Combined protocol of cell therapy for chronic spinal cord injury. Report on the electrical and functional recovery of two patients

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Background
This is a preliminary report on successful results obtained during treatment of two patients with chronic spinal cord injury. The therapeutic approach was based on the generation of controlled inflammatory activity at the injury site that induced a microenvironment for the subsequent administration of autologous, BM-driven transdifferentiated neural stem cells (NSC).

Methods
BM mesenchymal stem cells (MSC) were cocultured with the patient’s autoimmune T (AT) cells to be transdifferentiated into NSC. Forty-eight hours prior to NSC implant, patients received an i.v. infusion of $5 \times 10^8$ to $1 \times 10^9$ AT cells. NSC were infused via a feeding artery of the lesion site. Safety evaluations were performed everyday, from the day of the first infusion until 96 h after the second infusion. After treatment, patients started a Vojta and Bobath neurorehabilitation program.

Results
At present two patients have been treated. Patient 1 was a 19-year-old man who presented paraplegia at the eight thoracic vertebra (T8) with his sensitive level corresponding to his sixth thoracic metamere (T6). He received two AT–NSC treatments and neurorehabilitation for 6 months. At present his motor level corresponds to his first sacral metamere (S1) and his sensitive level to the fourth sacral metamere (S4). Patient 2 was a 21-year-old woman who had a lesion that extended from her third to her fifth cervical vertebrae (C3–C5). Prior to her first therapeutic cycle she had severe quadriplegia and her sensitive level corresponded to her second cervical metamere (C2). After 3 months of treatment her motor and sensitive levels reached her first and second thoracic metameres (T1–T2). No adverse events were detected in either patient.

Discussion
The preliminary results lead us to think that this minimally invasive approach, which has minor adverse events, is effective for the repair of chronic spinal cord lesions.

Keywords
adult stem cells, MSC, NSC, protective autoimmunity, spinal cord injury, T-cell therapy, transdifferentiation.

Introduction
Moalem et al. [1] have proved that treatment with anti-myelin autoimmune T (AT) cells can induce restoration of experimental spinal cord injury in rats. This restoration effect is limited in time, presenting a window period of efficacy of 14 days post-trauma [2]. This window period is coincidental with the period during which the mobilization of BM mesenchymal stem cells (MSC) is promoted through the action of the inflammatory cytokines generated by the injured spinal cord [3].
Leone et al. [4] have independently shown that AT cells produce cytokines similar to those necessary to induce the in vitro transdifferentiation of MSC to neural stem cells (NSC). Furthermore, our group has shown that the coculture of a polyclonal cell suspension of AT cells against different central nervous system (CNS) proteins with BM MSC induces the transdifferentiation of the latter into NSC [5].

The term AT cells was first used by Niels K Jerne in 1974 [6]. This author observed that each somatic cell has corresponding T and B effector cells that are in turn compensated for by T- and B-specific suppressor cells. Both cell subsets constitute a regulation network between the aggression and tolerance of each organ. Later, in 1999, Moalem et al. [1] built up the concept of protective autoimmunity, when they showed that the AT cells, in their effector reaction, are essential for the repair process of damaged tissue.

Encouraged by these findings, we developed a therapeutic protocol (AT cell−NSC) in which the generation of a controlled inflammatory activity at the injury site would generate the micro-environment for the subsequent administration of the in vitro transdifferentiated NSC. The present communication is a preliminary report on the successful results obtained by treatment of two patients with chronic spinal cord injury using this approach.

Methods

Therapeutic procedure

The patients were received at Instituto Regina Mater, Buenos Aires, Argentina. A neurologist performed a physical evaluation of the patients and analyzed their MRI, angioMRI and somatosensory evoked potentials (SEP) to determine the type of lesion and vascular access of the lesion site and to assess their functional deficit. Informed consent was obtained from the two participant patients after the nature and possible consequences of the studies were explained to them.

