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(54) **Title:** METHODS FOR ASSESSING ANTIBODY-MEDIATED CYTOTOXICITY

(57) **Abstract:** The present invention is directed to methods and kits for the prognosis and diagnosis of autoimmune diseases. In particular, the present invention provides methods and kits suitable for measuring the neuronal toxicity of biological samples from subjects suspected of having a neurodegenerative disease, such as multiple sclerosis.

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METHODS FOR ASSESSING ANTIBODY-MEDIATED CYTOTOXICITY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of provisional patent application U.S. Serial No. 60/662,187, filed March 15, 2005, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The invention was made in part with government support from the National Institutes of Health, Grant No. NS46678-01. As such, the United States government has certain rights in the invention.

FIELD

The present invention is directed to methods and kits for the prognosis and diagnosis of autoimmune diseases. In particular, the present invention provides methods and kits suitable for measuring the neuronal toxicity of biological samples from subjects suspected of having a neurodegenerative disease, such as multiple sclerosis.

BACKGROUND

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Approximately 350,000 people in the United States have been diagnosed with MS, with a typical disease onset of 20 to 50 years of age (Lindsey and Wolinsky, Scientific American Medicine, WebMD Inc., Chapter 11, pp. IX.1-12, 2003). The origin of MS remains unknown, but both environmental factors and genetic background contribute to disease susceptibility (Ebers et al., Nat Genet, 13:472-476, 1996; Moore and Rodriquez, Neurology, 51: 742-747, 1998; and Haines et al., Nat Genet, 13:469-471, 1996).

In general, MS patients can expect to experience one of four clinical courses of disease, each of which can be classified as mild, moderate, or severe: relapsing-remitting; primary-progressive; secondary progressive, and progressive-relapsing. Relapsing-remitting MS (RRMS) is the most common form at the time of initial diagnosis (~85%), characterized by clearly defined flare-ups (also called relapses, attacks, or exacerbations). These episodes of acute worsening of neurologic function are followed by partial or complete recovery periods (remissions) free of disease progression. In contrast, primary

progressive MS (PPMS) is relative rare (~10%), and is characterized by a slow but nearly continuous worsening of disease, without distinct relapses or remissions. There are, however, variations in rates of progression over time, with occasional plateaus, and temporary minor improvements. Secondary progressive MS (SPMS) is characterized by an initial period of relapsing-remitting disease followed by a steadily worsening disease course with or without occasional flare-ups, minor recoveries (remissions), or plateaus. Before the introduction of "disease modifying" drugs, approximately 50% of patients with relapsing-remitting MS developed secondary progressive MS within 10 years of initial diagnosis. Lastly, progressive-relapsing MS (PRMS) is characterized by a steadily worsening disease course, with distinct acute relapse periods (attacks or exacerbations). In contrast to relapsing-remitting MS, the periods between relapses are characterized by continuing disease progression.

Like the clinical course of disease, the inflammatory infiltrate (*e.g.*, mononuclear cells, macrophages, antibody, and complement, as well as other effector molecules such as cytokines and toxic radicals) of MS lesions, varies between patients. This heterogeneity in pathology underlines the existence of multiple pathogenic processes (Lucchinetti et al., Ann Neurol, 47:707-717, 2000). However, to date a correlation between the different pathophysiological subtypes of MS and the known variable clinical presentations of this disease has not been substantiated. Such information can only be derived from experiments that directly address the functional properties of immune responses in patients, and in relevant experimental systems.

Thus what is needed in the art are methods for correlating immunopathology with clinical course for autoimmune disease of the nervous system. In particular, it would be desirable to have relatively noninvasive methods for the diagnosis and prognosis of MS.

SUMMARY

The present invention is directed to methods and kits for the prognosis and diagnosis of autoimmune diseases of the central nervous system. In particular, the present invention provides methods and kits suitable for measuring the neuronal toxicity of biological samples from subjects suspected of having a neurodegenerative disease, such as multiple sclerosis.

Specifically, the present invention provides methods for testing a biological sample, comprising: providing: i) a biological sample comprising antibodies from a subject suspected of having an autoimmune disease, ii) a target cell of the autoimmune disease, and iii) complement; contacting the target cell with the biological sample and the complement

under conditions suitable for affecting lysis of the target cell; and measuring the extent of lysis of the target cell. In some embodiments, the autoimmune disease is a neurodegenerative autoimmune disease and the target cell is a neuron. In some preferred embodiments, the neurodegenerative autoimmune disease is multiple sclerosis, and the neuron is a SK-N-SH cell. In further embodiments, the neuron is selected from but not limited to the group consisting of primary human neuronal cells, IMR-32 cells, SK-N-AS cells, SK-N-FI cells, SK-N-DZ cells, SH-SY5Y cells, BE(2)-M17 cells, BE(2)-C cells, MC-IXC cells, SK-N-BE(s) cells, and CHP-212 cells. In some embodiments, the measuring comprises a technique selected from but not limited to the group consisting of propidium iodide staining, trypan blue staining, tritiated thymidine incorporation, acid phosphatase release, tunnel assay and DNA fragmentation. In some particularly preferred embodiments, the measuring comprises propidium iodide staining and flow cytometry. The present invention also provides methods further comprising a step of correlating the extent of lysis with a clinical course of the autoimmune disease. In a subset of these embodiments, when the extent of lysis is significantly elevated in relation to that observed for a target cell contacted with a biological sample from a healthy subject, the clinical course is primary progressive multiple sclerosis or relapsing remitting multiple sclerosis. In some embodiments, the biological sample is selected from but not limited to the group consisting of whole blood, serum, plasma, cerebral spinal fluid, and urine. Moreover the present invention provides methods further comprising a step of suggesting the use of a therapy directed against or reactive with the humoral immune response of the subject.

The present invention also provides methods of monitoring progression of neurodegeneration mediated by autoimmunity in a subject, comprising: comparing extent of cell death of target cells to a control, wherein the extent of cell death is measured after the target cells have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and wherein the target cells are a type of neuronal cells. Additionally, the present invention provides methods of diagnosing neurodegeneration mediated by autoimmunity in a subject, comprising: comparing extent of cell death of target cells to a control, wherein the extent of cell death is measured after the target cells have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and wherein the target cells are a type of neuronal cells. The present invention further provides methods of aiding diagnosis of

multiple sclerosis in a subject suspected of having multiple sclerosis, comprising: comparing extent of cell death of target cells to a control, wherein the extent of cell death is measured after the target cell have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and wherein the target cells are a type of neuronal cells.

In some embodiments, the neuronal cells are selected from the group consisting of primary human neuronal cells, IMR-32 cells, SK-N-AS cells, SK-N-FI cells, SK-N-DZ cells, SH-SY5Y cells, BE(2)-M17 cells, BE(2)-C cells, MC-IXC cells, SK-N-BE(s) cells, CHP-212 cells and SK-N-SH cells. In some embodiments, the extent of cell death is measured by a cell lysis assay selected from the group consisting of lactate dehydrogenase release assay, ^{51}Cr release assay, acid phosphatase release, glyceraldehyde-3-phosphate dehydrogenase release assay, and BrdU labeled DNA fragmentation ELISA. In other embodiments, the extent of cell death is measured by an assay which differentially stains live and dead cells, wherein the assay is selected from the group consisting of propidium iodide staining, trypan blue staining, neutral red uptake, and calcein-AM/EthD-1 cell staining. In further embodiments, the extent of cell death is measured by a proliferation assay selected from the group consisting of reduction of resazurin dye, reduction of a tetrazolium compound (e.g., MTT, WST-1), and label (e.g., BrdU, tritiated thymidine) incorporation in DNA. In some embodiments, the extent of cell death is measured by an apoptosis assay selected from the group consisting of labeled (e.g., tritiated thymidine, BrdU) DNA fragmentation assay, tunnel assay, caspase activity assay, binding of fluorescent caspase inhibitor assay, annexin V binding assay, cytochrome C release assay, detection of the ratio of ATP/ADP assay, and changes in mitochondrial transmembrane potential assay. In some preferred embodiments, the extent of cell death is measured with propidium iodide staining and flow cytometry. In some embodiments, the subject has multiple sclerosis. In some embodiments, the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease and Lafora body disease. In addition, some embodiments of the present invention further comprising a step of correlating the extent of cell death with a clinical course of an autoimmune disease. In a subset of these, when the extent of cell death is significantly elevated in relation to the control, the clinical course is selected from the group consisting of primary progressive

multiple sclerosis, secondary progressive multiple sclerosis, relapsing progressive multiple sclerosis, relapsing remitting multiple sclerosis, neuromyelitis optica (Devic disease), pleomorphic xanthoastrocytoma (Kepes), clinically isolated syndrome, optic neuritis, relapsing-remitting optic neuritis, transverse myelitis, relapsing remitting transverse myelitis and Marburg's multiple sclerosis, wherein the control is a mean extent of cell death observed for the target cells contacted with biological samples from healthy subjects. In some embodiments, the biological sample is selected from the group consisting of whole blood, serum, plasma, cerebral spinal fluid, and urine.

In addition, the present invention provides kits for the prognosis of an autoimmune disease, comprising: a target cell of an autoimmune disease, complement; and instructions for contacting the target cell with a biological sample comprising antibodies, and the complement, under conditions suitable for affecting lysis of the target cell, instructions for measuring the extent of lysis of the target cell, and instructions for correlating the extent of lysis with a clinical course of the autoimmune disease. In some preferred embodiments, the autoimmune disease is a neurodegenerative autoimmune disease and the target cell is a neuron. In some particularly preferred embodiments, the neurodegenerative autoimmune disease is multiple sclerosis, and the neuron is a SK-N-SH cell. In some kits, the measuring comprises propidium iodide staining and flow cytometry. Also provided by the present invention are embodiments, in which when the extent of lysis is significantly elevated in relation to that observed for a target cell contacted with a biological sample from a healthy subject, the clinical course is primary progressive multiple sclerosis or relapsing remitting multiple sclerosis. In some kits, the biological sample is serum.

