuron diseases such as lower motor neuron disease, multifocal motor neuropathy and acute axonal neuropathies (Latov et al., 1988; Geisler et al., 1991). Anti-ganglioside GT1b IgG, associated with both sympathetic ganglia and adrenal medulla antibodies, is reported in IDDM patients with autonomic neuropathy (Rabinowe et al., 1991). Thus, ganglioside should be considered as an important candidate antigen in autoimmune-mediated diabetic neuropathy.
b. Myelin Basic Protein

Anti-myelin basic protein antibodies are reported in Guillain-Barré syndrome, multiple sclerosis and experimental allergic neuritis (EAN) induced in Lewis rats. Administration of myelin basic protein suppresses experimental autoimmune encephalomyelitis by neutralizing antibody (Khoury et al., 1990). Considerable evidence favors a role of anti-myelin antibody in mediating demyelinating polyneuropathy (Hays et al., 1987; Hafler et al., 1986; Steck et al., 1983). It remains to be determined whether anti-myelin antibody plays a role in diabetic neuropathy.

c. Glutamic acid decarboxylase (GAD)

GAD is an enzyme involved in the synthesis of the neurotransmitter gamma aminobutyric acid (GABA). Antibody against GAD is present in sera of patients with type 1 diabetes and GAD is considered to be an autoantigen in pancreatic islets (Clare-Salzler et al., 1992). GAD exists in two isoforms, 65kDa and 67kDa, that differ in their intraneuronal distributions (Kaufman et al., 1992). GAD67 is widely present in cell bodies, whereas GAD65 is prominent in many axon terminals (Esclapez, 1994). GAD67 and GAD65 are different gene products, with GAD67 the predominant form in neurons (Kaufman et al., 1992). Higher titers of anti-GAD antibodies have been reported in a small number of diabetic patients with autonomic neuropathy (Kaufman, 1992). Of interest, anti-GAD antibodies appear to cause the neurological disorder in Stiff-Man Syndrome, which is often accompanied by diabetes (Karlson et al., 1994). Because GAD antibodies may be involved in different diseases, the epitope on GAD recognized by antibodies may be critical to the physiological and disease-specific effects (Daw et al., 1996). Thus, anti-GAD antibody may play a role in diabetic neuropathy. However, several recent studies argue this may not be the case. Anti-GAD65 antibodies are present in a high proportion of patients with diabetic neuropathy, but are not exclusively associated with it. High prevalence of autoantibodies to GAD in long-standing IDDM is not a marker of symptomatic autonomic neuropathy (Zanone et al., 1994; Vinik et al., 1995). There is no
correlation between the presence of anti-GAD65 antibodies and the presence of autoantibodies to sympathetic ganglia, vagus nerve, or adrenal medulla structures identified by immunofluorescence. Nor are GAD-antibodies associated with disturbed autonomic nerve function (Sundkvist et al., 1994). Moreover, GAD is a cytoplasmic enzyme and access of the autoantibody to the protein is problematic. Thus, the role of anti-GAD antibodies in diabetic neuropathy remains elusive.

d. Nerve growth factor (NGF) and NGF receptor

Shared sequence homology between portions of the nerve growth factor (NGF) and pro-insulin molecules suggests an autoimmune mechanism acting through effects of anti-insulin antibodies modifying NGF activity (Schmidt, 1993). Anti-insulin antibodies are common in IDDM patients and cross-reaction with NGF could lead to the immunologically-mediated destruction of nerve tissues that are depend on NGF. NGF stimulates and maintains growth and differentiation of sensory and sympathetic nerves and dorsal root ganglion cells during early development. NGF is also necessary for neuronal survival, morphology and function in maturity. NGF receptor TrkA knockout mice show deficient C-fiber function and lack sympathetic nerve development. An immune attack on NGF might result in damage to autonomic nerves, leading to neuropathy. Autoantibodies to NGF have been reported in the sera of patients with autoimmune diseases and diabetes (Dicou et al., 1993; Faradji et al., 1990). However, a recent investigation found no significant difference in anti-NGF antibody level among groups of diabetic patients with neuropathy, diabetic control patients and normal control subjects (Zanone et al., 1994). Any relationship between NGF and autoimmunity remains to be determined.

e. Other membrane lipids/protein components

Membrane lipids, such as phospholipids have been thought to be important in diabetic neuropathy (Greene et al., 1989; McNeil et al., 1991; Vinik et al., 1995). Membrane proteins, such as the voltage-dependent L-type calcium channel, have been suggested as autoimmune targets in the neurodegenerative diseases Lambert-Eaton
myasthenic syndrome and amyotrophic lateral sclerosis (Leys, 1991; Smith, 1993). The frequency of anti-phospholipid antibody is significantly higher in diabetic neuropathy patients than in the general population or patients without neuropathy according to a recent study (Vinik et al., 1995). Anti-cardiolipin antibodies occur in autoimmune disease and a phospholipid-beta 2-glycoprotein I complex has been suggested to be the antigen (Hunt et al., 1992). A significance of anti-phospholipid and anti-cardiolipin antibodies in the pathogenesis of diabetic neuropathy has not been established.

1.3. Cellular mechanism of neuronal death

Recent studies indicate dysfunctional control of programmed cell death (apoptosis) is involved in the development of autoimmune diseases and neurodegenerative diseases (Thompson., 1995; Carson et al., 1993). One mechanism underlying autoimmunity is the defective ability of the immune system to delete self-reactive T and B lymphocytes by apoptosis, which could predispose to autoimmune disease. Some studies have demonstrated that apoptosis may also play a role in the pathogenesis of IDDM. It has been reported that IgM in sera from IDDM patients causes an increase in voltage-dependent L-type calcium channel activity of insulin-producing cells. The subsequent increase in the concentration of free cytoplasmic Ca\(^{2+}\) was associated with DNA fragmentation and apoptotic cell death (Berggren et al., 1993). More recently, genetic analysis of non-obese diabetic (NOD) mice showed that the apoptosis related gene bcl-2 mapped close to the gene region which is associated with periinsulitis. The bcl-2 NOD (non-obese diabetic) mouse haplotype is linked to an elevated serum IgG. Activated T lymphocytes from NOD mice also showed a marked resistance to induction of apoptosis (Garchon et al., 1994). However, little is known about the relevance of apoptosis and the regulation of apoptosis to neuropathic complications in IDDM patients.

Apoptosis occurs through the activation of a cell-intrinsic program. The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells,
Neurons also have a programmed cell death pathway. Approximately half of the neurons produced during embryogenesis normally die before adulthood. Apoptotic morphological changes and DNA fragmentation have been described in 3-day-old cultured cortical neurons exposed to glutamate (Kure et al., 1991). Target-derived neurotrophic factors are known to be major determinants of programmed neuronal cell death (Garcia et al., 1992). Sympathetic neurons undergo RNA-and protein synthesis-dependent programmed cell death when deprived of nerve growth factor (Deckwerth et al., 1993). In diabetic neuropathy, the most important pathological change is loss of myelinated and unmyelinated axons (Chopra et al., 1971; Brewster et al., 1994). This is similar to a wide variety of neurological diseases, such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, and various forms of cerebellar degeneration, which are characterized by the gradual loss of specific sets of neurons but without an obvious inflammatory reaction. In these diseases, apoptosis has been suggested to be the mechanism of neuronal cell death (Thompson, 1995).

Apoptosis is characterized by condensation of cytoplasm, compacting of chromatin, formation of a convoluted plasma membrane and extensive fragmentation of chromosomal DNA into oligomers. The DNA ladder that forms during apoptosis results from the activation of an endogenous endonuclease. An increase of cytosolic calcium at an early phase of apoptosis may contribute to the activation of the endonuclease (Joseph et al., 1993). Meanwhile, there is no tissue inflammation. The suicide program is regulated by many extrinsic and intrinsic signals, and the threshold of apoptotic cell death is also dynamically regulated by multiple inducers and inhibitors of gene products (Steller, 1995). Removal of trophic factors, excessive exposure to excitatory amino acid neurotransmitters, oxidative stress, calcium toxicity and mitochondrial defects have all been postulated to contribute to the pathogenesis of apoptotic neuronal death.

Several apoptosis-related oncogene products are expressed and regulated in
neurons. Bcl-2, a cell death suppressor, is found in the mitochondrial membrane, the nucleus and the endoplasmic reticulum. A high level of expression of bcl-2 in sympathetic neurons prevents cell death induced by deprivation of nerve growth factor (Garcia et al., 1992).

Another important regulator of neuronal apoptosis is the low-affinity NGF receptor, p75, which appears to be important for neuronal cell death and survival (Rabizadeh et al., 1993). p75 has been shown to increase the affinity of TrkA for NGF and to enhance the specificity of the Trk family of receptors for neurotrophins (Barbara et al., 1991). It is now apparent that p75 is yet another cellular death signaling protein (Finkel, 1996). p75 has some sequence similarity to the tumor necrosis factor receptors (TNFR-I and TNFR-II), the human cell surface antigen Fas (APO-1), and the B cell antigen CD40, all of which mediate cell death through an intracellular "death domain" (Bazzoni, 1996). Because of structural and functional homology between the NGFR/TNFR system, the hypothesis that p75 serves as a constitutive cell death-promoting molecule that is inhibited by NGF binding was put forward (Finkel, 1996). Evidence suggested that because p75 enhanced the survival of neurons from developing embryos, it might act as a death gene for older neurons. The type of cell death induced by p75 was apoptosis and neural cell survival was enhanced by binding of NGF to p75 (Rabizadeh et al., 1993). Recent studies have further supported the concept of p75 as a constitutive regulator of apoptosis and suggested that inhibiting p75 expression with antisense oligonucleotides can rescue neurons from death (Finkel, 1996).