AT cell purification

AT cells of patients suffering from spinal cord lesions and amyotrophic lateral sclerosis were isolated from peripheral blood. This procedure is a modification of a previously described technique [7]. Briefly, we performed an apheresis using a Cobe Spectra (Gambro BCT, CO, USA) apheresis machine. We processed about two blood volumes for each patient using theuffy coat program. The obtained Buffy coat suspension had a composition of 85% MNC, 1.8 × 10⁶/µL RBC and 6 × 10⁷/µL platelets. To purify this MNC sample we seeded it on a Ficoll−Hypaque gradient (Beckman Coulter Inc, Fullertont, CA, USA) (1.077 density). The obtained cells were washed in DBSS without Ca²⁺ and Mg²⁺. After this procedure, the composition of the ring was approximately 98% MNC, 0.2 × 10⁵/µL RBC and 1 × 10⁶/µL platelets.

This MNC suspension was cultured for 4 days in DMEM enriched with a partial hydrolyzate of bovine brain and a partial hydrolyzate of bovine spinal cord (Laboratorios Villar, Santa Fe, Argentina ). After 2 days in cell culture, we added IL-2 (Laboratorio Gautier, Buenos Aires, Argentina) until it reached a final concentration of 100 IU/mL.

At day 5, CD3⁺ lymphocytes were isolated by negative selection using a MAb cocktail against the undesired cells (MAb against CD14, CD16, CD19, CD56 and glycophorin A). This first incubation was followed by a second incubation with a solution of Ab attached to a paramagnetic Teflon bead (Stem Sep Kit, Stem Cell Technology. Vancouver, Canada). After the immune labeling of the cell suspension, the lymphocytes were passed through a powerful magnetic field, which allowed the passage of CD3⁺ cells and retained the rest of the cells. The CD3⁺ cells enriched the purity of the purified cell suspension up to 96%.

This CD3⁺-enriched suspension was then marked with an anti-CD25 MAb solution (Stem Sep Kit). The cells were attached to a paramagnetic Teflon bead for a new selection, thus obtaining two different cell population phenotypes, one CD3⁺ CD25⁻ and one CD3⁺ CD25⁺. The proportion of these two subsets varied depending on the particular condition of each donor.

MSC harvest, purification and expansion

A sample of approximately 600/cm³ BM mononuclear cells was obtained through a needle aspiration of iliac crest BM. This sample presented approximately 8−10 × 10⁸ total MNC. Its composition was 20% lymphocytes, 5% MID cells and 75% granulocytes, with 1.6 × 10⁵/µL platelets and 4−5 × 10⁷/µL RBC. This sample showed an immune CD133 labeling of approximately 1.5−2 × 10⁸ total cells.

The MSC were purified from the BM cell suspension through negative selection using a MAb cocktail (Rosette Sep Kit, Stem Cell Technology) and a Ficoll−Hypaque
gradient. Briefly, the unprocessed BM suspension was concentrated by centrifugation at 300 g. Cells were suspended in a human MSC-enriched cocktail composed of MAb against CD3, CD4, CD19, CD38, CD66b and glycophorin A. The MAb cocktails produced cross-links between the unwanted cells and the RBC in the suspension, forming immune rosettes. This particular situation increased the density of the unwanted cells so that they pelleted along with the free RBC when they were centrifuged over a buoyant density medium such as Ficoll–Hypaque (1.077 density). After this incubation, MSC remained unlabelled and unclamped, therefore they could easily be collected as a highly enriched population at the interphase between the plasma and the buoyant density medium. By these means we were able to increase the concentration of MSC by 8-fold. The total amount of cells suspended in the ring ranged from 1.5 to 2 × 10^6.

The obtained MSC were then cultured in Mesencult medium (Stem Cell Technology) for 4–6 weeks at a concentration of 1 × 10^6 cells/mL in 25-mm² plastic Petri dishes at 37°C and at an atmosphere of 95% O₂, 5% CO₂ in a Thermo Forma® CO₂ incubator. The culture was controlled using a phase-control inverted microscope (Nikon Eclipse TS 100). MSC reached a semi-confluent stage at approximately 15 days. At that moment, the selected cells were removed and reseeded on different plastic Petri dishes. We repeated this process twice during a 4–6-week period.