The present invention also provides kits for monitoring progression of neurodegeneration mediated by autoimmunity in a subject, comprising: target neuronal cells; complement; and instructions for carrying out the method of monitoring progression of neurodegeneration mediated by autoimmunity described herein. Moreover, the present invention provides kits for diagnosing neurodegeneration mediated by autoimmunity in a subject, comprising: target neuronal cells; complement; and instructions for carrying out the method of diagnosing neurodegeneration mediated by autoimmunity describe herein. The present invention further provides kits for aiding diagnosis of multiple sclerosis in a subject suspected of having multiple sclerosis, comprising: target neuronal cells; complement; and instructions for carrying out the method of aiding diagnosis of multiple sclerosis described herein. In some embodiments, the subject has multiple sclerosis, and the target neuronal cells are SK-N-SH cells. In some preferred embodiments, the extent of cell death is

measured by staining the target neuronal cells with propidium iodide and followed by flow cytometry. In further embodiments, the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease and Lafora body disease. In some preferred embodiments, the instructions further comprise software configured for data analysis.

Moreover, the present invention provides methods for screening a test compound, comprising: providing: i) a biological sample comprising antibodies from a primary progressive multiple sclerosis patient, ii) a neuron, iii) complement, and iv) a test compound; contacting the target cell with the biological sample and the complement in the presence and absence of the test compound, under conditions suitable for affecting lysis of the target cell; and measuring the extent of lysis of the target cell. Some preferred methods further comprise a step of comparing the extent of lysis of the target cell in the presence and absence of the test compound.

The present invention also provides methods for screening a test compound, comprising: contacting target neuronal cells with a biological sample comprising an antibody from a subject with autoimmunity and complement in the presence and absence of the test compound, under conditions suitable for affecting cell death of the target neuronal cells; and measuring the extent of cell death of the target neuronal cells. The present invention also provides methods for screening a test compound, comprising measuring extent of cell death of target neuronal cells, wherein the target neuronal cells have been contacted with a biological sample comprising an antibody from a subject with autoimmunity and complement, in the presence and absence of the test compound, under conditions suitable for affecting cell death of the target neuronal cells. Some embodiments further comprise comparing the extent of cell death in the presence or absence of the test compound, wherein a decrease of the extent of cell death in the presence of the test compound compared to the extent of cell death in the absence of the test compound indicates that the test compound inhibits cytotoxicity. In some preferred embodiments, the test compound is an antibody that inhibits the binding of the antibody in the biological sample to the target neuronal cells.

DESCRIPTION OF FIGURES

Figure 1 illustrates the results of a flow cytometric analysis of SK-N-SH neuronal cell mortality. FIG. 1A is a scattergram of the SK-N-SH cells showing gating of analyzed cells. FIG. 1B is a scattergram of untreated SK-N-SH cells (negative control) showing background mortality of 4.21% in the upper left quadrant (UL). FIG. 1C is a scattergram of SK-N-SH cells treated with human serum from a PPMS patient, showing a three fold increase in cell mortality (12.67% in the UL) as compared to the negative control in panel B. FIG. 1D is a flow histogram with dead cells numbers (PI positive cells, M2) from a PPMS patient (dark-filled trace) overlaid with dead cells numbers (PI positive cells, M2) of the negative control (white trace) depicting the difference between MS serum mortality and background mortality).

Figure 2 illustrates that treatment of SK-N-SH cells with serum from a PPMS patient increases neuronal mortality in a dose-dependent manner. FIG. 2A graphically depicts the increase in SK-N-SH cell mortality upon incubation with serum at decreasing dilutions. Cell mortality was analyzed by flow cytometry, and each column represents the mean +/- SEM from three independent, replicate experiments, with values normalized to 100% mortality at a dilution of 1:50. FIG. 2B provides immunofluorescence analysis (IFA) images of SK-N-SH cells incubated with the same PPMS serum as used in the experiments shown in panel A (dilution 1:50), while FIG. 2C provides IFA images of SK-N-SH cells incubated with a secondary antibody in the absence of treatment with MS patient serum.

Figure 3 depicts the level of serum neuronal toxicity assessed by SK-N-SH mortality in various experimental groups. FIG. 3A is a dot plot demonstrating heightened neuronal mortality in the primary progressive (PP) and relapsing remitting (RR) forms of MS as compared to control subjects (HuC), and to a lesser extent in patients with clinically-isolated syndromes (CIS). The horizontal line corresponds to the highest level of neuronal killing observed in the control group. FIG. 3B graphically depicts SK-N-SH mortality expressed as the mean mortality for each subgroup normalized to 100%, as defined by the highest mortality observed (PPMS group). Statistically significant differences from the controls are indicated for each subgroup, with the most significant difference observed for PPMS ($p<0.0001$).

Figure 4 illustrates that pre-absorption of MS serum on SK-N-SH cells, suppresses binding to and serum-induced mortality of SK-N-SH cells. FIG. 4A graphically depicts neuronal mortality *in vitro* induced by treatment of SK-N-SH cells with MS serum that was pre-absorbed on SK-N-SH cells (Preabs), in comparison with neuronal mortality observed

upon treatment with un-absorbed serum (Serum positive control) and non-bound serum. For these experiments, the immunoglobulin G (IgG) fraction was removed from MS serum by Protein A-affinity chromatography, leaving a flowthrough (Non bound) component. Data are represented as the mean ± SEM of three independent (Non bound), and triplicate experiments (Preabs). FIG. 4B is an IFA image of SK-N-SH cells stained with the IgG fraction of MS serum absorbed to and then eluted from Protein A, while FIG. 4C is an IFA image of SK-N-SH cells incubated with the Non-bound Protein A fraction, and FIG. 4D is an IFA image of SK-N-SH cells stained with the secondary antibody alone.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

The term "autoimmune disease" as used herein refers to the loss of function or destruction of normal tissue that arises from humoral or cellular immune responses to the body's own tissue constituents. Non-limiting examples of such autoimmune diseases include: type I diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis, Guillain-Barré syndrome, autoimmune uveitis, Crohn's disease, ulcerative colitis, primary biliary cirrhosis, autoimmune hepatitis, autoimmune hemolytic anemia, pernicious anemia, autoimmune thrombocytopenia, Grave's disease, autoimmune oophoritis, autoimmune orchitis, temporal arteritis, anti-phospholipid syndrome, Wegener's granulomatosis, Behcet's disease, scleroderma, polymyositis, dermatomyositis, ankylosing spondylitis, Sjogren's syndrome, psoriasis, dermatitis herpetiformis, pemphigus vulgaris, vitiligo, psoriatic arthritis, osteoarthritis, steroid-resistant asthma, chronic obstructive pulmonary disease, and atherosclerosis.

As used herein, the term "neurodegeneration" refers broadly to a defect involving or relating to the gradual and progressive loss of neural tissue. As used herein, the terms "neurodegenerative disorder" or "neurodegenerative disease" refer broadly to disorders or diseases that affect the nervous system, including but not limited to multiple sclerosis, Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. As used herein, the term "neurodegeneration is reduced" refers to the improvement in the neurodegenerative condition, such that the degree of neurodegeneration is lessened.

The term "subject suffering from a neurodegenerative disease" as used herein, refers to both humans and mammals displaying symptoms normally associated with a disease that affects the nervous system. The terms "mammal" and "mammalian" refer animals of the class *mammalia*, which nourish their young by fluid secreted from mammary glands of the mother, including human beings (*e.g.*, mice, rats, pigs, monkeys, humans, etc.). Typically, the terms "subject" and "patient," are used interchangeably.

As used herein, the terms "sample" and "biological sample" refers broadly to all types of samples obtained from humans and other animals, including but not limited to, body fluids such as urine, blood (also serum or plasma), fecal matter, cerebrospinal fluid (CSF), semen, and saliva, as well as solid tissue. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "multiple sclerosis," "MS," "disseminated sclerosis," "focal sclerosis," "insular sclerosis," and "sclerose en plaques" refer to a disease in which there are foci of demyelination (scleroses) of various sizes throughout the white matter of the central nervous system, sometimes extending into the gray matter. Typical symptoms of white matter lesions include but are not limited to weakness, incoordination, paresthesias, speech disturbances, and visual complaints. The course of the disease is usually prolonged, so that the term "multiple" also refers to remissions and relapses that occur over a period of many years.

As used herein, the term "subject suspected of having multiple sclerosis" refers to a subject that presents with one or more symptoms indicative of MS (*e.g.*, vision loss, double vision, nystagmus, speech difficulties, tremor, clumsiness of the hands, unsteady gait, weakness, spasticity, numbness, bladder, bowel, and/or sexual dysfunction, loss of short term memory, depression, fatigue, etc.), or is being screened for MS (*e.g.*, during a routine physical exam). A subject suspected of having MS may also have one or more risk factors (*e.g.*, Caucasian, female, although a subject suspected of having MS has generally not yet been tested for MS. The term "subject suspected of having MS" encompasses an individual who has received an initial diagnosis (*e.g.*, evidence of two or more distinct CNS lesions by observation of clinical symptoms or by magnetic resonance imaging), but for whom the stage or clinical course has is not known. The term also includes subjects that have once had MS (*e.g.*, subject in a remission).

The phrase "diagnosed with multiple sclerosis" refers to evidence for dissemination of lesions within the central nervous system both in space and in time. This means that not only must there exist evidence of at least two distinct lesions as determined by observation

of clinical symptoms or CNS lesions by MRI, there must also be evidence of an occurrence of new symptoms or lesions within a time interval of at least 30 days. A lumbar puncture done to collect a sample of cerebrospinal fluid is useful for providing evidence of chronic inflammation of the central nervous system, often indicated by oligoclonal banding. Nerve conduction studies of optic, sensory and motor nerves can provide further evidence for MS, as the process of demyelination results in reduced nerve conduction velocities. The diagnostic process is completed by several laboratory tests to exclude other diseases that can mimic MS, such as sarcoidosis, vasculitis, and Lyme disease.

As used herein, the term "providing a prognosis" refers to providing information regarding the impact of the presence of an autoimmune disease on a subject's future health (*e.g.,* for MS this may include categorization of a subject in one of the four clinical courses and/or determination of the likelihood of relapses or remissions, etc.).

As used herein, methods for "aiding diagnosis" refer to methods that assist in making a clinical determination regarding the presence, or nature, of multiple sclerosis, and may or may not be conclusive with respect to the definitive diagnosis. Accordingly, for example, a method of aiding diagnosis of MS can comprise measuring the extent of lysis of a target neuronal cell after the target neuronal cell has been contacted with in a biological sample comprising an antibody from a subject and complement under conditions suitable for affecting lysis of the target neuronal cell in the presence of an antibody against the target cell.

As used herein, the term "staining" refers to any number of processes known to those in the field that are used to better visualize, distinguish or identify a specific component(s) and/or feature(s) of a cell or cells. In some preferred embodiments, the term staining refers to immunofluorescence analysis. The terms "immunofluorescence analysis" and "IFA" refer an immunoassay employing a fluorochrome-labeled antibody or antigen, which can be visualized with an appropriate instrument (*e.g.,* fluorescent microscope, FACS, etc.).

