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Plenary article

Prophylactic neuroprotection with A91 improves the outcome of spinal cord injured rats

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ABSTRACT

Iatrogenic injury to the spinal cord (SC) is not an uncommon complication of spinal surgery. In an attempt to establish a preventive therapy for anticipated SC injury, we tested the effect of a single dose (SD) vaccine vs. the addition of a booster dose (BD) of a neural-derived peptide (A91) prior to SC contusion. Immunization with A91 immediately after SC injury has demonstrated to induce significant tissue protection and motor recovery. After injury, only the BD vaccination schedule had a neuroprotective effect. It was capable of improving neurological recovery that was always significantly higher than the one observed in rats with SD immunization or those only treated with PBS. Toward the end of study, animals treated with an A91 BD presented a BBB score of 9.75 ± 0.17 (mean $\pm$ standard deviation) while rats treated with SD or PBS had a score of 6.6 ± 0.7 and 5.6 ± 0.6 respectively. In the next step we attempted to corroborate the neuroprotective effect induced by A91 immunization. For this purpose, we assessed the survival of rubrospinal neurons (RSNs) and ventral horn neurons (VHNs) sixty days after SC injury. BD vaccination induced a significant survival of both RSNs and VHNs after injury. Finally, the failure or success of this therapy (SD or BD respectively) was associated with a lower (SD) or higher (BD) A91-specific T cell proliferation. Prophylactic neuroprotection with an initial and subsequent booster dose of A91 may improve recovery after SC injury sustained during invasive spinal surgery procedures.

1. Introduction

Iatrogenic SC injury is an unfortunate complication of many surgical procedures that take place on or around the vertebral column. Recent reports suggest that the incidence of SC injury due to iatrogenic causes ranges from 0.59 to 14.7% [1,2]. Most of these happen during invasive procedures like scoliosis corrective surgeries (0.55%) and spinal fusions (0.14%) [1,2]. Several risk factors for iatrogenic SC injury have been identified such as: age, gender, history of hypertension, preoperative SC function, and the involved segments of the SC [3]. Considering the risk of SC injury surrounding certain procedures and patient comorbidities, a preventive treatment prior to surgery would be of great clinical value. Previous attempts have used erythropoietin, melatonin, cyclosporine-A, and even methylprednisolone (MP) but have not achieved successful prophylactic neuroprotection [4]. Immunizing against altered peptide ligands designed from nervous tissue proteins has shown to promote neuroprotection and neural restoration after SC injury [5,6]. This phenomenon is known as protective autoimmunity. An example of these is A91, a non-encephalitogenic peptide derived from myelin basic protein (MBP) sequence 87–99 but with a lysine residue changed to alanine at position 91. In line with this, previous studies in our laboratory have shown that immunization with a single dose of A91 after SC injury is capable of inducing significant tissue protection and motor recovery. A91 has also shown to induce a Th2 phenotype response that is capable of diminishing nitric oxide production and lipid peroxidation. This Th2 phenotype also releases neurotrophic factors after SC injury [7–9]. These effects explain, at least in part, the mechanisms by which protective autoimmunity ameliorates injury to the nervous system.

Abbreviations: BBB, Basso, Beattie & Bresnahan open-field test; BD, booster dose; CFA, complete Freund's adjuvant; CFSE, carboxifluorescein diester amine; MBP, myelin basic protein; MP, methylprednisolone; OVA, ovalbumin; RSNs, rubrospinal neurons; SC, spinal cord; SD, single dose; VHNs, ventral horn neurons.

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Since its use in a clinical setting is very realistic, we are now attempting to optimize its efficacy [10]. Regarding this, earlier studies have shown that A91 could be combined with MP therapy [11]. Furthermore, it was also demonstrated that the protective effects exerted by A91 can also be improved by adding an antioxidant in the form of combination therapy [12]. In the present work we explored the possible preventive use of A91 vaccination. The rationale that substantiates this idea is the fact that A91 induces a T cell response that before injury could create a microenvironment that promotes neuroprotection.

2. Materials and methods

2.1. Experimental animals

Female Sprague-Dawley (SPD) rats (21 days old) were supplied by the Animal Breeding Center of Camina Research Project. The rats were age-matched and housed in a light and temperature-controlled room. Efforts were made to minimize the number of animals used as well their suffering. All procedures were in accordance with the National Institutes of Health (US) Guide for the Care and Use of Laboratory Animals and the Mexican Official Norm on Principles of Laboratory Animal Care.