Coculture of AT cells and MSC
After 4–6 weeks of culture, when MSC reached the semi-confluent stage as previously described for the third time, they were reseeded on 22-mm² cover slips in two 6-well plates. Fifteen days later, the 12 wells were divided into three different groups of four wells each.

The first group was coincubated with unselected mononuclear cells from the patient and kept as a control. The second group was incubated with the CD3⁺ CD25⁻ lymphocytes from the patient. The third group was incubated with the CD3⁺ CD25⁺ lymphocytes from the patient.

Quality control and quality assurance of the cell process
All the cell processes were performed by highly trained personnel, according to the AABB Standards, in a clean room that followed the GTP final rules of the FDA part 1271 and the cGMP [8].

Microbiologic studies were carried out on samples taken from the BM cell suspension, aphaeresis products, selected cells and final product of each culture to detect contamination by bacteria, fungi or viruses. If any sample proved positive, the final product was disposed of and the procedure was repeated.

Flow cytometry analyzes were performed at the beginning, at each critical point of the cellular process and to the final product to corroborate the identity of the cell suspensions. Regarding the identity control of the cell suspension of the MSC and NSC, this was mainly based on the cytologic appearance of the tissue culture by observation through the phase-contrast inverted microscope. At least 90% of the MSC observed in a 10 high magnified field should have homogeneous cytoplasm with at least four nucleoli/nucleus. After 48 h of coincubation, 80% of the observed cells should have homogeneous granular cytoplasm (polarized or not) and a single large nucleus with not more than two prominent nucleoli.

Intravenous infusion of CD3⁺ CD25⁻ AT cells against CNS proteins
A suspension of CD3⁺ CD25⁻ AT cells against CNS proteins was infused at a dose of 5–10 × 10^8 cells/m² suspended in a 500-cc ringer solution for 8 h (at a rate of 63 mL/h) using an appropriate peristaltic pump. A program of preventive measures was established; this program incorporated the following measures.

Firstly, during the treatment the patients’ general clinical conditions (temperature, blood pressure and urinary output) were controlled. Secondly, 500 mg ASA and 10 mg loratadine were administered to the patients in 500-cc saline solution in parallel with the cell suspension. Thirdly, if the urinary output was reduced, the patients would receive 10 mg furosemide i.v. Fourthly, if the patients’ blood pressure dropped below 90 mm Hg, they would receive 500–1000 cc saline solution with free dripping. Fifthly, if nausea or vomiting was observed, the patients would receive 10 mg metoclopramide i.v.

These measures were adopted based on our previous cell therapy experience with similar, although different, treatments. [9]. No other adverse events were likely to occur if the above described conditions were observed.
Selective endovascular cell implant of MNC or NSC

The femoral artery was punctured 3 cm below the inguinal crease using Judkins’ technique. A Judkins’ catheter RCA 2 was placed through a 6-French introducer needle. The heparinization was performed locally at the puncture site.

The Adamkiewicz artery or first intercostal artery was later catheterized according to each case. The vertebral arteries were selectively catheterized. The catheter was set up in the dorsal area in order to reach the corresponding medullar area. The NSC suspension, in a total volume of 10 mL, was injected into each artery. The infusion pressure did not exceed 4.5 atmospheres.

Once this procedure was completed, the catheter and arterial guide were removed and homeostasis was performed through local compression. There were no severe complications. The patients remained in observation for 12 h.

Safety evaluations

The safety evaluations were performed in accordance with IND regulations 21CFR 312.32. Controls were performed prior to the administration of the T cells, 24 h after the administration of these T cells, on the day of the intra-arterial infusion of the NSC, and 24 and 96 h after this infusion. Thus we were able to monitor both early and late reactions and avoid toxic complications related to the administration of the different therapeutic agents. A complete clinical exam was performed at each control, consisting of the recording of the patient’s weight and vital signs, as well as records of the patient’s clinical, neurologic, cardiorespiratory, digestive, renal and endocrine activity.