As used herein, the term "control" refers to subjects or samples that provide a basis for comparison for experimental subjects or samples. For instance, the use of control subjects (*e.g.,* age-matched healthy subject) or samples permits determinations to be made regarding the efficacy of experimental procedures. In some embodiments, the term "control subject" refers to cells that receive a mock treatment (*e.g.,* treatment with serum from a normal subject or staining with only the secondary antibody).

In some embodiments, the present invention provides methods and kits for determining whether the extent of lysis is "significantly elevated" or "meaningfully elevated." As used herein, the term "elevated" indicates that the methods and kits are suitable for detection of increased cell mortality. In preferred embodiments, the term "meaningfully elevated" indicates that the extent of cell lysis upon incubation with an experimental (*e.g.*, MS patient serum) sample is at least two fold higher (*e.g.*, percent mortality normalized to lysis upon incubation of a target cell with a positive control) than that observed for a negative control sample (*e.g.*, serum from a healthy subject). In still further preferred embodiments, the term "elevated" indicates that the extent of lysis is at least three fold, more preferably at least four fold and most preferably at least five fold greater than the control or untreated target cells.

As used herein the terms "antigen" and "Ag" refer to a protein, glycoprotein, lipoprotein, lipid or other substance that is capable, under appropriate conditions, of inducing a specific immune response and of reacting with the products of that response, that is, with specific antibody or specifically sensitized T-lymphocytes, or both.

The term "target cell" refers to a cell that is destroyed or attacked by an autoimmune disease process. In some embodiments, the term "target cell" refers to a CNS cell (*e.g.*, neuron) that damaged in a subject or by a biological sample from a subject having MS.

As used herein, the term "antibody" typically refers to both polyclonal and monoclonal antibodies. Polyclonal antibodies which are formed in the animal as the result of an immunological reaction against a protein of interest or a fragment thereof, can then be readily isolated from the blood using well-known methods and purified by column chromatography. Monoclonal antibodies can also be prepared using known methods (*See, e.g.,* Winter and Milstein, Nature, 349:293-299, 1991). As used herein, the term "antibody" encompasses recombinantly prepared, and modified antibodies and antigen-binding fragments thereof, such as chimeric antibodies, humanized antibodies, multifunctional antibodies, bispecific or oligo-specific antibodies, single-stranded antibodies and F(ab) or $F(ab)_2$ fragments. The term "reactive" in used in reference to an antibody indicates that the antibody is capable of binding an antigen or target cell (*e.g.*, neuron) of interest.

The term "complement" as used herein refers to a group of about 20 distinct serum proteins, the activity of which is affected by a series of interactions resulting in enzymatic cleavages. In preferred embodiments, the term refers to the classical complement pathway comprising nine components (designated C1 through C9) that react in a definite sequence

upon activation by an antigen-antibody complex, resulting in the immune cytolysis of antibody-coated target cells.

The terms "lysis" and "cytolysis" refer to the pathological breakdown of cells by rupture of their outer membrane.

The term "humoral immune response" refers to an immune response mediated by antibody. In contrast, a "cell-mediated immune response" is an immune response mediated by T lymphocytes.

As used herein, the term "purified" or "to purify" refers to the removal of components (*e.g.*, contaminants) from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind to the target molecule or cell. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule, results in an increase in the percent of target-reactive immunoglobulins in the sample.

As used herein, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments can consist of, but are not limited to, test tubes and cell culture. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

The terms "test compound" and "candidate compound" refer to any chemical entity, pharmaceutical, drug, and the like that is a candidate for use to treat or prevent a disease, illness, sickness, or disorder of bodily function (*e.g.*, MS). Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention.

As used herein, the term "instructions for using said kit" includes instructions for using the reagents contained in the kit for the detection and characterization of an autoimmune disease in a sample from a subject. In some embodiments, the instructions further comprise the statement of intended use required by the U.S. Food and Drug Administration (FDA) in labeling *in vitro* diagnostic products. The FDA classifies *in vitro* diagnostics as medical devices and requires that they be approved through the 510(k) or analyte specific reagent (ASR) procedure. Information required in an application under 510(k) includes: 1) The *in vitro* diagnostic product name, including the trade or proprietary name, the common or usual name, and the classification name of the device; 2) The intended use of the product; 3) The establishment registration number, if applicable, of the

owner or operator submitting the 510(k) submission; the class in which the in vitro diagnostic product was placed under section 513 of the FD&C Act, if known, its appropriate panel, or, if the owner or operator determines that the device has not been classified under such section, a statement of that determination and the basis for the determination that the in vitro diagnostic product is not so classified; 4) Proposed labels, labeling and advertisements sufficient to describe the in vitro diagnostic product, its intended use, and directions for use. Where applicable, photographs or engineering drawings should be supplied; 5) A statement indicating that the device is similar to and/or different from other in vitro diagnostic products of comparable type in commercial distribution in the U.S., accompanied by data to support the statement; 6) A 510(k) summary of the safety and effectiveness data upon which the substantial equivalence determination is based; or a statement that the 510(k) safety and effectiveness information supporting the FDA finding of substantial equivalence will be made available to any person within 30 days of a written request; 7) A statement that the submitter believes, to the best of their knowledge, that all data and information submitted in the premarket notification are truthful and accurate and that no material fact has been omitted; 8) Any additional information regarding the *in vitro* diagnostic product requested that is necessary for the FDA to make a substantial equivalency determination. Additional information is available at the Internet web page of the U.S. FDA.

BRIEF DESCRIPTION OF INVENTION

Inflammatory cells and antibodies mediate multiple sclerosis (MS) pathogenesis. The mechanisms of early neuronal toxicity, as opposed to secondary Wallerian degeneration of axons, are not well understood and furthermore, have not been characterized in association with specific MS subtypes. During development of the present invention, a pathway of neuronal toxicity in MS involving serum antibody-complement mediated lysis has been elucidated. Specifically, fifty-eight sera from MS patients stratified as primary progressive (PPMS, n=27), relapsing-remitting (RRMS, n=17) (Poser criteria), or clinically isolated syndrome (CIS, n=14) were tested as was sera from healthy volunteers as negative controls (HC, n=31). The human neuronal cell line SK-N-SH, which has been observed to specifically bind MS serum, was used as a cell target. Neurons were incubated with MS serum and human complement, and cell death was measured by flow cytometry. As described herein, neuronal mortality was significantly increased upon incubation of cells with sera from PPMS patients, and to a lesser extent upon incubation of cells with sera from RRMS patients, as compared to controls. In contrast, no meaningful difference in neuronal

mortality was upon comparison of cultures incubated with sera from CIS patients versus cultures incubated with sera from control subjects. The toxic effect was found to be mediated by complement in an antibody-dependent fashion, as complement omission, selective removal of IgG by affinity chromatography or serum pre-absorption all inhibited neuronal toxicity. Thus, a serum/antibody-complement-mediated neuro-axonal toxicity is contemplated to occur in MS patients, particularly in patients diagnosed with PPMS. These findings explain the known clinical and radiological patterns of PPMS, which indicate that early brain atrophy may be associated with the observed resistance of PPMS to approved disease-modifying therapies that only target cellular-inflammatory components of MS pathophysiology. Accordingly, the inventors contemplate that therapies targeting humoral-inflammatory components of MS will be more affective in treating PPMS patients. Nonetheless, use of the present invention is not limited to particular mechanism(s).

DETAILED DESCRIPTION OF INVENTION

Methods of the Invention

More than one century ago, Charcot outlined a description of MS as syndrome that included not only demyelination and astrogliosis, but also axonal damage due to Wallerian degeneration, which is the progressive demise of axons after loss of their protective myelin sheaths (Charcot, Gaz Hopital (Paris), 41:554-556, 1868). Recently, axonal damage has been re-emphasized as an early component of MS pathology that may even precede demyelination, and/or be correlated with progression of irreversible neurological disability in MS patients (Arnold et al., Ann Neurol, 36:76-82, 1994; Davie et al., Brain, 117:49-58, 1994; Filippi et al., Brain, 118:1601-1612, 1995; Lovas et al., Brain, 123:308-317, 2000; and Bjartmar et al., Ann Neurol, 48:893-901, 2000). Moreover, axonal transection is described as a common pattern in MS, which correlates with the level of inflammation within the lesion (Trapp et al., J Neuroimmunol, 98:49-56, 1999). The biological mechanisms responsible for axonal damage are not well understood, and may include biomechanical vulnerability secondary to the loss of myelin-trophic support, or a direct and specific immune attack (*e.g.,* inflammatory cells, antibodies or cytokine-mediated toxicity) against axons.

The current study provides for the first time, direct evidence of antibody-mediated toxicity of MS serum towards human neurons. The inventors also confirm previous reports indicating that MS serum contains antibodies that bind to neuronal antigen determinants. Toxicity as measured by neuronal death is concentration dependent, and in some patients is

observed for dilutions as high as 1:500. The neuronal death appears to be dependent on complement-mediated lysis, and is not observed for sera of healthy control subjects, thereby confirming disease specificity. Furthermore, neuronal toxicity appears to be most frequently observed in the primary progressive and relapsing remitting forms of MS, whereas a reduced albeit significant proportion of patients with a clinically-isolated syndrome exhibit this property.

Studies in mice lacking expression of the myelin-associated glycoprotein or the proteolipid protein have shown that axonal degeneration may occur in the absence of an immune response against these myelin antigens. This has been attributed to a deficient trophic effect from oligodendrocytes (the myelin-forming cells) in these animals, and supports the hypothesis that axonal loss occurs as a consequence of primary demyelination (15, 16). On the other hand, acute axonal damage has been observed in acute inflammatory lesions of human MS and in active borders of sub-acute lesions. A correlation was found between acute axonal damage and infiltration by macrophages and/or CD8+ T cells, but not with commonly recognized soluble mediators of demyelination such as TNF-alpha and inducible nitric oxide synthase, suggesting that in MS, axonal damage may not be proportional to demyelinating activity. Examination of normal-appearing white matter from MS patients also revealed that axonal density is reduced by 19-57%, depending to the clinical subtype, disease duration, sex and area of the CNS investigated (9, 17, 19). The latter observation also supports the contention that axonal damage may occur independently of myelin damage or even inflammation, and could perhaps represent a primary pathological process for certain forms of MS.