2.2. Study design

Thirty-six Sprague Dawley rats were vaccinated either with phosphate buffered saline (PBS) ($n = 12$) or A91 ($n = 24$) at 21 days of age to assure immunological maturity. Animals vaccinated with A91 were subdivided into two groups (12 rats per group): group one only received the initial single dose (SD) of A91 and group two received a booster dose (BD) of A91 at 45 days old. The booster dose (only in the case of PBS and BD groups) was applied 24 days (45 days old) after the initial dose in order to assure that the antigenic recognition was only mediated by an adaptive memory response. All groups were subjected to a SC injury when the animals were 60 days old. After injury, motor recovery in all rats was evaluated weekly until they were euthanized 60 days later. The effect of protective autoimmunity on the morphohistology of the injured SC was evaluated by measuring the survival of rubrospinal neurons (RSNs; $n = 4$ per group) and ventral horn neurons (VHNs; $n = 4$ per group). Finally, successful immunization to the vaccine was corroborated in all groups ($n = 4$ per group) using T cell proliferation against A91 peptide.

2.3. Spinal cord injury

Rats were subjected to a moderate SC contusion as previously described [8]. Thirty minutes after an intramuscular injection of a mixture of ketamine (50 mg/kg, Probiomed, Mexico city, Mexico) and xylazine (10 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa) a laminectomy of the T9 vertebrae was done on all rats. For SC injury, 10 g rod was dropped onto the exposed spinal cord from a height of 25 mm, using the NYU impactor (NYU, New York). The contusion was inflicted at the T9 SC level.

2.4. Immunization

Animals were injected subcutaneously at the base of the tail with either 70 μg of the A91 peptide (Invitrogen Life Technologies, San Diego, CA) or 0.15 M, pH 7.4 PBS. All vaccines were emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma, St Louis, MO, USA) containing 0.5 mg/ml *Mycobacterium tuberculosis*. In the group of rats receiving the booster dose, the second vaccine had the same concentration of peptide (70 μg) as the initial dose.

2.5. Assessment of motor recovery

Behavioral recovery was assessed every week using the Basso, Beattie & Bresnahan (BBB) open-field test of locomotor ability [13]. Observers blinded to the treatment received by each rat performed the test.

2.6. Retrograde labeling of rubrospinal neurons (RSNs)

Sixty days after injury, 4 animals from each group were reanaesthetized and 5 μl of 10% tetramethylrhodamine dextran dye (FluoroRuby; Molecular Probes, Eugene, OR) in PBS was applied into both rubrospinal tracts of the proximal stump after a complete transection of the SC below the site of contusion, at T12, in order to evaluate the survival of RSNs. Five days later the rats were decapitated and their brains were excised, processed, and cryosectioned. Every other section (20 μm thick) of the red nuclei – an average of 44 sections – was qualitatively and quantitatively examined by fluorescence and confocal microscopy. Only large, well-stained cells with whole body labeling were counted. The total number of labeled cells was counted in every section from each brain. The number of labeled cells recorded for each brain is the sum of all the cells counted in each section. The number of labeled neurons in each rat is given by the average number of cells counted in its two red nuclei.

2.7. Number of surviving ventral horn neurons (VHNs)

Sixty days after injury another four rats from each group were anaesthetized and perfused intra-aortically with 100 ml of PBS, pH 7.4 plus heparin (1%) at 4 °C, followed by 400 ml of a fixative solution (4% paraformaldehyde in PBS, pH 7.4 at 4 °C). One centimeter of tissue at the lesion site was removed and incubated for 2 h in the same fixative solution and then cryoprotected in a 30% sucrose solution for at least 3 days. Afterwards, three sequential cryosections (10 μm thick) were cut at 0, 1, 2 and 3 mm caudal and rostral to the epicenter of injury. Hematoxylin & eosin-stained sections were analyzed for residual VHNs. The number of surviving VHNs was confirmed and counted by the presence of Nissl substance, a euchromatic nucleus and a nucleolus [14]. The number of neurons in each rat is given by the average number of cells counted in its three sequential sections.

2.8. T cell proliferation

Cells were pooled from excised inguinal lymph nodes 60 days after SC injury ($n = 4$ per group). The cells were cultured in quintuplicate flat-bottomed wells in 0.2 ml of RPMI-1640 medium (GIBCO, New York) supplemented with 10% fetal bovine serum (Gibco, New York) on a 96-well microtiter plate. Cells (2.5×10^5 cells per well) were cultured 72 h in antigen-free medium or together with A91 (10 μg/ml), ovalbumin (OVA; 10 μg/ml; Sigma), or concanavalin-A (ConA; 10 μg/ml; Sigma, St. Louis, MO) at 37 °C in 5% CO_2. After two washes with RPMI-1640, cells were labeled with carboxifluorescein diester amine (CFSE) (Molecular Probes) as previously described [7]. The proliferative response was determined by flow cytometry. Cells were also stained with phycoerythrin-labeled anti-CD4 monoclonal antibodies (BD Pharmigen, San Diego, CA). Cells stained with CFSE and CD4 were analyzed.

3. Results

3.1. Booster dose A91 vaccination induced a significant improvement in motor recovery

In an attempt to establish a preventive therapy for SC injury, we tested the effect of a single dose vs. the effect of an