Laboratory determinations, performed at these controls, enabled us to study the functions of BM, liver and kidneys as well as the most significant metabolic parameters, infection or systemic inflammatory activity, hydroelectrolytic balance and myocardial function through EKG. A total of 127 items was assessed at each test.

Adverse events were evaluated by the ‘Common terminology criteria for Adverse Events (AE), 2004’ developed by NIH. The definitions are available on the web at http://ctep.cancer.gov/reporting/ctc.html.

Although the patients did not develop major adverse events, precautions were taken. If patient developed signs compatible with acute hypersensitivity, anaphylaxis or systemic inflammatory response syndrome (SIRS), they would be treated with the appropriate supportive care, depending on the severity of the reaction, including admission into an intensive care unit. If there were any severe adverse events (SAE), the treating physician, in consultation with the IRB, would make any decisions regarding the continuation of therapy.

Results

Patient 1 was a 19-year-old man who had suffered a car accident 8 months before treatment. He presented with paraplegia and his sensitive level corresponded to his sixth thoracic metamere (T6). The patient had had two reparatory surgeries with poor results. After the second surgery the patient presented with an infection in his prosthesis. Because of this infection he had to receive antibiotics for a year. The patient received intense neurorehabilitation during this period. After 6 months, because he was considered a chronic patient, he started with a less intense neurorehabilitation program, thus developing severe muscle atrophy in his pelvic girdle and lower limbs. The patient presented with little possibility of fixing his trunk, thus being unable to rotate or make the movements necessary for intimate hygiene. He was, however, able to carry out simple tasks such as feeding or brushing his teeth by his own means.

Two months after his first treatment with AT cell−NSC, both his spine MRI and evoked potentials showed a clear improvement. An SEP of the posterior tibial nerves at ankle level of both legs had been performed on the patient prior to treatment. This study showed no cortical arrival. An SEP of the same area performed after treatment showed a significant improvement in wave reproducibility.

![Figure 1. Evoked potentials of patient 1, 2 months after spinal cord injury.](link)
and a normalization of the arrival rate to the spinal cord and brain cortex (N20 and P40) (Figures 1 and 2) at 39.0 microseconds. The MRI showed a significant increase of the transversal diameter of the spinal cord (Figures 3 and 4). Although the patient presented with a reappearance of spontaneous muscle contractions from the pelvic girdle to the knee, these contractions did not represent coordinated, effective movements. These signs of new progress led us to reinstitute a new intensive neurorehabilitation program as described in the Methods. After 3 days, he was able to move his lower limbs against gravity supported by the movements in his quadriceps, now recovered for the first time since his accident.

During the following 3 months the patient presented progressive improvement that slowly reached a plateau. Therefore we gave him a second AT cell–NSC treatment. After this last treatment he had achieved better coordination in all his movements and developed the ability to walk helped by two canes and short prostheses. Currently, the patient presents motor and sensitive levels corresponding to his first sacral metamere (S1). At present he continues with his neurorehabilitation program and a third treatment of AT cell–NSC has been programmed. No adverse events were detected.

Patient 2 was a 21-year-old woman who had suffered a car accident 30 months before she started her treatment at our institute. She suffered a spinal cord injury between her third and fifth cervical vertebrae (C3–C5). She presented no complications during her reparatory surgery. During the following 2 months the affected area extended to reach her seventh cervical vertebra (C7). She presented with severe quadriplegia according to her lesion in her third cervical vertebra (C3). Her sensitive level corresponded to her second cervical metamere (C2).

She followed an intensive neurorehabilitation plan for 28 months. This program enabled her to produce slight movements with her hands. These movements were not effective because she could not even hold an object; she could, however, hold her head up, with some effort. At the beginning of her treatment she presented with total

Figure 2. Evoked potentials of patient 1, 6 weeks after one treatment.

Figure 3. Patient 1’s MRI 3 days after the spinal cord injury.