Fas/APO-1(APO-1, CD95), another member of the NGFR/TNFR family, is a type I cell-surface receptor with a molecular weight of 35-40kDa. Binding of the receptor by ligand or antibody initiates cell death (Bazzoni, 1996). Fas-mediated apoptosis triggered by ligation of Fas molecule seems to be target dependent. For example, macrophages express Fas and undergo apoptosis when cultured with anti-Fas. In contrast, although endothelial cells can express the Fas molecule, Fas ligation is insufficient to
induce apoptosis suggesting differential regulation of Fas function among cells (Richardson et al., 1994). The susceptibility of Fas-bearing cells to Fas/APO-1 antibody-mediated cell killing is also modulated by cytokines. Fas dependent cytotoxicity were augmented by pre-exposure to interferon gamma and TNF alpha in malignant glioma cells (Weller et al, 1994).

Fas/APO-1 antigen was originally described in fibroblasts, some malignant tumor cell lines (Yonehara, 1989) and some cells of lymphocyte lineage (Nagata, 1995). Subsequently, a variety of normal cells have been found to express this cell surface glycoprotein. In mice, the mRNA coding for Fas antigen is present in the thymus, heart, liver and ovaries (Watanabe-Fukunaga et al., 1992). In human tissue, Fas/APO-1 is expressed by epithelial cells, satellite cells of the autonomic ganglia and some mesenchymal cells, such as fibroblasts and endothelial cells. Histiocytes and subsets of T- and B-lymphocytes, as well as a number of malignant cells, also express the Fas/APO-1 antigen (Owen-Schaub et al., 1993). However, Fas expression has not been reported in neurons, although Fas antigen was found in brains of patients with Alzheimer-type dementia, mainly expressed by a subset of reactive astrocytes. These astrocytes may undergo the Fas-mediated apoptotic process (Nishimura et al., 1995).

The role of Fas and Fas ligand in regulation of cytotoxic T lymphocytes, especially in the pathogenesis of autoimmune diseases, has been noted (Hanabuchi et al., 1994). For example, point mutation in the Fas cytoplasmic domain and Fas ligand in homozygous mice caused conditions termed lpr (lymphoproliferation) or gld (generalized lymphoproliferative disease), which are similar to systemic lupus erythematosus, a human autoimmune disease with neurological complications (Nagata, 1995).

Many cell cycle-regulating genes, including several cyclins, c-fos, c-myc, c-jun, cdc-2, RB and p53 have been suggested as mediators of cell survival, division or apoptotic death (Pandey et al., 1995). p53, the protein product of a tumor suppresser gene, and inhibitor of cell mitosis, acts as a direct effector causing apoptosis.
Overproduction of normal p53 protein in a myeloid leukemia cell line induces rapid apoptotic cell death (Yonish-Rouach et al. 1991). What their role in neuronal cell death is remains to be explored.

1.5. Rationale

Diabetic neuropathy is a life-threatening complication in a significant proportion of patients with all forms of diabetes mellitus, whose etiology is unclear. Studies indicate the appearance of autoantibodies before clinical presentation, thus, progressive target cell destruction may develop over several years (Boitard, 1992). On these bases, it may be possible to identify individuals at high risk for development of IDDM and its neuropathic complications using specific genetic and immunological assays. This raises the possibility that effective immunotherapies could be developed to prevent the target cell destruction, while the individual still has substantial target cell reserves. The presence of autoantibodies to antigens contained in islet cells has become a reliable means of predicting future development of IDDM, although islet cell antibodies are heterogeneous and some antibodies may be more predictive than others (Eisenbarth et al., 1988). As mentioned before, autoantibodies against neuronal tissue may not be correlated with ICA (islet cell cytoplasmic antigen) antibodies. A method to predict diabetic neuropathy by measurement of antibodies to specific neuronal autoantigens may provide a means to earlier identification of people at risk for developing neuropathy and allow for development of strategies for prevention.

In these studies, we used an adrenergic clone of a murine neuroblastoma tumor, N1E-115 (NB) as an autonomic neuron model to investigate the humoral factors in sera of diabetic patients with neuropathy on neuronal cell growth and differentiation. Neuroblastoma is a neoplasm of the adrenal medulla and components of the peripheral autonomic nervous system. They are widely used as a model for neuronal research, because they exhibit properties similar to differentiated sympathetic neurons such as neurite outgrowth, synthesis of neuropeptides or neurotransmitters and expression of receptors for
pharmacological agents (Sonnenfeld, 1982). Such a neuronal cell model might allow early prediction and prevention of diabetic neuropathy. It could also be a useful model to study the mechanism of neuronal death, autoimmune pathogenesis and possible immunotherapy as well as the protective effects of neurotrophic factors or other new drugs on diabetic neuropathy.
CHAPTER II. THE HUMORAL EFFECT OF DIABETIC PATIENTS' SERA ON N1E-115 CELL PROLIFERATION AND DIFFERENTIATION

Previous evidence showing that autoantibodies to autonomic nerve tissue components had a higher frequency in neuropathic patients with diabetes than in diabetic control subjects with disease of similar duration indicated that neuronal tissue autoantibodies have a role in the development of symptomatic autonomic neuropathy (Brown et al., 1989). However, it was unclear whether these autoantibodies can directly cause neuronal tissue damage or whether they were only a secondary manifestation, because of the exposure of autoantigen after neuronal tissue damage. Also, what was the possible mechanism by which these antibodies or other humoral factors besides immunoglobulins could cause neuronal death? To answer these questions, the humoral effect of diabetic patient sera (IDDM, NIDDM) and control subject sera on N1E-115 neuroblastoma cell growth and differentiation was investigated.

2.1. Diabetic patients' sera collection and N1E-115 neuroblastoma cell line as a model of sympathetic neurons

Serum was collected from patients in three categories: control subjects, patients with insulin-dependent diabetes mellitus (IDDM) and patients with non-insulin-dependent diabetes mellitus (NIDDM) (Table. 1). Patients with IDDM, or type I diabetes, were separated into groups with neuropathy and without neuropathy. Diabetic neuropathy was established by the presence of symptoms, signs, and quantitative electrophysiology, electromyography or other neuropathy tests, according to the recommendation of the American Diabetes Association and the American Academy of Neurology (1988).

The sex distribution between groups was well matched. Because of the nature of the disorders, patients with NIDDM were significantly heavier and older.
Table 1. Sera were collected from healthy controls and patients with either NIDDM or IDDM. IDDM patients were further separated into groups with neuropathy or without neuropathy. The serum glucose level, HbA1c level, duration of diabetes as well as cholesterol and lipid levels were determined by group. The age and weight of NIDDM patients was significantly higher (*, p<0.05) than any of the groups. The serum glucose and HbA1c measures were elevated in the diabetic groups compared to controls.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Type 1 diabetes</th>
<th>Type 1 diabetes</th>
<th>Type 2 diabetes</th>
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<tbody>
<tr>
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<td>9/9</td>
<td>0/7</td>
<td>9/10</td>
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<tr>
<td>Sex</td>
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<td>6F/3M</td>
<td>3F/4M</td>
<td>6F/4M</td>
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<tr>
<td>Age (yr)</td>
<td>41.2±2.6</td>
<td>36.6±3.1</td>
<td>37.6±3.1</td>
<td>87.7±5.2*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.1±6.1</td>
<td>70.7±4.9</td>
<td>78.1±3.1</td>
<td>217±22.0*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>89.4±1.3</td>
<td>226±37.6</td>
<td>128±29.3</td>
<td>230±19.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>222.4±18.3</td>
<td>220±18.3</td>
<td>221±19.3</td>
<td>183±32.4</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>97.3±15.4</td>
<td>100±14.0</td>
<td>87±11.3</td>
<td>147±17.1</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
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<td>64±4.0</td>
<td>59±2.5</td>
<td>50±4.9</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>141.7±17.4</td>
<td>144±22.2</td>
<td>145±18.4</td>
<td>8.5±0.7</td>
</tr>
<tr>
<td>HbA1c(%)</td>
<td>Range [3.4-6.1]</td>
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<td>10.4±2.4</td>
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<tr>
<td>Duration of Disease (yr)</td>
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<td>19.7±2.86</td>
<td>15.3±3.4</td>
<td>8.5±0.7</td>
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than the other groups. Serum glucose level and HbA1c in both IDDM and NIDDM patients were significantly higher than the normal range. The duration of diabetes was slightly longer in both IDDM groups than the NIDDM patients, but did not reach statistical significance. Cholesterol and lipid levels did not differ between groups. None of the subjects had clinical nephropathy. Serum urea and creatinine measurements were normal.

The N1E-115 neuroblastoma cell line was an adrenergic cell clone derived from the C1300 murine neuroblastoma tumor (Amano, 1972). This cell line had the capacity to develop the characteristics of adrenergic neurons when maintained in cell culture, and therefore was representative of sympathetic autonomic neurons. These cells could be induced to differentiate and extend neurites by removal of serum from the media.

2.2. The humoral effect of diabetic patients' sera (IDDM, NIDDM) and control sera on neuronal cell proliferation

Neuronal cell growth tests were performed by growing flasks of N1E-115 cells to confluence, lifting the cells with Pucks' D1 solution, and subculturing at 10^5 cells per 35mm² dish. The cultures were split into four groups (IDDM, NIDDM, control and FBS) of four plates each, cultured in DMEM (4,500mg/L D-glucose) supplemented with 10% human serum from each group. Each day for 4 consecutive days the cells from one plate in each group were lifted and the number of viable cells in each dish was determined using trypan blue exclusion criteria. The viable cell counts were taken over a 5-day period (including the day of plating), and the growth curves for each group were established.

