USE OF POLY-GLU,TYR FOR NEUROPROTECTIVE THERAPY

FIELD OF THE INVENTION

The present invention relates to compositions for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury, disorder or disease of the nervous system (NS). In particular, the invention relates to compositions comprising poly-Glu,Tyr to protect central nervous system (CNS) cells from glutamate toxicity, to promote nerve regeneration or to prevent or inhibit neuronal degeneration caused by injury or disease of nerves within the CNS or peripheral nervous system (PNS) of a human subject. The compositions of the present invention may be administered alone or may be optionally administered in any desired combination.

ABBREVIATIONS: CFA: complete Freund's adjuvant; **CNS:** central nervous system; **MBP:** myelin basic protein; **MHC:** major histocompatibility complex; **NS:** nervous system; **PBS:** phosphate-buffered saline; **pEY:** poly-Glu,Tyr; **PNS:** peripheral nervous system; **Poly-Glu,Tyr:** copolymer poly-$Glu^{50}Tyr^{50}$, a random heterocopolymer of L-glutamic acid and L-tyrosine; **RGC:** retinal ganglion cells.

BACKGROUND OF THE INVENTION

The nervous system comprises the central (CNS) and the peripheral nervous system (PNS). The CNS is composed of the brain spinal cord and visual system; the PNS consists of all of the other neural elements, namely the nerves and ganglia outside of the brain and spinal cord.

Damage to the nervous system may result from a traumatic injury, such as penetrating trauma or blunt trauma, or a disease or disorder including, but not limited to, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), diabetic neuropathy, senile dementia, stroke and ischemia.

Maintenance of CNS integrity is a complex "balancing act" in which compromises are struck with the immune system. In most tissues, the immune system

plays an essential part in protection, repair, and healing. In the CNS, because of its unique immune privilege, immunological reactions are relatively limited. A growing body of evidence indicates that the failure of the mammalian CNS to achieve functional recovery after injury reflects an ineffective dialog between the damaged tissue and the immune system. For example, the restricted communication between the CNS and blood-borne macrophages affects the capacity of axotomized axons to regrow, while transplants of activated macrophages can promote CNS regrowth.

Activated T cells have been shown to enter the CNS parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a CNS antigen seem to persist there (Hickey et al, 1991). T cells reactive to antigens of CNS white matter, such as myelin basic protein (MBP), can induce the paralytic disease experimental autoimmune encephalomyelitis (EAE) within several days of their inoculation into naive recipient rats (Ben-Nun, 1981). Anti-MBP T cells may also be involved in the human disease multiple sclerosis (Ota et al, 1990). However, despite their pathogenic potential, anti-MBP T cell clones are present in the immune systems of healthy subjects (Pette et al, 1990). Activated T cells, which normally patrol the intact CNS, transiently accumulate at sites of CNS white matter lesions (Hirschberg et al, 1998).

A catastrophic consequence of CNS injury is that the primary damage is often compounded by the gradual secondary loss of adjacent neurons that apparently were undamaged, or only marginally damaged, by the initial injury (McIntosh, 1993). The primary lesion causes changes in extracellular ion concentrations, elevation of amounts of free radicals, release of neurotransmitters, depletion of growth factors, and local inflammation. These changes trigger a cascade of destructive events in the adjacent neurons that initially escaped the primary injury (Lynch et al, 1994). This secondary damage is mediated by activation of voltage-dependent or agonist-gated channels, ion leaks, activation of calcium-dependent enzymes such as proteases, lipases and nucleases, mitochondrial dysfunction and energy depletion, culminating in neuronal cell death. The widespread loss of neurons beyond the loss caused directly by the primary injury has been called "secondary degeneration."

One of the most common mediators which cause self-propagation of the diseases even when the primary risk factor is removed or attenuated is glutamate, an excitatory amino acid capable of displaying dual activity: it plays a pivotal role in normal CNS functioning as an essential neurotransmitter, but becomes toxic when its physiological levels are exceeded. Elevation of glutamate has been reported in many CNS disorders. In its role as an excitotoxic compound, glutamate is one of the most common mediators of toxicity in acute and chronic (including optic nerve degeneration in glaucoma) degenerative disorders (Pitt et al., 2000). Endogenous glutamate has been attributed to the brain damage occurring acutely after status epilepticus, cerebral ischemia or traumatic brain injury. Endogenous glutamate may also contribute to chronic neurodegeneration in such disorders as amyotrophic lateral sclerosis and Huntington's chorea.

Intensive research has been devoted to attenuating the cytotoxic effect of glutamate by the use of locally acting drugs, such as N-methyl-D-aspartate (NMDA)-receptor antagonists. In humans, such compounds have psychotropic and other side effects that make them unsuitable as therapeutic agents. In addition, conventional therapy of this type is often unsatisfactory, since neutralization of the glutamate toxic effect is likely to interfere with its physiological functioning as a ubiquitous CNS neurotransmitter. Because glutamate activity is essential for normal physiological functioning, yet is potentially devastating after acute injury or in chronic CNS disorders, any attempt to neutralize its harmful effect should avoid eliminating its essential activity at other sites in the body.