Figure 4. Patient 1’s MRI 6 weeks after one treatment.
severe muscle atrophy in the upper limbs, back, trunk and lower limbs. She required the use of two belts to remain seated in her chair. She was not able to brush her teeth, eat or perform those tasks necessary for intimate hygiene by her own means.

After her first treatment with AT cell–NSC recovery was observed in her SEP, MRI and physical functions. The most significant change observed in her SEP was the latency changes in N20, which used to arrive at 50 microseconds, as shown in the SEP performed on the patient prior to treatment, and 8 weeks later had improved to 28 microseconds. Because of this phenomenon, it was believed that there had been a remyelinization of the injury. The MRI showed an increase of the transversal diameter partially as a result of certain inflammation in the lesion.

As regards functionality, the patient acquired the ability to hold her head, move her upper limbs, brush her own teeth, eat using special cutlery, paint and write helped by a cock-up orthosis. A cock-up orthosis is a wrist splint designed for assisting grip and pinch functions in the hand through the stabilization of the joint at about 30° dorsiflexion.

Although the patient currently presents voluntary movements in her trunk and lower limbs, her motor and sensitive levels correspond to her first and second thoracic metameres (T1 and T2). No adverse events were detected. A second treatment is scheduled to be applied to the patient.

**Discussion**

Some observations suggest that, although to a lesser extent, superior vertebrates present restoration processes similar to those, more significant and effective, presented by inferior vertebrates after traumatic or ischemic lesions of the CNS [1]. Studies performed on autopsies of female patients who had been transplanted with BM of histocompatible male donors showed that 1–2% of the CNS cells in the lesion site had a karyotype with the Y chromosome. This phenomenon suggests that the transplanted BM MSC were the only probable source of the Y carrier cells [10].

On the other hand, in 1999, Moalem et al. [1] established that the immune system plays a fundamental role in this repair process. They observed that after experimental spinal cord or optic nerve injury, an inflammatory reaction takes place. Macrophages and lymphocytes are predominant in this reaction. These lymphocytes are autoreactive and similar to those that produce encephalitis or autoimmune demyelization. This reaction is limited in time [2]. The intensity of this phenomenon is directly proportional to the restoration capacity presented by each species. Amphibians, such as toads and salamanders, have presented an inflammatory infiltration degree 10 times superior to that presented by rats after the same degree of spinal cord or optic nerve injury [1]. Schwartz’s group, helped by Irun Cohen [2], has experimentally proved that the exogenous administration of a considerable amount of anti-myelin lymphocytes to mammals enables the restoration of these lesions. This phenomenon could only take place in a window period of 15 days.

Other clinical data have shown that traumatic lesions in the heart and CNS are followed by a significant mobilization to blood of CD133 cells [4]. CD133 is an MSC marker of BM and fat tissue [11,12]. This mobilization reaches a peak within the week the injury took place. Studies by Kerschensteiner et al. [3] have shown that these anti-myelin AT cells secrete neurotrophic factors of the neurotrophin family, such as brain growth factor and nerve growth factor. All of these phenomena justify the presence of lymphocytes in the lesion site as inducers of the restoration through the transdifferentiation of the MSC, which were attracted to the site as a result of the inflammatory process [4].

Our laboratory has proved that the in vitro coculture of MSC and anti-CNS AT cells can induce the transdifferentiation of MSC into NSC [5]. Considering the above described phenomena, we developed this new therapeutic approach to promote the restoration of chronic spinal cord injuries. The approach is based on the combination of a treatment with autologous AT cells against the spinal cord, followed by i.v. administration (systemic or through selective catheterism) of autologous MSC transdifferentiated to NSC. Two patients received the above described treatment.

The rationale for a treatment with CD3+CD25− specific cells committed against CNS cells is mainly sustained by the fact that those AT cells have strong specificity to attach to CNS capillaries, break the blood–brain barriers and home in on the damaged area, increasing the local inflammation [1,2]. This specific and local inflammation would generate a neurotrophin and cytokine microenvironment [3,7,13,14], which in turn would attract circulating MSC and promote their transdifferentiation into NSC [5] to start the repair of the damaged area [15].
These T cells increase their inflammatory activity during the following 3 days [2]. Thus, at 48 h of the T-cell infusion, when the cells still need another 24 h to reach their action peak, we administered the NSC through an intravascular infusion.