Figure 1 shows the mean viable cell number in cultures treated with sera from 5 groups during five days culture period. Control group included 9 individuals; IDDM without neuropathy included 7 individuals; IDDM with neuropathy included 6 individuals; NIDDM included 10 individuals and 9 with neuropathy. The results show that N1E-115 neuroblastoma cells grow rapidly in regular 10% FBS-containing DMEM media (Fig.1; Fig.2-a). The viable cell numbers were reduced when cells were grown in media containing
Figure 1. Mean growth curves for N1E-115 NB cells in DMEM media with 10% serum from different population groups. The N1E-115 cell cultures were exposed to 10% fetal bovine serum (FBS) or control sera, sera from IDDM patients with and without neuropathy or NIDDM patients' sera with neuropathy for 5 days. The viable cells were counted by trypan blue exclusion criteria and expressed as mean±SEM. Cell growth was significantly inhibited (*p< 0.05) by sera from IDDM patients with neuropathy compared to cells grown in sera from control, NIDDM, and IDDM patients without neuropathy on days 4-5 of culture. Viable cell number was determined using trypan blue exclusion criteria. The control group included 9 individuals; IDDM without neuropathy included 7 individuals; IDDM with neuropathy included 6 individuals; NIDDM included 10 individuals. Statistical analysis was performed by analysis of variance and Wilcoxon rank sum test.
Figure 2. Representative photomicrographs of N1E-115 NB cell growth in DMEM media with 10% serum from different groups: (A). 10% FBS; (B). 10% serum from a control subject; (C). 10% serum from an IDDM patient with neuropathy; (D). 10% serum from an NIDDM patient with neuropathy. N1E-115 cells in A, B and D showed the normal cell appearance and most cells were health, while those treated with IDDM serum showed cytotoxic effects indicated by cell shrinkage, lifting from attachment and cell death (C).
10% human serum. There was no statistically significant difference between the healthy control group, NIDDM with neuropathy and IDDM without neuropathy. In contrast, viable cell numbers in cultures treated with serum from the IDDM patients with neuropathy were significantly lower than in the FBS cultures by day 2 (p<0.05) and cultures with NIDDM and IDDM serum without neuropathy by day 4 and 5 (p<0.05). In 3 of the 6 cases, IDDM serum associated with neuropathy appeared to cause total cell death within 3 days. However, this also occurred in response to one serum sample from a IDDM patient without neuropathy.

2.3. The humoral effect of diabetic patients' sera (IDDM, NIDDM) and control sera on neuronal cell differentiation

N1E-115 cells were subcultured into 12-well culture plates for 2 days in DMEM media supplemented with 10% FBS. The cultures were then exposed to individual serum from each group used in the cell growth studies for 24 hr. The serum was then removed from the culture media and the cells grown in serum-free DMEM for another 24 hr to induce differentiation. The differentiated cell was defined as one that had at least one neurite extended from the cell body by more than one cell diameter. Two hundred cells from each well were evaluated under phase contrast microscopy and the differentiation rate (%) and length of neurites were determined.

The results of the study show that the sera from the IDDM patients with neuropathy group significantly (p<0.05) inhibited neuroblastoma cell differentiation after 24 hr (differentiation rate = 18.7%) compared to NIDDM (30.0%) and control (42.4%) groups (Fig. 3, Fig. 4). However, once the cells were induced to differentiate, the neurite length extended did not show a significant difference after exposure to serum from the different groups (Fig. 5.).

2.4. The Effect of IDDM sera pre-absorbed by N1E-115 cells and mouse
liver acetone powder on cell proliferation.

To determine whether the inhibition of cell proliferation was due to the presence of a cytotoxic factor in the serum, or the absence of a supporting factor required for cell survival, human sera from 3 subject groups were pre-absorbed by N1E-115 neuroblastoma cells (Fig. 6) and mouse liver acetone powder. Aliquots of sera were pre-incubated with $10^6$ neuroblastoma cells or 1 mg of mouse liver acetone powder at $37^\circ$C for 20 min. After centrifugation for 10 min at 3000xg, the supernatant sera were applied to neuroblastoma cell cultures as a 10% concentration in DMEM for 24 hr and compared with cell cultures in 10% non-absorbed serum media. The growth inhibition of IDDM serum was totally reversed by pre-absorption to neuroblastoma cells but not mouse liver acetone powder pre-absorption. The results demonstrated that cell growth inhibition was caused by some 'cytotoxic factor' in IDDM serum that could be pre-absorbed by neuroblastoma cells but not other mouse tissue extracts prepared with organic solvents. It also indicates that the cell growth inhibition or death was not only caused by a deficiency of required factors in IDDM serum (Fig. 7).

Summary

Using N1E-115 neuroblastoma (NB) cells as an adrenergic neuronal model, we found that the serum from IDDM patients with neuropathy had greater effects and more frequently inhibited neuronal cell growth and differentiation than control sera and sera from non-neuropathic IDDM. In some cases, individual IDDM sera caused neuronal death. This inhibiting or neurotoxic effect of IDDM serum could be partially reversed by NB cell pre-absorption, which suggested that the cytotoxic effect was caused by a cytotoxic factor in the serum, although we have not excluded that neurotrophic factors in the serum may also have some effect. This cytotoxic effect could not be removed by pre-absorption with mouse liver powder prepared with an organic solvent, indicating that it could be a tissue specific effect or that the antigen was extracted by organic solvents.
Figure 3. N1E-115 NB cell differentiation rate after exposure to individual serum from different groups. N1E-115 neuroblastoma cells were exposed to 10% serum from different population groups for 24 hr and then induced to differentiate by removing serum from the medium for 24 hr. The cells extending neurites were counted, expressed as a percent of total cells and compared among different groups. There was a significant inhibition (*p<0.05) in the percentage of cells extending neurites in response to IDDM serum, but not in response to NIDDM serum. There were 9 individuals in control group, 10 individuals in IDDM group and 10 individuals in NIDDM group tested.
Figure 4. Representative photomicrographs of morphology of differentiated NB cells after treatment with different serum groups. N1E-115 neuroblastoma cells treated with 10% serum from different population groups for 48 hr and then cultured in serum-free medium to induce differentiation: (A) Control; (B) IDDM; (C) NIDDM. Serum from an IDDM patient with neuropathy (B) inhibited neurite extension.
Figure 5. The neurite length after exposure to sera from different groups. N1E-115 neuroblastoma cells were cultured in DMEM media containing 10% serum from individuals of different population groups for 48 hr and then cultured in serum free media to induce neurite extension (differentiation). The length of the longest neurite on each evaluated cell was measured under microscopy. At least 20 differentiated cells were measured from each individual. There were 9 individuals in control group, 10 individuals in IDDM group and 10 individuals in NIDDM group evaluated. The results show that there was no significant difference among different groups.
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Figure 6. The principle of immune pre-absorption. For pre-absorption studies, $10^6$ NB cells were collected and incubated with IDDM serum, which had shown a cytotoxic effect on NB cell, at 37°C for 30 min. The cells were precipitated, and the supernatant serum was added to the NB cell culture to examine the effect on cell growth and compare with serum before absorption. The absorbed NB cells were lysed and the protein components were collected and separated by SDS polyacrylamide gel electrophoresis to examine whether extra proteins were absorbed onto the cells.
Supernatant added to N1E-115 cell culture

Cells were precipitated and homogenized, and a Western-blot was run using anti-human IgG as second antibody.

Incubation

10^6 N1E-115 cells

Human serum

37°C 30 min

Non-absorbed IDDM serum

Pre-adsorbed IDDM serum

Non-absorbed IDDM serum
Figure 7. Photomicrographs of the effect of IDDM sera pre-absorbed by NB cells on cell growth. N1E-115 neuroblastoma cell cultures exposed to serum from a patient with IDDM before or after pre-absorption by neuroblastoma cells. (a) The IDDM patient serum inhibited NB cell growth and caused cell death. (b) The same IDDM patient serum absorbed by NB cells showed a complete reversal of the cytotoxic effect. These studies demonstrated the presence of a cytotoxic factor in the IDDM serum.
CHAPTER III. IDENTIFY THE NEURONAL TOXIC FACTORS IN THE SERA OF IDDM PATIENTS WITH NEUROPATHY

In the previous studies, we found that there was a cytotoxic factor in IDDM patients with neuropathy, but not in sera from the NIDDM group and control group. Because there was a different effect of IDDM and NIDDM sera on proliferation and differentiation, this suggested a difference in pathogenesis of neuropathy between IDDM and NIDDM groups. Based on this and previous studies by other investigators, an autoimmune-related humoral factor, such as autoantibodies, was considered in mediating the neuronal death effect of IDDM sera. Here, we investigated whether this cytotoxic effect was mediated by an immunoglobulin and/or other humoral factors.