Regardless of the timing of its occurrence and role in perpetrating CNS tissue damage, the exact mechanisms of axonal degeneration (excluding Wallerian degeneration) in MS are not well understood. Soluble factors from the cerebrospinal fluid (CSF) of patients with PPMS or RRMS, has been shown to cause axonal damage and neuronal apoptosis *in vitro* (19). In addition, glutamate excitotoxicity has also been incriminated as a cause for axonal damage as the use of an AMPA/kainite glutamate receptor antagonist in the murine EAE model was shown to lead to a reduction in axonal damage, as well as an induction of oligodendrocyte survival (20, 21).

Antibody-mediated toxicity is suspected as a major factor in the pathogenesis of MS, primarily because studies of EAE, and more recently MS, have identified demyelinating antibodies. Anti-MOG antibodies have been shown to correlate with myelin vacuolization within MS plaques (22, 23). In fact, studies on the marmoset EAE model have

unequivocally demonstrated that these antibodies are capable of inducing demyelination, and furthermore, that they are probably required for development of a fully demyelinated MS-like lesion in this model (24, 25). However, a few recent studies have focused on identifying antibodies that bind to axonal and neuronal components in MS. Antibodies to the 68-kd light neurofilament subunit, an axonal cytoskeletal protein, were found to be elevated in the CSF of patients with progressive forms of MS (PPMS and SPMS), but not in CSF of patients with RRMS. A correlation was also found between the accumulation of these antibodies in progressive form of MS, and disability and/or disease duration (26). The screening of cell surface determinants with MS sera has shown a surprising IgG and IgM binding to a neuronal cell line in about 70 % of SPMS sera tested, but in only 25% of the RRMS sera tested. This binding ability was not more than of 50% when both SPMS and RRMS sera were tested with either differentiated or precursor-oligodendrocyte cell lines, raising the question of a specific neuro-axonal antibody-mediated damage among a specific subgroup of MS patients (14). However, none of the binding studies of the prior art described a direct association between antibody responses and neuronal damage or brain atrophy, and subsequent disability in MS.

In this regard, during development of the present invention, a statistically significant difference between neuronal toxicity in the PPMS subgroup was compared to the RRMS, CIS and controls, was described. These observations also provide an explanation of the clinical and MRI presentation of PPMS versus other forms of MS (PPMS is characterized by progressive and often severe disability, predominance of non-enhancing lesions, and prominent brain and spinal cord atrophy). While the mean expanded disability status scale (EDSS) score (Kurtzke, Neurology, 33:1444-52, 1983), and age were higher in the PPMS cohort compared to the RRMS cohort, the disease duration was not meaningfully different between these two groups. In addition, in the control group, which was age-matched with the PPMS group, evidence of neuronal toxicity was not observed. Thus, the observed differences do not represent a natural consequence of aging or disease duration, but rather, indicate that antibody-mediated neuronal damage is a primary process in PPMS.

In some embodiments, the methods of the invention involve comparing the extent of target cell death in the presence of the biological sample to a control. Generally, a control value can be an absolute value; a relative value; a value that has an upper and/or lower limit; a range of values; an average value; a median value, a mean value, or a value as compared to a particular control or baseline value. A control value can be based on an individual sample value, such as for example, a value obtained from a sample from the individual with

an autoimmune disease, but at an earlier point in time, or a value obtained from a sample from a patient with an autoimmune disease other than the individual being tested, or a "normal" individual, that is an individual not diagnosed with an autoimmune disease. The control value can be based on a large number of samples, such as from autoimmune patients or normal individuals or based on a pool of samples including or excluding the sample to be tested.

Both cell lysis (or necrosis) and apoptosis mechanisms are involved in antibody-mediated cytotoxicity (Cole et al., Clin. Sci. (Lond.) 104:455-66, 2003; Fishelson et al., J. Immunol. Methods 207:43-51, 1997; Mathas et al., Cancer Res. 60:7170-6, 2000; Nauta et al., Eur. J. Immunol. 32:783-92, 2002; Attali et al., Eur. J. Immunol. 34:3236-45, 2004). Various techniques known in the art can be used to measure extent of cell death. These techniques include various assays for measuring cell lysis or necrosis, live/dead cell staining assays, proliferation assays, and apoptosis assays. For example, the lactate dehydrogenase release assay, ^{51}Cr release assay, acid phosphatase release assay, glyceraldehyde-3-phosphate dehydrogenase release assay, and BrdU labeled DNA fragmentation ELISA can be used for measuring cell lysis or necrosis. Various strategies for live/dead cell staining can be employed, such as propidium iodide staining, trypan blue staining, neutral red uptake, and calcein-AM/EthD-1 cell staining. Other types of methods such as proliferation assays that determine the total number of viable cells and therefore indirectly reveal cell death can also be used. For example, reduction of resazurin dye, reduction of the tetrazolium compounds MTT or WST-1, and measurement of tritiated thymidine or non-radioactive BrdU incorporation in DNA could be used. Different types of apoptosis assays include but are not limited to tritiated thymidine or BrdU DNA fragmentation assays, the tunnel assay (deoxynucleotidyl transferase-mediated dUTP nick end labeling assay), assays for caspase activity, binding of fluorescent caspase inhibitors, annexin V binding assays, cytochrome C release assays, detection of the ratio of ATP/ADP assays, and changes in mitochondrial potential could also be used.

For example, *aCella –TOX* assay that is available from Cell Technology Inc., (Mountain View, CA) may be used to quantitate cytotoxicity by determining the release of Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) from dead or dying neuronal cells (Corey et al., J. Immunol. Methods, 207:43-51, 1997). In this assay, GAPDH catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (GAP) to 1,3-Bisphosphoglycerate (1,3-BPG) within the glycolytic pathway. Then, 1,3-BPG is dephosphorylated by the enzyme phosphoglycerate kinase (PGK) to produce ATP, which is

detected via luciferase/luciferin bioluminescence. GAPDH is a natural enzyme that is an abundant component of all known living cells. This distinguishes the assay from methods that require prelabelling of cells, transfection, transformation, or other methods of introducing proteins or other molecules into the target cells. Other enzyme release assays, such as the Lactate Dehydrogenase (LDH) release assay, can suffer from low sensitivity due to interference by serum or phenol red in the media. The *aCella-TOX* assay requires one step with no washing and can be performed in a few minutes.

aCella –TOX assay can be performed briefly as follows. Enzyme Assay Reagent is a frozen 4X liquid concentrate, and prior to use, the vial is thawed and kept on ice. To make a 2X working stock, dilute the 4X concentrate 1:2 with the Enzyme Assay Diluent. Then 3.5mL of Vial C (G3P) is added to each mL of 2X Enzyme Assay Reagent (from above). For detection, a 1X dilution of Detection Assay Diluent is made with deionized H_2O. Aliquot and freeze the unused portion of the solution of the Detection Reagent (Luicferase/Luciferin) frozen 50X concentrate. Just before adding the detection reagent to the sample, dilute the Detection Reagent 1:50 with Detection Assay Diluent B, and then add 50 µl to each sample, and read in a luminometer. It could be necessary to incubate the sample for 2-5 minutes in order to achieve maximum signal. The percentage of cytotoxicity is determined by the signal for the experimental sample minus that of the negative control - divided by the positive control with maximum G3PDH minus the negative control. ACella-TOX also can be used to measure both cytotoxicity and cell viability (or proliferation) in the same sample well. The cytotoxicity measurement is first made as described above. A lytic agent (5 mL of 0.2% NP-40/100µL sample) is then added and the total cell count is read. Viability is represented by the difference between the two measurements.

Cell lysis can also be measured using a Cellular DNA Fragmentation ELISA that is commercially available from Roche Diagnostic (Mannheim, Germany) and is a photometric ELISA for the detection of BrdU-labeled DNA fragments in cell lysates or in cell culture supernatants. It is a non-radioactive alternative to the tritiated thymidine release assay. BrdU-labeled DNA fragments are released from cells in the early stages of lysis or the late stages of apoptosis into the supernatant that is analyzed. The remaining cells are lyzed in order to release apoptotic DNA fragments located in the cytoplasm.

Cell proliferation assays can be performed by measurement of cellular 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction. Cell proliferation analysis can be performed as follow for adherent cells in 96-well plates. First, a solution of 5 mg/ml MTT is made in PBS and filter sterilized. Then, 5 hours before the

end of the incubation period, 20 µl of MTT solution is added to each well containing cells. The plate is incubated in a CO_2 incubator at 37°C for 5 hours. The media are removed with a needle and syringe, and 200 µl of DMSO is added to each well and the solution is pipetted up and down to dissolve crystals. The plate is put back into the 37°C incubator for 5 minutes. The plate is transferred to a plate reader and the absorbance measured at 550 nm. The results reflect the number of viable cells per well.

In a very similar fashion to the MTT assay, the tetrazolium compound, WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Roche), can be used to measure the number of viable cells. The compound's dehydrogenase activities are useful in the colorimetric determination of the bioreducibility of cells. Compared with the MTT assay, the WST-1 assay could be more sensitive, more convenient, and more accurate. To perform the WST-1 assay, cells (5×10^4) are seeded into wells of a 96-well microtiter plate and incubated overnight at 37°C. The next day, cells are washed with PBS, then human sera or other preparations are diluted at the described concentration in RPMI 1640, added to the wells and incubated for 90 min at 37°C. The cells are then gently washed with PBS and incubated 3 hours at 37°C with human complement (Sigma-Aldrich) diluted in RPMI at 1:100. The plate is incubated at 37°C in 5% CO_2 for 24 or 72 hours. The number of cells per well are determined by adding 10 µl of reagent WST-1 (Roche) to each well and incubating the plates at 37°C for 90-240 min. The absorbance of the formazan product is measured after shaking the plate for 1 min between 420-480 nm with a reference wavelength of more than 600 nm with an ELISA plate reader. Cell numbers calculated based on standard curves for each cell line. Assays are done in quadruplicate on two occasions.

Apoptosis can be assessed with the commercially available Apo-One ELISA available from Promega (Madison, WI) that measures the fluorescence of a substrate of caspase 3/7. Increased caspase 3/7 activity defines mechanistic toxicity that is indicative of apoptotic induction. Target cells, such as SK-N-SH neuroblastoma cells that can be purchased from the American Type Culture Collection (ATCC), are cultured in monolayers in a 96-well tissue culture plate (Corning Inc.) at 37°C in a 5% CO_2 humidified incubator overnight. Culture media consist of RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. The next day, cells are washed with PBS, then human sera or other preparations are diluted at the described concentration in RPMI 1640, added to the wells and incubated for 90 min at 37°C. The cells are then gently washed with PBS and incubated 3 hours at 37°C with human complement (Sigma-Aldrich) diluted in RPMI at 1:100. The

instructions of the vendor are followed in use of the Apo-One ELISA. Finally, the plate is incubated for 2 hours at RT with shaking, and the fluorescence of each well measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence plate reader.