Another tragic consequence of CNS injury is that neurons in the mammalian CNS do not undergo spontaneous regeneration following an injury. Thus, a CNS injury causes permanent impairment of motor and sensory functions.

Spinal cord lesions, regardless of the severity of the injury, initially result in a complete functional paralysis known as spinal shock. Some spontaneous recovery from spinal shock may be observed, starting a few days after the injury and tapering off within three to four weeks. The less severe the insult, the better the functional outcome. The extent of recovery is a function of the amount of initially undamaged

tissue minus the loss due to secondary degeneration. Recovery from injury would be improved by neuroprotective treatment that could reduce secondary degeneration. For example, alleviation of the effect of glutamate is a frequent target of neuroprotective drug development. Among the drugs which are being developed for this purpose are N-methyl-D-aspartate (NMDA)-receptor or alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-receptor antagonists. These drugs will inevitably have severe side effects as they interfere with the functioning of NMDA and AMPA receptors, which are crucial for normal CNS activity. One of the most intensely studied NMDA-receptor antagonists is MK801, which provides effective neuroprotection but with severe side effects. In animal models of cerebral ischemia and traumatic brain injury, NMDA and AMPA receptor antagonists protect from acute brain damage and delayed behavioral deficits. Such compounds are undergoing testing in humans, but therapeutic efficacy has yet to be established. Other clinical conditions that may respond to drugs acting on glutamatergic transmission include epilepsy, amnesia, anxiety, hyperalgesia and psychosis (Meldrum, 2000).

In the laboratory of the present inventors, it has recently been discovered that activated T cells that recognize an antigen of the NS of the patient confer neuroprotection. Reference is made to US Applications Serial Nos. 09/218,277 and 09/314,161 and PCT Publication WO 99/60021, the entire contents of which is hereby incorporated herein by reference. More specifically, T cells reactive to MBP were shown to be neuroprotective in rat models of partially crushed optic nerve (see also Moalem et al, 1999) and of spinal cord injury (see also Hauben et al, 2000). Until recently, it had been thought that immune cells do not participate in NS repair. Furthermore, any immune activity in the context of CNS damage was traditionally considered detrimental for recovery. It was quite surprising to discover that NS-specific activated T cells could be used to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or PNS. The mechanism of action of such NS-specific T cells has yet to be discovered, but the massive accumulation of exogenously administered T cells at the site of CNS injury suggests that the presence of T cells at the site of injury plays a

prominent role in neuroprotection. It appears, however, that the accumulation, though a necessary condition, is not sufficient for the purpose, as T cells specific to the non-self antigen ovalbumin also accumulate at the site, but have no neuroprotective effect (Hirschberg et al, 1998).

In addition to the NS-specific activated T cells, the above-referenced US applications and PCT publication WO 99/60021 disclose that therapy for amelioration of effects of injury or disease of NS can be carried out also with a natural or synthetic NS-specific antigen such as MAG, S-100, β-amyloid, Thy-1, P0, P2, a neurotransmitter receptor, and preferably human MBP, human proteolipid protein (PLP), and human oligodendrocyte glycoprotein (MOG), or with a peptide derived from an NS-specific antigen such as a peptide comprising amino acids 51-70 of MBP or amino acids 35-55 of MOG.

More recently, it has been discovered in the laboratory of the present inventors that a high molecular weight synthetic basic random copolymer consisting of L-Ala, L-Glu, L-Lys and L-Tyr residues with an average molar fraction of 0.141, 0.427, 0.095 and 0.338, designated Copolymer 1 or Cop 1 and being the active ingredient of COPAXONE® (Teva Pharmaceuticals Ltd., Israel), a medicament for the treatment of multiple sclerosis, is able to prevent or inhibit neuronal degeneration, or to promote nerve regeneration, in the CNS or PNS, as well as to protect CNS cells from glutamate toxicity. Reference is made to copending US Applications Serial Nos. 09/487,793, 09/620,216, and 09/765,644, and PCT Publications WO 01/52878 and WO 01/93893, the entire contents of which is hereby incorporated herein by reference. More specifically, Cop 1-specific activated T cells were shown to accumulate in both injured and non-injured neuronal tissues and to be protective in the injured optic nerve against the destructive effect of secondary degeneration, and immunization with Cop 1 was shown to protect against glutamate toxicity.

Oral administration of autoantigen in order to obtain "oral tolerance" has been disclosed for the treatment of various autoimmune diseases. For example, EP 359 783 discloses the oral administration of MBP for the treatment of multiple sclerosis, and PCT International Publications WO 91/12816, WO 91/08760 and WO 92/06704

disclose the treatment of other autoimmune diseases using the oral tolerance method with a variety of autoantigens. Treatment of multiple sclerosis by ingestion or inhalation of Copolymer 1, to achieve suppression of the autoimmune T cell response to myelin antigens, has been disclosed in PCT publication WO 98/30227.