3.1. Effect of IDDM sera pre-absorbed by protein A on cell growth.

Affi-Gel protein A agarose (Bio-Rad, Inc., Richmond, CA) was washed with PBS and added to the test serum at a 2:1 (v/v) dilution. After incubation for 30 min in a 37°C water bath, the mix was centrifuged at 10,000xg for 30 min to precipitate the protein A gel. Identical untreated sera were tested in parallel with the protein A precipitated sera for both control and IDDM groups. The groups tested included 6 IDDM patients with neuropathy or 6 control subjects. The results showed that precipitation of immunoglobulin (Ig) from control sera had little effect on cell growth (8.6±1.3 $\times 10^3$ cells before precipitation and 9.4±1.8 $\times 10^5$ cells after Ig precipitation). In contrast, depletion of Ig from IDDM serum significantly ($p<0.05$, one way ANOVA) improved cell growth (3.7±1.5 $\times 10^5$ cells before precipitation and 8.0±1.2 $\times 10^5$ cells after precipitation). There was an 3/4 recovery from 57% inhibition before protein A treatment to 15.2% inhibition after protein A treatment, indicating that the protein A-agarose was able to remove about 75% of the cytotoxic effect in IDDM serum (Fig. 8).
Figure 8. The effect of protein A precipitated sera on N1E-115 cell growth. Sera were precipitated with protein A from 6 IDDM and 6 control subjects. Equal numbers of N1E-115 cells were cultured in DMEM media supplemented with 10% of one of four test sera: control serum, IDDM serum, protein A-absorbed control serum or protein A-absorbed IDDM serum. After 72 hr the viable cells were counted and the mean for each group was determined. There was a significant reduction (*p<0.05) in cell number in cultures treated with IDDM sera compared to cultures treated with control sera. However, after protein A absorption, IDDM sera were able to support cell growth similarly to untreated control sera. There was a 73.3% recovery from inhibition before protein A treatment, indicating that the protein A-agarose was able to remove the cytotoxic factor in IDDM serum in large portion. There was no significant change in the ability of control sera to support cell growth after protein A absorption.
3.2. Effects of immunoglobulin fractions extracted from diabetic sera (IDDM, NIDDM) and control sera on cell growth.

Immunoglobulin was precipitated from sera of 10 subjects from each of the IDDM, NIDDM and control groups. Immunoglobulin was precipitated from the test sera by gradually adding saturated \((NH_4)_2SO_4\) to the serum to a final concentration of 40%, and the slurry was stirred at 4°C overnight. The solution was centrifuged at 10,000xg for 30 min. The supernatant was removed and the pellet was resuspended in pH 7.4 PBS and dialyzed with 3 changes of 10 volumes of PBS to remove \((NH_4)_2SO_4\). Immunoglobulin fractions isolated from serum from each subject were tested separately in neuroblastoma cell cultures, at a concentration of 10% serum equivalents in DMEM plus 10% pooled human serum (Gibco, Grand Island, NY). Viable cells were counted for 5 consecutive days to determine the effect of the Ig fractions on cell growth. When the immunoglobulins precipitated were applied to the cell culture media in the absence of serum, the cells did not grow well because of the lack of serum and began differentiating. However, when the Ig isolated from IDDM serum was added to medium containing 10% of commercial pooled human serum (Gibco, Grand Island, NY), cell growth was significantly (one way ANOVA, \(p<0.01\)) inhibited by day 4 and 5 of culture, compared to the growth in the presence of Ig precipitated from control serum (Fig. 9). The growth of cells in media with Ig from the control group sera did not differ from that of media containing 10% pooled serum alone (Fig. 9).

3.3. Effects of heat inactivated IDDM and control sera on cell growth.

Test sera from IDDM and the healthy control groups were heated in a 56°C water bath for 30 min to inactivate the complement to test the effect on neuroblastoma cell growth. Heat inactivated serum was then added to the DMEM media and incubated for 72 hr as described before. Non-heated serum was tested in parallel with the heated...
Figure 9. The effects of immunoglobulin fractions extracted from different groups on N1E-115 cell growth. The effect of immunoglobulin-containing fractions precipitated from serum of 10 subjects in each of the IDDM, NIDDM and control groups on NB cell growth, expressed as mean±SEM. Cells were grown in DMEM media supplemented with 10% of a pooled normal human serum. Immunoglobulin isolated from sera in the test groups were added to cell cultures for 5 days. The viable cell number was counted by trypan blue exclusion criteria. There was a significant (*p<0.05) reduction in cell numbers in cultures treated with Ig isolated from IDDM sera by day 5 compared with control and NIDDM groups.
Figure 10. Effects of IDDM and control sera treated by heat inactivation on N1E-115 cell growth. Equal numbers of N1E-115 NB cells were cultured in DMEM media supplemented with 10% of test sera from 4 different groups: 6 control sera, 6 IDDM sera, and the same sera heat inactivated (56°C for 30 min). After 72hr culture the viable cells were counted. The cytotoxic effect of IDDM sera was reversed by heat inactivation. There was a 66.2% recovery from cytotoxicity (48.5% inhibition before heat treatment and 16.4% inhibition after heat treatment). Paired t-test showed statistical significance (p< 0.05). There was no significant difference before and after heat treatment in the control group.
counterparts in all studies. The results indicated that heat treatment of control serum had little effect on cell growth compared to that of untreated serum. In contrast, for IDDM serum that had cytotoxic effects on cell proliferation, heat inactivation significantly (p<0.05, paired t-test) reversed the inhibiting effect on the cells in all cases (Fig. 10), with a 2/3 recovery of the cytotoxic serum effect (48.5% inhibition before heat treatment and 16.4% inhibition after heat treatment).

3.4. Identification of the factor in IDDM serum against N1E-115 cells by immunocytochemistry

A number of autoantigens have been proposed as candidates for the targeting of autoimmune immunoglobulin in diabetic neuropathy, including glutamic acid decarboxylase (GAD), gangliosides and phospholipids. Because GAD is cytoplasmic in neuronal cells and gangliosides and phospholipids are membrane components, we felt that identification of the topography of the antigens recognized would be helpful. The IDDM and control sera were tested for binding to the surface or cytoplasm of the neuroblastoma cells by immunocytochemistry. Anti-GAD antibodies, including anti-carboxy terminal of GAD-65, anti-amino terminal of GAD-65 and anti-GAD 67, all were provided by Dr. Åke Lernmark (Karolinska Institutes, Stockholm, Sweden). Neuroblastoma cells were plated at 10^4 cells in 35mm^2 tissue culture dishes on sterile coverslips and grown for 2 days. The cells were fixed in 4% paraformaldehyde in PBS for 60 min, blocked by 10% normal goat serum in PBS for 30 min, incubated with a 1:100 dilution of test human serum for 90 min, and finally exposed to a fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG secondary antibody (Sigma Chemical Co., St. Louis, MO) for 60 min. After immunostaining, the cells were mounted on slides using o-phenylenediamine (1mg x ml⁻¹) in 90% glycerol and examined by fluorescence microscopy. To examine cytoplasmic immunostaining, the cells were permeabilized after fixation by immersion in acetone for 7 min prior to the addition of 10% goat serum.
**Figure 11.** Indirect immunofluorescence showing the IgG binding pattern of IDDM serum on N1E-115 cells (1). Indirect immunofluorescence (IF) was performed on N1E-115 cells using individual serum from different groups as primary antibody and FITC-labeled anti-human IgG as secondary antibody. (A) Representative photomicrograph of IF with IDDM serum which induced cell death as primary antibody on N1E-115 cells fixed on cover slide. (B) Representative photomicrograph of IF with IDDM serum as primary antibody on differentiated cells. (C) Representative photomicrograph of IF with NIDDM serum as the primary antibody. (D) IF with control serum as the primary antibody. The IF with IDDM serum treated slides showed a fluorescent ring, indicating binding at the cell membrane (arrows), but not in cells treated with any of the other sera.
Figure 12. Indirect immunofluorescence showing the IgG binding pattern of IDDM serum on N1E-115 cells (2). Indirect immunofluorescence was performed on N1E-115 cells using IDDM (A), or control serum (B) as primary antibody and FITC-labeled anti-human IgG as secondary antibody and observed at high magnification. There was light diffuse fluorescent staining in cells exposed to control serum (B). In contrast, cells exposed to IDDM serum showed a specific bright halo of fluorescence (A, arrows), indicating the recognition of a cell surface antigen by immunoglobulin IgG in IDDM serum.
The results showed a characteristic cell surface binding pattern with a bright rim of immunofluorescence when cells were labeled with serum from IDDM patients with neuropathy (Fig. 11-A; Fig. 12-A), indicating specific recognition of a membrane antigen. This pattern was not seen in cells treated with control serum (Fig. 11-D, Fig. 12-B) or NIDDM serum (Fig. 11-C). Three monoclonal anti-GAD antibodies only show weak cytoplasmic binding and no cell surface binding (picture not shown).

Summary

The properties of the cytotoxic factor present in IDDM serum were examined. These studies indicate that the factor was likely to be an immunoglobulin. The neurotoxic effect could be reduced by 75% by protein A precipitation. The cytotoxic factor was present in the immunoglobulin containing fraction precipitated from IDDM patients' sera, evidenced by the inhibition of growth of cells treated with immunoglobulins extracted from the IDDM sera. Indirect immunofluorescence using IDDM sera showing cytotoxicity as primary antibody and FITC labeled anti-human IgG as secondary revealed a specific cell-surface IgG binding pattern, further supporting the hypothesis that there was immunoglobulin IgG binding to the cell surface. Moreover, the cytotoxic effect was reversed about 2/3 by heat inactivation, suggesting the possible involvement of complement, but not excluding other heat-sensitive factors.
CHAPTER IV. STUDIES OF THE MECHANISM OF NEURONAL DEATH CAUSED BY SERUM OF IDDM PATIENTS

There are two forms of cell death with very different characteristics, i.e., necrosis and apoptosis. These differ both morphologically and biochemically. Apoptosis is an active process of neuronal cell death with specific, defining morphologic and molecular features, such as cell shrinkage with preservation of organelles, membrane blebbing, condensation of cytoplasm, compacting of chromatin, fragmentation of DNA into oligomers, detachment from surrounding cells and the formation of apoptotic bodies (Carson, 1993). Necrosis is an event with passive cell swelling, membrane lysis and rapid collapse of internal homeostasis. Apoptosis as a mechanism of cell death has been suggested in many neurodegenerative diseases.