To increase sensitivity of the assay, target cells may be synchronized in cell division cycles, or may be treated with nitric oxide, glutamate, tumor necrosis factor or other toxic factor(s) to increase neuronal cell fragility. The target cell population may be homogenized in terms of the level of expression of complement receptor proteins, for example, by sorting the target cells according to their level of expression of complement receptors.

The inventors contemplate repeating the observations disclosed herein, by testing the pathogenic properties of anti-neuronal antibodies found in sera from MS subjects, by using passive antibody transfer in the marmoset EAE system. In addition, the inventors contemplate that therapies targeting B-cell mediated mechanisms of disease will positively impact disability in patients with PPMS, and that this impact will correlate with changes in serum neuronal toxicity.

The SK-N-SH cell line (ATTC No. HTB-11) was used in the proof of concept studies described herein because of the recent demonstration that MS sera specifically binds to these neuronal cells, and because it is known that neurons are predisposed to complement attack (27). Although the absolute differences in neuronal mortality found between patients and controls were somewhat low, a clear increase in neuronal cell death in the presence of a combination of serum and complement was demonstrated. Nonetheless, the present invention is not limited to the use of SK-N-SH cells, as neuronal cells with similar properties (*e.g.*, preferential binding of sera from MS patients) are also suitable. These cells include but are not limited to primary neuronal cultures (*e.g.*, human, primate, rodent, etc.), SH-SY5Y neuroblastoma cells, and IMR-32 neuroblastoma cells. In addition the inventors contemplate that other types of CNS and PNS cells will also find use in the methods and kits described herein. These cells include for instance, primary oligodendrocyte cell lines or oligodendroglioma cells (TC 620, HOG, M03-13), Schwann cells, Schwannoma cells, astrocytes and culture derived lines (CRT), and glial or neuronal stem or precursor cells. Likewise, this type of approach is contemplated to also find use in the diagnosis and prognosis of other autoimmune diseases (*See,* Table 1) suspected of having a humoral pathogenic component.

The methods of the invention may also be used to identify individuals at risk of multiple sclerosis and/or to indicate pre-symptomatic development of multiple sclerosis.

Table 1. Exemplary Autoimmune Disease and Target Cell Combinations

Autoimmune Disease(s)	Target Cell(s)
Diabetes	Islet cells
Lupus	Kidney cells
Vasculitis	Endothelial cells
Rheumatoid arthritis	Synovial cells
Dermititis	Epithelial cells
Myositis	Myocytes, Myoblasts
Thyroiditis	Endocrine cells
Addison's	Adrenal cells
Anemia	Erythrocytes
Immune deficiency syndromes	T cells, B cells, NK cells, etc.
Thrombocytopenia	Megakaryocytes
Hepatitis	Hepatocytes
Inflammatory bowel diseases (Crohn's, colitis)	Epithelial cells of the intestine and stomach
Parkinson's disease	Basal ganglia and substantia nigra neurons
Alzheimer's disease	Temporal lobe and hippocampal neurons
ALS	Spinal cord motor neurons
Sensory neuropathy	Dorsal ganglia neurons

Kits

The invention provides kits for carrying out any of the methods described herein. For example, the invention provides kits comprising one or more components for measuring cell death mediated by complement and antibodies. Kits of the invention may comprise at least one target cell (such as a neuronal cell), and complement, and may further comprise instructions for carrying out a method described herein. Kits may also comprising reference samples useful for determining a control value. Kits may further comprise one or more reagents for measuring cell lysis, such as propidium iodide or trypan blue.

The kits may include more than one target cells, and the target cells may be in the form of a cell array. Thus, cytotoxicity to more than one cell type may be measured at the same time.

The instructions relating to the use of the kit for carrying out the invention generally describe how the contents of the kit are used to carry out the methods of the invention. Instructions may also include information as sample requirements (e.g., form, pre-assay processing, and size), steps necessary to measure cell lysis, and interpretation of results. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable. In certain embodiments, machine-readable instructions comprise software for a programmable digital computer for comparing the measured values obtained using the reagents included in the kit.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: U (units); N (normal); M (molar); mM (millimolar); µM (micromolar); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams); µg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers); nm (nanometers); °C (degrees Centigrade); FBS (fetal bovine serum); PBS (phosphate buffered saline); PI (propidium iodide); FACS (fluorescence activated cell sorter); and IFA (immunofluorescence analysis).

EXAMPLE 1

Assessment of Neuronal Toxicity Mediated by Serum from Subjects with Multiple Sclerosis

Subjects

Patients and healthy controls were recruited from the UCSF outpatient clinics, neurology wards and multiple sclerosis treatment trials with the authorization of the local research Institutional Review Board. Blood samples were obtained after patients signed information consent. Clotted sera were stored at -40°C until use. None of the patients or controls involved in the study was treated with any MS disease-modifying therapy or

steroids at the time of the blood draw. Healthy volunteers (HuC, n=31) were randomly chosen, consented and enrolled in the study as controls. All MS patients had clinically definite multiple sclerosis as defined by the Poser criteria (Poser et al., Ann Neurol, 13:227-231, 1983). Briefly, the Poser criteria define clinically definite MS as: 2 attacks and clinical evidence of 2 separate lesions, or 2 attacks and clinical evidence of one lesion and paraclinical evidence of a separate lesion. Laboratory supported definite MS is defined as: 2 attack, clinical or paraclinical evidence of one lesion, and CSF immunologic abnormalities, or 1 attack, clinical evidence of 2 separate lesions, and CSF abnormalities, or 1 attack, clinical evidence of one lesion and paraclinical evidence of a separate lesion, and CSF abnormalities. Clinically probable MS is defined as: 2 attacks and clinical evidence of one lesion, or 1 attack and clinical evidence of 2 separate lesions, or 1 attack, clinical evidence of one lesion and paraclinical evidence of a separate lesion. Lastly, laboratory-supported probable MS is defined as: 2 attacks and CSF abnormalities.

Patients were classified as either primary progressive (PPMS, n=27) or relapsing-remitting (RRMS, n=17) multiple sclerosis by clinical history. Clinically isolated syndrome (CIS, n=14) was defined by an acute or subacute episode suggestive of demyelination in a patient with no history of previous neurological symptoms. As shown in Table 2, clinical information was recorded including age, disease duration and current disability measured by the Expanded Disability Status Score (12).

Table 2. Characteristics of Study Subjects*

Subject Group	Number	Male/Female	Median Age (range)	Median EDSS	Median Disease Duration (range)
Healthy Controls	31	15/16	55.5 (28-69)	N/A	N/A
CIS Patients	14	4/10	35 (24-52)	1.0 (0-2.0)	0.75 (0.3-4.0)
RRMS Patients	17	4/13	45 (26-59)	1.5 (0-3.5)	4.0 (1-17.0)
PPMS Patients	27	11/16	53 (41-65)	5.0 (2.5-8.0)	6.0 (0.75-24.0)
Total Patients	58	19/39	47.5 (24-65)	3.0 (0-8.0)	4.0 (0.3-24.0)

* CIS = clinically isolated syndrome, RRMS = relapsing-remitting multiple sclerosis, PPMS = primary progressive multiple sclerosis, and N/A not applicable. Both the median age and median disease duration are shown in years.

SK-N-SH cell-death assay

SK-N-SH neuroblastoma cells were purchased from the American Type Culture Collection (ATCC) and grown in monolayers in a plastic culture flask (Corning Inc.) at 37°C in a 5% CO2 humidified incubator. Culture media consisted of RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. The cell death assay was adapted from Jensen and Schell (13). In short, about 200,000 SK-N-SH cells were plated into 6 wells plates or 24 wells plates, and incubated overnight at 37°C. The next day, cells were washed with PBS, then human sera or other preparations were diluted at the described concentration in RPMI 1640, added to the wells and incubated for 90 min at 37°C. The cells were then gently washed with PBS and incubated 3 hours at 37°C with human complement (Sigma-Aldrich) diluted in RPMI at 1:100. Cells were further analyzed with flow cytometry to assess the mortality rate.

Flow cytometry analysis

First supernatants were removed from SK-N-SH incubations and added to FACS tubes. Cells were trypsinized and washed with FACS buffer consisting of PBS with 2% FBS and 0.1% sodium axide, then added to the respective FACS tubes and centrifuged at 1100 rpm for 5 min at 4°C. The pellets were resuspended in 0.25ml of FACS buffer containing propidium iodide (PI) (Molecular Probes) at 2μg/ml and gently shaken. FACS tubes were kept on ice and analyzed by gating the selected cell population (10^4 cells) immediately after harvesting using an analytical flow cytometer (Becton-Dickinson FACScan). Dead cells were defined as the cells that were detected in the upper left quadrant (UL) or M2.

Pre-absorption of serum

About $2x10^7$ SK-N-SH cells were plated in a 6 wells plate (Falcon) overnight at 37°C. After washing with PBS, cells were incubated with serum from MS patient (dilution 1:150) for one hour at 37°C. After repeating this procedure two times with fresh SK-N-SH cells, supernatant was collected and incubated with new cells. Neuronal mortality was then analyzed via FACS, as described above.

Antibody purification by affinity chromatography

Serum from an MS patient was diluted 1:5, and IgG were purified using a Nab Protein A Spin Purification Kit (Pierce Biotechnology) following the manufacturer's recommendations. The purification process was repeated three times, each time with a new column. The eluted fraction was obtained from the first column and was neutralized with neutralization buffer consisting of 1M Tris-HCL (pH 9). The non-bound component was collected when the third column was used. Before use, elution fraction and non-bound component were purified and concentrated using a 50 μm centrifugal filter device (Millipore Corporation) following the manufacturer's recommendation.