The copolymer poly-$Glu^{50}Tyr^{50}$, formerly often termed polyGT and hereinafter also called pEY, is a random heterocopolymer of L-glutamic acid and L-tyrosine, with an average length of 100 amino acids and a capacity to elicit strong immune response in certain mouse strains (Vidovic et al., 1985; Vidovic and Matzinger, 1988). More than 20 years ago, it was shown that several inbred as well as congenic resistant strains of mice, which fail to respond to pEY, developed specific plaque-forming cell (PFC) responses when stimulated by pEY complexed to an immunogenic carrier such as methylated bovine serum albumin (MBSA); pre-immunization with pEY had a tolerogenic effect on the response to pEY-MBSA in some mouse strains and this tolerance could be transferred to normal, syngeneic recipients by spleen cells or thymocytes of pEY-primed animals (Debre et al., 1975). More recently, pEY was shown to activate murine epidermal V gamma 5/V delta 1-TCR(+) T cell lines (Seo et al., 2001). None of these publications describes or suggests any useful biological activity of pEY and, particularly, not the use of pEY for neuroprotection.

Citation or identification of any reference in this section or any other part of this application shall not be construed as an admission that such reference is available as prior art to the invention.

SUMMARY OF THE INVENTION

It has now been found, in accordance with the present invention, that poly-Glu,Tyr can protect nerve cells from glutamate toxicity and from undergoing secondary degeneration following spinal cord contusion. The spontaneous appearance of T-cells specific to MBP and T-cells specific to poly-Glu,Tyr was examined in rats after spinal cord contusion. In addition, active immunization with

poly-Glu,Tyr was used to attenuate neuronal degeneration induced by glutamate toxicity or by mechanical injury to the spinal cord.

The present invention thus relates, in one aspect, to the use of poly-Glu,Tyr for the preparation of a pharmaceutical composition which is useful for neuroprotection, namely for preventing or inhibiting neuronal degeneration, or for promoting nerve regeneration, in the CNS or PNS, particularly for treating an injury, disorder or disease of the CNS or PNS that results in, or is accompanied by, axonal damage.

In a further embodiment of the invention, the pharmaceutical compositions are useful for protecting CNS or PNS cells from glutamate toxicity, particularly for treatment of an injury, disorder or disease of the CNS or PNS that is caused or exacerbated by glutamate toxicity.

In one embodiment, the injury, disorder or disease comprises spinal cord injury, blunt trauma, penetrating trauma, brain coup or contrecoup, hemorrhagic stroke, or ischemic stroke. In one preferred embodiment, the injury is spinal cord injury.

In another embodiment, the injury, disorder or disease comprises diabetic neuropathy, senile dementia, Alzheimer's disease, Parkinson's disease, facial nerve (Bell's) palsy, Huntington's chorea, amyotrophic lateral sclerosis (ALS), vitamin deficiency, epilepsy, amnesia, anxiety, hyperalgesia, psychosis, seizures, oxidative stress, or opiate tolerance and dependence and other disorders and diseases as described hereinafter.

In a further embodiment, the injury, disorder or disease is associated with the eye and includes optic neuropathy, age-related macular degeneration, a retinal disorder such as retinal degeneration or disease associated with abnormally elevated intraocular pressure such as glaucoma. In one preferred embodiment, the disorder or disease is glaucoma.

In still another embodiment, the injury, disorder or disease is an autoimmune disease.

An "an effective amount" is defined herein as an amount which is effective to ameliorate the effects of an injury, disorder or disease of the CNS or PNS.

As used herein, the term "neuroprotection" refers to the prevention or inhibition of degenerative effects of an injury, disorder or disease in the CNS or PNS, including protection from the secondary neurodegenerative effects which persist even when the primary risk factor is removed or attenuated. This includes protection of both white matter and gray matter.

Furthermore, as poly-Glu,Tyr protects from glutamate toxicity, it must also have a regulatory activity, such as by creating regulatory cells or regulatory substances. In view of this regulatory activity, the poly-Glu,Tyr vaccination is expected also to protect white matter and gray matter from damage caused by oxidative stress and other sources of damage to neural cells. In addition, because of this regulatory activity, the present invention can also be used to protect neural cells from autoimmune diseases.

The pharmaceutical compositions of the present invention comprise a therapeutically effective amount of poly-Glu,Tyr to inhibit or ameliorate the effects of an injury, disorder or disease of the CNS or PNS.

Additionally, oral administration of poly-Glu,Tyr may be effective for neuroprotection after priming with poly-Glu,Tyr administered in adjuvant. Thus, oral poly-Glu,Tyr can be used to boost the activity of the T cells, subsequent to primary activation of such poly-Glu,Tyr, preferably in adjuvant, to build up a critical T cell response immediately after injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the results of proliferation assay of splenocytes in response to different antigens: ovalbumin (Ova), copolymer 1(Cop 1), myelin basic protein (MBP), the MBP peptide p87-99, poly-Glu,Tyr (pEY) and concanavalin A (Con A). The assay was carried out in splenocytes isolated from SPD rats 8-10 days after the rats were subjected to spinal cord contusion. The index was determined in comparison to proliferation of splenocytes in medium not containing any antigen (SI=1).