In previous studies, we found that serum from IDDM patients inhibited N1E-115 cell growth and differentiation as well as caused cell death in some cases. In attempting to define the mechanism and events leading to neuronal death induced by IDDM patient serum, we examined the morphologic, biochemical and genomic DNA change and intracellular Ca\textsuperscript{2+} change of N1E-115 cells after exposure to IDDM serum. Further, we tested one regulator of apoptosis, Fas/APO-1, as a mediator of the apoptotic effects observed in response to IDDM sera.

4.1. Morphology change of N1E-115 cells after exposure to IDDM serum

We previously observed that some sera of IDDM patients with neuropathy induce N1E-115 neuroblastoma cell death as early as 4 hr. Furthermore, after exposure to sera from IDDM patients, the morphological changes of NB cells under phase contrast microscopy showed eccentric cytoplasmic condensation, cell shrinkage and lifting from attachment. Some cells had an intact membrane ballooned around the condensed cytoplasm,
Figure 13. Morphology change of N1E-115 cells after exposure to IDDM serum. N1E-115 neuroblastoma cells were split and grown in DMEM medium with 10% serum from control subjects (A) or IDDM patients (B). After exposure to serum from an IDDM patient, the morphological changes of NB cells under phase contrast microscopy show eccentric cytoplasmic condensation, cell shrinkage and lifting from attachment (all indicating an apoptotic cell death). Some cells have intact cell membrane around condensed cytoplasm (arrow).
all suggesting apoptotic cell death (Fig. 13). The viable cell number was reduced significantly as counted with trypan blue exclusion criteria, indicating some manner of cell removal or death. It should be noted that the number of viable cells might be overestimated by this procedure, due to the intact cell membranes of apoptotic cells, which would also exclude trypan blue and could be counted as viable.

4.2. Intracellular calcium change induced by IDDM serum

One of the early cellular changes described with apoptosis is a rise in intracellular Ca\(^{2+}\). To measure the changes of intracellular free calcium in response to IDDM serum, \(10^6\) NB cells/ml were loaded with the fluorescent intracellular calcium indicator, Fura-2 (5\(\mu\)M final conc.), mixed with pluronic acid (0.02%) to facilitate entry into the cell. After incubation at 37°C for 60 min, extracellular dye was removed by centrifugation and the NB cells were resuspended in PBS buffer with 1% bovine serum albumin. An aliquot of cells was added to a cuvette, which was constantly stirred, and measurements were taken by alternate excitement with 340nm and 380nm light and emission read at 540nm. The sensitivity of Fura-2 loaded NB was checked with histamine and calibrated for minimum and maximum fluorescence during each experiment. Serum from IDDM, NIDDM and control subjects was added directly to the cuvette, individually at 15% final concentration. The emission was recorded for 5-10 min and then analyzed and graphed using the software SPEX-DM 3000 according to the equations of Grynkiewicz et al., (1985). Results showed that five of the eight IDDM sera caused a sustained increase of intracellular calcium concentration, starting within 10 seconds after the initial exposure to IDDM serum and continuing to increase at least up to 10 min (Fig.14 ; Fig.15. 1-4), while with most control sera (4/5), there was an early peak and then return to base line (Fig. 15. 5-6). There was a significant (Chi-square, p< 0.05) difference in the frequency of increased intracellular Ca\(^{2+}\) in response to sera from the IDDM and control groups. The early transient peak was considered to be an artifact of adding serum. These results suggested that the elevation of
Figure 14. Intracellular calcium changes induced by IDDM serum (1). Typical patterns of cytosolic free calcium change in response to IDDM and control serum in NB cells loaded with the intracellular calcium indicator, Fura-2 are shown in this and figure 16. Results showed that five of the eight IDDM sera caused a sustained increase of intracellular calcium concentration, starting within 10 seconds after the initial exposure to IDDM serum and continuing to increase at least up to 10 min, while with most control sera (4/5), there was an early transient peak and then concentrations returned to baseline. This was a significant (Chi-square, p< 0.05) difference in the frequency of increased intracellular Ca^{2+} in response to serum from the IDDM and control groups.
Chapter 4. The Mechanism of Neuronal Death

3.4 W -0 7 -

IDDM Serum

\([\text{Ca}^{2+}]_i\)

Control Serum

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Figure 15. Intracellular calcium changes induced by IDDM serum (2). The representative changes of cytosolic free calcium in response to IDDM serum (1-4) and control serum (5-6) in NB cells loaded with the intracellular calcium indicator, Fura-2 were shown. IDDM sera caused a sustained increase of intracellular calcium concentration, starting 10 seconds after initial exposure to IDDM serum and continuing to increase up to 10 min (1-4), while with control sera, there was an early peak and then a return to base line (6) or a sustained reduction in intracellular calcium (5).
intracellular calcium may play a role in the process of cell death by apoptosis.

4.3. Examine genomic DNA fragmentation of N1E-115 cells after exposure to IDDM serum

IDDM sera showing cytotoxicity to neuroblastoma cells were added to neuroblastoma cell cultures as 15% in DMEM. Suspended and attached cells were collected separately at 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr after exposure to IDDM serum. The genomic DNA from cells collected at each time point was isolated with a gentle process using a Micro TurboGen kit (Invitrogen, San Diego). The cells were lysed with lysing buffer and RNase solution from the kit. After the lysates were incubated at 65°C for 5 minutes, the protein fraction was precipitated by adding chloroform and separated from the aqueous layer containing DNA by centrifugation. The DNA was precipitated, washed and quantitated, and finally resuspended in pH 8.0 TE (Tris-EDTA) buffer. The purified genomic DNA was separated by electrophoresis on 1% agarose gel and stained with ethidium bromide. The gel was examined under ultraviolet illumination to check for the DNA pattern. The genomic DNA of cells treated with control serum was purified and run on gel electrophoresis in parallel. Results showed that a DNA fragmentation ladder, a marker of apoptotic cell death, was revealed from suspended cells (Fig. 16, A lanes) as early as 4 hr after NB cell exposure to cytotoxic IDDM serum. The DNA fragmentation patterns and time of appearance were similar for the IDDM sera tested (Fig.16-A and B, lanes A). The cells retaining attachment show no pattern of DNA fragmentation (Fig. 16-A and B, B lanes) reflecting a non-apoptotic state.

4.4. Studies of apoptosis-related proteins (Fas, p75) expression in N1E-115 NB cells

Indirect immunofluorescence (IF)

IF was performed to examine the expression of the apoptosis-related proteins.
Figure 16. Cellular DNA fragmentation ladder of N1E-115 cells induced by IDDM serum. IDDM serum showing cytotoxicity to neuroblastoma cell was added to a set of neuroblastoma cell cultures as 15% in DMEM. Suspended and attached cells were collected separately at 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr. The genomic DNA was isolated at each time point and separated by electrophoresis on a 1% agarose gel. A lanes were DNA extracted from suspended cells and B lanes were DNA extracted from attached cells. A DNA fragmentation ladder, characteristic of apoptotic cell death, was revealed in cells that have lost attachment as early as 4 hr after NB cell exposure to cytotoxic IDDM serum but not with control serum. The DNA fragmentation ladder was shown in suspended cells but not attached cells. The DNA fragmentation pattern and time course are similar with two individual IDDM sera.
Figure 17. Indirect immunofluorescence detecting Fas expression and changes in N1E-115 cells. Photomicrographs of indirect immunofluorescence using hamster anti-mouse Fas monoclonal antibody (mAb) (MBL RK8) as primary antibody and FITC labeled anti-mouse IgG secondary antibody are shown. The results demonstrate that Fas is expressed on the N1E-115 NB cell surface (a) as well as on differentiated N1E-115 NB cells (b, arrows). The surface staining pattern is totally blocked by IDDM serum (c), suggesting that Fas might be one of the surface antigens recognized by immunoglobulin from IDDM serum (c). Fas staining on NB cells showed a cluster pattern after culture with IDDM serum 24 hr prior to fixation (d). The residual yellow fluorescence is autofluorescence from the cell nuclei.
(Fas, p75, bcl-2, p53) and their possible change in N1E-115 cells after exposure to IDDM serum. p75 and bcl-2 were expressed weakly on N1E-115 cells (data not shown), while Fas was strongly expressed on the cell surface (Fig. 17). Because Fas was known as a surface receptor mediating apoptotic cell death, we hypothesized that IDDM serum might induce cell death through a death signal from Fas. Whether the toxic factor in IDDM serum might block anti-Fas antibody binding to the cell surface was also examined. The procedure of immunofluorescence was performed as described before. Briefly, N1E-115 cells were plated at \(10^4\) cells in 35mm² tissue culture dishes on sterile coverslips and grown for 2 days. The cells were fixed in 4% paraformaldehyde in PBS for 60 min, blocked by 10% mouse serum in PBS for 30 min, incubated with a 1:100 dilution of hamster anti-mouse Fas (MBL, RK8) serum for 60 min, washed 3 times with PBS and exposed to secondary fluorescent isothiocyanate (FITC)-labeled mouse anti-hamster IgG secondary antibody (Pharmingen, San Diego) for 30 min. After immunostaining, the cells were mounted on slides using o-phenylenediamine (1mg x ml⁻¹) in 90% glycerol and examined by fluorescence microscopy. To examine whether IDDM serum blocked anti-Fas mAb binding, fixed NB cells were first exposed to IDDM serum for 30 min then to the anti-Fas mAb and FITC-labeled secondary antibody. To examine the change of Fas expression on NB cells after culture with IDDM serum, the NB cells were cultured in DMEM containing 10% IDDM serum for 24 hr, then IF was performed.