Immunofluorescence (IFA)

SK-N-SH cells were plated (~20,000 to 30,000) on slide coverslip chambers (Nalge Nunc Int.) overnight at 37°C. After washing with PBS, cells were fixed with 100% ice-cold methanol at –20C for 10 min. Slides were air-dried, washed once with PBS then blocked with PBS-2% bovine serum albumin (BSA)-2% fetal bovine serum (FBS) for 30 min at RT. After washing, cells were incubated with MS serum (1:50), Eluted Igs from the Protein A purification assay from an MS patient (1:15) or non-bound (flow through) compound (1:15) obtained from same Protein A purification assay, were incubated with the blocked cells for 1 hour at RT. To detect bound antibodies, a fluorescein isothiocyanate (FITC)-conjugated secondary antibody to human IgG (1:100) (Sigma-Aldrich) was incubated with the cells for 1 hour at RT after three washes with PBS. For controls, cells were incubated with the secondary (FITC)-conjugated antibody only. Pictures were taken with an optical microscope (Nikon Eclipse E600) at 400X magnitude.

Statistical analysis

Statistical differences between groups were analyzed with ANOVA and Bonferroni's multiple comparison tests (Graph Pad Software). For each test, $P<0.05$ was considered significant.

MS serum induces neuron mortality in vitro in a dose-dependent manner

Sera from MS patients have been shown to bind with a high affinity the SK-N-SH neuroblastoma cell line (14). We used this system to assess the capacity of serum to induce neuronal toxicity by quantifying cell killing in the presence of complement, by measuring

cellular PI intake by flow cytometry (FACS). For each sample, 10,000 events were analyzed and the upper left quadrant was considered as positive for dead cells (Figure 1). The background mortality was measured in each experiment with a cell sample incubated only in the presence of complement without serum, and subtracted in each case.

One PPMS patient found to induce a more than 2 times neurons mortality was used to further control for specificity and quality control (Figure 2A). The serum from this patient was showed to induce neuronal mortality in a concentration-dependent manner, up to a 1:500 dilution. The inter-assay coefficient of variation was 15.95%, and the intra-assay coefficient of variation was 9.33%, indicating the robustness and reproducibility of this assay. A pre-requisite for pathogenicity is that antibodies must be capable of binding to exposed epitopes of the neurons. We verified by immunostaining of the SK-N-SH cell line with serum (Figure 2B).

Neuronal pathogenicity of serum in clinical multiple sclerosis subtypes.

The characteristics of patients used in the study are summarized in Table 2. The median age in the control group (55.5 years, range 28-69) was lower than the RRMS group (median 45, range 26-59) and the CIS group (median 35 years, range 24-52) but was matched with that of the PPMS group (median 53 years, range 41-65). As it is known that with age, the risk to develop humoral autoimmunity increases, we chose as a control a cohort that matched the greatest median age among the patient groups. As would be expected, the CIS group had a lower EDSS score (median 1, range 0-2) than the RRMS group (median 1.5, range 0-3.5) and the PPMS group (median 5, range 2.5-8).

Sera were incubated with SK-N-SH cells and analyzed as described above (Figure 3). None of the control sera induced neuron mortality rate above 4%. Fifty two percent (14/27) of PPMS, 41% (7/17) of the RRMS, and 27% (3/11) of the CIS patients showed increased mortality rate compared to the highest value measured for the control group. The neuronal mortality rate compared to the control group was found to be statistically higher for the PPMS group ($p<0.0001$) than for the RRMS group ($p=0.0044$) or the CIS group ($p=0.0046$).

It is noteworthy that individual sera devoid of neuron-killing properties were identified in all groups of patients. Thus, we analyzed subgroups of patients after stratification for pathogenicity to neurons. The threshold for pathogenicity (killing) was the value observed for the control sample with the highest cell mortality (horizontal line, Figure 3A).

Statistical analysis was done to assess any possible difference in disease characteristics comparing the sera that showed ability to kill neurons (represented above the line on Figure 3A) and those that did not (below the line). No statistical correlation was found when these data were matched with the disease duration, or EDSS. Normalization of the mean SK-N-SH mortality for the PPMS group and comparison with control group showed a nearly 4-fold increase of the mean cell mortality rate for the PPMS group (PPMS=100%), a 3-fold increase for the RRMS group (RRMS 81.8%) and a 2.4-fold increase for the CIS group (63.4%) compared to the group control (HuC=26.7%) (Figure 3B).

Suppression of serum-induced neuronal mortality by pre-absorption or IgG removal

To assess whether the antibodies present in the serum played a role in the capacity of serum to kill neurons, two different techniques were used. First, diluted serum from the control PPMS patient was pre-absorbed on SK-N-SH cells as described above. Supernatant was further used to assess the ability to induce neurons mortality. Pre-absorbed serum was diluted at concentrations similar to those used for the experiment showed on the Figure 2A. No effect was seen at any of the dilution used as shown on figure 4A (left panel) compared to the non pre-absorbed serum (control, middle panel). Then, serum IgG from the same patient were removed on protein A columns (3 passes), the non-bound component from the last column was further concentrated and assayed for its potential to induce neuronal death. As shown in Figure 4A (right panel) no pathogenic effect of the non-bound component was seen. To confirm the efficiency of the protein A purification, the IgG fraction from the first column was eluted and preserved. IFA of SK-N-SH cells demonstrated that this eluted IgG fraction clearly retained binding properties (Figure 4B), whereas the non-bound fraction used with the same dilution showed no or very minimal binding (Figure 4C).

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All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention, which are obvious to those skilled in the relevant fields, are intended to be within the scope of the following claims.

CLAIMS

We claim:

1. A method of monitoring progression of neurodegeneration mediated by autoimmunity in a subject, comprising:

comparing extent of cell death of target cells to a control,

wherein the extent of cell death is measured after the target cells have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and

wherein the target cells are a type of neuronal cells.

2. The method of Claim 1, wherein the neuronal cells are selected from the group consisting of primary human neuronal cells, IMR-32 cells, SK-N-AS cells, SK-N-FI cells, SK-N-DZ cells, SH-SY5Y cells, BE(2)-M17 cells, BE(2)-C cells, MC-IXC cells, SK-N-BE(s) cells, CHP-212 cells, and SK-N-SH cells.

3. The method of Claim 1, wherein the neuronal cells are SK-N-SH cells.

4. The method of Claim 1, wherein the extent of cell death is measured by a cell lysis assay selected from the group consisting of lactate dehydrogenase release assay, ^{51}Cr release assay, acid phosphatase release, glyceraldehyde-3-phosphate dehydrogenase release assay, and BrdU labeled DNA fragmentation ELISA.

5. The method of Claim 1, wherein the extent of cell death is measured by an assay which differentially stains live and dead cells, wherein the assay is selected from the group consisting of propidium iodide staining, trypan blue staining, neutral red uptake, and calcein-AM/EthD-1 cell staining.

6. The method of Claim 1, wherein the extent of cell death is measured by a proliferation assay selected from the group consisting of reduction of resazurin dye, reduction of a tetrazolium compound, and label incorporation in DNA.

7. The method of Claim 1, wherein the extent of cell death is measured by an apoptosis assay selected from the group consisting of labeled DNA fragmentation assay, tunnel assay, caspase activity assay, binding of fluorescent caspase inhibitor assay, annexin V binding assay, cytochrome C release assay, detection of the ratio of ATP/ADP assay, and changes in mitochondrial transmembrane potential assay.

8. The method of Claim 1, wherein the extent of cell death is measured with propidium iodide staining and flow cytometry.

9. The method of Claim 1, wherein the subject has multiple sclerosis.

10. The method of Claim 1, wherein the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease, and Lafora body disease.

11. The method of Claim 1, further comprising a step of correlating the extent of cell death with a clinical course of an autoimmune disease.

12. The method of Claim 11, wherein the extent of cell death is significantly elevated in relation to the control, said clinical course is selected from the group consisting of primary progressive multiple sclerosis, secondary progressive multiple sclerosis, relapsing progressive multiple sclerosis, relapsing remitting multiple sclerosis, neuromyelitis optica, pleomorphic xanthoastrocytoma, clinically isolated syndrome, optic neuritis, relapsing-remitting optic neuritis, transverse myelitis, relapsing remitting transverse myelitis, and Marburg's multiple sclerosis, wherein the control is a mean extent of cell death observed for the target cells contacted with biological samples from healthy subjects.

13. The method of Claim 1, wherein said biological sample is selected from the group consisting of whole blood, serum, plasma, cerebral spinal fluid, and urine.

14. The method of Claim 1, wherein said biological sample is serum.

15. A method of diagnosing neurodegeneration mediated by autoimmunity in a subject, comprising:

comparing extent of cell death of target cells to a control,

wherein the extent of cell death is measured after the target cells have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and

wherein the target cells are a type of neuronal cells.

16. The method of Claim 15, wherein the neuronal cells are selected from the group consisting of primary human neuronal cells, IMR-32 cells, SK-N-AS cells, SK-N-FI cells, SK-N-DZ cells, SH-SY5Y cells, BE(2)-M17 cells, BE(2)-C cells, MC-IXC cells, SK-N-BE(s) cells, CHP-212 cells, and SK-N-SH cells.

17. The method of Claim 15, wherein the neuronal cells are SK-N-SH cells.

18. The method of Claim 15, wherein the extent of cell death is measured by a cell lysis assay selected from the group consisting of lactate dehydrogenase release assay, ^{51}Cr release assay, acid phosphatase release, glyceraldehyde-3-phosphate dehydrogenase release assay, and BrdU labeled DNA fragmentation ELISA.

19. The method of Claim 15, wherein the extent of cell death is measured by an assay which differentially stains live and dead cells, wherein the assay is selected from the group consisting of propidium iodide staining, trypan blue staining, neutral red uptake, and calcein-AM/EthD-1 cell staining.

20. The method of Claim 15, wherein the extent of cell death is measured by a proliferation assay selected from the group consisting of reduction of resazurin dye, reduction of a tetrazolium compound, and label incorporation in DNA.

21. The method of Claim 15, wherein the extent of cell death is measured by an apoptosis assay selected from the group consisting of labeled DNA fragmentation assay, tunnel assay, caspase activity assay, binding of fluorescent caspase inhibitor assay, annexin

V binding assay, cytochrome C release assay, detection of the ratio of ATP/ADP assay, and changes in mitochondrial transmembrane potential assay.

22. The method of Claim 15, wherein the extent of cell death is measured with propidium iodide staining and flow cytometry.

23. The method of Claim 15, wherein the subject has multiple sclerosis.

24. The method of Claim 15, wherein the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease, and Lafora body disease.

25. The method of Claim 15, further comprising a step of correlating the extent of cell death with a clinical course of an autoimmune disease.