Fig. 2 is a graph showing how immunization with pEY attenuates significantly retinal ganglion cells (RGCs) death induced by glutamate. The number of labeled (surviving) RGC/mm^2 in retinas excised from C57BL/6J mice who had been immunized with an emulsion of pEY in complete Freund's adjuvant (CFA- pEY) or with PBS in CFA (CFA-PBS), 7 days prior to intravitreal glutamate injection, and 7 days later was counted. Bars represent mean ± sem of percentage of RGC death compared to the naïve retina.

Fig. 3 depicts the effects of pEY/CFA immunization on the recovery of rats from spinal cord contusion. The graph presents the mean ± sd of hindlimbs motor activity scores in open field (BBB test as described in Example 3.1 hereinafter) with time after spinal cord injury in two groups of SPD rats immunized with pEY/CFA (squares) or CFA-PBS (control; triangles) immediately after spinal cord injury.

Fig. 4 depicts the effects of adoptive transfer of splenocytes activated with pEY on spinal cord injury recovery. The graph presents the mean ± sd of the hindlimb motor activity scores in open field with time after spinal cord injury in two groups of SPD rats injected intraperitoneally with CFA-pEY-activated T cells (SPc+pEY; squares) or CFA-PBS-treated T cells (control; triangles) immediately after spinal cord injury.

DETAILED DESCRIPTION OF THE INVENTION

The compositions of the invention comprising poly-Glu,Tyr may be used to promote nerve regeneration or to prevent or inhibit secondary degeneration which may otherwise follow primary NS injury, e.g., spinal cord injury, blunt trauma such as those caused by participation in dangerous sports, penetrating trauma such as gunshot wounds, brain coup or contrecoup, hemorrhagic stroke, ischemic stroke, cerebral ischemia, or damages caused by surgery such as tumor excision.

In addition, such compositions may be used to ameliorate the effects of disease that result in a degenerative process, e.g., degeneration occurring in either gray or white matter (or both) as a result of various diseases or disorders, including, without limitation, an injury, disorder or disease selected from a senile dementia including

Alzheimer's disease, Parkinsonian syndrome including Parkinson's disease, facial nerve (Bell's) palsy, Huntington's chorea, a motor neuron disease including amyotrophic lateral sclerosis, a prion disease including Creutzfeldt-Jakob disease, Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, status epilepticus, carpal tunnel syndrome, intervertebral disc herniation, vitamin deficiency, epilepsy, amnesia, anxiety, hyperalgesia, psychosis, seizures, oxidative stress, opiate tolerance and dependence, an autoimmune disease, or a peripheral neuropathy associated with a disease such as amyloid polyneuropathy, diabetic neuropathy, uremic neuropathy, porphyric polyneuropathy, hypoglycemia, Sjogren-Larsson syndrome, acute sensory neuropathy, chronic ataxic neuropathy, biliary cirrhosis, primary amyloidosis, obstructive lung diseases, acromegaly, malabsorption syndromes, polycythemia vera, IgA and IgG gammapathies, complications of various drugs such as nitrofurantoin, metronidazole, isoniazid and toxins such as alcohol or organophosphates, Charcot-Marie-Tooth disease, ataxia telangiectasia, Friedreich's ataxia, adrenomyeloneuropathy, giant axonal neuropathy, Refsum's disease, Fabry's disease, or lipoproteinemia.

In addition, in light of the findings with respect to the glutamate protective aspect of the present invention, other clinical conditions that may be treated in accordance with the present invention include epilepsy, amnesia, anxiety, hyperalgesia, psychosis, seizures, abnormally elevated intraocular pressure e.g. glaucoma, oxidative stress, and opiate tolerance and dependence. In addition, the glutamate protective aspect of the present invention, i.e., treating injury or disease caused or exacerbated by glutamate toxicity, can include post-operative treatments such as for tumor removal from the CNS and other forms of surgery on the CNS.

In view of the fact that poly-Glu,Tyr immunization has been surprisingly found useful in protecting against glutamate toxicity, it is expected that poly-Glu,Tyr treatment in accordance with the present invention will be effective in the treatment of the above listed conditions not only in a late phase when myelin is being affected, but also in the early stages in which the neurons are being attacked by factors which cause an elevation in glutamate levels to toxic levels. Thus, the present invention is

useful for any indication, i.e., chronic or acute neurodegeneration, which is caused or exacerbated by an elevation in glutamate levels, including the early stages of ischemic stroke, Alzheimer's disease, etc.

In a preferred embodiment, the immunization composition comprising poly-Glu,Tyr of the present invention is used to treat diseases or disorders where promotion of nerve regeneration or prevention or inhibition of secondary neural degeneration is indicated.

In a preferred embodiment, the present invention contemplates the use of poly-Glu,Tyr administered in adjuvants. Oral administration of poly-Glu,Tyr for neuroprotection, if possible, is contemplated always subsequent to primary activation with poly-Glu,Tyr, preferably in adjuvant. Thus, oral poly-Glu,Tyr can be used to boost the activity of the T cells subsequent to primary activation with poly-Glu,Tyr.