The immunofluorescence studies revealed that Fas was expressed on the N1E-115 NB cell surface in a characteristic non-homogeneous staining pattern (Fig. 17-a). Fas was also expressed on differentiated N1E-115 NB cells, which were induced to differentiate by culture in serum free media for 24 hr (Fig. 17-b). The surface staining pattern with anti-Fas mAb was completely blocked by cytotoxic IDDM serum, suggesting that Fas might be one of the surface antigens recognized by IDDM serum (Fig. 17-c). After NB were cultured in DMEM media with 10% IDDM serum for 24 hr, Fas staining on NB cell showed a clustering pattern, indicating possible cross-linking by antibody or soluble Fas
ligand in IDDM serum, a prerequisite for Fas activation and apoptosis.

Expression of p75 was also examined by IF in N1E-115 NB cells. For p75 detection, anti-p75 monoclonal antibody was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN). However, there was only a weak fluorescence signal observed (results not shown). p53 expression was abundant in the cell nucleus and perinuclear cytoplasmic region with IF (results not shown), but because of the cytoplasmic localization of this nuclear protein, the significance was uncertain. Because N1E-115 cell is a tumor cell line, the p53 could be mutated causing inactivation and dislocated from the nuclear to the cytoplasm region. Bcl-2 expression was not detectable in this cell line either in proliferating or differentiating stages.

**N1E-115 cell membrane protein extraction, immunoprecipitation and Western blotting**

Protein chemistry techniques were employed to confirm the presence of Fas in NB cells. NB cells \((10^7)\) were lysed by nitrogen cavitation (PARR Instrument Company, Moline, IL) in PBS. Cell membrane and cytosol fractions were separated by gradient centrifugation. The tissue homogenate was first centrifuged at 500 \(x\) g to remove crude nuclear material, then 10,000 \(x\) g to remove mitochondria and 100,000 \(x\) g for 60 min to get the microsomal fraction containing cell membranes. Membrane protein fractions were solubilized with 1% Triton-X100 detergent in buffer containing 150mM NaCl, 10mM Tris pH7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium vanadate and 0.5%NP-40 (Tiruppathi, 1986) and the protein content was quantitated with a Micro BCA Protein Assay kit (Pierce Co., IL). Fifty \(\mu\)l of total lysate containing 100 \(\mu\)g of membrane protein fractions were mixed with 10 \(\mu\)l of anti-Fas mAb (MBL, RK8 clone about 5 \(\mu\)g) at 4°C for 30 min followed by 50 \(\mu\)l of 10% protein A beads for 30 min. The protein A beads were spun down and washed with PBS for 3 times, then the pellet was resuspend in electrophoresis sample buffer, and boiled for 5 minutes. The supernatant was loaded onto a 12% SDS-
polyacrylamide gel. After Coomassie blue staining, a major protein band at mouse Fas MW 40kDa was revealed (Fig. 18-A).

For Western blotting, the NB cell membrane proteins and whole cell homogenates were solubilized with 1% Triton-X100 detergent in buffer containing 150mM NaCl, 10mM Tris pH7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium vanadate and 0.5% NP-40 for 30 min, and the protein content was quantitated with the Micro BCA Protein Assay kit (Pierce Co., IL). Fifty μl total lysate containing 100 μg membrane protein fractions were mixed with 100 μl of IDDM serum at 4°C for 30 min followed by 50 μl of protein A beads for 30 min. The protein A beads were centrifuged and washed with PBS 3 times, then the pellet resuspended in electrophoresis sample buffer and boiled for 5 minutes. The supernatant was loaded onto a 12% SDS-polyacrylamide gel and separated by electrophoresis, then transferred to PVDF membrane. The membrane was exposed to anti-Fas monoclonal antibody (MBL, RK8) at a 1:200 dilution at room temperature (RT) for 30 min and washed 3 times with PBS, then exposed to a secondary peroxidase-labeled anti-IgG secondary antibody at RT for 30 min and developed by enzyme chemiluminescence (ECL) (Amersham Life Science, Arlington, IL) with Kodak X-Omat AR5 film (Kodak Co., Rochester, NY) (Fig. 18-B). These studies demonstrated that NB cell membrane proteins and whole cell proteins immunoprecipitated with 3 individual IDDM sera, separated by SDS-polyacrylamide gel electrophoresis and revealed by Western blot with anti-Fas mAb, showed Fas protein. The results indicated that Fas could be one of the proteins recognized by IDDM serum. However, more studies are needed to demonstrate that anti-Fas antibody or Fas ligands are present in IDDM serum and could cause neural cell death.

**The effects of Anti-Fas mAb on neuroblastoma cell growth**

If N1E-115 cells express Fas, activating anti-Fas mAb may have an inhibiting or lethal effect on cell growth in culture. RK8 anti-Fas mAb, which had been shown to activate the Fas mechanism in other cells, was added to the N1E-115 cell culture in three
Figure 18. Immunoprecipitation and western blot of Fas from N1E-115 cells. 18-A. Proteins from the membrane fraction of NB cells were immunoprecipitated with anti-Fas mAb and run on SDS polyacrylamide gel, showing a major protein band at 40kDa, the mouse Fas molecular weight. Figure 18-B: Proteins from the NB cell membrane and whole cell preparations were immunoprecipitated with IDDM serum, separated by SDS-PAGE and Western blot performed with anti-Fas mAb. Protein in lanes 1, 2/3 and 4 were precipitated by serum from 3 different patients. Lanes 2 and 3 were precipitated with the same serum, but performed with whole cell protein or membrane protein as indicated (WP - whole cell protein; MP - cell membrane protein). Protein recognized by the anti-Fas antibody was precipitated from NB cells by all 3 IDDM sera tested.
Figure 19. The effects of anti-Fas mAb on N1E-115 cell growth. To examine the effects of anti-Fas mAb on NB cell growth, different doses of anti-Fas mAb (1000 ng/ml, 500 ng/ml, 100 ng/ml) were added to the N1E-115 cell culture media for 72 hr. The number of viable cells was determined by trypan blue exclusion criteria as described before. The maximal dose of anti-Fas mAb (1000 ng/ml) had 49.3% inhibition compared with 10% FBS control.
doses (100 ng/ml, 500 ng/ml and 1000 ng/ml). Anti-Fas mAb caused the loss of NB cells in a dose-dependent manner within 72 hr of culture. The maximal dose of anti-Fas mAb (1000 ng/ml) had 43.8% inhibition compared with control (Fig. 19). This observation further supported the presence of membrane-bound Fas on NB cells and the possibility that anti-Fas antibody in IDDM serum could cause inhibition of cell growth.

**Summary**

Apoptosis as the possible mechanism of neuronal death induced by IDDM serum was examined in the N1E-115 NB cell model. The morphological change of NB cells after exposure to IDDM serum exhibited a characteristic apoptotic cell death pattern: cytoplasmic condensation, cell shrinkage and lifting. A DNA fragmentation ladder, a marker of apoptotic cell death, was evident within 4 hr after exposure to cytotoxic IDDM serum. A sustained increase in intracellular calcium concentration was noted in the presence of 5 of 8 IDDM sera in Fura-2 loaded NB cells, also characteristic of apoptosis. These investigations support the hypothesis that the death of NB cells caused by IDDM serum occurred by a mechanism of programmed cell death.

Apoptosis-related protein, including Fas and p75 expression in N1E-115 cells was examined. Fas was expressed on the NB cell surface in a characteristic non-homogeneous distribution, as determined by immunofluorescence staining with anti-mouse Fas mAb. The low affinity NGF receptor, p75, was expressed weakly in N1E-115 cells. Immunoprecipitation of cell membrane protein with anti-mouse Fas mAb revealed a major protein band of MW 40kDa, approximately the size of murine Fas. Cell-surface membrane immunofluorescence staining by anti-Fas antibody was completely blocked by IDDM sera which were previously shown to induce apoptotic neuronal death. Fas distribution on the cell surface was found in a clustering pattern after 24 hr in culture with IDDM serum, which may indicate activation of Fas receptors by cross linking with anti-Fas antibody or soluble Fas ligand in IDDM serum. Anti-Fas mAb inhibited NB cell growth in a dose
dependent manner. The maximal dose of anti-Fas mAb (1000 ng/ml) had 43.8% inhibition compared with control. The studies indicated that Fas could be one of the cell surface antigens recognized by humoral factors in IDDM serum. Whether it is the mediator inducing apoptotic cell death in response to IDDM serum needs to be confirmed.
CHAPTER V. SUMMARY AND DISCUSSION

Summary

The possibility that autoimmune-related humoral factors contribute as a cause of diabetic neuropathy was tested by in vitro application of serum from patients with IDDM and NIDDM to adrenergic murine neuroblastoma cells in culture. In these studies, we have demonstrated that the serum from patients with IDDM and neuropathy could cause inhibition of growth and differentiation in N1E-115 neuroblastoma cells and, in some cases, induced cell death. The growth-inhibition, or cytotoxic effect, was more prominent in IDDM patients with neuropathy but varied in IDDM patients with no neuropathic symptoms. This effect was not reproduced in NIDDM patients with neuropathy, suggesting a heterogeneous pathogenesis of diabetic neuropathy in these two type of diabetes. The neuronal toxic effect of IDDM serum could be abolished by pre-absorption with neuroblastoma cells, indicating that the effect was mediated by humoral factors that could bind to neuroblastoma cells.