Because we were successful when we used a peripheral blood vessel in one of the patients, we inferred that there could be a preferential attraction of the NSC to the damaged areas for the above described reasons [15]. In this way we could overcome the failures observed by other groups that had used systemic administration, because NSC preferentially homed in on the areas where the CD3⁺ CD25⁻ had previously arrived. However, we observed that if this treatment was performed by selective catheterism the almost complete delivery of these cells into the CNS damaged area was ensured. Moreover, this combined treatment program could resemble and improve the physiologic pathway to restoration of a trauma or injury of the CNS during its acute phase [1,2]. The sequential administration of AT cells to prepare the microenvironment, followed by a large number of pre-differentiated NSC, not only reproduces the process but also saves time and body energy during the new critical window period that we developed in each patient.

The important sensitivity and motor level recovery observed in both patients, as well as the modifications observed in the SEP, enabled us to believe that both glia cell and neuron fiber recovery, took place in the previously injured area. This recovery was also coincidental with clinical progress observed in both patients.

The fact that patient 1 recovered effective and coordinated motor function after starting neurorehabilitation led us to think that this program was essential for recovering the motor–neuron anagram of the CNS. The rehabilitation program that patient 1 had received for 8 months prior to treatment was based on the development of those muscles that remained innervated after the accident. These muscles could compensate for the rest of the lost movement as much as possible, for example using the movements in his trunk, the patient could move his lower limbs through the use of a long orthosis that fixed the joints of his hips, knees and ankles in a way similar to that used to move an artificial limb prosthesis.

The methods developed by Vojta and Bobath are based on the double stimulation of the muscle sets, through the nervous reflex and muscle–muscle stimulus, connected like the links in a chain. Thus the efficient stimulus of the previously stimulated muscle fiber adds to the neurologic impulse received by the re-innerved muscle fiber. This phenomenon enables the reconstitution, by retrograde stimulus, of the ability to move the whole body from the neck to the lower limbs, resembling a child's neurodevelopment.

Patient 2 received a neurorehabilitation program similar to that described above. However, we have observed that after 6 months of this kind of, and similar, program the patients reach a plateau in their muscle development. The subsequent rehabilitation programs are destined to maintain the patients’ achievements as well as optimize the recovered functions.

It is evident that patient 2 had developed new functions after the reinnervation of new muscle groups. The improvements in her current condition may be mainly attributed to the association of the NSC treatment and the neurorehabilitation program.

We need to stress that, even though we observed and handled 127 different safety assessments, which included clinical and laboratory data, we could not observe any adverse events that would have forced us to suspend or interrupt the treatment. Finally, we would like to highlight some advantages of the present approach over others published at the moment [15–20]. Firstly, because we used adult NSC, we eliminated the potential risk of neoplasic transformation, as described in animal experimentation with the use of embryonic NSC [10]. Secondly, as the cells used were autologous, we eliminated the risks and problems inherent with donor selection and graft rejection, as well as the need for immnosuppressant drugs [21]. Thirdly, being this an intravascular, minimally invasive procedure, we eliminated the risks of neurosurgery or even an intradural injection, as referred to by Satake et al. [17] and several other authors [16,18–20]. As a preliminary conclusion, the results described in the present report have led us to think that the use of this combined immunotherapy scheme may induce repair of the areas affected by CNS acute and chronic trauma with an approach that is minimally invasive and presents minimal or no adverse events.

**Acknowledgements**

The spiritual support of Ms Maricel Brandolino and Dr Ernesto Goberman, technical assistance of Ms Corina González Pulido, and the economic support of Fundación 208 GA Moviglia et al.
Regina Mater and Fundación Don Roberto Fernandez Viña are gratefully acknowledged.

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