Poly-Glu,Tyr may also be used to ameliorate the degenerative process caused by neoplasms, without using immunotherapy processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

The following exemplification of carriers, modes of administration, dosage forms, etc., are listed as known possibilities from which the carriers, modes of administration, dosage forms, etc., may be selected for use with the present invention. Those of ordinary skill in the art will understand, however, that any given formulation and mode of administration selected should first be tested to determine that it achieves the desired results. Thus, for example, when the active principle is poly-Glu,Tyr, the particular formulation and mode of administration must permit the active principle to act as a vaccine so as to raise T cells activated thereagainst *in vivo*. If such an immune response is not obtained, then that particular formulation and mode of administration should not be used in accordance with the present invention.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. The carriers in the pharmaceutical composition may comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatin, starch, lactose or lactose monochydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art. Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration, the compositions may take

the form of tablets or lozenges formulated in conventional manner. When poly-Glu,Tyr is introduced orally, it may be mixed with other food forms and consumed in solid, semi-solid, suspension, or emulsion form; and it may be mixed with pharmaceutically acceptable carriers, including water, suspending agents, emulsifying agents, flavor enhancers, and the like. In one embodiment, the oral composition is enterically-coated. Use of enteric coatings is well known in the art.

The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen free water, before use.

The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. Poly-Glu,Tyr may also be administered nasally in certain of the above-mentioned forms by inhalation or nose drops. Furthermore, oral inhalation may be employed to deliver poly-Glu,Tyr to the mucosal linings of the trachea and bronchial passages.

In a preferred embodiment, compositions comprising poly-Glu,Tyr are formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

Pharmaceutical compositions comprising poly-Glu,Tyr may optionally be administered with an adjuvant in the usual manner for immunization. Non-limiting examples of such adjuvants include alum and incomplete Freund's adjuvant. Metabolizable lipid emulsions, such as Intralipid or Lipofundin may also be used as vehicles for the poly-Glu,Tyr therapy in the manner disclosed in WO 97/02016, the entire contents of which being hereby incorporated herein by reference. While these materials are known to cause a TH1 to TH2 cytokine shift, there is no reason to believe that TH2 cytokines will not be operable, and perhaps even preferable, for the purpose of the present invention.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

In a preferred embodiment, the pharmaceutical compositions of the invention are administered to a mammal, preferably a human, shortly after injury or detection of a degenerative lesion in the NS.

In one embodiment, the compositions of the invention are administered in combination with one or more of the following: (a) mononuclear phagocytes, preferably cultured monocytes (as described in WO 97/09985, WO 98/41220, US 5,800,812, US 6,117,424 and US 6,267,955, which are incorporated herein by

reference in their entirety), that have been stimulated to enhance their capacity to promote neuronal regeneration; and (b) a neurotrophic factor such as acidic fibroblast growth factor;

In another embodiment, mononuclear phagocyte cells according to WO 97/09985, WO 98/41220, US 5,800,812, US 6,117,424 and US 6,267,955, are injected into the site of injury or lesion within the CNS, either concurrently, prior to, or following parenteral administration of poly-Glu,Tyr.

In another embodiment, poly-Glu,Tyr may be administered as a single dose or may be repeated, preferably at 2 week intervals, and then at successively longer intervals once a month, once a quarter, once every six months, etc. The course of treatment may last several months, several years or occasionally also through the life-time of the individual, depending on the condition or disease which is being treated. In the case of a CNS injury, the treatment may range between several days to months or even years, until the condition has stabilized and there is no or only a limited risk of development of secondary degeneration. In chronic human disease or Parkinson's disease, the therapeutic treatment in accordance with the invention may be for life.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g., gender, weight, etc.) of the individual, as well as on various other factors, e.g., whether the individual is taking other drugs, etc.

The following examples illustrate certain features of the present invention but are not intended to limit the scope of the present invention.

EXAMPLES

Materials and Methods

Animals. All animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee (IACUC). Mice of the C57BL/6J strain, aged 8–13 weeks, and adult male SPD rats aged 8–12 weeks were supplied by the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, Israel)

and housed in light- and temperature-controlled rooms. The rats were matched for age and size in each experiment. Prior to the experiments, animals were anesthetized by intraperitoneal administration of ketamine 80 mg/kg and xylazine 16 mg/kg.

Antigens. MBP from the spinal cords of guinea pigs and ovalbumin (OVA), poly-Glu,Tyr and Con-A were purchased from Sigma (St. Louis, MO). Cop 1 was purchased from Teva Pharmaceuticals (Petah Tikva, Israel). The MBP p87-99 peptide was synthesized at the Weizmann Institute of Science (Rehovot, Israel).

Immunization. Mice or rats were immunized with 100 μg of poly-Glu,Tyr emulsified with an equal volume of CFA containing 0.5 mg/ml *Mycobacterium tuberculosis*. The emulsion (total volume 0.1 ml) was injected subcutaneously at one site in the flank in the mice and in the upper back in the rats. Control mice and rats were injected with PBS in CFA (Difco, Detroit, Michigan, USA).