The identity of the cytotoxic factor in IDDM patients serum was examined. These studies indicated that the toxic factor was likely to be an immunoglobulin. The neurotoxic effect of IDDM serum could be reduced by protein A pre-absorption (the inhibition was reduced by 3/4 comparing before and after protein A treatment). An immunoglobulin-containing fraction of IDDM serum exerted the inhibitory effect when pooled human serum was added, but not the immunoglobulin containing fraction from NIDDM and control groups. The cytotoxic effect was partially reversed by heat inactivation (which reversed the inhibiting effect by about 2/3), suggesting the involvement of complement but not excluding other heat sensitive factors. However, none of the treatments could totally reverse the inhibition, suggesting that multiple mechanisms may be involved.

In our study, examination of N1E-115 cells with indirect immunofluorescence using IDDM serum as the primary antibody and FITC-labeled anti-human IgG as secondary
antibody revealed a specific cell surface binding pattern even when the serum was diluted from 1:50 to 1:200 (Fig. 11; Fig. 12), which demonstrated the binding of immunoglobulin IgG in IDDM serum to a surface antigen on neuroblastoma cells.

The morphological changes of the neuronal death caused by IDDM serum, such as cytoplasmic condensation, cell shrinkage and lifting (possibly caused by cell membrane blebbing) suggested an apoptotic cell death, which could happen as early as 4 hr after exposure to IDDM serum. Further study showed that the cell death caused by IDDM serum was associated with an increased intracellular free calcium concentration within 10 min in some samples. The cytosolic free calcium rise rapidly, as early as 10 sec after exposure to IDDM serum and continued to rose for up to 10 min. A DNA fragmentation ladder, which can be found in apoptotic cell death, was observed within 4 hr after exposure to cytotoxic IDDM serum. The DNA fragmentation ladder was found in cells that had lifted from attachment with dishes, but not the cells still attached, suggesting loss of attachment was also an early event in apoptotic cell death in vitro. Those unattached cells could be washed and collected by low speed centrifugation, indicating the integrity of the membranes was still intact.

Selected classic apoptosis-related proteins, p75, Fas, bcl-2 and p53, were examined in this cell line. NGF low affinity receptor p75 was too weakly expressed in NB cells to evaluate. However, immunofluorescence with anti-mouse Fas mAb and immunoprecipitation with anti-Fas mAb indicated Fas antigen was expressed on the surface of N1E-115 NB cells. The cell-surface Fas immunofluorescence binding pattern was totally blocked by IDDM serum, indicating that Fas might be one surface antigen recognized by IDDM serum. The results from immunoprecipitation with IDDM serum followed by Western blot (Fig. 19-B) further supported the possibility that Fas antigen was recognized by humoral factors in IDDM serum and was potentially involved in serum toxicity.


**Discussion**

Antibodies against different neuronal tissue components have been reported in many neurodegenerative diseases. However, most studies have only demonstrated immunoglobulin binding in neuronal tissues, or that there was a higher titer of antibodies against neuronal antigens in the circulation of neuropathy patients. It has been unclear whether these antibodies truly play a primary role in causing neuronal tissue damage or whether they are a secondary response, resulting from the exposure of neuronal antigen by damaged tissue. Furthermore, another question is: "what might the mechanism be of neuronal tissue damage by these antibodies?" Neuronal cell culture of N1E-115 cells provided a model to study both the toxic potential of serum as well as the mechanism of neuronal death induced by humoral factors in IDDM serum.

This study demonstrated that humoral factors such as immunoglobulins in IDDM patients' sera could inhibit neuronal cell growth and differentiation, indicating humoral immune components could directly cause neuronal target tissue damage, even though activation of humoral immune components could be a secondary response to unknown stimulation. However, none of the studies of immunoglobulin effects, such as protein A precipitation to remove immunoglobulins, heat inactivation of complement or testing the effect of an immunoglobulin-containing fraction on cell growth, could completely (>75%) account for the cytotoxic effect of IDDM serum. Therefore, other possible contributing factors, such as low levels of neurotrophic growth factors in the serum of IDDM patients or activation of the programmed cell death pathway by another initiator, should also be considered.

The different effects of sera on neuronal cell growth and differentiation between IDDM and NIDDM patients with neuropathy support the possible role of humoral immunopathogenesis in neuropathy of IDDM patients, but not NIDDM. If the humoral factors, such as anti-neuronal tissue antibodies, were a secondary manifestation because of neuronal tissue destruction and antigen exposure, the test results should be expected to be
the same in IDDM and NIDDM patients with neuropathy. These inhibitory or toxic effects were more prominent in IDDM patients with neuropathy, but inconclusive in IDDM patients with no neuropathy. One explanation was that the patients were in different stages of neuronal damage with the non-symptomatic patients in a "pre-neuropathic" stage. They had not yet had sufficient neuronal loss to cause symptomatic neuropathy. Inhibition was not reproduced in NIDDM patients with neuropathy, suggesting a possible heterogeneous pathogenesis of diabetic neuropathy in the two type of diabetes.

These studies indicate that the IDDM serum exerted cytotoxic effects on N1E-115 neuroblastoma cells by activation of a programmed cell death cascade, apoptosis. From temporal analysis in this model, cytosolic free calcium elevation preceded DNA fragmentation and cell death. Intracellular calcium elevation had been hypothesized to be an early primary event in both programmed cell death and hypoxic-ischemic neuronal injury (Kirino, 1994). The consistent finding that IDDM serum caused an increase in cytosol free calcium concentration followed by apoptotic cell death, indicated that calcium ion plays a critical role in the neurotoxic effect. Increasing evidence indicates that calcium could activate latent enzymes that can contribute to the structural changes of apoptosis (Jones et al, 1989; Trump et al, 1992). These enzymes include a calcium-dependent nuclear endonuclease that can cleave DNA, and a transglutaminase that can crosslink cytosolic proteins. Calcium-dependent proteases may also degrade the cytoskeleton. All of the enzymes together appear to be responsible for nuclear degradation and cell morphology change. It has been reported that IgM autoantibody in IDDM serum can interact with L-type Ca\(^{2+}\) channels to increase Ca\(^{2+}\) influx and cause apoptotic cell death in insulin-secreting cells (Berggren et al., 1993). It remains to be determined in our model that the increased cytosolic calcium was induced by immunoglobulin (IgG or IgM) and whether the increased cytosolic calcium stimulated by IDDM serum resulted from calcium influx through membrane calcium channels or a release from intracellular calcium stores. Different calcium channel blockers can be studied to determine the type of calcium channel involved and their possible role in apoptotic cell death.
death.

Homeostasis of multicellular organisms can be controlled not only by the proliferation and differentiation of cells but also by cell death. Recent evidence clearly indicates that apoptosis may be positively or negatively regulated by certain genes. Many growth factors and hormones could regulate cell survival, growth or differentiation. Otherwise, cytokines such as Fas ligand or TNF as well as proto-oncogene products and transcription factors like c-mys, c-fos, c-jun, p53, bcl-2 may regulate or signal the intrinsic cell death program by inducing or inhibiting apoptosis, which functions to remove unwanted or non functional cells (Pandey, 1995). Abnormality in the regulation of the cell death program may contribute to the pathogenesis of many neurodegenerative diseases, autoimmune or neoplastic diseases.

Fas/APO-1, a type I cell surface receptor signaling apoptotic cell death pathway, is expressed in many cell lines and mediates apoptosis of susceptible target cells (Weller et al, 1994). Fas/Fas ligand interaction serves as an important regulatory mechanism in the development and function of the immune system. Most studies on Fas show Fas-mediated apoptosis in the cytotoxicity of susceptible T-lymphocytes, suggesting that Fas might play a critical role in the effector phase of T-dependent immune responses (Hanabuchi et al, 1994). Some growth factors and hormones, when bound to their receptors, induce dimerization (homodimerization or heterodimerization), which then triggers an intracellular signaling cascade with subsequent gene expression. Similarly, the apoptotic cell death-signaling pathway from Fas requires the cross-linking of Fas receptors, either with specific antibodies to Fas or by Fas ligand trimerization. This is consistent with our observations of Fas localization on NB cells treated with cytotoxic IDDM sera.

In our immunocytochemistry study, the cluster of Fas antigen expression on NB cells surface in response to IDDM serum stimulation might represent Fas molecular cross-linking by the toxic factor in IDDM serum, inducing the cell apoptosis cascade. Treatment with anti-Fas mAb caused cell death in a dose-dependent fashion, supporting the hypothesis
that anti-Fas antibody could induce cell death. Whether Fas expression in NB cells was altered by exposure to IDDM serum or there was increased circulating Fas ligand in IDDM serum remains to be determined. The observation that Fas receptor is expressed on the N1E-115 cell surface and is possibly recognized by IDDM serum suggests that Fas could be one of the cell surface antigens recognized by the cytotoxic factor in IDDM serum and thereby mediate apoptotic cell death.

Fas antigen expression has not been reported in neurons and has not been examined in adrenal medulla, although Fas antigen has been described in some tumor cell lines (Yonehara, 1989) and satellite cells of the autonomic ganglia (Owen-Schaub et al., 1993). Fas was found in brains of patients with Alzheimer-type dementia, mainly expressed by a subset of reactive astrocytes. Such astrocytes may undergo the Fas-mediated apoptotic process (Nishimura et al., 1995). The existence and the role of Fas in neuronal cell death induced by IDDM serum needs to be further confirmed. Fas is a member of the NGF receptor/TNF receptor family, which all have a cysteine-rich extracellular domain. It is unclear whether the anti-Fas monoclonal antibody will cross react with other members in the NGFR/TNFR family on N1E-115 cells because of the homology of the extracellular domain and thereby induce cell death, also creating a link between the hypothetical apoptotic mechanism and the growth factor deficiency.