26. The method of Claim 25, wherein the extent of cell death is significantly elevated in relation to the control, said clinical course is selected from the group consisting of primary progressive multiple sclerosis, secondary progressive multiple sclerosis, relapsing progressive multiple sclerosis, relapsing remitting multiple sclerosis, neuromyelitis optica, pleomorphic xanthoastrocytoma, clinically isolated syndrome, optic neuritis, relapsing-remitting optic neuritis, transverse myelitis, relapsing remitting transverse myelitis, and Marburg's multiple sclerosis, wherein the control is a mean extent of cell death observed for the target cells contacted with biological samples from healthy subjects.

27. The method of Claim 15, wherein said biological sample is selected from the group consisting of whole blood, serum, plasma, cerebral spinal fluid, and urine.

28. The method of Claim 15, wherein said biological sample is serum.

29. A method of aiding diagnosis of multiple sclerosis in a subject suspected of having multiple sclerosis, comprising:

comparing extent of cell death of target cells to a control,

wherein the extent of cell death is measured after the target cell have been contacted with a biological sample comprising an antibody from the subject and complement under conditions suitable for affecting cell death of the target cells in the presence of an antibody against the target cells, and

wherein the target cells are a type of neuronal cells.

30. The method of Claim 29, wherein the neuronal cells are selected from the group consisting of primary human neuronal cells, IMR-32 cells, SK-N-AS cells, SK-N-FI cells, SK-N-DZ cells, SH-SY5Y cells, BE(2)-M17 cells, BE(2)-C cells, MC-IXC cells, SK-N-BE(s) cells, CHP-212 cells, and SK-N-SH cells.

31. The method of Claim 29, wherein the neuronal cells are SK-N-SH cells.

32. The method of Claim 29, wherein the extent of cell death is measured by a cell lysis assay selected from the group consisting of lactate dehydrogenase release assay, ^{51}Cr release assay, acid phosphatase release, glyceraldehyde-3-phosphate dehydrogenase release assay, and BrdU labeled DNA fragmentation ELISA.

33. The method of Claim 29, wherein the extent of cell death is measured by an assay which differentially stains live and dead cells, wherein the assay is selected from the group consisting of propidium iodide staining, trypan blue staining, neutral red uptake, and calcein-AM/EthD-1 cell staining.

34. The method of Claim 29, wherein the extent of cell death is measured by a proliferation assay selected from the group consisting of reduction of resazurin dye, reduction of a tetrazolium compound, and label incorporation in DNA.

35. The method of Claim 29, wherein the extent of cell death is measured by an apoptosis assay selected from the group consisting of labeled DNA fragmentation assay, tunnel assay, caspase activity assay, binding of fluorescent caspase inhibitor assay, annexin

V binding assay, cytochrome C release assay, detection of the ratio of ATP/ADP assay, and changes in mitochondrial transmembrane potential assay.

36. The method of Claim 29, wherein the extent of cell death is measured with propidium iodide staining and flow cytometry.

37. The method of Claim 29, further comprising correlating a significantly elevated extent of cell death in relation to the control to a clinical course selected from the group consisting of primary progressive multiple sclerosis, secondary progressive multiple sclerosis, relapsing progressive multiple sclerosis, relapsing remitting multiple sclerosis, neuromyelitis optica, pleomorphic xanthoastrocytoma, clinically isolated syndrome, optic neuritis, relapsing-remitting optic neuritis, transverse myelitis, relapsing remitting transverse myelitis, and Marburg's multiple sclerosis, wherein the control is a mean extent of cell death observed for the target cells contacted with biological samples from healthy subjects.

38. The method of Claim 29, wherein said biological sample is selected from the group consisting of whole blood, serum, plasma, cerebral spinal fluid, and urine.

39. The method of Claim 29, wherein said biological sample is serum.

40. A kit for monitoring progression of neurodegeneration mediated by autoimmunity in a subject, comprising:

a) target neuronal cells;

b) complement; and

c) instructions for carrying out the method of Claim 1.

41. The kit of Claim 40, wherein the subject has multiple sclerosis, and said target neuronal cells are SK-N-SH cells.

42. The kit of Claim 40, wherein the extent of cell death is measured by staining the target neuronal cells with propidium iodide and followed by flow cytometry.

43. The kit of Claim 40, wherein the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease and Lafora body disease.

44. The kit of Claim 40, wherein said instructions further comprise software configured for data analysis.

45. A kit for diagnosing neurodegeneration mediated by autoimmunity in a subject, comprising:

a) target neuronal cells;

b) complement; and

c) instructions for carrying out the method of Claim 15.

46. The kit of Claim 45, wherein the subject has multiple sclerosis, and said target neuronal cells are SK-N-SH cells.

47. The kit of Claim 45, wherein the extent of cell death is measured by staining the target neuronal cells with propidium iodide and followed by flow cytometry.

48. The kit of Claim 45, wherein the subject has a disease selected from the group consisting of Behcet's syndrome, frontotemporal dementia, Alzheimer's disease, Parkinson's disease, lupus cerebritis, progressive supranuclear palsy, amyotrophic lateral sclerosis, neurosarcoidosis, Sjogren's syndrome, Pick's Lewie body disease and Lafora body disease.

50. The kit of Claim 45, wherein said instructions further comprise software configured for data analysis.

51. A kit for aiding diagnosis of multiple sclerosis in a subject suspected of having multiple sclerosis, comprising:

a) target neuronal cells;

b) complement; and

c) instructions for carrying out the method of Claim 29.

52. The kit of Claim 51, wherein said target neuronal cells are SK-N-SH cells.

53. The kit of Claim 51, wherein the extent of cell death is measured by staining the target neuronal cells with propidium iodide and followed by flow cytometry.

54. The kit of Claim 51, wherein said instructions further comprise software configured for data analysis.

55. A method for screening a test compound, comprising:

a) contacting target neuronal cells with a biological sample comprising an antibody from a subject with autoimmunity and complement in the presence and absence of said test compound, under conditions suitable for affecting cell death of the target neuronal cells; and

b) measuring the extent of cell death of the target cells.

56. The method of Claim 55, further comprising step c) comparing the extent of cell death in the presence or absence of the test compound, wherein a decrease of the extent of cell death in the presence of the test compound compared to the extent of cell death in the absence of the test compound indicates that the test compound inhibits cytotoxicity.

57. The method of Claim 56, wherein the test compound is an antibody that inhibits the binding of the antibody in the biological sample to the target neuronal cells.

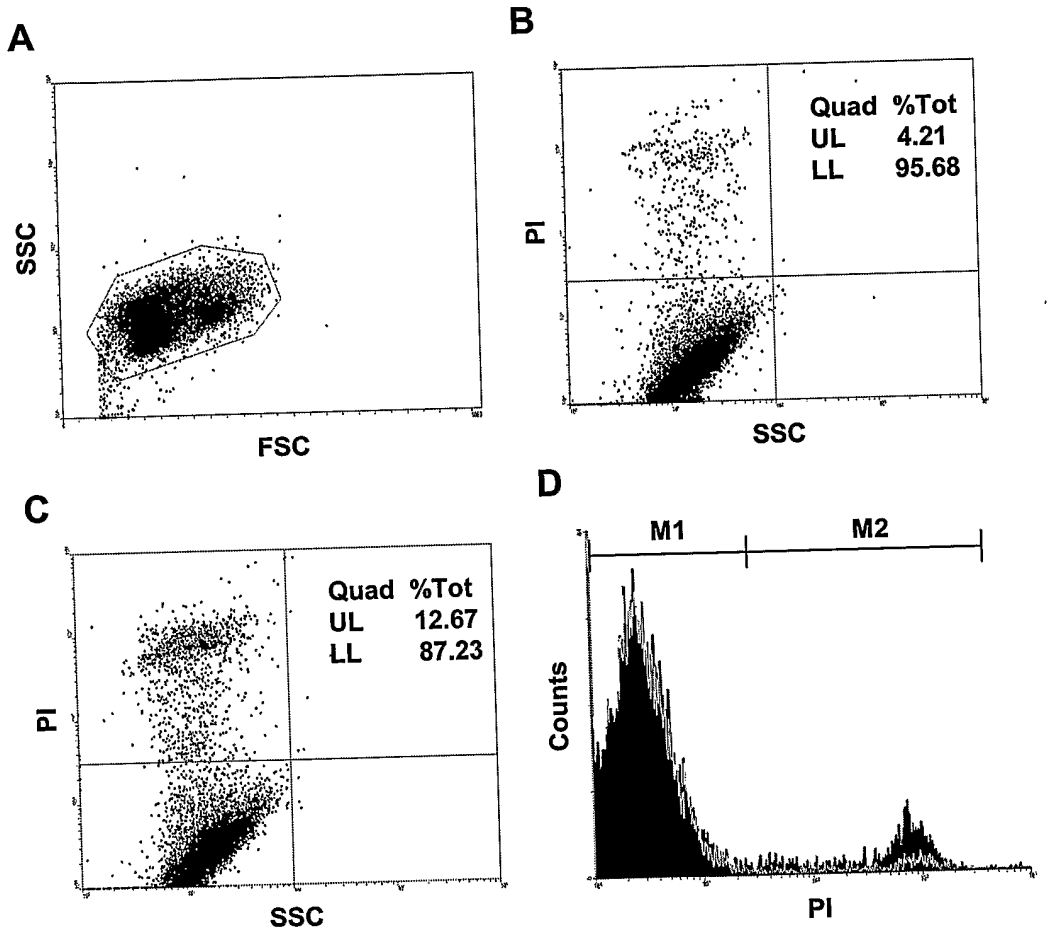
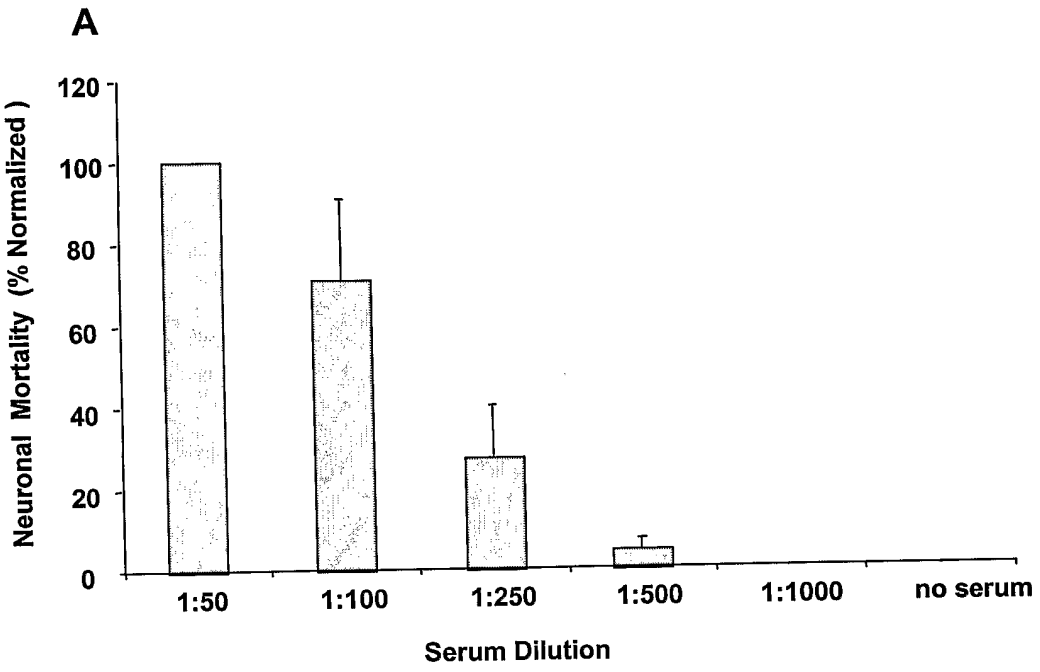