Glutamate injection. The right eye of the anesthetized mouse or rat was punctured with a 27-gauge needle in the upper part of the sclera, and a 10-μl Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Mice were injected with a total volume of 1 μl (200 nmole) of L-glutamate dissolved in saline.

T Cell Lines. T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens (Ben-Nun et al, 1981). The antigen was dissolved in PBS (1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, MI) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and their draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 μg/ml) in stimulation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), non-essential amino acids (1

ml/100 ml), and autologous serum 1% (volume/volume). After incubation for 72 hours at 37°C, 98% relative humidity and 10% CO_2, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (FCS) (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A (ConA)-stimulated spleen cells. Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 μg/ml) in the presence of irradiated (2000 rad) thymus cells (10^7 cells/ml) in stimulation medium. The T cell lines were expanded by repeated stimulation and propagation (Ben-Nun et al, 1982).

Crush Injury of Optic Nerve: (a) The optic nerve was subjected to crush injury. Briefly, rats were deeply anesthetized by intraperitoneal (i.p.) injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA). Using a binocular operating microscope, lateral canthotomy was performed in the right eye, and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a crush injury 1–2 mm from the eye. Mild and severe crush injuries were inflicted for short-term trials (two weeks), as this time period was shown to be optimal for demonstrating secondary degeneration and its response to treatment (Yoles, 1998). The uninjured contralateral nerve was left undisturbed; ***(b)*** Mice or rats were anesthetized and subjected to graded crush injury in the intraorbital portion of the optic nerve, 1–2 mm from the eyeball. With the aid of a binocular operating microscope, the conjunctiva was incised and the optic nerve exposed. Using cross-action calibrated forceps and taking special care not to interfere with the blood supply, the nerve was crushed for 2 s (mice) or 30 s (rats).

Measurement of Secondary Degeneration in the Rat following Optic Nerve Crush, by Retrograde Labeling of RGCs. Secondary degeneration of the optic nerve axons

and their attached RGCs was measured by post-injury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the lesion site, two weeks after crush injury. Because only axons that are intact can transport the dye back to their cell bodies, application of the dye distally to the lesion site after two weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach enabled differentiation between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, because only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled RGCs reliably reflects the number of still-functioning neurons. Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Five days after dye application the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy.

Labeling of retinal ganglion cells (RCGs) in mice. RCGs were labeled 72 hours before the end of the experiment. Mice were anesthetized and placed in a stereotactic device. The skull was exposed and kept dry and clean. The bregma was identified and marked. The designated point of injection was at a depth of 2 mm from the brain surface, 2.92 mm behind the bregma in the anteroposterior axis and 0.5 mm lateral to the midline. A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was then applied (1 μl, at a rate of 0.5 μl/min in each hemisphere) using a Hamilton syringe, and the skin over the wound was sutured.

Assessment of RGC survival in mice. Mice were given a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in paraformaldehyde (4% in PBS). Labeled cells from 4–6 selected fields of identical size (0.7 mm^2) were counted. The selected fields were located at approximately the same distance from the optic disk (0.3 mm) to overcome the variation in RGC density as a function of distance from the optic disk. Fields were counted under the fluorescence microscope (magnification ×800) by observers blinded to the treatment received by the mouse. The average number of RGCs per field in each retina was calculated.

Assessment of RGC survival in rats. Survival of RGCs in rats was measured after post-injury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the optic nerve head. Labeling and measurement were carried out as follows: the optic nerve was exposed without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the optic nerve head and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the formed axotomy. Five days after dye application the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy. In the IOP experimental animals, the ganglion cells were labeled by retrograde transport dextran tetramethylrhodamine (DTMR) (Molecular Probes, OR). Crystals of 3000 MW DTMR were applied to the cut end of the optic nerve about 2 to 3 mm from the globe. Twenty-four hours later the retinas were whole-mounted and labeled ganglion cells in 8 regions, 2 in each quadrant, (0.66 to 1.103mm from the edge of the optic disk) were counted with 400x magnification.

Histological analysis. Seven days after glutamate or saline injection the mice were killed by injection of a lethal dose of pentobarbitone (170 mg/kg) and their eyes were

removed and fixed in formaldehyde (4% in PBS) for 48 h at 4 °C. Sections (10 μm thick) were embedded in paraffin and stained with hematoxylin and eosin (H&E).

Generation of ocular hypertension in rats/Elevation of intraocular pressure in rats. Male Lewis rats were anesthetized with a mixture of ketamine (15 mg/kg), acepromazine (1.5 mg/kg), and xylazine (0.3 mg/kg). An increase in intraocular pressure (IOP) was achieved by laser photocoagulation of the limbal and episcleral veins. Rats received 2 laser treatments, 1 week apart, with a blue-green argon laser (1 watt for 0.2 s, delivering a total of 130–150 spots of 50 μm in the 2 treatments; Coherent, Palo Alto, CA). IOP was measured once a week using TONO-PEN (Mentor, Norwell, MA), after injecting the rats intramuscularly with the veterinary tranquilizer acepromazine 3.0 mg/kg and applying procaine 0.5% topically on the eyes to anesthetize the cornea.