We were unable to detect bcl-2 in N1E-115 cells by immunofluorescence. The apparent lack of bcl-2 in the NB-cell line might simply reflect the lack of bcl-2 in neuroblastomas, its replacement by another member of the bcl-2 family, eg, bcl-X, or it may result from gene loss during tumorigenesis. Furthermore, the lack of bcl-2 may be associated with an increased propensity for apoptosis in this cell line. p53 was abundant in this cell line as indicated by immunofluorescence, but cytoplasmic distribution raises questions about detection of this nuclear protein. However, it is also possible that p53 could be mutated because the N1E-115 cell line is an adrenergic cell clone derived from a neuroblastoma tumor (Amano et al, 1972).
Our study cannot exclude the possibility that other unknown antigens or death factors, such as p75 low affinity NGF receptor, or TNF and its receptors, might also be involved. Thus, their role, if any, in the apoptotic neuronal death induced by IDDM humoral factors remains to be elucidated. The low-affinity NGF receptor, p75, appears to be important for neuronal cell death and survival (Rabizadeh et al., 1993). p75 has some sequence similarity to the tumor necrosis factor receptors (TNFR-I and TNFR-II), the human cell surface antigen Fas (APO-1), and the B cell antigen CD40, all of which mediate cell death through an intracellular "death domain" (Bazzoni, 1996). Lowering of NGF concentration, overexpression of p75, or expression of less trkA, the high affinity NGF receptor, causes p75 to signal death (Finkel 1996). The type of cell death induced by p75 is apoptosis, and neural cell survival is enhanced by binding of NGF to p75 (Rabizadeh et al., 1993). Recent studies have further supported the concept of p75 as a constitutive regulator of apoptosis and suggested that inhibiting p75 expression with antisense oligonucleotides can rescue neurons from death (Finkel, 1996). In the N1E-115 cell model, p75 was detected weakly, while in preliminary studies trkA expression was strong with immunofluorescence assay. More sensitive and quantitative methods will be required to examine the possible change of p75 or trkA in response to diabetic sera and their role, if any, in induction of apoptosis by IDDM sera.

These studies demonstrated that serum of IDDM patients with neuropathy had a greater ability to inhibit neuronal cell growth and differentiation and in some cases induced cell death in cultured adrenergic neurons. They indicates that autoimmune-related humoral factors inducing apoptotic neuronal death could be one mechanism involved in the pathogenesis of diabetic neuropathy. Other investigators have confirmed the appearance of autoantibodies before clinical presentation, thus, progressive target cell destruction may develop over several years (Boitard, 1992). On this basis, it may be possible to identify individuals at high risk for development of IDDM and its complications using specific genetic and immunological assays, including the N1E-115 cell model. This raises the
possibility that effective immunotherapies could be developed to prevent the target cell destruction, while the individual still has substantial target cell reserves. As mentioned before, autoantibodies against neuronal tissue may not be correlated with islet cytoplasmic antibodies (ICA). A method to predict diabetic neuropathy with a more specific neuronal cell model may provide a more sensitive, earlier and more accurate diagnosis.

Because the N1E-115 neuroblastoma cell line revealed a neurotoxic effect in response to antibodies in IDDM serum, which was correlated with a diagnosis of clinical neuropathy and was significantly different from the results in response to NIDDM and control serum, this cell culture model might allow early prediction and immunotherapy for prevention of diabetic neuropathy. This cell line may also prove to be a useful model to further study the mechanism of neuronal death, autoimmune pathogenesis and may allow studies of the possible neuronal protective effect of new drugs, neurotrophic factors like NGF and to evaluate immune therapy intervention, like intravenous immunoglobulin, for treatment of diabetic neuropathy. It is possible in the N1E-115 cell assay that those IDDM patients without clinical neuropathy symptoms, but whose sera exhibit neurotoxicity, are in a pre-neuropathic state. Thus, this assay may identify those patients at risk for developing symptomatic neuropathy. Further longitudinal studies would help to establish the significance of these findings. Supporting the possibility that autoimmunity might be important in the pathogenesis of diabetic neuropathy was the observation that N1E-115 cytotoxicity correlates with symptomatic improvement in patients treated with intravenous immunoglobulin (IVIg), which has been studied by other investigators in our research institutes (unpublished data).

The N1E-115 cell model, like all in vitro cell culture systems, is an advance in cellular level biomedical research. It provides a living model to study and observe changes of cell morphology, growth, differentiation and related regulating mechanisms in both physiological and pathological conditions. The significance of this study lies in determining that humoral immune components in sera of IDDM patients can inhibit
neuronal cell growth and differentiation inhibition and result in cell death, possibly through inducing apoptosis. The difference between these studies and most other immunopathogenesis studies in neuropathy is that the others showed that antibodies could recognize neuronal tissue but could not demonstrate that these antibodies actually damage neuronal tissue. In contrast, in the N1E-115 cell model we have shown both neuronal toxicity and immunoglobulin causation.

However, the N1E-115 neuroblastoma cell line is a murine tumor cell line. Its regulating machinery for cell growth and differentiation might already be altered. Some oncogenes could be constitutively turned on while others could be mutated or deleted. Another disadvantage is that the sera tested are an unknown mixture. There are components which could influence the growth and differentiation of the cells but may not be related to diabetes and with varying quantities in the serum among individuals. Thus we could not control for all effects of serum in the model. Finally, this model could not reveal the possible intrinsic change of diabetic neuronal tissue which may play a role in the process of immunopathogenesis of diabetic neuropathy, especially in IDDM patients. Only external effectors could be evaluated in these studies.

**Conclusion**

- Sera from IDDM patients with neuropathy had a higher frequency to inhibit growth and differentiation of an adrenergic neuronal cell line, N1E-115 neuroblastoma cells, but not sera from NIDDM and control subjects. In some cases, the humoral factors in sera of IDDM patients with neuropathy induced neuronal death in vitro.
- The neuronal toxicity appeared to be mediated by immunoglobulin in the serum of IDDM patients and at least partially through a complement-related mechanism.
- The neuronal death caused by IDDM serum seemed to be an apoptotic cell death, which is characterized by cell shrinkage, cytoplasmic condensation, cell lifting, increased...
intracellular calcium change and genomic DNA fragmentation.

- Fas, an initiator of apoptosis, could be one cell surface molecule recognized by humoral factors in IDDM serum and able to activate the neuronal death pathway.

- Our study supports the hypothesis that immunopathogenesis may be important in the development of diabetic neuropathy, thus, indicating that immunotherapy such as IVIg treatment could be a practical and effective treatment. Other possible strategies based on autoimmune pathogenesis aimed to block the neuronal death pathway should be considered and further investigated.
Manuscripts


Abstracts

- Pittenger GL, Liu D and Vinik AI, The neurotoxic factor in IDDM serum is IgG which may contribute to the pathogenesis of diabetic autonomic neuropathy. *Diabetes* 43(suppl 1):65A, 1994
- Pittenger GL, Liu D, Newlon PG and Vinik AI, Neurotoxicity of serum from patients with insulin-dependent diabetes (IDDM) is associated with increased Trk-A expression and is partially reversed by NGF. *Abstracts* 25th Annual Meeting, Society for Neuroscience, 21: 1740 (679.15), 1995


Boitard C: The differentiation of the immune system towards anti-islet autoimmunity.
Clinical Prospects 35:1101-1112, 1992


Deckwerth TL and Johnson EM Jr: Temporal analysis of events associated with

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programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor


Dicou E, Hurez D and Nerriere V: Natural autoantibodies against the nerve growth factor in autoimmune diseases. *J Neuroimmunol* 47:159-168, 1993


Graham AR and Johnson PC: Direct immunofluorescence findings in peripheral nerve from patients with diabetic neuropathy. *Ann Neurol* 17:450-454, 1985


Hofmann K and Tschopp J: The death domain motif found in Fas and TNF receptor is present in proteins involved in apoptosis and axonal guidance. *FEBS Lett* 371:321-323,
1995


Joseph R, Li W and Han E: Neuronal death, cytoplasmic calcium and internucleosomal DNA fragmentation: evidence for DNA being released from cells. *Brain Res Mol Brain Res* 17:70-76, 1993


Kirino T: Cerebral ischemia and neuronal death. *No To Hattatsu* 26: 130-135, 1994


Nayak RC, Omar MAK and Rabizadeh A: "Cytoplasmic" islet cell antibodies evidence that
the target antigen is a sialoglycoconjugate, *Diabetes* 34:617-619. 1985


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Skyler JS and Mancard G: Clin. Imm. 1:15-42, 1993

Smith RG, Hamilton S: Science Publishing Co., 1988


Smith RG, Hamilton S, Hofmann F, Schneider T, Nastainozky M, Birnbaumer L, Stefani


Tiruppathi C, Alpers DH and Seetharam B: Phase separation of rat intestinal brush border


Vives M, Somoza N and Soldevilla G: Reevaluation of autoantibodies to islet cell membrane in IDDM. *Diabetes* 41:1624-1631, 1992


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PUBLICATION

HONORS
- Shanghai Second Grade Scientific and Technical Award 1982
- The American Society for Cell Biology 35th annual meeting student travel award. 1995; The abstract is chosen by peer review from thousands as one of the fifteen representing the most exciting research in cell biology today.