A
SSC
FSC
B
Quad %Tot
UL 4.21
LL 95.68
PI
SSC
C
Quad %Tot
UL 12.67
LL 87.23
PI
SSC
D
M1
M2
Counts
PI

FIG. 1


A
120
100
80
60
40
20
0
Neuronal Mortality (% Normalized)
1:50
1:100
1:250
1:500
1:1000
no serum
Serum Dilution

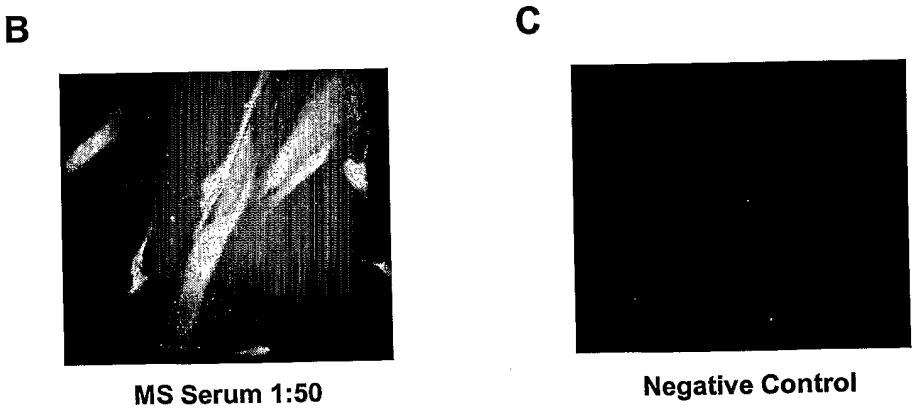

B
C
MS Serum 1:50
Negative Control

FIG. 2

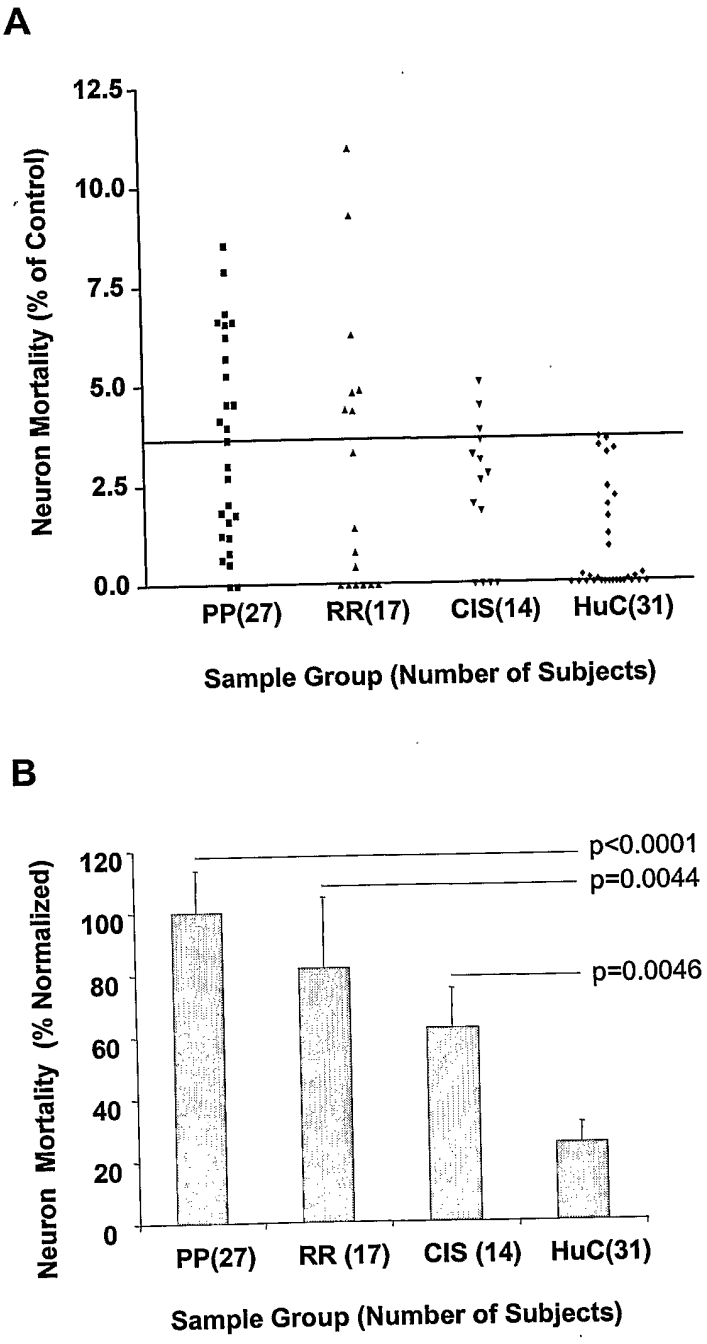

A
12.5
10.0
7.5
5.0
2.5
0.0
Neuron Mortality (% of Control)
PP(27)
RR(17)
CIS(14)
HuC(31)
Sample Group (Number of Subjects)
B
120
100
80
60
40
20
0
Neuron Mortality (% Normalized)
p<0.0001
p=0.0044
p=0.0046
PP(27)
RR (17)
CIS (14)
HuC(31)
Sample Group (Number of Subjects)

FIG. 3

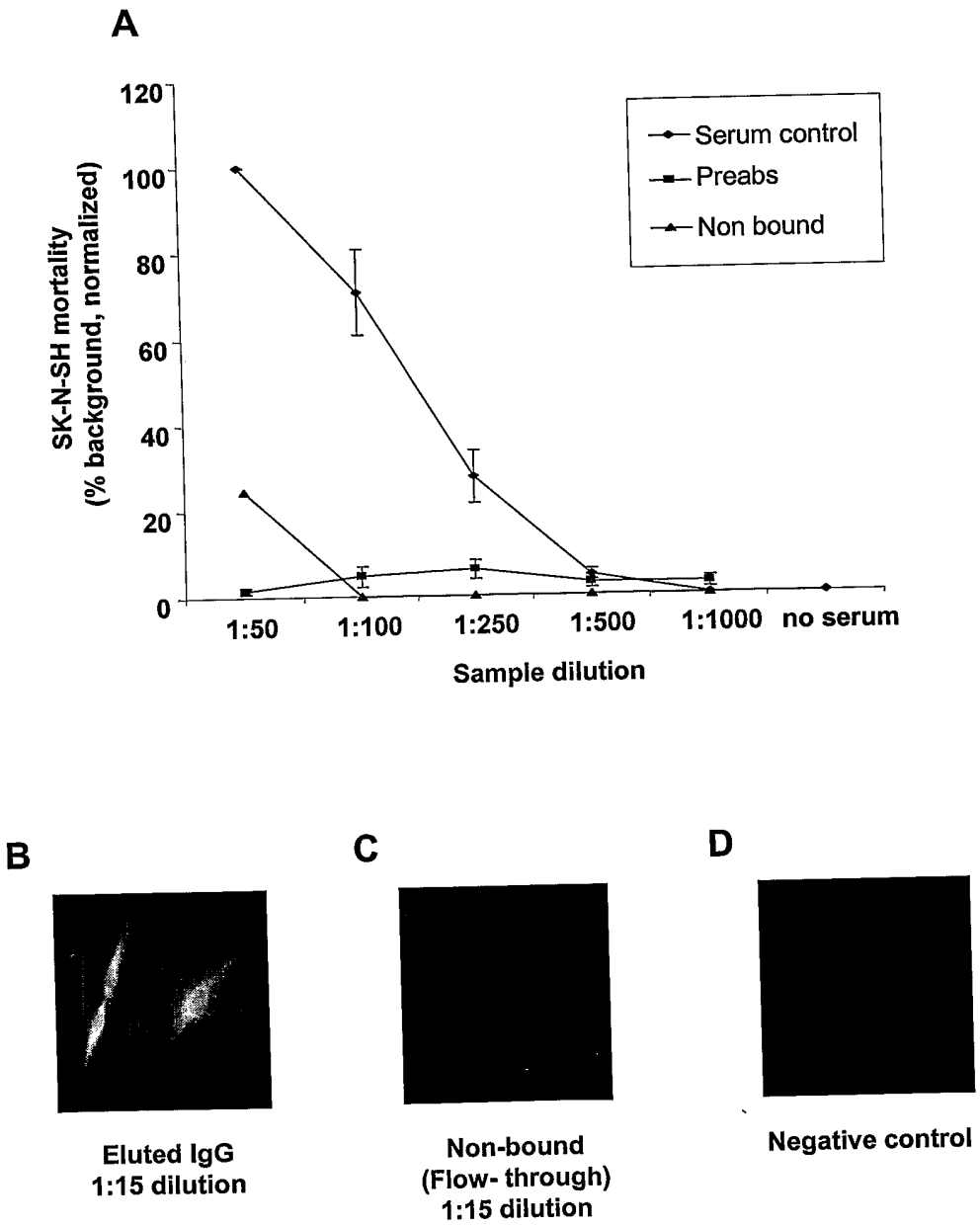

A
120
100
80
60
40
20
0
SK-N-SH mortality
(% background, normalized)
Serum control
Preabs
Non bound
1:50
1:100
1:250
1:500
1:1000
no serum
Sample dilution
B
Eluted IgG
1:15 dilution
C
Non-bound
(Flow- through)
1:15 dilution
D
Negative control

FIG. 4