EXAMPLE 1. Physiological T-cell repertoire in contused animals.

SPD rats were anesthetized and their spinal cords were exposed by laminectomy at the level of T8. One hour after induction of anesthesia, a 10-g rod was dropped onto the laminectomized cord from a height of 50mm, using the NYU impactor (Basso et al., 1995 and 1996).

Rats were killed 8-10 days after spinal cord contusion and their spleens were excised and pressed trough a fine wire mesh. The washed cells ($2x10^6$/ml) were cultured in triplicate in flat-bottomed microtiter wells in 0.2 ml proliferation medium containing DMEM supplemented with L-glutamine (2 mM), 2-mercaptoethanol ($5x10^{-5}$ M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), non-essential amino acids, and autologous rat serum 1% (vol/vol) with the antigen (15 μg/ml) or Con A (1.25 μg/ml), and irradiated thymocytes (2000 rad, $2x10^6$ cells/ml). The proliferative response to different antigens namely Ova, Cop 1, MBP, 87-99, poly-Glu,Tyr and Con A, was determined by measuring the incorporation of [^{3}H]thymidine (1 μCi/well), which was added for the last 16h of a 72h culture. The splenocyte proliferation index (SI) was determined as compared to

the proliferation of the splenocytes in medium with no antigen (SI=1 indicates no proliferation response to the antigen above the proliferation without any antigen). This parameter is indicative of the physiological T-cell repertoire in contused animals. Con-A is the positive control. The results in Fig. 1 indicate that in the spinally contused rats there is a high occurrence of T-cells reactive to poly-Glu,Tyr, more than to Cop-1 or to MBP.

EXAMPLE 2. Protection of optic nerve fibers from glutamate toxicity

In order to find out whether poly-Glu,Tyr can impart a more general neuroprotection from hostile environmental conditions caused by glutamate-induced toxicity, the following experiment was conducted.

Injection of the excitatory neurotransmitter glutamate into the vitreal body of C57Bl/6J mice eye causes dose-dependent death of the cell bodies of optic nerve neurons. A previous study showed that the onset of RGC death is delayed (by more than 24 hours after glutamate injection) and is apoptotic-like.

In the present experiment, 8-week-old male C57Bl/6J mice were immunized subcutaneously with 100 μg poly-Glu,Tyr emulsified in CFA, 7 days prior to glutamate injection. A group of mice immunized at the same time with PBS emulsified in CFA to rule out a non-specific effect of the immunization and a group of non-immunized mice served as controls. Mice in all three groups received an injection of glutamate (400 nmole) into the vitreous of the right eye. The left eye received no injection and was used as an intact control. Seven days after glutamate injection, the eyes were excised and RGC survival was determined.

The average number of RGCs per mm^2 counted in the intact retinas of the poly-Glu,Tyr-immunized, the PBS-immunized, and the non-immunized mice were 2796±165, 2874±197 and 2807±42, respectively, indicating that immunization had no effect on survival of RGCs in the contralateral intact eye. These average values of RGCs per mm^2 in intact retina in all 3 experimental groups were therefore combined and taken as 100% RGC survival (0% toxicity).

The results depicted in Fig. 2 show that immunization of the mice with poly-Glu,Tyr in CFA (CFA-EY) significantly attenuated the glutamate-induced RGC death

compared to immunization with PBS (*t*-test, p=0.007) or to non-immunization (*t*-test, p=0.01). There was no difference in RGC survival between the 2 latter groups (*t*-test, p= 0.71).

EXAMPLE 3. Neuroprotection in spinal cord injury

Acute incomplete spinal cord injury at the low thoracic levels causes an immediate loss of hindlimb motor activity that spontaneously recovers within the first 12 days post-injury and stabilizes on deficient movement abilities. The amount of motor function restoration is the sum up effect of the positive recovery from spinal shock and the negative effect of longitudinal and ventral spread of damage. A therapeutic approach aiming at reducing the spread of damage through neuroprotection will result in a better recovery in terms of hind limb motor activity.

In the following experiments, the effect of active or passive immunization with poly-Glu,Tyr on motor activity of the hind limb after spinal cord contusion, was tested.

3.1 Active immunization with poly-Glu,Tyr : the effect of poly-Glu,Tyr/CFA immunization on rat recovery from spinal cord contusion

A contusive injury of the spinal cord was inflicted on anesthetized 12 SPD male rats by using the NYU impactor device to drop a 10-g rod from a height of 50 mm onto the exposed laminectomized spinal cord at level T8. The NYU impactor device used allowed, for each animal, measurement of the trajectory of the rod and its contact with the exposed spinal cord to allow uniform lesion.

Due to the spinal shock, the motor skills of the rats' hindlimbs initially disappeared, but recovered with time to reach a steady state of deficient motor activity. The amount of this deficiency caused by the injury can be reduced with adequate neuroprotective treatment.

The rats were divided into 2 groups (6 each) according to their impact errors to achieve similar groups. In one group, the rats were SC immunized in their upper back with PBS/CFA (triangles). In the other group, the rats were SC immunized with

pEY/CFA (100 μg/rat, squares). Both groups were immunized immediately after the injury and 7 days later both groups received a second immunization identical to the first one. The hind limb motor skills of the animals were scored using a scoring method developed by Basso et al., 1995 (the locomotor activity is scored (range of 0-21) according to the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale) following the kinetics and amount of hind limb motor activity in the two experimental groups. Approximately twice a week, locomotor activity of the trunk, tail and hind limbs in an open field was evaluated by placing the rat for 4 min in the middle of a circular enclosure made of molded plastic with a smooth, non-slip floor (90 cm diameter, 7 cm wall height). The results depicted in Fig. 3 show that rats treated with pEY (squares) showed a tendency to recover better than PBS-treated rats.

3.2 Passive immunization with poly-Glu,Tyr

In order to examine whether poly-Glu,Tyr-specific T cells also provide neuroprotection after spinal cord injury, the following experiment was conducted.

For the preparation of the T cells, four SPD rats were SC immunized in their lower back with pEY/CFA (125 μg/rat). Seven days later their splenocytes were harvested and a single cell suspension was prepared by pressing the spleens against a metal mesh using the plunger of a syringe. The splenocytes were activated in culture for 3 days with pEY (10 μg/ml). The cells were harvested, washed in PBS and counted.

Another group of 12 male SPD rats went trough surgery and their spinal cord contused at T7 level using 10-g weight drop from 50 mm height as described in Example 3.1 above. Immediately after the contusion, the rats were divided to 2 equal groups according to their impact errors. One group received intravenously 0.5 ml of PBS and the other group received splenocytes activated with pEY ($30x10^6$/0.5ml PBS/rat). The rats were followed for their recovery of function using the open field BBB score.

The results depicted in Fig. 4 show that the rats treated with splenocytes activated with pEY (squares) recovered better than the control group (triangles).

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CLAIMS:

1. Use of poly-Glu,Tyr for the preparation of a pharmaceutical composition for preventing or inhibiting neuronal degeneration in the central nervous system (CNS) or peripheral nervous system (PNS), for promoting nerve regeneration in the CNS or PNS, for treating an injury in the CNS or PNS or a disorder or disease caused or exacerbated by glutamate toxicity.

2. Use according to claim 1, wherein the pharmaceutical composition is for treating an injury in the CNS or PNS in order to prevent or inhibit neuronal degeneration or for promoting nerve regeneration.

3. Use according to claim 2, wherein said injury is spinal cord injury, blunt trauma, penetrating trauma, brain coup or contrecoup, hemorrhagic stroke, or ischemic stroke.

4. Use according to claim 3, wherein said injury is spinal cord injury.

5. Use according to claim 1, wherein the pharmaceutical composition is for treating a disorder or disease caused or exacerbated by glutamate toxicity.

6. Use according to claim 5, wherein said disorder or disease is a senile dementia including Alzheimer's disease, Parkinsonian syndrome including Parkinson's disease, facial nerve (Bell's) palsy, Huntington's chorea, a motor neuron disease including amyotrophic lateral sclerosis, a prion disease including Creutzfeldt-Jakob disease, Alper's disease, Batten disease, Cockayne syndrome, Lewy body disease, status epilepticus, carpal tunnel syndrome, intervertebral disc herniation, vitamin deficiency, epilepsy, amnesia, anxiety, hyperalgesia, psychosis, seizures, oxidative stress, opiate tolerance and dependence, an autoimmune disease, or a peripheral neuropathy associated with a disease such as amyloid polyneuropathy, diabetic neuropathy, uremic neuropathy, porphyric polyneuropathy, hypoglycemia, Sjogren-

Larsson syndrome, acute sensory neuropathy, chronic ataxic neuropathy, biliary cirrhosis, primary amyloidosis, obstructive lung diseases, acromegaly, malabsorption syndromes, polycythemia vera, IgA and IgG gammapathies, complications of various drugs such as nitrofurantoin, metronidazole, isoniazid and toxins such as alcohol or organophosphates, Charcot-Marie-Tooth disease, ataxia telangiectasia, Friedreich's ataxia, adrenomyeloneuropathy, giant axonal neuropathy, Refsum's disease, Fabry's disease, or lipoproteinemia.

7. Use according to claim 5, wherein said disorder or disease is associated with the eye and includes non-arteritic optic neuropathy, age-related macular degeneration, a retinal disorder such as retinal degeneration or a disease associated with abnormally elevated intraocular pressure.

8. Use according to claim 7, wherein said disease associated with abnormally elevated intraocular pressure is glaucoma.

9. Use according to any one of claims 1 to 8, wherein said poly-Glu,Tyr is poly-$Glu^{50}Tyr^{50}$.

ABSTRACT

Poly-Glu,Tyr is used for the preparation of a pharmaceutical composition for preventing or inhibiting neuronal degeneration, or for promoting nerve regeneration, in the central nervous system (CNS) or peripheral nervous system (PNS), or for treating an injury, disorder or disease in the CNS or PNS caused or exacerbated by glutamate toxicity.